EVALUATION OF THE GASTROINTESTINAL MICROBIOTA IN RESPONSE TO DIETARY AND THERAPEUTIC FACTORS IN CATS AND DOGS USING MOLECULAR METHODS

A Dissertation

by

JOSE FRANCISCO GARCIA-MAZCORRO

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

DOCTOR OF PHILOSOPHY

December 2011

Major Subject: Biomedical Sciences

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Approved by:

Chair of Committee,	Jan S. Suchodolski
Committee Members,	Albert E. Jergens
	Joerg M. Steiner
	Sara D. Lawhon
	Yanan Tian
Head of Department,	Sandee Hartsfield

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ABSTRACT

Evaluation of the Gastrointestinal Microbiota in Response to Dietary and Therapeutic Factors in Cats and Dogs Using Molecular Methods. (December 2011)

Jose Francisco Garcia-Mazcorro, M.V.Z, Universidad Autonoma de Nuevo Leon

Chair of Advisory Committee: Dr. Jan S. Suchodolski

The gastrointestinal (GI) tract of cats and dogs is inhabited by many different types of microorganisms, known as the GI microbiota. Mounting evidence suggests that the administration of certain dietary and/or therapeutic agents can alter the composition and activity of the GI microbiota, thus influencing gastrointestinal health and disease. The aim of this study was to evaluate the gastrointestinal microbiota in response to dietary and therapeutic interventions in cats and dogs. A multi-species synbiotic formulation, containing a total of $5x10^9$ colony forming units of a mixture of seven probiotic bacterial strains and a blend of prebiotics, was administered daily for 21 days to healthy cats and dogs. Fecal samples were collected before, during, and up to three weeks after discontinuation of the administration of the synbiotic. The fecal microbiota was analyzed using 454-pyrosequencing, denaturing gradient gel electrophoresis, quantitative real-time PCR, and 16S rRNA gene clone libraries. The results showed that the synbiotic led to increased concentrations of probiotic bacteria in the feces but did not alter the predominant bacterial phyla. Additionally, we investigated the effect of age, body weight, and baseline abundance of probiotic related bacterial genera, as potential

predictors of intestinal colonization by the ingested microorganisms. The results suggested that cats having a low abundance of fecal probiotic genera before consuming probiotics may have a higher concentration of the probiotic groups in feces during consumption of the synbiotic formulation. Also, a proton-pump inhibitor, aimed at suppressing the secretion of gastric acid, was administered daily for 15 days to healthy dogs. Changes in the GI microbiota were analyzed using 454-pyrosequencing, fluorescent *in situ* hybridization, and quantitative real-time PCR. The results suggested that inhibition of gastric acid secretion can alter the abundance of several gastric, duodenal, and fecal bacterial groups. However, these changes were not associated with major qualitative modifications of the overall composition of the GI microbiota. These studies showed that dietary and therapeutic agents can alter the composition of the GI microbiota and suggest that these changes could be associated with particular characteristics of the host. The clinical significance of these results needs further investigation.

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1. INTRODUCTION

1.1 The gastrointestinal microbiota

The gastrointestinal (GI) tract of cats and dogs as well as other mammals is a long muscular canal extending from the mouth to the anus where assimilation of nutrients takes place. Perhaps due in part to the continuous supply and variety of nutrients, the GI tract of mammals is one of the most heavily populated microbial ecosystems known (Xu & Gordon, 2003; Ley *et al.*, 2006). For example, a recent estimation of the cultivable fraction of the human GI microbiota included 442 bacterial, three archaeal, and 17 eukaryotic species (Rajilic-Stojanovic, 2007). However, the GI tract may harbor more than 1,200 distinct microorganisms, most of which have yet to be cultured successfully (Rajilic-Stojanovic *et al.*, 2007). Despite the presence of other types of microorganisms, bacteria make up the most abundant and functionality diverse microbial group in the GI tract of mammals.

1.2 Characterization of the GI microbiota

1.2.1 Culture methods

Traditional culture methods for the identification of bacteria rely on phenotypic characterization, including the assessment of the morphology of bacterial cells and colonies, their growth requirements, as well as their fermentation profiles and other bio-

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This dissertation follows the style of *FEMS Microbiology Ecology*.

chemical characteristics. Culture techniques have the advantages of being relatively inexpensive, widely available, and suitable for physiological and biochemical studies (Furrie, 2006). Although culture techniques remain indispensable to conduct detailed metabolic and functional studies on the GI microbiota (Duncan *et al.*, 2007), their utility in contemporary microbial ecology has been questioned because of their overall lack of representative results concerning microorganisms identifiable in a complex microbial ecosystem (Ritz, 2007), such as the GI tract.

1.2.2 Molecular methods

In spite of their usefulness in conducting functional studies of the GI microbiota, classic culture techniques are generally time consuming and labor-intensive for analysis of complex microbial communities (Furrie, 2006). Furthermore, culture is by definition restricted to cultivable organisms, and the selection of growth media can greatly affect results (Hartemink & Rombouts, 1999). As the large majority of bacterial species present in the intestinal tract are not cultivable (Pace, 1997; Eckburg *et al.*, 2005), it is difficult to perform a detailed examination of the composition of the GI microbiota using traditional culture techniques.

The advent of genetic-based molecular technologies by the end of the 20th century allowed the recognition of many novel molecular bacterial phylotypes within the mammalian gut (Suau *et al.*, 1999). Most molecular methods to identify bacteria rely on the detection of the 16S rRNA gene, which is readily isolated and universally distributed, displays consistency of function, and appears to have undergone a relatively

slow change in base pair composition throughout evolution (Fox *et al.*, 1980; Baker *et al.*, 2003). To date, molecular techniques have been successfully employed in studies evaluating the microbial composition of the GI tract of cats and dogs (see below). The following is a brief overview over the most commonly used molecular techniques.

1.2.2.1 Molecular fingerprinting

This technique is used to separate a mixture of polymerase chain reaction (PCR) amplicons based on differences in base pair composition. Molecular fingerprinting techniques include denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), and terminal restriction fragment length polymorphism analysis (T-RFLP) (Muyzer, 1999; Kitts, 2001). Despite the usefulness of these techniques at evaluating microbial diversity quickly and reproducibly (Muyzer *et al.*, 1993; Suchodolski *et al.*, 2004), fingerprinting techniques such as DGGE or TGGE have a limited ability to resolve different phylotypes because the different PCR amplicons can have a similar melting behavior (Nikolausz *et al.*, 2005). Also, there is a high frequency of additional secondary terminal restriction fragments in T-RFLP analysis (Egert & Friedrich, 2003), which could lead to an overestimation of microbial diversity. Moreover, the fingerprinting profiles may vary depending on the initial DNA template concentration and the type of DNA polymerase used during PCR, primer specificity, number of PCR cycles, as well as the annealing temperature (Jackson *et al.*, 2000; Osborn *et al.*, 2000; Egert & Friedrich, 2003).

1.2.2.2 16S rRNA gene clone libraries and sequencing

For identification of individual bacterial phylotypes, PCR amplicons generated using group-specific or universal bacterial primers can be identified using sequencing, a procedure which can be carried out using 16S rRNA clone libraries (Suchodolski *et al.*, 2008), or by automated high-throughput sequencing platforms (e.g., 454-pyrosequencing, Illumina) (Handl *et al.*, 2011). A 16S rRNA clone library is an approach to separate and identify PCR by ligating PCR amplicons into plasmid vectors, which are subsequently separated by transformation of vectors into *E. coli*. Plasmids can then be repurified and sequenced for identification. On the other hand, pyrosequencing, or sequencing by synthesis, relies on the detection of DNA polymerase activity by an enzymatic luminometric inorganic pyrophosphate (PPi) detection assay (ELIDA) to sequence DNA (Nyrén, 1987), where a successful nucleotide incorporation by the DNA polymerase is detected as emitted photons (Ronaghi *et al.* 1998). This pyrosequencing chemistry occurs by a DNA polymerase-driven generation of PPi, with the formation of ATP and ATP-dependent conversion of luciferin to oxyluciferin. This generation of oxyluciferin causes the emission of light pulses, and the amplitude of each signal is directly related to the presence of one or more nucleotides (Petrosino *et al.* 2009). The recently developed sequencer by 454 Life Sciences and Roche uses an emulsion PCR followed by this pyrosequencing chemistry of clonally amplified beads in a PicoTiterPlate (Pettersson *et al*. 2009). Although 454-pyrosequencing is capable to sequence 25 million bases in one four-hour run (Margulies *et al*. 2005), during last years Illumina and Applied Biosystems have introduced other sequencing systems with even

higher throughput than the 454-pyrosequencing, capable of sequencing billions of bases in a single run (Pettersson *et al*. 2009).

1.2.2.3 Quantitative real-time PCR (qPCR)

Traditional end-point PCR yields qualitative information, i.e., presence or absence of a given genomic target. Quantitative real-time PCR (qPCR) allows for the quantification of unknown genomic targets. This is made possible by including a fluorescent molecule in the PCR reaction that can be detected in real-time as the fluorescence increase is proportional to the increase in the amount of DNA generated. The fluorescent tools employed for this purpose include DNA-binding dyes and fluorescently labeled sequence-specific probes (Mackay, 2004).

1.2.2.4 Fluorescent *in situ* hybridization (FISH)

As mentioned before, qPCR can be utilized to quantify genomic targets (e.g., the 16S rRNA gene). However, the accuracy of qPCR-based quantification to enumerate microorganisms is limited because of the following reasons: bacteria contain different copy numbers of the 16S rRNA gene (Candela *et al.*, 2004; Lee *et al.*, 2008), there is preferential amplification of certain genomic targets (Sipos *et al.*, 2007), and also fast growing bacterial cells have a higher amount of genomic DNA because new rounds of DNA replication have started before the cell has actually divided (Champness, 2007). Also, as mentioned before, the initial DNA template concentration and the type of DNA polymerase used in the PCR, primer specificity, number of PCR cycles, as well as the

annealing temperature, can bias the utilization of the PCR towards certain genomic targets. Unlike qPCR, FISH is a molecular technique that does not depend on PCR amplification. FISH detects nucleic acid sequences by fluorescently labeled oligonucleotides that hybridize to its complementary target sequence within the intact cell (Moter & Gobel, 2000). Limitations of FISH include the need of specialized equipment and training, and issues with sensitivity, as the use of this technique to detect low abundant microorganisms is very time-consuming and impractical.

1.2.2.5 Metagenomics and transcriptomics

As mentioned before, the detection of a single genomic target (e.g., 16S rRNA gene) allows for the identification and/or quantification of the GI microbiota. However, this approach only yields phylogenetic information (i.e., it does not provide information about the metabolic or functional properties of the microbiota). In contrast, new high-throughput sequencing platforms allow for the identification of total genomic DNA or mRNA, thus allowing a more accurate characterization of the microbiota because there is no bias towards specific genes (for example, the 16S rRNA gene). These techniques have been recently used to study community structure as well as the functional properties of the canine fecal microbiota (Swanson *et al.*, 2011).

1.3 The canine GI microbiota

Clapper & Meade (1963) attempted one of the first characterizations of bacteria and fungi present in the lower intestine, nose, and throat of dogs using twelve different types

of culture media. Using rectal swabs from 25 healthy Beagle dogs, the authors isolated and identified 20 species of bacteria and 10 species of fungi. Years later, Balish *et al.* (1977) isolated a total of 53 bacterial genera (187 species of microorganisms) in feces of male Beagle dogs housed either in an isolated germ-free environment (n=7 dogs, 129 bacterial species) or in an open environment (n=2 dogs, 58 bacterial species). These results were among the first to suggest a potential environmental effect (i.e., housing conditions) on canine fecal microbial diversity. Using the same dogs, Davis *et al.* (1977) showed that the quantitative and qualitative distribution of bacteria varies along the different segments of the canine GI tract. The authors confirmed the effect of housing conditions on the canine GI microbiota by showing several qualitative and quantitative changes along the GI microbiota between the two groups of dogs (i.e., isolated and open environment). Also, the authors showed that the ileum of dogs possesses heterogeneous populations of bacteria, and that their abundance in this intestinal region is more variable than in the cecum or the colon. Also, using culture techniques, Devriese *et al.* (1992) reported that *Enterococcus faecalis* was the most frequently isolated *Enterococcus* spp. from anal swabs of healthy dogs (n=60), whereas *Streptococcus canis* and *S. bovis* were the most frequently isolated *Streptococcus* spp. Notably, the culture techniques utilized in this study could not identify 16% of all the isolates (47/288) as either *Enterococcus* or *Streptococcus* spp., suggesting a limitation of culture methods in identifying bacterial groups in the canine GI tract. Also, using culture techniques, Mentula *et al.* (2005) showed that the aerobic/facultative anaerobic and anaerobic bacteria were equally represented in the jejunum of Beagle dogs (n=22), whereas anaerobes predominated in

feces. Despite lower numbers of total bacterial organisms in the jejunum (10^2-10^6 cfu/g wet weight of intestinal fluid) than in feces (10^8-10^{11} cfu/g feces), *Staphylococcus* spp. and non-fermentative gram-negative rods were more prevalent in the small intestine. Similarly to the observations made by Davis *et al.* (1977), the authors also showed that the small intestine of dogs contains only few bacterial species at a time with vastly fluctuating counts, whereas the results obtained from the colon showed that the major bacterial groups remain relatively constant over time (Mentula *et al.*, 2005). Also, using traditional culture methods, Buddington *et al.* (2003) showed that the entire GI tract of Beagle dogs (n=110) was colonized by day 1 after birth, and that postnatal development was associated with changes in the relative proportions of the various groups of bacteria with anaerobic groups increasing in absolute and relative numbers.

The recent use of molecular technologies revealed that traditional culture techniques were underestimating the composition of the GI microbiota (Vaughan *et al.*, 2000). Greetham *et al.* (2002) were among the first in using both a culture and a genotyping approach to describe the composition of the canine GI microbiota. By sequencing the 16S rRNA gene from cultured isolates, they were able to identify 157 out of 171 bacterial colonies (14 colonies were not recovered due to technical difficulties) from the feces of one Labrador retriever. However, the gene sequences of many of the isolates (29% of total) did not correlate with known sequence information deposited in the Ribosomal Database Project at the time, suggesting the presence of novel bacterial phylotypes in the canine GI tract. The authors concluded that traditional culture methods failed to reflect the bacterial diversity in feces of Labrador retrievers. Years later,

Suchodolski *et al.* (2004) applied a fingerprinting technique (DGGE) to study the intestinal bacterial diversity in dogs (n=14). In this study, the obtained banding profiles suggested that dogs harbored a highly individualized and previously uncharacterized bacterial profile in the duodenum. A year later, the same research group expanded these results and showed that dogs had a higher bacterial diversity in the large intestine (i.e., colon and rectum) when compared with all sections of the small intestine (i.e., duodenum, jejunum, and ileum) (Suchodolski *et al.* 2005). More recently, Jia *et al.* (2010) investigated the abundance of selected groups of the fecal microbiota of healthy dogs (n=8) and dogs with chronic diarrhea (n=9) using fluorescence *in situ* hybridization, and showed that the *Atopobium* cluster (mainly the genera *Atopobium* and *Coriobacterium*), *Lactobacillus-Enterococcus* group, and *Clostridium* cluster XIVa were major fecal bacterial groups in dogs. More recently, Middelbos *et al.* (2010) for the first time used a new generation 454-pyrosequencing to describe the composition of the fecal microbiota in dogs (n=6), and showed that Fusobacteria (23-40%), Firmicutes (14-28%), and Bacteroidetes (31-34%) were the co-dominant fecal bacterial phyla. These results were similar to the results of another recent 454-pyrosequencing approach to evaluate the canine fecal microbiota where Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria made up >99% of total bacteria (Handl *et al.*, 2011). However, Firmicutes was by far the most abundant phylum (>90%). This would suggest that differences in DNA extraction procedures and/or primer selection may yield different abundance results between studies. Finally, a recent study used a phylogenetic and gene-centric metagenomics approach (i.e., analysis of a community from the viewpoint of its

component genes rather than its component organisms) to study the intestinal microbiome of dogs (n=6) and revealed similarities between canine, human, and mouse intestinal metagenomes (Swanson*, et al.*, 2011).

1.4 The feline GI microbiota

Osbaldiston & Stowe (1971) were among the first to investigate the intestinal microbiota of cats. The authors isolated a variety of microorganisms in the colon of healthy cats (n=12), including *Enterococcus*, *Streptococcus*, *Staphylococcus*, *Escherichia*, *Clostridium*, and *Lactobacillus* spp. Another early study compared the fecal microbiota of conventional cats and specific pathogen free cats using culture techniques (Itoh *et al.*, 1984). Sparkes *et al.* (1998) detected a total of 66 bacterial groups (species or genera, 43 aerobes and 23 anaerobes) in the duodenum of healthy cats (n=12). In addition to this higher qualitative abundance of aerobes in the feline duodenum, the authors showed that the numbers of aerobic bacteria were also higher than the numbers of anaerobic bacteria. Also in this study, only five bacterial groups (*Enterococcus faecalis*, *Clostridium perfringens*, *Bacteroides*, *Pasteurella*, and *Streptococcus* spp.) were present in more than 50% of all intestinal aspirates. Similarly, Johnston *et al.* (1999) isolated a mixture of aerobic, anaerobic, and microaerophilic bacteria, including *Bacillus*, *Bacteroides*, *Clostridium*, *Corynebacterium*, *Lactobacillus*, *Neisseria*, and *Streptococcus* spp. from the duodenal aspirates of healthy cats (n=7).

Using fluorescent *in situ* hybridization, Inness *et al.* (2007) evaluated the composition of the fecal microbiota in cats and showed that the feces of healthy animals

(n=34) contained an average of $10.3x10^{10}$ bacterial cells per gram of feces, whereas cats with inflammatory bowel disease (n=11) had a slightly lower average of $10.0x10^{10}$. As in dogs, recent studies using cloning and sequencing of the 16S rRNA gene have shown a higher bacterial diversity in the large intestine of healthy cats when compared with the small intestine (Ritchie *et al.*, 2008). Desai *et al.* (2009) sequenced the gene encoding the universal 60 kDa chaperonin (cpn60) and showed that the fecal microbiota of cats (n=9) was dominated by Actinobacteria (particularly *Bifidobacterium*) and Firmicutes (particularly *Lactobacillus*). The authors confirmed these results using quantitative real-time PCR and showed that, although there was substantial animal-to-animal variation, most targets were detected in all cats. Finally, Ritchie *et al.* (2010) studied the diversity of the fecal microbiota of healthy cats (n=15) using universal 16S rRNA gene primers and showed that the majority of the sequences were assigned to the phylum Firmicutes (87.3%), followed by Proteobacteria (7.9%), Bacteroidetes (2.4%), Actinobacteria (2.3%), and Fusobacteria (0.2%). These proportions of bacterial phyla are somewhat in agreement with a more recent evaluation of the feline fecal microbiota using 454-pyrosequencing (Handl *et al.*, 2011), which also showed a high proportion (~90%) of Firmicutes and a low proportion (< 10%) of all other phyla in fecal samples of healthy cats.

1.5 Host health and the GI microbiota

The GI microbiota is intimately related to gastrointestinal and overall health of the host. One of the main physiological phenomena linking host health and the GI microbiota is

microbial fermentation in the large intestine, where anaerobic bacteria break down undigested carbohydrates to short chain fatty acids (SCFA), which are rapidly absorbed by the colonic epithelium (Herschel *et al.*, 1981; Wong *et al.*, 2006). This symbiosis enables the host to salvage nutrients that would otherwise be lost by excretion from the digestive tract. In cats, one study showed that concentrations of volatile fatty acids were greatest in the proximal and distal portion of the colon when compared to the upper GI tract (Brosey *et al.*, 2000), suggesting the presence of a different microbiota in the different segments of the GI tract. SCFA have long been known to affect colonic epithelial cell transport, metabolism, growth, and differentiation of colonocytes, and hepatic metabolism of lipids and carbohydrates. They also provide energy to muscle, kidney, heart, and brain (Cummings & Macfarlane, 1997; Cook & Sellin, 1998; McOrist *et al.*, 2008). The GI microbiota is also known to stimulate the development of the immune system in young animals (Bauer *et al.*, 2006). Furthermore, intestinal bacteria can degrade free amino acids and small peptides, thus contributing to the recycling of nitrogen (Metges, 2000; Bergen & Wu, 2009). Moreover, certain bacterial populations in the GI tract are known to produce metabolic substrates for the host, such as *E. coli* and various *Bacteroides* spp. who produce menaquinones (Vitamin K_2) (Ramotar *et al.*, 1984), and *Enterococcus* spp. that are capable of synthesizing folate (Camilo *et al.*, 1996). Colonic bacteria such as *Lactobacillus* and *Bifidobacterium* spp. may also contribute to the salvage of bile acids that escape active transport in the distal ileum (Ridlon *et al.*, 2006). Yet other intestinal bacteria, such as *Enterococcus casseliflavus* and *Eubacterium ramulus* may aid in the bio-transformation of polyphenols (Schneider

et al., 1999), a group of compounds that may confer a health benefit to the host due to their high antioxidant activities (Lambert *et al.*, 2007). Finally, SCFA produced during fermentation have been shown to stimulate motility of the feline colon (Rondeau *et al.*, 2003) and the canine ileum in a concentration-dependent manner (Kamath *et al.*, 1987), suggesting a role of colonic bacteria in the overall digestive process of the GI tract.

1.6 The GI microbiota in disease

Batt and McLean (1987) performed one of the first studies to explore the relationship between the composition of the intestinal microbiota and intestinal mucosal damage in dogs with either aerobic or anaerobic bacterial overgrowth in the jejunum. Traditionally, small intestinal bacterial overgrowth (SIBO) was defined as a clinical syndrome characterized by an abnormal accumulation of bacteria in the small intestine (Johnston, 1999). However, this definition has changed over the years. SIBO has also been known synonymously as antibiotic-responsive diarrhea (German *et al.*, 2003) or tylosin-responsive diarrhea (Westermarck *et al.*, 2005). Today, there is little doubt that the intestinal microbiota plays an important role in GI disease (Cave, 2003; Marks & Kather, 2003; Stecher & Hardt, 2008), but the nature of the relationship is still elusive. For example, one study showed that only a combination of methods (ELISA and culture) can provide credible evidence for the presence of enterotoxigenic *C. perfringens* in the feces of dogs with diarrhea (Marks *et al.*, 2002). Also, while some authors believed that bacterial culture of duodenal juice remains necessary for definite diagnosis of SIBO (Rutgers *et al.*, 1995), it has been shown that SIBO, as assessed by quantitative culture,

is associated with mucosal damage not detected on histological examination of intestinal biopsies (Rutgers *et al.*, 1996), and one study did not find strong evidence of a relationship between duodenal bacterial numbers and a clinical response to antibiotics, questioning the utility of quantitative duodenal juice culture for the diagnosis of SIBO in canine patients with gastrointestinal signs (German, *et al.*, 2003). Despite this controversy regarding the relationship between intestinal bacteria and clinical disease, a higher prevalence of enteropathogenic *E. coli* was observed in feces of dogs with acute (n=57) and chronic (n=82) diarrhea when compared to healthy controls (n=122) (Sancak *et al.*, 2004). Also, a strain of *E. coli*, with similar phenotypic behavior of the Crohn's disease-associated strain *E. coli* LF 82, has been linked to granulomatous colitis in Boxer dogs with this disease (n=13) when compared to healthy controls (n=38) (Simpson *et al.*, 2006). Moreover, one study showed that total bacteria, *Bifidobacterium*, and *Bacteroides* counts in feces were all significantly higher in healthy cats (n=34) when compared to cats with IBD (n=11), whereas *Desulfovibrio* spp. (producers of toxic sulphides) numbers were significantly higher in the cats with IBD (Inness, *et al.*, 2007). Also, diarrheic episodes have been associated with increased fecal levels of *C. perfringens*, *Enterococcus faecalis*, and *Enterococcus faecium* in dogs (n=12) (Bell *et al.*, 2008). In addition, changes in microbiota composition have been associated with alteration of the host immune response. For example, the expression of several Toll-like receptors (TLRs), which are capable of recognizing microbe-associated molecular patters, has been shown to be upregulated in dogs with IBD (Burgener *et al.*, 2008; McMahon *et al.*, 2010). Similarly, one study showed that the expression of some of these TLRs was

increased in all intestinal segments (i.e., duodenum, colon, and ileum) in German Shepherd dogs with chronic enteropathies (Allenspach *et al.*, 2010).

The relationship between the GI microbiota and GI disease may involve specific groups of microorganisms (e.g., *E. coli*, *C. perfringens*) or groups of microorganisms. One study evaluated the relationship of duodenal mucosal bacteria to intestinal inflammation and clinical disease in cats with IBD, showing that the number of mucosa-associated Enterobacteriaceae was higher in cats with signs of GI disease (n=17) than in healthy control cats (n=10) (Janeczko *et al.*, 2008). Also, one study showed that dogs with IBD (n=7) had a higher abundance of all three main bacterial classes within the phylum Proteobacteria in the duodenum, when compared with healthy dogs (n=7) (Suchodolski *et al.*, 2010). In addition to bacteria, there could also be an involvement of fungal organisms in chronic intestinal enteropathies (Suchodolski *et al.*, 2008), but more research is needed to study these organisms (Handl, *et al.*, 2011).

1.7 Factors influencing the GI microbiota

The composition and function of the GI microbiota is affected by numerous factors associated with both the host and the surrounding environment. Early studies by Balish *et al.* and Davis *et al.* in the 1970's were among the first to suggest that different housing conditions could be associated with differences in the GI microbiota of dogs. Also, one study examined the GI microbiota of two age groups of dogs and showed that in all regions of the large intestine (i.e., cecum, colon, and rectum), the levels of *Bacteroides, Eubacterium, Peptostreptococcus, Bifidobacterium, Lactobacillus*, and *Staphylococcus*

spp. in the elderly dogs (n=8, older than 11 years of age) were lower than those in younger animals (n=8, younger than one year of age) (Benno *et al.*, 1992). Similarly, Simpson *et al.* (2002) studied the effect of age, breed, and diet on fecal bacterial populations of dogs (n=18). In this study, selected aerobic and anaerobic plate counts showed significant effects of breed and age, while no significant effect of diet was found. High dietary protein concentrations may also lower the fecal abundance of *E. coli*, *Bifidobacterium* and *Lactobacillus* spp. in young cats (n=17) (Vester *et al.*, 2009). In addition, the authors of this study showed that the numbers of *C. perfringens* and *Streptococcus* spp. were higher in elderly animals when compared to younger dogs. Moreover, Zentek *et al.* (2003) reported an increase in fecal culture counts of *Clostridium perfringens* in Beagle dogs (n=6) fed a high protein diet (66.3%), but other bacterial groups, such as *Streptococcus* and *Lactobacillus* spp. were not significantly affected. In this study, fecal ammonia concentrations also increased significantly with the high protein diet. Similarly, a high protein diet (52.9% protein in the diet) has also been shown to increase counts of *C. perfringens* in feces of healthy cats (n=8) (Lubbs *et al.*, 2009), as assessed by quantitative real-time PCR. In this study there was also a decrease in *Bifidobacterium* spp. during consumption of the high protein diet, but *Lactobacillus* and *E. coli* were not significantly affected by the treatment and fecal ammonia concentrations were not investigated.

Prebiotics and/or probiotic microorganisms are also known to modify the composition and function of the GI microbiota of cats and dogs. Sunvold *et al.* (1995) investigated the in vitro effect of dietary fiber on the metabolic behavior of the fecal

microbiota. The authors showed in dogs that a non-fermentable fiber (8.0% in the diet) yielded a lower in vitro organic matter disappearance and a higher acetate-to-propionate ratio when compared with a fermentable fiber (14.5% in diet) (Sunvold *et al.*, 1995). Similarly, the authors also showed that in cats a diet containing no supplemental fiber yielded a greater acetate-to-propionate ratio when compared with a diet containing beet pulp (12.5% in diet). Later, Sparkes *et al.* (1998) studied the effect of ingested fructooligosaccharides (0.75% in diet) on duodenal bacteria of healthy cats (n=12), but did not observe significant changes in aerobic, anaerobic, or total bacteria, as assessed by culture methods. Similarly, Willard *et al.*, also using culture methods (2000), did not observe changes in fecal concentrations of *Clostridium* spp. or *E. coli* after the addition of 1.0% FOS to the diet of healthy dogs (n=6). Marshall-Jones *et al.* (2006) evaluated the effect of orally administered *Lactobacillus acidophilus* (strain DSM13241, 10^8 cfu/day) on the fecal microbiota of healthy cats (n=15) for a period of 4.5 weeks. Culture techniques revealed that the fecal concentrations of *Lactobacillus* spp. were not increased during administration of the probiotic, but the treatment was associated with lower counts of *Clostridium* spp., total coliforms, and *Enterococcus* spp. In contrast to the results obtained with culture methods, fecal *Lactobacillus* spp. were significantly increased during probiotic administration in both absolute numbers and as a percentage of the total bacterial population as assessed by FISH. In contrast, using FISH, *Bifidobacterium* spp. and *Enterococcus faecalis* were decreased during administration of the probiotic (Marshall-Jones, *et al.*, 2006). One study showed that low-level fructan supplementation enhances digestion in dogs (n=5), but does not alter fecal microbial

populations (Barry *et al.*, 2009). Another study showed that dietary cellulose, fructooligosaccharides, and pectin modify protein catabolites and microbial populations in the feces of adult cats (n=12) (Barry *et al.*, 2010). In addition to the level of protein in the diet, probiotics and prebiotics, other environmental factors such as antibiotics (Suchodolski *et al.*, 2009; Gronvold *et al.*, 2010), and therapeutic drugs such as proton pump inhibitors (Lombardo *et al.*, 2010) have been shown to lead to alterations in the abundance of intestinal bacterial groups. Overall, these studies show that fluctuations in the GI microbiota of cats and dogs in response to environmental factors can be analyzed using culture-independent molecular techniques. Finally, as discussed before, disease episodes can lead to changes in the GI microbiota. Xenoulis *et al.* (2008) used 16S rRNA gene clone libraries and reported that the duodenum of dogs with IBD (n=10) may be composed of distinct microbial communities when compared with healthy dogs (n=9), especially within the members of the family Enterobacteriaceae.

1.8 Hypotheses and research objectives

The hypotheses of this study are that: 1) a multi-species synbiotic formulation will change the composition of the fecal microbiota in healthy cats and dogs; 2) age, baseline bacterial populations, and body weight will serve as significant predictors of intestinal colonization by ingested probiotic bacteria in healthy cats and dogs; and 3) the proton-pump inhibitor omeprazole will change the composition of the gastric, duodenal, and fecal microbiota in healthy dogs.

The objectives of the proposed research project were 1) to evaluate the effect of a multi-species synbiotic formulation on fecal bacteria of healthy cats and dogs, 2) to investigate the effect of age, baseline bacterial populations, and body weight as predictors of intestinal colonization by ingested probiotic bacteria in healthy cats and dogs, 3) to evaluate the effect of the proton-pump inhibitor omeprazole on gastric, duodenal, and fecal bacterial populations of healthy dogs, and 4) to characterize the abundance of predominant bacterial groups in feces of healthy dogs using fluorescence *in situ* hybridization.

2. EFFECT OF A MULTI-SPECIES SYNBIOTIC ON FECAL MICROBIOTA OF HEALTHY CATS AND DOGS

2.1 Overview

The effect of a multi-species synbiotic on the fecal microbiota of healthy cats (*n*=12) and dogs (*n*=12) was evaluated. The synbiotic (containing $5x10^9$ colony-forming units of a mixture of seven probiotic strains, and a blend of fructooligosaccharides and arabinogalactans) was administered daily for 21 days. Fecal and serum samples were collected before, during, and up to three weeks after administration. Changes in the fecal microbiota were analyzed using denaturing gradient gel electrophoresis, 16S rRNA gene libraries, quantitative real-time PCR, and 16S rRNA gene 454-pyrosequencing. Probiotic species were detectable in 10/12 dogs and 11/12 cats during product administration. Abundances of *Enterococcus* and *Streptococcus* spp. were significantly increased in at least one time point during administration, and returned to baseline abundance after treatment was discontinued. No changes in the major bacterial phyla were identified on 454-pyrosequencing. No adverse gastrointestinal effects were recorded and no significant changes in gastrointestinal function or immune markers were observed during the study period. This study shows that while the ingestion of probiotics and prebiotics does not appear to alter the predominant bacterial phyla present in feces, supplementation with the investigated synbiotic leads to an increased abundance of probiotic bacteria in the feces of healthy cats and dogs.

2.2 Introduction

Probiotics are live microorganisms, which when consumed in adequate amounts confer a health benefit on the host (FAO/WHO, 2002). Prebiotics are defined as selectively fermented ingredients that result in changes in the composition and/or activity of the gastrointestinal microbiota, thus also conferring health benefits on the host (Gibson *et al.*, 2010), and synbiotics are preparations containing both probiotics and prebiotics. Formulations containing probiotics and/or prebiotics are increasingly used in human and veterinary medicine, as they could potentially be useful to treat and/or prevent gastrointestinal as well as extra-gastrointestinal disorders (Roberfroid *et al.*, 2010; Wolvers *et al.*, 2010).

Probiotics can enhance intestinal health by several mechanisms, including displacement of intestinal pathogens (Lee *et al.*, 2003), production of antimicrobial substances (Jones & Versalovic, 2009), and/or enhancement of immune responses (Pagnini *et al.*, 2010). The success of these mechanisms in promoting health is thought to be dependent on an increased abundance of probiotic organisms in the intestinal tract (Kailasapathy & Chin, 2000). This hypothesis has led to numerous investigations addressing the survival (Bezkorovainy, 2001; Elli *et al.*, 2006) and colonization (Valeur *et al.*, 2004; Pagnini *et al.*, 2010) properties of probiotics after oral ingestion as enhancers of health.

The increased abundance of probiotics in the intestinal tract after oral ingestion has traditionally been thought to modify the composition of the intestinal microbiota (Fuller, 1989). However, to date most investigations have only studied the effect of

probiotics on select intestinal bacterial groups (Sauter *et al.*, 2006; Biagi *et al.*, 2007; Saulnier *et al.*, 2008), in part due to the challenges associated with the characterization of highly complex microbial ecosystems. Recently developed cost-effective high throughput technologies (e.g., microarray based methods or massive parallel pyrosequencing techniques) allow a deeper phylogenetic coverage of the intestinal microbiota (Zoetendal *et al.*, 2008; Suchodolski *et al.*, 2009; Middelbos *et al.*, 2010; Swanson *et al.*, 2011; Handl *et al.*, 2011) and, therefore, may be useful to assess the effect of probiotics and/or prebiotics on the overall composition of the intestinal microbiota (Middelbos *et al.*, 2010).

The effect of probiotics on intestinal and overall health has been studied in humans (Culligan *et al.*, 2009), but much more limited data are available for veterinary species (Callaway *et al.*, 2008). While probiotics and prebiotics are administered to dogs and cats with increasing frequency, only few investigations have evaluated the effect of these preparations on intestinal microbial composition and immune function of these animal species (Baillon *et al.*, 2004; Marshall-Jones *et al.*, 2006; Kelley *et al.*, 2009; Ogue-Bon *et al.*, 2010). Because extrapolations of the *in vivo* effect of probiotics among animal species are inherently weak, the effect of probiotic preparations on the intestinal microbiota of the target animal population deserves investigation. The objective of this study was to evaluate the effect of a multi-species synbiotic preparation designed for use in cats and dogs on the fecal bacterial microbiota of these animal species. Changes in fecal bacterial groups were evaluated using denaturing gradient gel electrophoresis (DGGE), comparative 16S rRNA gene analysis, quantitative real-time PCR (qPCR)

assays, as well as massive parallel 16S rRNA gene 454-pyrosequencing. Selected markers of gastrointestinal and immune function were also evaluated to investigate potentially beneficial effects due to the consumption of the synbiotic.

2.3 Materials and methods

2.3.1 Synbiotic description

Proviable®-DC (Nutramax Laboratories, Inc. Edgewood, MD) is a commercially available multi-species synbiotic formulation designed for use in cats and dogs that contains a blend of fructooligosaccharides (FOS), arabinogalactans, and a total of five billion ($5x10^9$) colony-forming units of a mixture of seven bacterial species per capsule (Table 1). The exact proportions of each component in the formulation (bacterial strains and prebiotics) are proprietary.

2.3.2 Animal subjects and study design

Privately owned healthy cats (*n*=12) and dogs (*n*=12) of different breeds and ages were enrolled (Table 2). None of the enrolled subjects had a history of antibiotic use or any other medication known to influence the intestinal microbiota for at least three months before the beginning of the study. All animal subjects were fed different commercial diets.

Baseline blood and fecal samples were collected before synbiotic administration (day 0). All cats and dogs then received one capsule of the formulation daily for 21 days.

Table 1. Probiotic bacterial strains in Proviable®-DC

Bacterium	Strain
Enterococcus faecium	NCIMB 30183
Streptococcus salivarus subsp. *thermophilus*	NCIMB 30189
Bifidobacterium longum	NCIMB 30179
Lactobacillus acidophilus	NCIMB 30184
Lactobacillus casei subsp. *rhamnosus*	NCIMB 30188
Lactobacillus plantarum	NCIMB 30187
Lactobacillus delbrueckii subsp. *bulgaricus*	NCIMB 30186

Owners were allowed to administer the capsule orally, mix the capsule into food, or open the capsule and mix the preparation contained in the capsule into the food, depending on the particular way of their pets to accept medications (Table 2). Owners (mainly students of Veterinary Medicine at Texas A&M University) were instructed to maintain the usual diet and asked to complete a daily questionnaire during the 21 days of synbiotic administration to record clinical signs of gastrointestinal discomfort such as diarrhea, vomiting, and/or abdominal pain. Additional serum samples were obtained on day 21 (last day of synbiotic administration) and day 42 (three weeks after cessation of administration of the synbiotic). Additional fecal samples were collected every 3 to 4 days during and up to three weeks after administration of the synbiotic. All subjects were maintained on their typical diet during the study period. The study protocol was approved by the Clinical Research Review Committee of Texas A&M University and written informed consent was obtained from the owners of all enrolled animals.

Table 2. Summary statistics for the enrolled cats and dogs. Information about the breed, age, weight, body condition score (BCS), type of food consumed, and the mode of administration of the synbiotic formulation are provided. Probiotic species were detected during the administration period either using universal bacterial primers (DGGE) or genus-specific primers (16S rRNA gene clone libraries) for *Bifidobacterium* and *Lactobacillus* spp

Cat	Breed	Age (years)	Weight (pounds)	BCS	Food	Administration of probiotic	DGGE	Clone libraries
1	DSH	5.0	13	7	dry	powder in food	yes	N/A
2	Mixed	0.7	9.5	5	dry	capsule in food	yes	yes
3	DSH	2.5	12	6	dry	capsule by mouth	yes	no
4	DSH	6.7	11	nn	dry	powder in food	yes	yes
5	DSH	1.3	12.7	5	dry	capsule by mouth	yes	yes
6	DLH	1.7	9.2	4	dry	powder in food	yes	no
7	DSH	1.4	9.3	5	dry	powder in food	no	no
8	Persian	1.2	8.3	4	raw	powder in food	yes	yes
9	DSH	4.8	11.5	5	dry	powder in food	yes	yes
10	DMH	2.8	11.1	5	dry	powder in food	yes	yes
11	DSH	4.2	11	6	dry	powder in food	yes	yes
12	DSH	3.0	10.8	6	dry/can	powder in food	yes	yes
							92%	73%
Dog								
1	Mixed	10.2	63	6	dry	capsule by mouth	yes	no
2	Mixed	0.8	10.2	4	dry	capsule by mouth	no	yes
3	Blue Heeler	3.7	59.5	8	dry	capsule by mouth	yes	yes
4	Lab Retriever	1.9	55.5	5	dry	capsule by mouth	no	yes
5	Min Dachshund	3.5	11.5	5	dry	capsule by mouth	N/A	N/A
6	Mixed	2.8	69	4	dry	capsule by mouth	no	yes
7	Mixed	3.7	10	6	dry	capsule in food	no	no
8	Corgi	0.9	24.8	5	dry	powder in food	no	yes
9	Mixed	0.7	35	4	dry	capsule by mouth	no	yes
10	Boxer	5.0	75	7	dry	powder in food	yes	yes
11	Chesapeake	7.9	64	5	dry	capsule by mouth	yes	yes
12	Lab Retriever	6.9	81.5	5	dry	capsule by mouth	yes	yes
							45%	82%

2.3.3 Assessment of fecal microbiota

Extraction of DNA. An aliquot of 100 mg (wet weight) of each fecal sample was mixed with 500 µL of lysis buffer (Purege® cell lysis solution, Gentra Systems, Minneapolis, MN) and 100 µL of 0.1 mm-diameter zirconia beads (BioSpec Products Inc., Bartlesville, OK). This mixture was vortexed for 5 min at maximum speed on a standard vortex. After centrifugation (7 min at 12,000 ***g***), the supernatant was transferred into a

sterile tube and mixed with 500 μL of a solution of phenol-chloroform-isoamyl alcohol (Applied Biosystems, Foster City, CA). Further steps of DNA extraction and purification were performed as previously described (Suchodolski *et al.*, 2005).

Denaturing Gel Gradient Electrophoresis (DGGE). To first investigate whether the synbiotic led to a noticeable change on the fecal microbiota, qualitative changes in fecal bacterial communities were evaluated by DGGE at baseline, day 21 (last day of synbiotic administration) and day 38 (two weeks after treatment) with some modifications to a protocol described previously (Suchodolski *et al.*, 2004). Briefly, universal bacterial primers F341 and R518 (Table 3) were used to amplify the variable V3 region of the 16S rRNA gene. A GC clamp (CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGG) was incorporated into the forward primer to prevent the complete dissociation of the DNA double strand during the subsequent DGGE analysis. PCR amplicons were loaded on 8% (w/v) polyacrylamide gels in TAE buffer with a linear denaturing gradient of 40% to 60%. Electrophoresis was performed in TAE buffer at 60 °C for 16 h at 70 V. Gels were stained with ethidium bromide for 12 min, destained in water two times for 30 min and visualized under UV light.

16S rRNA gene clone libraries. In addition to a change in the qualitative composition of the fecal microbiota, it was also of interest to investigate in feces the presence of closely related probiotic genera (see qPCR below) and species over time. Changes in fecal *Bifidobacterium* and *Lactobacillus* spp. were assessed using 16S rRNA gene clone libraries at baseline, during the last week of synbiotic administration (days

14-17), and two weeks after discontinuation of treatment (days 32-38). Genus-specific primers (Table 3) were used to amplify a 308 and 480 bp amplicon of the 16S rRNA gene of *Lactobacillus* and *Bifidobacterium* spp., respectively, as described previously (Ritchie *et al.*, 2010; Handl *et al.*, 2011).

Table 3 Oligonucleotides used for the amplification of bacterial targets

Target	Sequence (5'-3')	Annealing Temperature (°C)	Reference
Bifidobacterium spp.	F- TCGCGTCCGGTGTGAAAG R- CCACATCCAGCATCCAC	60	Rinttila *et al.* (2004)
Enterococcus spp.	F- CCCTTATTGTTAGTTGCCATCATT R- ACTCGTTGTACTTCCCATTGT	61	Malinen *et al.* (2005)
Lactobacillus spp.	F- AGCAGTAGGGAATCTTCCA R- CACCGCTACACATGGAG	58	Malinen *et al.*, (2005)
Streptococcus spp.	F- TTATTTGAAAGGGGCAATTGCT R- GTGAACTTTCCACTCTCACAC	54	Furet *et al.* (2004)
All bacteria	F- CCTACGGGAGGCAGCAG R- ATTACCGCGGCTGCTGG	57	Muyzer *et al.* (1993)

Quantitative real-time PCR (qPCR). Quantitative changes in the fecal abundance of probiotic groups were assessed by qPCR before, during (days 1, 8, and 17 in cats and days 3, 5, and 17 in dogs) and after (days 26, 29, and 38 in cats and days 23, 26, and 38) synbiotic administration, depending on the availability of fecal DNA samples. Bacterial DNA was amplified using bacterial universal and 16S rRNA gene genus-specific primers (Table 3) for all four probiotic genera (*Enterococcus, Lactobacillus,*

Bifidobacterium, and *Streptococcus* spp.) using SYBR Green-based assays (Biorad Laboratories, Hercules, CA, USA). Standard curves using 1:10 dilutions of DNA (ranging from 2 ng to 0.2 pg) from lyophilized bacterial species of each genus and canine fecal DNA for universal bacteria were used to calculate the unknown bacterial genomic targets. All samples and standards were run in duplicate. A commercial real-time PCR thermocycler (iCycler iQ, Biorad) was used for all experiments with the following PCR protocols: 95 °C for 3 min for enzyme activation, 35 cycles consisting of 10 s at 95 °C, 15 s at optimized annealing temperature (Table 3) and extension for 10 s at 72 °C. The PCR mixture (25 μL) contained 12.5 μL of iQ™ SYBR® Green Supermix (Biorad), 9.7 μL of sterile water, 0.4 μL of each primer (final concentration: 160 nmol) and 2 μL of DNA (~ 5 ng μL^{-1}). After all PCR cycles were completed, a melt-curve analysis was performed for all assays under the following conditions: 1 min denaturation at 95 °C, 1 min annealing at 55 °C, and 80 cycles of 0.5 °C increments (10 s each) beginning at 55 °C. The $\log_{10}$ 16S rRNA gene copies from each bacterial genera was normalized to the $\log_{10}$ 16S rRNA gene copies of all bacteria ($\log_{10}$ 16S rRNA gene copies from each bacterial group divided by the $\log_{10}$ 16S rRNA gene copies of all bacteria) for statistical comparisons (Frank *et al.*, 2007).

Massive parallel 16S rRNA gene pyrosequencing. Fecal bacterial communities were evaluated using pyrosequencing at baseline (day 0), after five days of feeding the synbiotic (day 5) and after two days of discontinuation of synbiotic administration (day 23) using a bacterial tag-encoded FLX-titanium 16S rRNA gene amplicon pyrosequencing (bTEFAP) as described previously for canine and feline fecal samples

(Handl *et al.*, 2011). Sequences with identity scores greater than 97% identity (< 3% divergence) were resolved at the species level, between 95% and 97% at the genus level, between 90% and 95% at the family level, and between 80% and 90% at the order level. To assess the diversity of the fecal microbiota, the Shannon-Weaver diversity index was calculated. High values for this diversity index indicate higher bacterial diversity. To investigate potential clustering of the microbial communities before, during, and after administering the synbiotic, principal component analysis (PCA) based on the phylogeny-based Unifrac method (Lozupone & Knight, 2005) was applied using the data generated by pyrosequencing.

2.3.4. Parameters of GI and immune function

Parameters in serum. All serum assays were measured before synbiotic administration (day 0), on day 21 (last day of synbiotic administration), and 42 (three weeks after end of treatment). A complete blood count and serum chemistry profile were analyzed at the Texas Veterinary Medical Diagnostic Laboratory (College Station, TX). Serum concentrations of IgA were measured using a commercial ELISA assay (Bethyl Laboratories, Montgomery, TX). Serum concentrations of cobalamin (competitive immunoassay, Immulite 2000 Vitamin B12®, Siemens, Los Angeles, CA), folate (competitive immunoassay, Immulite 2000 Folic acid®, Siemens), trypsin-like immunoreactivity (TLI, for dogs a radioimmunoassay (RIA) from Siemens, for cats an in-house RIA), and pancreatic lipase immunoreactivity (PLI, for dogs Spec cPL® from

Idexx Laboratories Westbrook, ME, for cats an in-house RIA) were analyzed at the Gastrointestinal Laboratory (College Station, TX).

Parameters in feces. Fecal IgA concentrations were measured in canine feces before (day 0), during (day 21, last day of synbiotic administration), and after (day 42) synbiotic administration using an ELISA that has been validated for measurement of IgA concentrations in canine feces (Tress *et al.*, 2006). The analysis of fecal IgA in cats was not performed because to date there is no validated assay available. Canine and feline fecal α_1-proteinase inhibitor (α_1-PI) concentrations were measured at day 0, day 21, and day 42 using species specific in-house immunoassays (Melgarejo *et al.*, 1998; Fetz *et al.*, 2004), respectively.

2.3.5 Statistical analysis

A repeated measures analysis of variance (ANOVA) or its non-parametric counterpart, Friedman's test, was used to compare the following dependent variables: the results of gastrointestinal and immune function tests, serum chemistry, complete blood counts, changes in microbial composition based on 16S rRNA gene pyrosequencing, bacterial abundance based on qPCRs, and Shannon-Weaver diversity indices, across different levels of the independent variable time (before, during and after synbiotic administration) using Prism5 (GraphPad Software Inc., San Diego, CA). The assumption of normality was tested using the D'Agostino and Pearson normality test (GraphPad Software Inc.). For all tests, a $P<0.05$ was considered statistically significant to reject the null hypotheses (time points are all equal). Multiple comparisons were adjusted by the

Tukey-Kramer method (ANOVA) and Dunns' test (Friedman's test). To adjust for falsely rejected null hypotheses, the Benjamin-Hochberg critical values were calculated (Benjamini & Hochberg, 1995) and compared with the *P* values from the comparison of proportions of pyrosequencing tags at each phylogenetic level separately.

2.4 Results

2.4.1 Qualitative assessment of the fecal microbiota

Due to insufficient DNA material, DGGE analysis was performed in all 12 cats but only in 11 of the 12 dogs, while 16S rRNA gene clone libraries were constructed for 11 dogs and 11 cats each. In 11/12 (92%) cats and in 5/11 (45%) dogs, DGGE bands were observed that appeared during synbiotic administration, but were absent at baseline and also after discontinuation of synbiotic administration (Table 2). The DNA from these bands was purified and re-amplified using universal bacterial primers as described above. Sequencing of these amplicons identified sequences matching the 16S rRNA gene of *Enterococcus faecium*. Using 16S rRNA gene clone libraries, at least one probiotic species (*Bifidobacterium longum*, *Lactobacillus acidophilus*, *L. plantarum*, *L. delbrueckii*, or *L. rhamnosus*) was detected during synbiotic administration in 8/11 (73%) cats and 9/11 (82%) dogs (Table 2), but were undetectable before or after synbiotic administration.

2.4.2. Quantitative real-time PCR

Analysis by qPCR showed an increase in the abundance of probiotic groups in feces during synbiotic administration and return to baseline abundance after conclusion of probiotic administration (Fig. 1), as determined by increased concentrations of target DNA from probiotic groups. However, the increase in abundance of probiotic bacteria in feces differed depending on the bacterial genus. Fecal abundance of *Enterococcus* and *Streptococcus* spp. were found to be significantly increased in at least one time point during synbiotic administration when compared with baseline abundances of these bacteria, in both cats and dogs (Fig. 1). In cats, *Lactobacillus* and to a lesser extent *Bifidobacterium* spp. increased during synbiotic administration, but the difference among time points did not reach statistical significance, with the exception of samples collected on day 17 showing significantly higher counts than those in samples collected on day 38 (two weeks after cessation of synbiotic administration). In dogs, counts of fecal *Lactobacillus* spp. increased by day 3 of synbiotic treatment, although this difference was not statistically significant. *Lactobacillus* spp. counts returned to baseline abundance values by day 8, while fecal abundance of *Bifidobacterium* spp. was not significantly altered (Fig. 1).

2.4.3 Massive parallel pyrosequencing

454-Pyrosequencing in cats. A total of 187 396 pyrosequencing reads were generated: 75 350 at baseline, 60 355 on day five of synbiotic administration, and 51 691 on day 23 (2 days after end of synbiotic administration). The most abundant phylum was Firmicutes

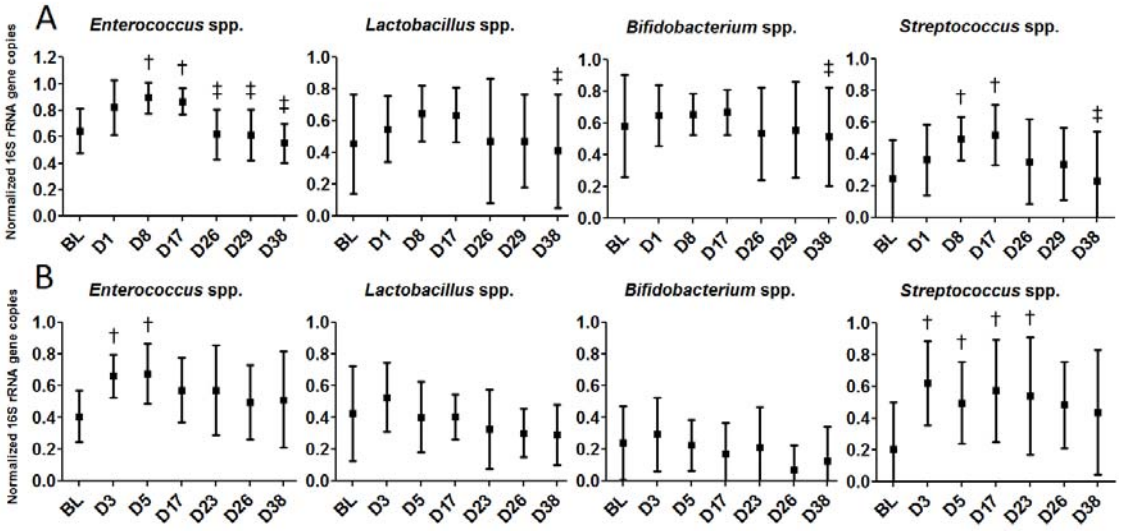

Fig. 1. Quantitative real-time PCR results for cats (A) and dogs (B) at baseline, during and after synbiotic administration. Baseline (BL), days (D) 1 or 3, 5 or 8, and 17 during synbiotic administration and days 23 or 26, 26 or 29, and 38 after synbiotic administration for all four probiotic genera: *Enterococcus, Lactobacillus, Bifidobacterium*, and *Streptococcus*. Error bars represent mean normalized $\log_{10}$ 16S rRNA gene copies and standard deviation. † = significantly higher than baseline; ‡ = significantly lower than day 17 ($P<0.05$).

followed by Actinobacteria (Fig. 2). Within these main phyla, Clostridiales, Lactobacillales and Erysipelotrichales (Firmicutes) and Coriobacteriales (Actinobacteria), were the most abundant orders (Fig. 3). After adjustment for falsely rejected null hypotheses, there were no significant differences in relative proportions of pyrosequencing tags belonging to the phyla Firmicutes, Actinobacteria, Bacteroidetes, Proteobacteria, and Fusobacteria across the three time points evaluated (Supplementary Table A1). Also, there was no statistically significant difference in any class, order, family, or genus within these phyla. Based on unadjusted *P* values, the genus *Collinsella*

(Actinobacteria) was found to be significantly higher at baseline (median: 2.7% of all sequences) when compared with both during (1.7%) and after synbiotic administration (1.2%) ($P<0.01$). Also, *Lactobacillus* (Firmicutes) was found to be higher during synbiotic administration (0.4%) than before or after administration (median: 0%) ($P<0.0390$); however, multiple comparisons did not reach statistical significance. Differences in proportions of pyrosequencing reads among and within cats before, during, and after synbiotic administration were visualized by plotting a heatmap at the family level (Fig. 4), using a gplots library (Warnes, 2010) in the R software (R, 2004). These heatmaps showed a high variability among cats and a comparably much lower degree of variation within cats (i.e., proportions for time points within a subject were usually clustered together).

454-Pyrosequencing in dogs. A total of 201 642 pyrosequencing reads were generated: 87 737 at baseline, 56 852 on day five of synbiotic administration, and 57 053 on day 23 (2 days after end of synbiotic administration). The most abundant phylum was Firmicutes, followed by Actinobacteria and Bacteroidetes (Fig. 2). Within these main phyla, Clostridiales, Erysipelotrichales, and Lactobacillales (Firmicutes), and Coriobacteriales (Actinobacteria) were the most abundant orders (Fig. 3). After adjustment for falsely rejected null hypotheses, there were no statistically significant differences in the relative proportions of pyrosequencing tags belonging to any phylogenetic level (Supplementary Table A2). Based on unadjusted *P* values, the proportion of organisms belonging to the family Eubacteriaceae (Firmicutes) was found

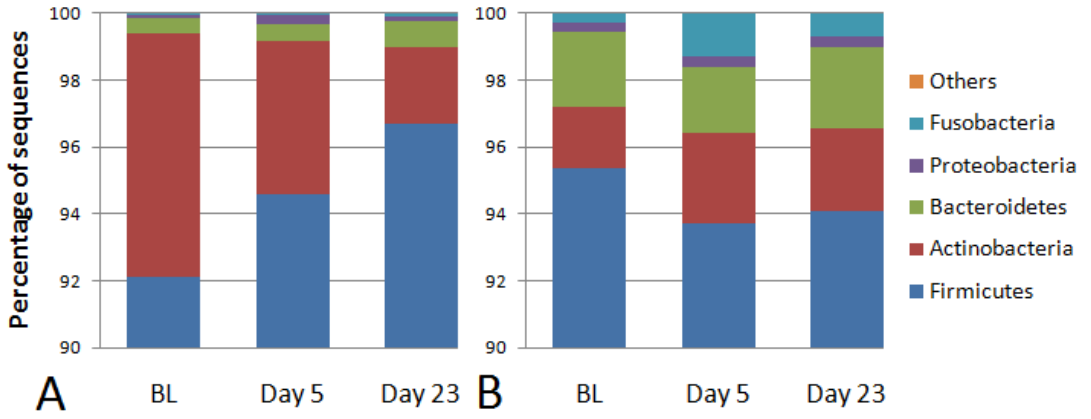

Fig. 2. Composition of the fecal bacterial microbiota for cats (A) and dogs (B) at baseline, during and after synbiotic administration at the bacterial phylum level. Baseline (BL), Day 5 during synbiotic administration, and Day 23 after synbiotic administration. The y axis (average percentage of sequences) was modified to show the low abundant phyla.

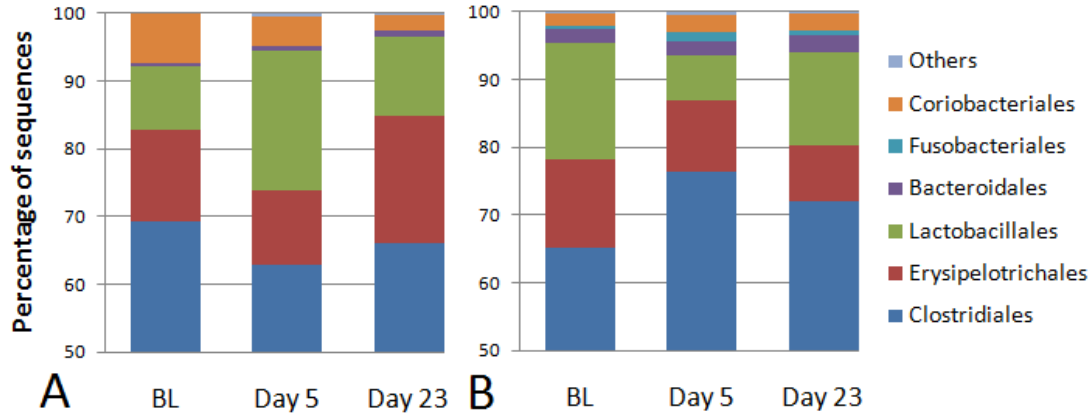

Fig. 3. Composition of the fecal bacterial microbiota for cats (A) and dogs (B) at baseline, during and after synbiotic administration at the bacterial order level. Baseline (BL), Day 5 during synbiotic administration, and Day 23 after synbiotic administration. The y axis (average percentage of sequences) was modified to show the low abundant orders.

to be significantly increased during synbiotic administration (median: 0.38% for all sequences) when compared with baseline values (0.02%; $P<0.05$). Likewise, the proportion of organisms belonging to the genus *Eubacterium* was found to be significantly higher during synbiotic administration (median: 0.38% of all sequences) when compared to baseline results (0.02%; $P<0.05$). Also, the proportion of organisms belonging to the genus *Roseburia* (Firmicutes) was found to be significantly lower two days after cessation of synbiotic administration (median: 3.3% of all sequences) when compared with the proportions at both baseline (5.1%) and on day 5 of synbiotic administration (4.5%; $P<0.05$). Also, based on unadjusted *P* values, relative proportions of sequencing tags belonging to the phylum Fusobacteria and all phylogenetic levels down to the genus *Fusobacterium* were found to be significantly higher during synbiotic administration (median: 0.77% of all sequences) when compared to baseline values (0.10%; $P<0.05$). Differences in proportions of pyrosequencing reads among and within dogs before, during, and after synbiotic administration were also visualized by plotting a heatmap at the family level (Fig. 4). Similarly to results observed in the cats, these heatmaps showed a high variability among individual dogs and a comparably lower variation within dogs (i.e., proportions for time points within a subject were usually clustered together).

2.4.4. Principal Component Analysis (PCA)

Principal component analysis, based on the Unifrac distance metric, did not show any clustering of the fecal microbiota at any time point during the study period for either cats or dogs (data not shown).

2.4.5 Diversity indices for pyrosequencing bacterial tags

At 1% dissimilarity, no significant differences were found between the Shannon-Weaver diversity indices across the evaluated time points in the dogs. Cats had a significantly higher Shannon-Weaver diversity index before administration of the synbiotic (mean ± SD: 4.9 ± 0.3) when compared to the indices at five days of feeding the synbiotic (4.3 ± 0.6, $P<0.05$) or at two days after the end of administration (4.3 ± 0.6, $P<0.01$), respectively.

2.4.6. Effect of the synbiotic on GI and immune function

With the exception of one cat and one dog that vomited once during synbiotic administration, all enrolled animals were reported by the owners to eat and behave normally during synbiotic administration. Similarly, fecal consistency was reported as normal by the owners with the exception of two cats and two dogs that were recorded to have pulpy feces for two days during the administration period. No flatulence was recorded in any cat during the 21 days of synbiotic administration. In contrast, owners reported that seven dogs had some flatulence (scores: 0 = no flatulence: 5 dogs; 1= some

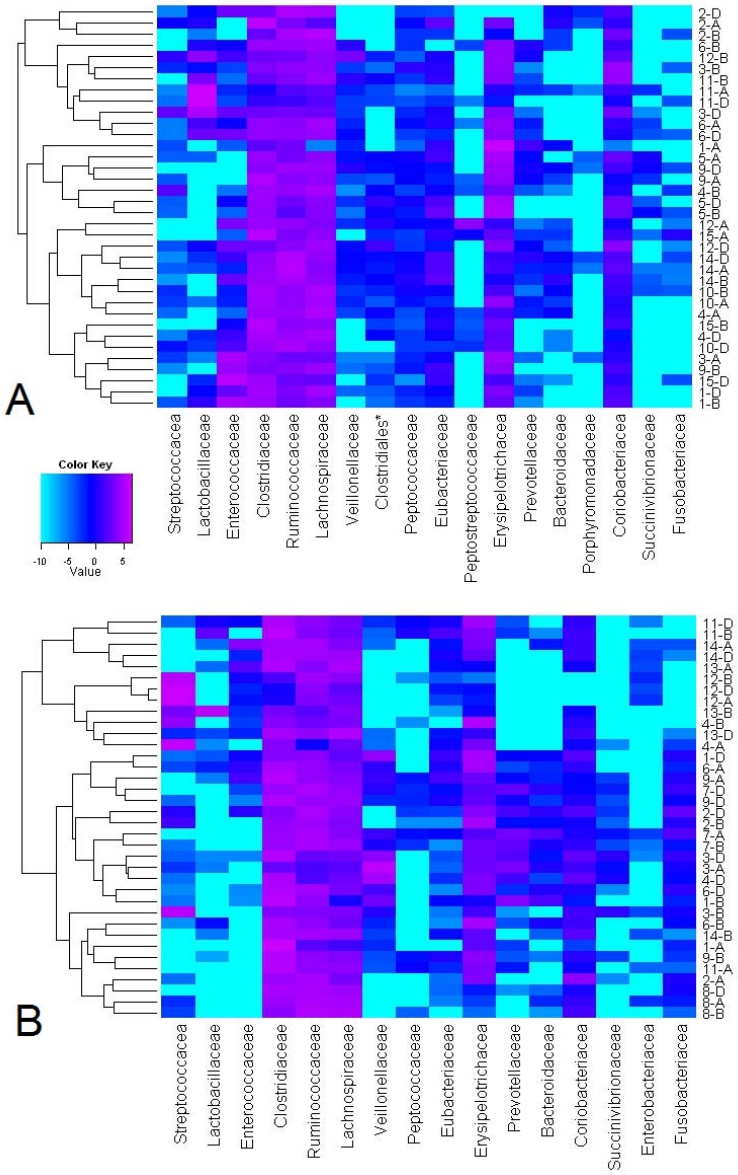

Fig. 4. Heatmap showing log2 transformed proportions of pyrosequencing tags for cats (A) and dogs (B) at baseline, during and after synbiotic administration at the bacterial family level. The ordering of the corresponding dendrogram is by the mean value of the rows. The identification for the subjects is shown on the right y-axis, where B, D, and A indicate before, during, and after synbiotic administration, respectively. Only bacterial families that were detected in a sample from at least one time point (before, during, or after synbiotic administration) in at least half of the animal subjects are shown. Clostridiales* in panel A indicates Clostridiales Family XIII incertae sedis.

flatulence: n = 7 dogs; 2 = frequent flatulence: n = 0 dogs) for at least one day during synbiotic administration. Other clinical signs of gastrointestinal health such as appetite, defecation frequency, and volume of feces in both cats and dogs were judged to be normal by the owners during treatment with the synbiotic (21 days). With the exception of lymphocytes in the cats and neutrophils in the dogs, which were decreased, although not significantly, during synbiotic administration, none of the evaluated serum (cobalamin, folate, IgA, TLI, and PLI) or fecal (IgA and α_1-PI) markers of gastrointestinal and immune function changed significantly after three weeks of administration or three weeks after discontinuation of the preparation (Table 4).

2.5 Discussion

Despite the numerous applications of prebiotics, probiotics, and synbiotics in veterinary medicine, to date little is known about the *in vivo* effects of these agents on the composition of the intestinal microbiota of cats and dogs. Several studies have used traditional culture techniques to evaluate the effects of probiotics and prebiotics on fecal microbial composition of dogs (Swanson *et al.*, 2002; Vahjen & Manner, 2003). Also, culture techniques have been used to assess the effect of fructooligosaccharides on duodenal bacterial populations of cats (Sparkes *et al.*, 1998). However, it is well acknowledged today that traditional culture techniques have limitations in fully characterizing complex microbial communities (Ritz, 2007) like those found in the mammalian gastrointestinal tract (Eckburg *et al.*, 2005). Molecular tools are now widely

Table 4 Mean concentrations (± standard deviation) of relevant blood, serum, and fecal markers obtained before (baseline), during (day 21), and after (day 42) synbiotic administration. *P* values are given for the comparison of means (ANOVA) or ranks (Friedman's test)

Parameters in cats	Control range	Baseline	Day 21	Day 42	*P*
Serum Cobalamin	290-1500 ng L^{-1}	1620.0±536.7	1680.0±532.8	1657.0±582.3	0.8845
Serum Folate	9.7-21.6 µg L^{-1}	20.8±7.8	19.6±6.0	19.8±6.2	0.5458
Serum fPLI	2.0-6.8 µg L^{-1}	5.8±2.1	5.8±2.8	7.4±2.6	0.1682
Serum fTLI	12.0-82.0 µg L^{-1}	29.7±19.3	33.4±13.4	27.7±21.5	0.4032
Serum IgA (mg/dl)	nn	94.8±55.9	97.0±62.9	106.0±91.2	0.7979
Total Lymphocytes	1500-7000 µL^{-1}	4111±1924	3097±1279	3918±1589	0.0519
Total Neutrophils	2500-12500 µL^{-1}	3644.0±1874.0	4289.0±1693.0	4739.0±3028.0	0.2192
Total Eosinophils	<1500 µL^{-1}	622.2±460.2	910.8±932.5	564.0±284.8	0.8302
RBC	5-10x10^6 µL^{-1}	8.8±1.3	8.5±0.9	8.7±1.2	0.4913
Fecal α_1-PI	0.04-1.6 µg g^{-1}	2.9±2.0	2.3±2.1	2.4±1.9	0.4355
Parameters in dogs					
Serum Cobalamin	251-908 ng L^{-1}	562.5±199.8	595.4±186.4	597.7±170.4	0.3919
Serum Folate	7.7-24.4 µg L^{-1}	11.5±2.3	12.4±2.6	12.3±3.1	0.3343
Serum cPLI (Spec cPL®)	0-200 µg L^{-1}	48.7±33.7	58.3±64.6	60.1±58.	0.8302
Serum cTLI	5.7-45.2 µg L^{-1}	10.2±3.2	11.7±2.4	9.9±3.9	0.3209
Serum IgA(mg/dl)	nn	53.6±52.0	65.2±62.8	63.1±59.2	0.9382
Fecal IgA	0.22-3.24 mg g^{-1}	1.2±0.7	1.1±1.0	1.1±0.9	0.9583
Total Lymphocytes	1500-7000 µL^{-1}	2172.0±1255.0	2347.0±1135.0	2643.0±1686	0.2979
Total Neutrophils	2500-12500 µL^{-1}	5874.0±2562.0	4306.0±2520.0	5284.0±2342.0	0.0621
Total Eosinophils	<1500 µL^{-1}	538.5±479.3	423.2±368.7	452.7±259.0	0.3508
RBC	5-10x10^6 µL^{-1}	6.8±0.7	6.9±0.8	6.9±0.8	0.3755
Fecal α_1-PI	2.2-18.7 µg g^{-1}	7.6±6.7	7.7±4.2	8.3±6.2	0.8438

available to identify intestinal microbial phylotypes (Furrie, 2006). Molecular methods, such as fluorescent *in situ* hybridization, have been used to evaluate the effect of the probiotic *Lactobacillus acidophilus* DSM13241 on fecal bacterial populations in cats (Marshall-Jones *et al.*, 2006) and dogs (Baillon *et al.*, 2004). Also, one study has recently evaluated the effect of the probiotic *Enterococcus faecium* SF68 on fecal microbial diversity of cats with feline herpesvirus infection using DGGE (Lappin *et al.*, 2009). However, the application of traditional molecular tools to fully characterize the

composition of the GI microbiota can also be technically and economically challenging. Recently developed high throughput technologies allow a more in depth phylogenetic coverage of the intestinal microbiota (Zoetendal *et al.*, 2008; Suchodolski *et al.*, 2009) and thus could be useful in evaluating the overall effect of prebiotic, probiotic, or synbiotic formulations on intestinal microbial communities.

This study used various complementary molecular tools to evaluate the effect of a synbiotic formulation on fecal bacterial composition of healthy cats and dogs. Our results indicate that while the administration of the multi-species synbiotic preparation for 21 days induced several changes in the abundance of specific probiotic groups in both cats and dogs, the fecal microbiota was not altered on higher phylogenetic levels as evidenced by 454-pyrosequencing. We observed the appearance of DGGE bands in 11/12 cats and 5/11 dogs that were not present before or after synbiotic administration. Sequence analysis identified these bands as *Enterococcus faecium*, suggesting that these organisms were more abundant in feces during the ingestion of the synbiotic. Similarly to these results obtained with DGGE, the analysis of 16S rRNA gene clone libraries revealed that bacterial species contained in the administered synbiotic, such as *Bifidobacterium longum* and various *Lactobacillus* spp. matching those contained in the synbiotic formulation, were present in the feces of most cats (8/11) and dogs (9/11) during the administration of the synbiotic, but were undetectable at baseline or after administration was discontinued. This observation further suggests an increase in fecal abundance of the ingested probiotic organisms during the administration period. However, the phylogenetic coverage of 16S rRNA gene clone libraries is generally

limited to the species level (Suchodolski *et al.*, 2008). Therefore, we cannot confirm if the appearance of the bacteria in feces were truly the ingested probiotic strains contained in the synbiotic, or if they belonged to the same bacterial species but were in fact different strains. Nonetheless, the fact that these particular species increased during the administration period, but were not detectable before and after the administration period in most of the animals, strongly suggests that the administration of the product led to an increase of these probiotic groups in the feces of the enrolled cats and dogs. Of further interest is that the detection of the probiotic species by the 16S rRNA gene clone libraries was not equal among individual cats and dogs. For instance, in some animals only one probiotic species was detected during the administration period, while in other animals several probiotic species could be identified during administration of the synbiotic. These observations suggest a highly individualized response of the host to administered probiotic species.

The fact that the probiotic groups were undetectable at baseline suggests that the bacterial species contained in the synbiotic were not present in the gastrointestinal tract of the enrolled cats and dogs. The probiotic strains in the synbiotic preparation have in fact been derived mostly from human and dairy sources. While it has been suggested that canine-derived bacterial species exhibit host specificity (McCoy & Gilliland, 2007), to date there are no studies confirming this assumption. In fact, probiotics may not need to be native to colonize the intestinal tract of the recipient host, because the adherence of these agents to intestinal mucus has been shown not to be host-specific (Rinkinen *et al.*, 2003). Furthermore, studies have shown that canine-derived probiotic strains can

successfully transit the murine gastrointestinal tract (O'Mahony *et al.*, 2009) and can adhere to both human and canine intestinal mucus in a similar fashion (Strompfova *et al.*, 2004). To our knowledge there are only a few probiotic strains that have been derived from dogs (Strompfova *et al.*, 2004; Biagi *et al.*, 2007; McCoy & Gilliland, 2007, O'Mahony *et al.*, 2009; Martin *et al.*, 2010), and no feline-specific probiotic strains have been described in the literature to date.

Similarly to the findings using DGGE and 16S rRNA gene clone libraries, quantitative real-time PCR analyses showed increases in fecal abundance of probiotic bacterial groups within a few days after the subjects started consuming the synbiotic preparation (Fig. 1), with abundances returning to baseline levels after discontinuation of synbiotic administration. Several studies in humans have shown that qPCR can be useful in detecting quantitative increases of ingested probiotics in feces (Bartosch *et al.*, 2005; Vitali *et al.*, 2010), although an inter-individual host response to the dietary intervention is frequently noticed. This variation among subjects was also noticeable in our study, as evidenced by the wide variability in fecal abundances of the target groups within each time point (Fig. 1). Interestingly, in the current study the observed quantitative increases also varied among the four bacterial genera contained in the synbiotic preparation. The fecal abundance of *Lactobacillus* and *Bifidobacterium* spp. was not significantly increased during synbiotic administration, while abundances of *Enterococcus* and *Streptococcus* spp. were significantly increased during at least one time point during synbiotic administration in both cats and dogs. This observation is likely due to the unequal proportions of each probiotic strain contained in the synbiotic preparation

evaluated. It is common for commercially available multi-species probiotic formulations to contain unequal quantities of microorganisms. For instance, a multi-species probiotic for use in humans (VSL#3, VSL Pharmaceuticals) is known to contain different concentrations of at least one probiotic strain (Pagnini *et al.*, 2010).

In spite of the observed increases of the ingested probiotic groups in feces using DGGE, 16S rRNA gene clone libraries, and qPCR, pyrosequencing of the 16S rRNA gene did not reveal major changes in the proportions of the most abundant fecal bacterial phyla such as Firmicutes, Actinobacteria, Bacteroidetes, Proteobacteria, and Fusobacteria (Supplementary Tables A1 and A2). This observation is in agreement with other studies showing that ingestion of probiotics leads to fecal colonization by the ingested groups but does not alter the major bacterial groups in the intestine (Venturi *et al.*, 1999). This may be due in part to the low abundance of the targeted probiotic groups (i.e., *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, and *Streptococcus* spp.), as representatives of these groups accounted for less than 1% of all identified sequences in both cats and dogs at baseline (Supplementary Tables A1 and A2). Also, the effect of probiotic/prebiotic formulations on the intestinal tract may be more evident at the functional level (i.e., production of lactic acid and/or short chain fatty acids) rather than or in addition to changes in the abundance of intestinal microbial groups. For example, it has been shown that as low as 0.2 or 0.4% of inulin or short-chain FOS can be effective at modifying fecal concentrations of short-chain fatty acids in dogs, while minimally altering the abundance of fecal bacterial populations (Barry *et al.*, 2009). Also, in humans, the ingestion of two probiotic strains (i.e., *Lactobacillus helveticus* Bar13 and

Bifidobacterium longum Bar33) led to significant increases of acetic and valeric acids but did not modify the overall structure of the fecal microbiota, as assessed by DGGE (Vitali *et al.*, 2010). More studies are needed to investigate the effect of synbiotics on the functional (metabolic) activities of the GI microbiota as a supplement to phylogenetic analysis.

For pyrosequencing analysis, we selected day 5 and day 23 because, based on DGGE and qPCR results, we expected eventual changes in the microbiota to occur quite rapidly (within 2-3 days) after beginning the administration of the synbiotic and after discontinuation of treatment. However, it is possible that alterations in the microbiota in response to synbiotics require several days, and by selecting days that were close to the transition periods we may have missed some changes that may have occurred past the analyzed time points. Ideally multiple days should be analyzed by sequencing to detect temporal changes in the microbiota. As sequencing costs continue to decrease, such multiple samplings could be performed in future studies.

In this study we also showed that 21 days of oral administration of a synbiotic preparation did not lead to adverse gastrointestinal effects and may not interfere with markers of gastrointestinal (e.g., serum cobalamin and folate concentrations) or immune (e.g., fecal and serum IgA concentration) function. Similarly, one study showed that the administration of the probiotic *Enterococcus faecium* SF68 for six weeks did not change fecal IgA concentrations in adult dogs with chronic giardiasis (Simpson *et al.*, 2009). In contrast, an increase in fecal IgA in young dogs has been described after oral administration of the same probiotic strain (i.e., *E. faecium* SF68), although this effect

was only evident after 30 weeks of probiotic administration (Benyacoub *et al.*, 2003). Possible explanations for the discrepancy between this study and our investigation may include a shorter period of probiotic administration (3 versus 30 weeks) and also a more age-heterogeneous group of individuals, as age-related differences in immunological parameters have been observed in both cats (Campbell *et al.*, 2004) and dogs (Blount *et al.*, 2005).

Limitations of this study include the fact that each individual animal was fed a different diet, lived in a different environment, and/or received the formulation in a different manner, all factors that could have potentially influenced our ability to detect major alterations in the fecal microbiota due to the administration of probiotics. Moreover, all animals were fed the same dose of probiotic bacteria ($5x10^9$ cfu) with no regards to differences in body weight (for example the body weight among the dogs differed up to 8-fold). While there is growing evidence suggesting a dose-dependent clinical effect of probiotics (Pagnini *et al.*, 2010), to our knowledge no information about the efficacy of various doses of synbiotic have been published for dogs and cats. While preliminary data in our laboratory suggest that the body weight of dogs may not be a significant predictor to forecast an increased abundance of ingested probiotics in the feces (unpublished observations), clearly more studies are needed to evaluate if there is a dose-dependent effect of synbiotics on the microbiome of dogs and cats. Also, the separate contribution of each component in the synbiotic preparation (i.e., prebiotics and probiotic bacteria) to the changes observed in the fecal bacterial populations was not assessed independently. This is important because it has been shown in both humans

(Worthley *et al.*, 2009) and dogs (Swanson *et al.*, 2002) that the probiotic and the prebiotic component of a synbiotic formulation, when administered separately, may have different effects on fecal bacterial populations. Finally, molecular methods generally cannot confirm the viability of bacteria, and therefore it is possible that the isolated fecal bacterial DNA belonged to both viable and non-viable microorganisms (Palka-Santini *et al.*, 2003).

In summary, this study shows that oral administration with the multi-species synbiotic Proviable®-DC leads to increased concentrations of probiotic bacteria in the feces of healthy cats and dogs. Moreover, the results add to the increasing body of literature showing that probiotics and prebiotics may not lead to significant changes in the abundance of major intestinal bacterial groups of healthy animals. Further studies are warranted to assess the effects of the investigated synbiotic formulation on the intestinal microbiota of animals with gastrointestinal disease.

3. *IN VIVO* PREDICTORS OF INTESTINAL COLONIZATION BY INGESTED PROBIOTIC BACTERIA IN HEALTHY CATS AND DOGS

3.1 Overview

A better understanding of host-microbe interactions has the potential to improve the use of beneficial probiotic microorganisms to treat gastrointestinal disorders. This study analyzed the effect of age, bodyweight, and baseline fecal concentrations of related probiotic bacterial genera, as potential in vivo predictors of intestinal colonization by ingested probiotic microorganisms. Privately-owned healthy cats (*n*=12) and dogs (*n*=12) of different ages and breeds were enrolled. A multi-species synbiotic formulation (containing $5x10^9$ cfu of seven probiotic bacteria, and a blend of arabinogalactans and fructooligosacharides) was administered daily for 21 days. Fecal samples were obtained before synbiotic administration (baseline samples), and every 3 to 4 days during and up to three weeks after synbiotic administration.

As assessed by quantitative real-time PCR targeting the 16S rRNA gene, cats having a lower abundance of fecal probiotic genera at baseline had a higher fecal abundance of probiotic genera during the administration period when compared to the period after administration of probiotics. In contrast, cats having a higher fecal abundance of probiotic genera at baseline showed a similar abundance of probiotic genera during and after the administration period. Older cats may also have a higher

abundance of probiotic genera during the administration period when compared to the period after administration. Body weight was not a significant predictor in the cats. Age, body weight, and baseline bacteria were not significant predictors in the dogs. More research is needed to confirm these results using other probiotic formulations and to identify further predictors to forecast colonization outcomes by ingested probiotics.

3.2 Introduction

Probiotics are live microorganisms, which when administered in adequate amounts may confer a health benefit on the host (FAO, 2002) by enhancing immune responses (Pagnini *et al.*, 2010), displacing intestinal pathogens (Lee *et al.*, 2003), and/or producing antimicrobial substances (Jones & Versalovic, 2009). Because of these properties, the use of probiotics is gaining popularity for both the prevention and treatment of a variety of diseases in humans (Gareau *et al.*, 2010) and also in veterinary species (Callaway *et al.*, 2008). The success of probiotics in promoting health is thought to depend on an increased abundance of these agents in the intestinal tract after administration (Kailasapathy & Chin, 2000), a hypothesis that has led to the investigation of the survival and intestinal colonization properties of probiotic microorganisms (Bezkorovainy, 2001; Valeur *et al.*, 2004).

The intestinal microbiota of cats and dogs has been studied in some detail (Suchodolski, 2010; Handl *et al.*, 2011). As in other animal species, probiotics are also increasingly being used in cats and dogs in an effort to increase the abundance of beneficial microorganisms in the intestinal tract. Although several investigations have

evaluated the in vivo effect of probiotics on the intestinal microbiota of dogs (Swanson *et al.*, 2002; Manninen *et al.*, 2006; Biagi *et al.*, 2007; O'Mahony *et al.*, 2009) and cats (Marshall-Jones *et al.*, 2006; Veir *et al.*, 2007; Lappin *et al.*, 2009), the possibility of predicting colonization outcomes after ingestion of probiotics has not been explored in these animal species. This is important because an improved understanding of host-microbe interactions has the potential to improve the therapeutic use of probiotics to treat disorders of the GI tract, such as inflammatory bowel disease (Shanahan, 2004). Here we investigated the effect of three potential in vivo predictors of intestinal colonization by ingested probiotic bacteria in healthy cats and dogs. Additionally, and because this is the first attempt to identify in vivo predictors of intestinal colonization by probiotics in cats and dogs, we built upon the obtained results and investigated in dogs quantitative fluctuations of fecal bacteria shed in feces over time, and the relationship between the body weight and the amount of feces excreted, as plausible hypotheses that may partly explain our findings.

3.3 Materials and methods

3.3.1 Probiotic study protocol

The study protocol was reviewed and approved by the Clinical Research Review Committee of Texas A&M University (CRRC#07-38). Written consent was obtained from the owners of all enrolled animals.

Privately-owned clinically healthy pet cats (n=12) and dogs (n=12) of different breeds were prospectively enrolled. On average, cats were 2.8 years old (median: 2.0,

range: 0.6-7.6 years old) and weighted 4.9 kg (median: 5.0, range: 3.8-5.9 kg); dogs were 3.8 years old (median: 3.4, range: 0.6-10.5 years old) and weighted 21.1 kg (median: 26.1, range: 4.6-36.9 kg). Naturally passed fecal samples were obtained by the owners (mainly students of Veterinary Medicine at Texas A&M University) before starting the study (baseline samples). Then dogs and cats received one capsule of Proviable®-DC (Nutramax Laboratories, Inc.) orally at home once daily for 21 days. Proviable®-DC is a synbiotic formulation for use in cats and dogs containing a blend of fructooligosaccharides, arabinogalactans, and a minimum of $5x10^9$ cfu probiotic bacteria (Table 1). The proportions of each probiotic strain in Proviable®-DC are proprietary. In an effort to mimic a real-life situation where owners administer the probiotic preparation at home, owners were allowed to administer the capsule directly in the mouth, mix the capsule into the food, or open the capsule and mix the synbiotic preparation into the food, depending on the particular manner their pets generally accept oral medications. Additional fecal samples were collected every 3-4 days during and after probiotic administration until day 42 (three weeks after cessation of the administration of the probiotic formulation).

3.3.2 DNA extraction and quantitative real-time PCR analysis

Fecal samples (~1 gram) were collected and stored at -20°C until analysis. DNA was extracted from a 100 mg aliquot of each fecal sample using a bead beating phenol-chloroform-based method described in detail elsewhere (Suchodolski *et al.*, 2008). Fecal abundance of the four bacterial genera contained in the probiotic preparation (i.e.,

Enterococcus, *Streptococcus*, *Lactobacillus*, and *Bifidobacterium* spp.) were estimated using SYBR-based (Biorad Laboratories) quantitative real-time PCR (qPCR) using a commercial real-time PCR thermocycler (iCycler iQ, Biorad Laboratories) and previously published oligonucleotide primers (Table 3). Standard curves using five 1:10 dilutions of DNA (ranging from 2.0 ng to 0.2 pg) from lyophilized bacterial species of each genus and canine fecal DNA for universal bacteria were used to calculate the unknown bacterial genomic targets. All samples and standards were run in duplicate fashion. Care was taken to minimize inter-assay variability by amplifying all samples from a given animal subject (baseline sample, samples collected during the administration period, and samples collected after probiotic administration) in the same 96 well PCR plate. The PCR mixtures (25 µl) contained 12.5 µl of iQ™ SYBR® Green Supermix (100 mM KCl, 40 mM TRis-HCl pH 8.4, 0.4 mM of each dNTP, 50 units/ml of iTaq DNA polymerase, 6mM MgCl2, SYBR Green I, and 20 nM fluorescein, Biorad Laboratories), 9.7 µl of sterile water, 0.4 µl of each primer (final concentration: 160 nM) and 2 µl of DNA (~ 5 ng/µl). After the PCR was finished, a melt-curve profile was generated to analyze the specificity of the primers. Expected length of the amplicons was verified using agarose gel electrophoresis.

3.3.3 Statistical analysis

Statistical analysis was performed using SAS® 9.2 with Enterprise Guide® 4.2. For all analyses, we used normalized $\log_{10}$ 16S rRNA gene copies ($\log_{10}$ 16S rRNA gene copies from each particular bacterial genus divided by the $\log_{10}$ 16S rRNA gene copies from all

bacteria) as our dependent variable to account for variations in total bacterial DNA obtained during the extraction procedure (Frank *et al.*, 2007). In order to perform the analysis, raw data was organized separately for dogs and cats, and a new variable "bacterial group" was created for each animal species to indicate which bacterial genus each observation related to. This approach resulted in the creation of a total of 288 observations for cats and 278 observations for dogs (qPCR results for *Streptococcus* spp. for one canine subject (all time points) and one time point from three other dogs were not available because of lack of DNA samples and were treated as missing values). The variable "time" was treated as a fixed variable with six levels (days 1, 8, and 17 during, and days 26, 29, and 38 after probiotic administration in cats, and days 3, 5, and 17 during, and days 23, 26, and 38 after probiotic administration in dogs). Statistical models were built separately for cats and dogs. An example of a full statistical model as well as the corresponding SAS code is available upon request.

Additionally, we also investigated the effect of the covariates age and body weight when baseline bacteria is included as the first time point (day 0) instead of as a covariate, resulting in time having seven levels. Based on the nature of the data (time points being unequally spaced but same across subjects), three covariance structures were attempted: Compound Symmetry (CS), Heterogeneous Compound Symmetry (CSH), Spatial Exponential (SPexp), as well as using no repeated statement in the MIXED procedure. The final covariance structure was chosen based on the Bayesian Information Criterion (lower is better). Model diagnostics showed that the studentized residuals seemed to be random and had a distribution very close to normal for both cats

and dogs, thus indicating valid models. Backward variable selection was performed as the next step by removing non significant terms ($P>0.05$) one by one. All post-test comparisons were adjusted by the Tukey-Kramer method. Restricted/residual maximum likelihood was used for all final analyses. A P value of less than 0.05 was considered statistically significant.

3.3.4 Quantitative fluctuations of fecal bacteria over time

To investigate quantitative fluctuations of fecal bacteria shed in feces over time among dogs of different ages, we collected one naturally passed fecal sample from five consecutive days (one stool per day) from a total of 22 clinically healthy dogs of different breeds: 10 dogs younger than five years old (median: 1.7 years, range: 3 months to 3.8 years), and 12 dogs older than five years old (median: 8.3 years, range: 5.9-12 years). DNA extraction and qPCR were performed as described above. We analyzed the fecal abundance of the genus *Enterococcus* because in our experience this bacterial group can be found in detectable concentrations by qPCR in the feces of most dogs.

3.3.5 Relationship between the body weight and the amount of feces excreted

To investigate the relationship between the amount of feces excreted and the bodyweight, we collected the total amount of one naturally passed fecal sample per day from two to three consecutive days (one stool per day) from 15 clinically healthy dogs of different breeds with a median bodyweight of 19.5 kg (range: 2.1-33.6 kg). Fecal

samples were weighed and these weights were used to calculate the mean amount of feces (grams of wet weight) excreted per day.

3.4 Results

3.4.1 Results for cats

Using baseline bacterial abundance of related probiotic genera as a predictor, at least one time point and one bacterial group were different from the others ($P<0.0001$ and $P=0.0099$, respectively), and there was a significant interaction between baseline and time ($P<0.0001$), indicating that the differences in fecal abundance of probiotic genera among time points differed upon different baseline values. Cats that had a lower baseline abundance of related probiotic genera before starting to consume the probiotics had a higher abundance of the fecal probiotic genera during probiotic administration when compared with the post-administration period (Fig. 5). In contrast, cats that had a higher baseline abundance of these bacteria also had a similar abundance of probiotic genera both during and after the administration period (Fig. 5). Moreover, there was a significant interaction between baseline and bacterial group ($P=0.0272$), indicating that the baseline effect was different in at least one of the bacterial genera contained in the probiotic formulation (Fig. 6). Age and bodyweight were not significant predictors.

Using baseline bacterial abundance of related probiotic genera as a response (day 0), at least one time point and one bacterial group were different from the others ($P=0.0011$ and $P<0.0001$, respectively), as before. Also, there was an interaction between age and time ($P=0.0291$), indicating that the differences in fecal abundance of

probiotic genera differed based on the age of the cats. Older cats had a higher fecal abundance of probiotic genera during the consumption of probiotics when compared with the period after administration (Fig. 7). However, after removing the oldest cat from the statistical analysis, the age was no longer a significant predictor. As before, bodyweight was not a significant predictor.

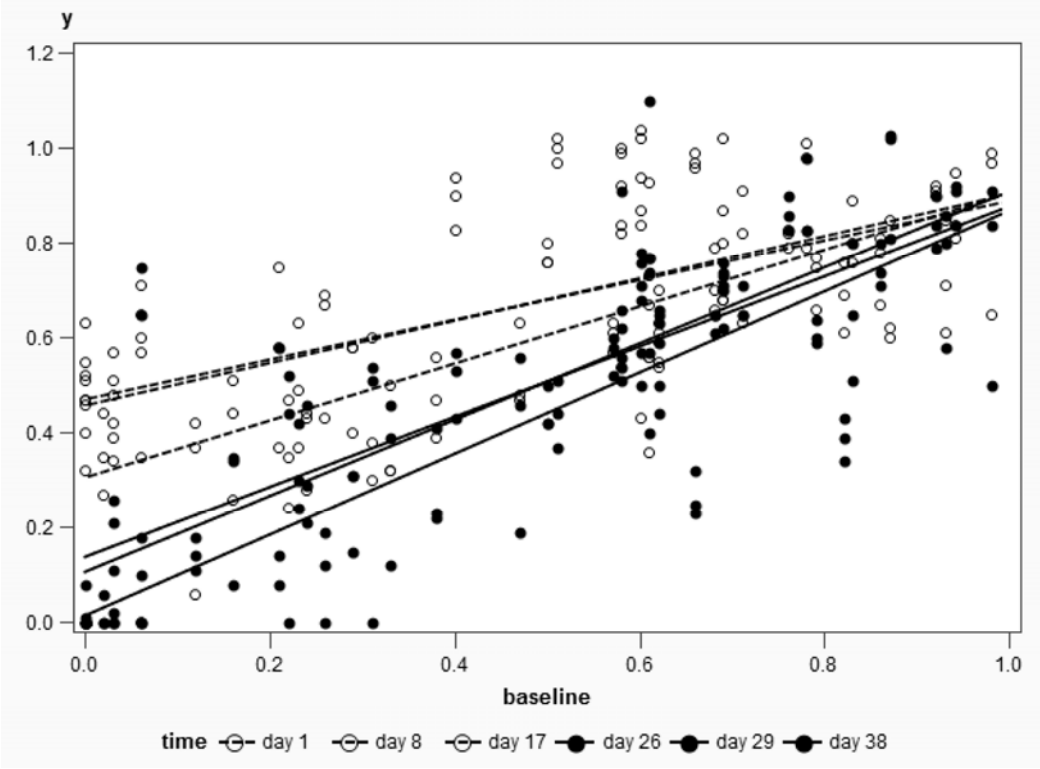

Fig. 5. Effect of baseline fecal abundance of probiotic bacterial genera on the abundance of these bacteria during and after synbiotic administration in cats. Days 1, 8, and 17 during synbiotic administration, Days 26, 29, and 38 after synbiotic administration. The dots in the graph represent the abundance of all probiotic genera in feces, as determined by qPCR. Cats that had a lower fecal baseline abundance of the probiotic genera (x axis) had a higher abundance of the probiotic genera during synbiotic administration (days 1, 8 and 17) when compared to the period after administration (days 26, 29, and 38).

3.4.2 Results for dogs

Using baseline bacterial abundance of related probiotic genera as a predictor, at least one time and one bacterial group were different than the others (P=0.0003 and P<0.0001, respectively). Differences of least squares means revealed that the fecal abundance of probiotic species on day 3 were higher than on day 26 (P=0.0004) and day 38 (P=0.0008). Although fecal colonization with the probiotic preparation was also affected by the bacterial group administered (P<0.0001), multiple comparisons among the different bacterial groups were not performed because the results from different qPCR assays are not directly comparable to each other. The estimate for the common slope for the effect of baseline bacteria (0.21 ± 0.07) was significantly different from zero (P=0.0023), suggesting a positive relationship between the fecal bacterial abundance of related probiotic genera at baseline and at subsequent time points during and after probiotic administration. Age and bodyweight were not significant predictors.

Using baseline bacterial abundance of related probiotic genera as a response (day 0), at least one time point and one bacterial group were different than the others (P<0.0001 and P<0.0001, respectively), as before. Differences of least squares means revealed that probiotic genera on day 3 during probiotic administration were higher than those at baseline (P=0.0005), on day 26 (P=0.0002), or on day 38 (P=0.0004). As before, age and bodyweight were not significant predictors.

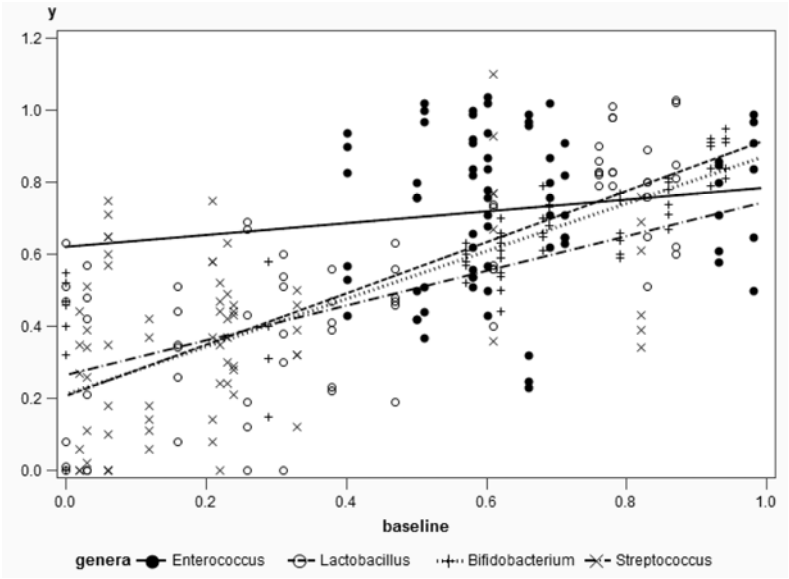

Fig. 6. Effect of baseline fecal abundance of probiotic bacterial genera on the abundance of these bacteria at all subsequent time points in cats. With the exception of the genus *Enterococcus*, there was a positive linear relationship between the abundance of probiotic genera at baseline (x axis) and the abundance of these bacteria at all subsequent time points.

3.4.3 Quantitative fluctuations of fecal bacteria over time

Using two-tailed unpaired Student's *t* test for groups with unequal variances, there was no significant difference in the fecal abundance of *Enterococcus* between the two age groups (younger and older than five, P=0.6966). Also, there was no difference in the percentage coefficient of variation (P=0.1783) and in the population variability (P=0.2313) across the 5 sampled days between the two age groups. Unexpectedly, however, there was a marginally significant difference in the standard deviations across

the five sampled days between the younger (mean standard deviation: 0.24 ± 0.14) and the older (mean standard deviation: 0.15 ± 0.07) group of dogs (*P*=0.0618).

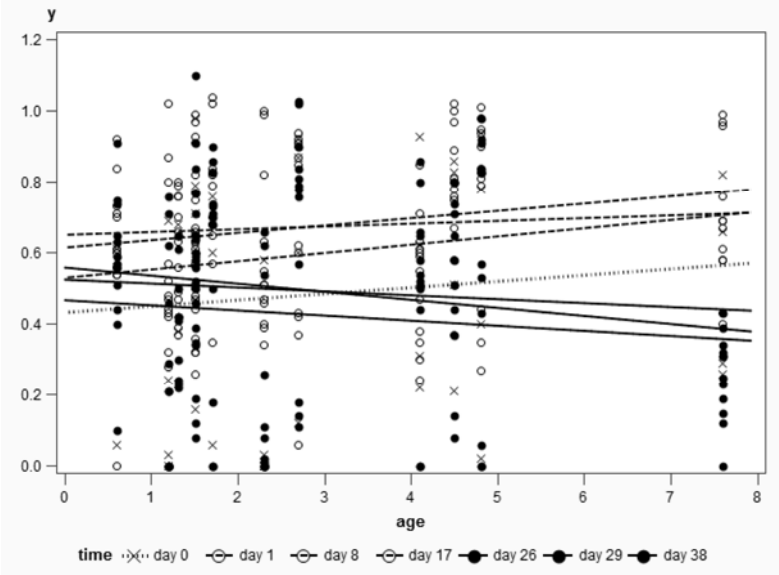

Fig. 7. Effect of age on fecal abundance of probiotic bacterial genera at baseline, during and after synbiotic administration in cats. Baseline (day 0), during synbiotic administration (days 1, 8, and 17) and after administration (days 26, 29, and 38). Although the divergence of the regression lines suggests that age can be a significant factor to explain difference in fecal abundance of the probiotic genera during and after synbiotic administration, this effect was mainly related with the oldest cat.

3.4.4 The relationship between the bodyweight and the amount of feces excreted

The total amount of a total of 35 fecal samples from 15 dogs was weighed and these weights were used to calculate the mean amount of feces (grams of wet weight) excreted per day. This mean (in grams) was plotted against the bodyweight in kilograms (Fig. 8).

There was a significant positive linear relationship between bodyweight and the amount of feces excreted (P=0.0001). The 90% predictive interval of this relationship was ~ 70 grams of feces.

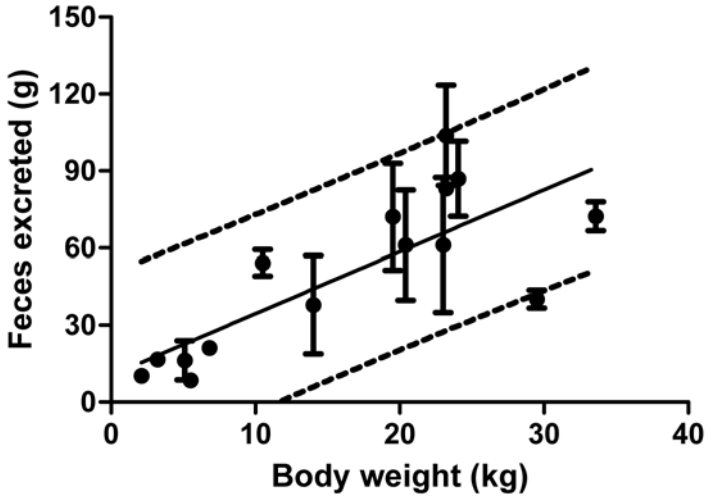

Fig. 8. Linear relationship between the body weight (in kilograms) of dogs and the amount of feces excreted (in grams, wet weight). Error bars represent mean with standard deviation. Dashed lines represent the 90% confidence interval of the linear slope.

3.5 Discussion

Increasing evidence suggests that ageing may be associated with changes in the composition of the intestinal microbiota (Woodmansey, 2007), but the role of age on colonization outcomes by ingested probiotics has rarely been explored. A recent study

showed, using microbial microcosms, that when the communities in an ecosystem are highly uneven, or there is dominance by one or a few species, the function of the ecosystem as a whole is less resistant to environmental stress (Wittebolle *et al.*, 2009). This observation led us to speculate that the opposite may also be true: that a stable ecosystem (the intestinal microbiota) will contain steady bacterial numbers over time, and may therefore be less likely to change (e.g. less prone to displacement and/or competition by probiotic bacteria). Hence, we hypothesized that dogs of different age would excrete similar amounts of fecal bacterial organisms over time (i.e. would have a stable intestinal microbial ecosystem over time regardless of the age) because if true, this may help explain the observed non-significant effect of age as a predictor of colonization by the ingested probiotics in the dogs. In contrast to our original hypothesis, however, the findings suggest that fecal shedding of *Enterococcus* over a period of five days may be more variable in dogs younger than five years old when compared with dogs older than five. However, this observation may or may not apply to other members of the fecal microbiota. Also, it has been shown in dogs that age-related changes in the relative proportions of intestinal bacterial groups coincide with changes in diet and physiological processes (Buddington, 2003) and dogs of different age are known to harbor different microbial populations, especially in the large intestine (Benno *et al.*, 1992). More research is needed to investigate the effect of age on intestinal colonization outcomes by ingested probiotics.

The finding that the bodyweight of the dogs was not a significant predictor of colonization by the ingested probiotics was interesting because one would expect that

heavier (bigger) dogs would defecate proportionally a higher amount of feces than lighter (smaller) dogs. More importantly, most investigations have shown that while the consumption of probiotics leads to increased concentrations in the feces, these increases generally disappear within days after ending its administration (Tannock et al., 2000; Baillon et al. 2004; Elli et al., 2006), suggesting that the presence of probiotics in feces is merely due to the excretion of the ingested microorganisms. Thus, we hypothesized that if bodyweight correlates with the amount of feces excreted, an ingested probiotic mixture could also be more or less diluted in feces depending on the bodyweight of the animal, and this parameter could be used to predict fecal colonization by ingested probiotics. The findings showed that indeed there is a positive relationship between the bodyweight and the amount of feces excreted, but the 90% predictive interval was found to be considerably large (about 70 grams of feces). This was partly due to the wide day-to-day variation in the amount of feces excreted in six out of the 15 dogs. Interestingly, the owners of five of these six dogs revealed that their pets eat a fixed amount of food per meal and also a fixed amount of meals per day. Hence, these results suggest that dogs excrete variable amounts of feces every time, depending on their unique metabolism. Therefore, we propose that the same dose of ingested probiotics (e.g. $5x10^9$ cfu) may not get diluted in feces proportionally to the bodyweight of the dog, and this may partly explain the observed non-significant effect of bodyweight as predictor of fecal colonization in the probiotic study. In this study, however, we did not investigate whether the relationship between bodyweight and feces excreted can be extrapolated to the actual numbers of fecal bacteria. Also, bodyweight may not be an accurate parameter

to estimate the size of an animal. Other parameters to estimate the size of an animal (e.g. height at the cross and/or length of body) would be interesting to investigate as potential predictors of fecal colonization by ingested probiotics.

The effect of native intestinal microbial populations on colonization outcomes by ingested probiotics is an interesting phenomenon rarely discussed in the literature. Stecher *et al.* (2010) reported that mice with a high abundance of fecal *Lactobacillus* spp. were more efficiently colonized by a commensal *Lactobacillus reuteri* strain after oral inoculation. However, this conclusion was based only on a linear relationship between fecal *Lactobacillus* at baseline (i.e. before oral inoculation) and fecal *L. reuteri* after oral inoculation. A similar linear relationship was also found in the current study between the fecal concentrations of probiotic genera at baseline and during all subsequent time points during and after probiotic administration (Fig. 6). However, in this study this observation only reflects that cats that had a lower fecal bacterial abundance at baseline also maintained a lower fecal bacterial abundance during and after probiotic administration, and cats that had a higher fecal bacterial abundance at baseline also maintained a higher bacterial abundance during and after administration of the probiotic (Fig. 6). Here, we investigated beyond this observation and showed that fecal abundance of the ingested probiotic genera was higher in cats that had a lower fecal baseline abundance of related bacteria (Fig. 5). Others have also suggested that intestinal colonization by probiotic bacteria may be higher in subjects having lower concentrations of these bacteria before ingestion of probiotics (Vitali et al., 2010). Indeed, more

research is needed to study the effect of native intestinal microbial populations on colonization outcomes by ingested probiotics.

Finally, in this study the animals were fed different diets and received the probiotic preparation in a different manner, variables that could have influenced our ability to identify a significant effect of the investigated predictors. However, the study design was intended to mimic a real-life scenario where owners administer probiotics at home and therefore our results may still hold relevant to the veterinary field. The prebiotic components in the synbiotic formulation could also have had an effect on the quantitative changes observed in fecal bacteria (Worthley *et al.*, 2009), but this effect could not be tested separately in this study.

In summary, cats having a lower fecal abundance of related probiotic genera before consuming probiotics may have a higher fecal abundance of ingested probiotics during consumption of these agents. The body weight was not a significant predictor of colonization in the dogs, maybe due to the wide variation in the linear relationship between bodyweight and the amount of feces excreted. The age in cats may also influence colonization outcomes by ingested probiotics but more studies are needed to confirm this effect.

4. THE EFFECT OF THE PROTON-PUMP INHIBITOR OMEPRAZOLE ON GASTROINTESTINAL BACTERIA OF HEALTHY DOGS

4.1 Overview

The effect of a proton-pump inhibitor on gastrointestinal microbiota was evaluated. Eight healthy 9-month-old dogs (4 males and 4 females) received omeprazole (1.1 mg/kg) orally twice a day for 15 days. Fecal samples and endoscopic biopsies from the stomach and duodenum were obtained on days 30 and 15 before omeprazole administration, on day 15 (last day of administration), and 15 days after administration. The microbiota was evaluated using 16S rRNA gene 454-pyrosequencing, fluorescence *in situ* hybridization, and qPCR. In the stomach, pyrosequencing revealed a decrease in *Helicobacter* spp. during omeprazole (median 92% of sequences during administration compared to >98% before and after administration; $p=0.0336$), which was accompanied by higher proportions of Firmicutes and Fusobacteria. FISH confirmed this decrease in gastric *Helicobacter* ($p<0.0001$) and showed an increase in total bacteria in the duodenum ($p=0.0033$) during omeprazole. However, Unifrac analysis showed that omeprazole administration did not significantly alter the overall phylogenetic composition of the gastric and duodenal microbiota. In feces, qPCR showed an increase in *Lactobacillus* spp. during omeprazole ($p<0.0001$), which was accompanied by a lower abundance of *Faecalibacterium* spp. and *Bacteroides-Prevotella-Porphyromonas* in the male dogs. This study suggests that omeprazole administration leads to quantitative changes in gastrointestinal microbiota of healthy dogs.

4.2 Introduction

The secretion of gastric acid is one of the first defense mechanisms in the body to avoid the introduction of potentially harmful infectious agents into the intestinal tract. Gastric acid is secreted by the parietal cells and is regulated by complex paracrine, endocrine, and neural pathways (Yao & Forte, 2003).

Proton pump inhibitors (PPIs) are compounds of widespread therapeutic use in human and veterinary medicine. PPIs inhibit the secretion of gastric acid by blocking the H^+/K^+-ATPase in gastric parietal cells (Howden *et al.*, 1984, Sachs *et al.*, 1995). In humans, a recent retrospective study of 125 patients showed that advanced age, low serum albumin concentrations, and concomitant use of PPIs were significant risk factors for *Clostridium difficile*-associated diarrhea (Kim *et al.*, 2010), an important disease with increasing rates of mortality (Dawson *et al.*, 2009). Likewise, a recent study involving 5,387 elderly subjects, and a systematic review of 2,948 patients, has linked the use of PPIs with an increased risk of diarrhea (Pilotto *et al.*, 2008), and a higher risk of enteric infections (Leonard *et al.*, 2007), respectively.

The mechanisms by which the suppression of gastric acid secretion predisposes patients to an increased risk of gastrointestinal (GI) disease are not well understood. For example, while there is mounting evidence suggesting an association between the use of PPIs and *C. difficile*-associated disease (Dial, 2009, Pant *et al.*, 2009), gastric acid does not kill *C. difficile* spores (Rao *et al.*, 2006), which are believed to be crucial for the transfer of the microorganism (Dawson *et al.*, 2009). Also, a large case-control cohort study of more than 170,000 users of acid-suppressing drugs, including PPIs, showed no

association of antacid use with bacterial gastroenteritis (Garcia Rodriguez & Ruigomez, 1997) and a recent review of the literature indicates that bacterial overgrowth during PPIs administration rarely leads to clinical disease (Williams & McColl, 2006). These observations illustrate the possibility that the development of GI disorders in patients that are treated with gastric acid inhibitors is a multi-factorial phenomenon rather than an isolated association (Canani & Terrin, 2010).

The GI tract of mammals is home to a vast number of different microbial groups, all acting in close symbiosis with one another and with their host (Neish, 2009). Despite the widespread medical use of PPIs and its potential involvement in intestinal dysbiosis (Vesper *et al.*, 2009), only a few studies have explored the effect of these compounds on GI microbial communities, mainly using culture techniques for specific microorganisms (e.g. *Helicobacter pylori*) (Sharma *et al.*, 1984, Fried *et al.*, 1994, Saltzman *et al.*, 1994, Verdu *et al.*, 1994, Logan *et al.*, 1995, Thorens *et al.*, 1996). However, culture techniques are by definition restricted to cultivable microorganisms, a group representing an insignificant proportion of all GI microbiota (Eckburg *et al.*, 2005, Rajilic-Stojanovic *et al.*, 2007). Culture independent, 16S rRNA gene-based techniques have greatly enhanced our knowledge of intestinal microbial inhabitants, but these techniques have rarely been used to evaluate the effect of gastric acid inhibition on the overall composition of the GI bacterial microbiota (Williams & McColl, 2006, Vesper *et al.*, 2009).

As in humans, PPIs and other inhibitors of gastric acid secretion are frequently used in dogs with disorders of the upper GI tract. However, the effect of omeprazole or

any other suppressor of gastric acid secretion on the GI bacterial microbiota of dogs has not been investigated and was the primary objective of this study.

4.3 Materials and methods

4.3.1 Study design

Eight intact clinically healthy mixed-breed dogs, four male and four female, were entered into this study. All dogs were nine months old, of similar weight (18.6 ± 2.0 kg) and were fed once a day a commercial diet (8755 Teklad: 21% protein, 4% fiber, Harlan). Omeprazole capsules (Zegerid, Santarus) were administered orally at an average dose of 1.1 ± 0.1 mg/kg twice a day (8 am and 8 pm) for 15 days. Immediately after administration of omeprazole, all dogs were given 20 ml of water orally. Multiple mucosal biopsy specimens from the gastric body and the proximal duodenum (12-15 from each site) were obtained from all dogs on Days 30 (Day -30) and 15 (Day -15) before omeprazole administration, on the last day of omeprazole administration (Day 15), and 15 days after the end of omeprazole administration (Day 30). Biopsies were collected by endoscopy under general anesthesia (sedation with butorphanol 0.2 mg/kg IM 15 minutes before induction with thiopental IV 15 mg/kg followed by endotracheal intubation and maintenance of anesthesia with sevoflurane in 100% oxygen via a circle system). For both stomach and duodenum, 3 biopsies were flash frozen in liquid nitrogen for DNA extraction, and 6-7 biopsies were harvested and placed into 10% formalin for FISH analysis and histological assessment according to the guidelines of the World Small Animal Veterinary Association (Day *et al*., 2008). Gastric juice (~2 mL) was

obtained before each endoscopic procedure via an endoscopic catheter and the pH measured immediately with a pH paper (EMD Chemicals) and a pH meter. Fecal samples were collected by rectal palpation on Days -30, -15, 15, and 30, and stored at -80 °C until analysis. The study protocol was approved by the Institutional Animal Care and Use Committee of the University of Illinois (approval number: 08261).

4.3.2 DNA extraction

Genomic DNA was extracted from the biopsies and feces using a bead-beating phenol-chloroform method as described elsewhere (Suchodolski *et al.*, 2010).

4.3.3 Massive parallel 454-pyrosequencing

The gastric and duodenal mucosa-adherent microbiota were evaluated using pyrosequencing of samples collected on Days -30 and -15 (before omeprazole administration), on Day 15 of omeprazole administration, and on Day 30 (after discontinuation of omeprazole administration) using a bacterial tag-encoded FLX-Titanium 16S rRNA gene amplicon pyrosequencing (bTEFAP) as described previously for canine intestinal samples (Handl *et al.*, 2011). Sequences with identity scores to known or well characterized 16S rRNA gene sequences greater than 97% identity (< 3% divergence) were resolved at the species level, between 95% and 97% at the genus level, between 90% and 95% at the family level, and between 80% and 90% at the order level.

4.3.4 Quantitative real-time PCR (qPCR)

The abundance of total bacteria, *Helicobacter*, *Lactobacillus*, and *Enterococcus* was estimated by qPCR in the obtained DNA samples from the gastric and duodenal biopsies using published oligonucleotides (Supplementary Table A3). TaqMan reaction mixtures (total 10 µL) contained 5 µL of TaqMan® Fast Universal PCR master mix (2x), No AmpErase® UNG (Applied Biosystems), 1 µL of water, 0.4 µL of each primer (400 nmol final concentration), 0.2 µL of the probe (200 nmol final concentration), 1 µL of 1% bovine serum albumin (BSA, final concentration: 0.1%), and 2 µL of DNA (1:10 or 1:100 dilution), and the PCR conditions were: 95°C for 20 s, and 40 cycles at 95°C for 5 s, and 10 s at the optimized annealing temperature (Supplementary Table A3). SYBR-based reaction mixtures (total 10 µL) contained 5 µL of SsoFast™ EvaGreen® supermix (Biorad Laboratories), 1.6 µL of water, 0.4 µL of each primer (final concentration: 400 nmol), 1 µL of 1% BSA (final concentration: 0.1%), and 2 µL of DNA (1:10 or 1:100 dilution). PCR conditions were 95°C for 2 min, and 40 cycles at 95°C 5 s and 10 s at the optimized annealing temperature. A melt curve analysis was performed for SYBR-based qPCR assays under the following conditions: 1 minute at 95°C, 1 minute at 55°C, and 80 cycles of 0.5°C increments (10 s each). Amplicons were also visualized in an agarose gel (1%) to confirm the presence of one band of the expected molecular size. The qPCR data for *Helicobacter*, *Lactobacillus*, and *Enterococcus* spp. was normalized to the qPCR data for total bacteria and all samples were run in duplicate.

The abundance of total bacteria, *Bifidobacterium*, *Lactobacillus*, the *Bacteroides-Prevotella-Porphyromonas* group, gamma-Proteobacteria (Class), Firmicutes (Phylum), *Clostridium perfringens*, as well as *C. difficile* and the *C. difficile* gene encoding toxin B, was evaluated in feces using published oligonucleotides (Supplementary Table A3). The abundance of Ruminococacceae (Family) and *Faecalibacterium* was also evaluated using family and genus-specific oligonucleotides (as assessed by 16S rRNA gene clone libraries) recently developed at our laboratory. SYBR-based qPCR assays were performed as described above (without BSA) at the optimized annealing temperature. A commercial real-time PCR thermal cycler (CFX96^{TM}, Biorad Laboratories) was used for all qPCR assays. The DNA concentration of all fecal samples was adjusted to 5 ng μL^{-1}.

4.3.5 Fluorescence in *situ* hybridization (FISH)

An average of 6 biopsies (range: 4-7 per organ evaluated) were obtained at each time point and from each dog, fixed in neutral-buffered 10% formalin, and embedded in paraffin. Histological sections (4 μm) were evaluated using FISH with oligonucleotide probes 5'-labeled with 6-FAM or Cy-5 targeting the 16S rRNA of total bacteria and *Helicobacter* (Supplementary Table A3) as described previously (Jergens *et al.*, 2009). Gastric and duodenal bacteria were quantified every 3-5 microscopic fields throughout the mucosal perimeter of each biopsy, depending on the unique morphology of each specimen, using a Zeiss Stallion digital confocal microscope (Carl Zeiss Microimaging). To facilitate the quantification of bacteria at different levels of the glass slide, at least three consecutive pictures were taken sequentially throughout the vertical z axis (each

picture separated from one another by 0.5 μm) from each microscopic field. A C-apochromat (63x water correction) objective lens was used for all FISH analyses.

4.3.6 Statistical analysis

To assess the diversity of the GI microbiota, the Shannon-Weaver diversity index was calculated. Alterations of microbial communities before, during, and after omeprazole administration, was investigated using principal component analysis (PCA) based on the phylogeny-based Unifrac distance metric (Lozupone & Knight, 2005).

Parametric analyses. A general linear mixed model using the MIXED procedure of SAS 9.2 (SAS Institute, Inc.) was used to analyze the qPCR data with *time*, *gender*, and *time*gender* interaction as fixed effects. The inclusion of the interaction between time and gender is justified by the fact that all dogs were the same age, had a very similar body weight, and were subjected to the same diet and environmental conditions. In addition, *time* was also used in the REPEATED statement to model the repeated measures (before, during, and after omeprazole administration) and *dog* was included as a random effect. The $\log_{10}$ gastric *Helicobacter* FISH counts were analyzed using a general linear mixed model in SAS 9.2 and the same approach described for qPCR data. Post-hoc multiple comparisons were performed using the Tukey-Kramer method. All model residuals showed a distribution very close to normal, thus indicating valid models.

Non-parametric analyzes. The Friedman's test in Prism5 (GraphPad Software, CA) was used to compare the pyrosequencing data (percentage of sequences) for each bacterial group separately, gastric non-*Helicobacter* total FISH counts, and the indexes

of bacterial richness and diversity. Post-hoc multiple comparisons were performed using the Dunn's post test. The NPAR1WAY procedure in SAS 9.2 was used to compare intragastric pH and duodenal bacterial FISH counts. A p value of less than 0.05 was considered to be statistically significant for all analyses.

4.4 Results

4.4.1 Side effects of omeprazole administration and intragastric pH

All dogs remained clinically healthy throughout the study. Intragastric pH was significantly increased during omeprazole administration (median pH: 7.4, interquartile range: 7.2-7.9) when compared with intragastric pH on Days -30 (1.7, 1.5-1.9) and -15 (1.8, 1.5-2.1) before administration, and Day 30 after omeprazole administration (1.5, 1.4-6.8) ($p=0.0037$). The pH measurements did not correlate linearly or quadratically with gastric or duodenal bacterial FISH counts, pyrosequencing or qPCR data (results not shown).

4.4.2 Pyrosequencing

A total of 142,026 (stomach) and 133,449 (duodenum) sequences (~4,000 per sample evaluated) were analyzed. With the exception of the gastric microbiota of two male dogs at only one different time point each, the gastric and duodenal microbiota formed completely separated phylogenetic clusters (Supplementary Fig. A1), suggesting a distinctive microbiota in each of the evaluated sections of the gastrointestinal tract.

4.4.3 Pyrosequencing in gastric biopsies

In the stomach, a median of 34 operational taxonomic units (OTUs >97% sequence identity) was detected per dog per time point. There was a significantly higher bacterial richness in the stomach during omeprazole administration but bacterial diversity was not significantly modified (Table 5). While we observed significant changes in specific bacterial groups in response to omeprazole administration (see below), the constructed dendrograms based on the Unifrac distance metric did not reveal an obvious clustering of animals according to treatment period (Supplementary Fig. A2). The great majority (>90% on average at baseline) of the obtained sequences from the stomach were classified as Proteobacteria, a phylum that decreased during omeprazole administration ($p=0.0427$, Supplementary Table A4). This effect was more evident on the genus *Helicobacter* ($p=0.0336$). The median percentage of *Helicobacter* spp. during omeprazole was 92%, the median percentage before and after omeprazole was >98%. This decrease in *Helicobacter* spp. during omeprazole administration was accompanied by an increase in other genera of the phyla Proteobacteria (especially *Actinobacillus*), Firmicutes (especially *Streptococcus*) and Fusobacteria (Supplementary Table A4).

4.4.4 FISH in gastric biopsies

Gastric *Helicobacter* and non-*Helicobacter* bacteria were counted throughout the mucosal side of a total of 155 gastric biopsies from a similar number of microscopic fields (Supplementary Table A5). There was a significant effect of omeprazole on the abundance of gastric *Helicobacter* ($p<0.0001$) and there was no difference in abundance

Table 5 Median (interquartile range) indexes of bacterial richness (OTUs 3%) and diversity (Shannon Weaver 3%) before, during and after omeprazole administration. Day -30 and Day -15 before omeprazole administration, Day 15 last day of omeprazole administration, and Day 30 after omeprazole administration (Day 30). *P* values were obtained by non-parametric Friedman's tests

	Stomach				
	Day -30	Day -15	Day 15	Day 30	p
Shannon	1.1 (0.8/1.5)	1.5 (1.0/1.8)	1.2 (0.8/2.1)	0.6 (0.2/1.2)	0.0658
OTU	32 (17/37)[a]	35 (20/46)	63 (36/82)[a,b]	22 (14/33)[b]	0.0021
	Duodenum				
Shannon	2.8 (2.7/3.5)	2.9 (2.6/3.7)	2.8 (2.7/3.4)	2.7 (2.0/3.3)	0.8254
OTU	156 (114/176)	206 (151/278)	145 (123/181)	156 (86/239)	0.2407

[a,b] Same superscripts indicate statistically significant difference ($p<0.05$, Dunn's multiple comparison).

of gastric *Helicobacter* between the male and female dogs (p=0.3161). Also, there was a significant interaction between time and gender (p=0.0323), suggesting that the change in gastric *Helicobacter* organisms over time was different between the male and female dogs (Fig. 9). Also, in the stomach, non-*Helicobacter* bacteria were observed more frequently during omeprazole administration (median: 3, range: 0-20) than on Day -30 (median: 0, range: 0-3) and Day -15 (median: 1, range: 0-6) before omeprazole administration, and 15 days after omeprazole administration on Day 30 (median: 0, range: 0-2) (p=0.0300).

4.4.5 Quantitative real-time PCR in gastric biopsies

There was not a significant effect of omeprazole administration on gastric total bacteria ($p=0.0687$), there was no difference in bacterial abundance between the male and female dogs ($p=0.7566$), but there was a significant interaction between time and gender ($p=0.0001$) (Fig. 10). In the male dogs, there was a higher bacterial abundance during omeprazole administration on Day 15 ($p=0.0093$) and on Day 30 after discontinuation of omeprazole administration ($p=0.0007$) when compared with that on Day -30 before omeprazole administration (Fig. 10). There was no significant effect of omeprazole administration on total gastric bacteria in the female dogs and there was no significant effect of omeprazole administration on the abundance of gastric *Helicobacter* and *Lactobacillus* spp. (Fig. 10).

4.4.6 Pyrosequencing in duodenal biopsies

In the duodenum, a median of 163 OTUs (>97% sequence identity) was detected per dog per time point. Omeprazole administration was not associated with significant differences in the indexes of bacterial richness and/or diversity (Table 5). While we observed significant changes in specific bacterial groups in response to omeprazole administration (see below), the constructed dendrograms based on the Unifrac distance metric did not reveal an obvious clustering of animals according to treatment period (Supplementary Fig. A3). Bacterial representatives of at least seven different phyla were identified in the duodenum (Supplementary Table A6). The majority of the obtained sequences from the proximal duodenum were classified as Firmicutes, followed by

Proteobacteria and Bacteroidetes. On average, these three bacterial phyla represented more than 80% of all sequences at all time points. Omeprazole administration was associated with a higher abundance of *Enterococcus* (p=0.0137) and a lower abundance of *Helicobacter* (p=0.0287) and *Porphyromonas* (p=0.0316), but there was no statistically significant difference in all the rest of the analyzed bacterial groups analyzed (Supplementary Table A6).

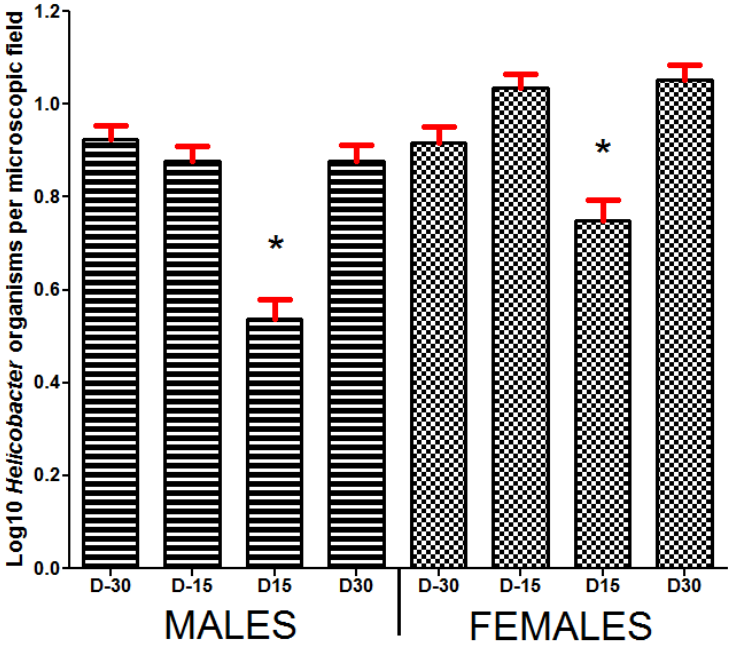

Fig. 9. Log_{10} *Helicobacter* FISH counts per microscopic field before, during and after omeprazole administration. Day 30 (D-30) and Day 15 (D-15) before omeprazole administration, the last day of omeprazole administration (D15), and 15 days after completion of omeprazole administration (D30) in the male (left) and the female (right) dogs. The error bars represent the mean and the standard error. Within each gender, there was a significant decrease in gastric *Helicobacter* during omeprazole administration at Day 15 (*, D15) when compared to all other time points ($p<0.0001$).

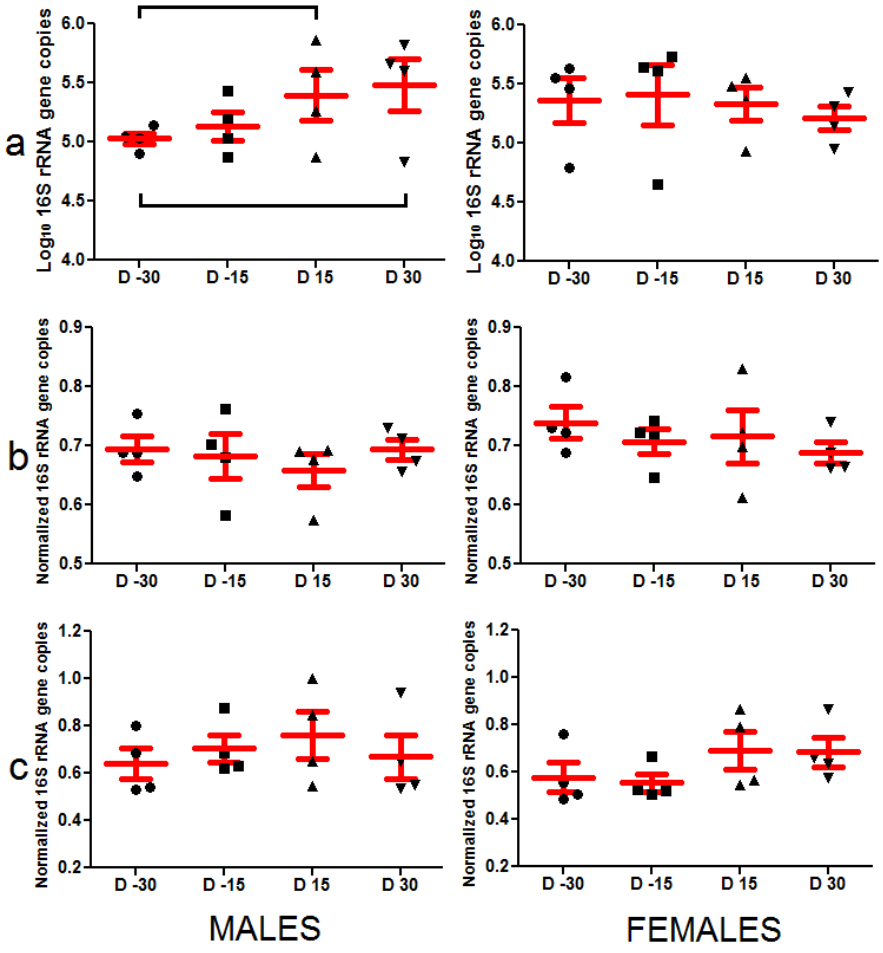

Fig. 10. Quantitative real-time PCR results for total gastric bacteria (a), gastric *Helicobacter* spp. (b), and gastric *Lactobacillus* spp. (c) before, during and after omeprazole administration. Day 30 (D -30) and Day 15 (D -15) before omeprazole administration, the last day of omeprazole administration (D 15), and 15 days after completion of omeprazole administration (D 30) in the male (left) and the female (right) dogs. Error bars represent the mean and the standard error. Horizontal brackets represent statistical significance ($p<0.05$).

Interestingly, the effect of omeprazole administration on duodenal *Lactobacillus* spp. was noticed only in the male dogs (Supplementary Fig. A4). All four male dogs had an increase in the Class Bacilli (Phylum Firmicutes) during omeprazole administration (all had >70% during omeprazole administration while only two had more than 10% at either baseline evaluation) (Supplementary Fig. A4). This effect was also evident at the order Lactobacillales and the genera *Enterococcus* and *Lactobacillus* in three of the four male dogs. This consistent increase in Bacilli during omeprazole administration in the male dogs was associated with a lower abundance of other bacterial phyla (especially Proteobacteria and Bacteroidetes) during omeprazole administration. In the female dogs, no such consistent changes in the proportions of duodenal bacteria were observed.

4.4.7 FISH in duodenal biopsies

Duodenal total bacteria were counted in a total of 132 biopsies from a similar number of microscopic fields (Supplementary Table A5). While the median number of bacteria per microscopic field was zero for all time points (range: 0-3), non-parametric analyzes revealed higher numbers of bacteria during omeprazole administration ($p=0.0033$). The sum of all counted bacteria during omeprazole was 40 bacteria (male dogs only: 34), while the median sum of all other time points was 8 bacteria. All the observed bacteria were morphologically similar (i.e., rod-shaped, 2-3 μm long).

4.4.8 Quantitative real-time PCR in duodenal biopsies

There was a significant effect of omeprazole administration on the abundance of total duodenal bacteria ($p=0.0003$), but there was no difference between genders and there was no significant interaction between omeprazole administration and gender. Regardless of gender, there was a higher bacterial abundance on Day 15 during omeprazole administration when compared to Day -15 before omeprazole administration ($p=0.0295$). Also, there was a higher bacterial abundance in the duodenum on Day 30 after omeprazole administration when compared to that on Day -30 ($p=0.0040$) and Day -15 ($p=0.0009$) before omeprazole administration (Fig. 11). In contrast to the pyrosequencing results that showed a decrease of *Helicobacter* spp. in the duodenum during omeprazole administration, the genus *Helicobacter* was detected only at six isolated time points in the duodenum of five dogs (three male and two female dogs). *Enterococcus* spp. was detected only in two male dogs during omeprazole administration on Day 15. There was a significant effect of omeprazole on duodenal *Lactobacillus* ($p<0.0001$) with male dogs having a higher abundance of duodenal *Lactobacillus* when compared with female dogs ($p=0.0168$). Also, there was a significant interaction between omeprazole administration and gender ($p<0.0001$) (Fig. 11). The male dogs had a significantly higher abundance of *Lactobacillus* during omeprazole administration when compared to all time points before and after omeprazole administration ($p<0.005$ for all multiple comparisons) (Fig. 11).

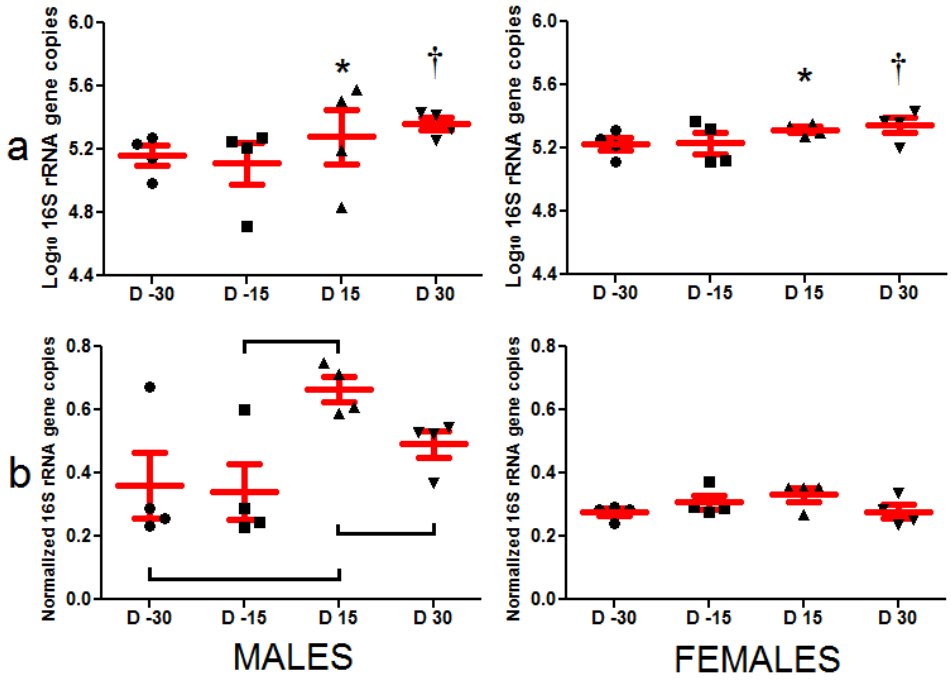

Fig. 11. Quantitative real-time PCR results for total duodenal bacteria (a) and *Lactobacillus* spp. (b) before, during and after omeprazole administration. Day 30 (D -30) and Day 15 (D -15) before omeprazole administration, the last day of omeprazole administration (D 15), and 15 days after completion of omeprazole administration (D 30). Horizontal brackets represent statistical significance ($p<0.05$). *Significantly different ($p<0.05$) than Day 15 before omeprazole administration (D -15), regardless of gender. † Significantly different ($p<0.01$) than Day 15 (D -15) and Day 30 (D -30) before omeprazole administration, regardless of gender.

4.4.9 Fecal microbiota

One fecal DNA sample (from one female dog, Day -15 before omeprazole administration) was not available and was treated as a missing value. All time points in all dogs were PCR negative for *C. difficile* and the *C. difficile* gene encoding toxin B. *C.*

perfringens was detected in all female dogs only on Day -30 before omeprazole administration. Regardless of gender, there was a significant increase in fecal *Lactobacillus* during omeprazole administration when compared with all other time points ($p<0.05$, Supplementary Fig. A5). This decrease in *Lactobacillus* was accompanied, in the male dogs, by a decrease of *Faecalibacterium* and the *Bacteroides-Prevotella-Porphyromonas* group (Supplementary Fig. A5).

4.5 Discussion

PPIs and other suppressors of gastric acid secretion are used extensively in both human and veterinary patients with suspected disorders of the upper GI tract. Despite the widespread use of these compounds in dogs and the cumulative evidence suggesting an association between PPI use and GI infections in human patients, there are no studies to date that have evaluated the effect of PPIs or any other gastric acid suppressor on the composition of the canine GI microbiota. The results of this study suggest that orally administered omeprazole at a dose of 1.1 mg/kg twice a day for 15 days can alter the composition of the gastric, duodenal, and fecal bacterial microbiota of healthy dogs.

In this study, omeprazole administration led to a decrease in gastric *Helicobacter* spp., an effect which was more evident on the quantitative FISH analysis. While a growing number of investigations suggest that PPIs can also lead to a decrease in the abundance of gastric *Helicobacter pylori* in humans, most studies have evaluated the effect of PPIs on this bacterium only in combination with other pharmaceuticals such as antibiotics (Graham & Fischbach, 2010, Luther *et al.*, 2010, Wu *et al.*, 2010). Also, the

histological density of *H. pylori* in the gastric body and antrum of humans was reduced after four weeks of omeprazole treatment, while it was increased in the fundus (Logan *et al.*, 1995). Other studies have confirmed this phenomenon (Ishihara *et al.*, 2001). This is important because in the current study we only collected biopsies from the gastric body and antrum, and therefore we cannot confirm an overall decrease in gastric *Helicobacter* in all regions of the stomach. Moreover, the quantitative real-time PCR assay used in this study did not confirm the decrease in gastric *Helicobacter* spp. abundance during omeprazole administration, an effect suggested by both pyrosequencing and FISH. It is possible that the qPCR assay used here does not detect all canine gastric species and strains of *Helicobacter*. For instance, while both the reverse primer and the oligo probe detect all *Helicobacter* spp. that have been isolated from the stomach in dogs (Neiger & Simpson, 2000), the forward primer may not detect *H. bilis* and *Flexispira rappini*. The latter may be especially relevant as it includes multiple *Helicobacter* taxa (Dewhirst *et al.*, 2000). These observations raise the interesting question of whether the effect of omeprazole is different among different species and/or strains of gastric *Helicobacter*, a hypothesis that is indirectly supported by a recent study showing that the effect of pantoprazole (another PPI) on growth and morphology of bacteria was different among several strains of oral *Lactobacillus* spp. (Altman *et al.*, 2008).

The mechanism by which omeprazole leads to a decrease in gastric *Helicobacter* is unclear and controversial in the literature (Canani & Terrin, 2010). Omeprazole could have an indirect effect by means of raising intragastric pH, which in turn could allow other non-*Helicobacter* bacteria to thrive. Alternatively, omeprazole may act directly by

means of a direct bactericidal effect. For instance, it has been shown that omeprazole inhibits the growth of gram-positive and gram-negative bacteria in vitro, including *H. pylori* (Jonkers *et al.*, 1996). More recent studies also support a direct effect of PPIs on *H. pylori* (Suzuki *et al.*, 2003, Nakamura *et al.*, 2007). This effect may be due to a direct effect on the proton-pumps of the bacteria, as these enzymes have been identified at least in *H. pylori* (Melchers *et al.*, 1998) and *Streptococcus pneumoniae* (Hoskins *et al.*, 2001). Thus, it has been hypothesized that these enzymes of bacterial origin may serve as extrinsic sites of action for PPI therapy (Vesper *et al.*, 2009). However, while much research has focused on *H. pylori*, dogs do not harbor this species in the stomach but other *Helicobacter* spp. such as *H. felis* and *H. heilmannii* (Neiger & Simpson, 2000, Shinozaki *et al.*, 2002). To date, the effect of PPIs on other non *H. pylori* gastric *Helicobacter* spp. has not been investigated.

The decrease in gastric *Helicobacter* abundance during omeprazole administration was accompanied by a higher abundance of other bacteria, especially *Streptococcus*, *Lactobacillus, Fusobacterium*, and *Actinobacillus*, whose abundances returned to baseline levels after discontinuation of omeprazole administration. It is likely that other, non-*Helicobacter* bacteria were able to thrive in the stomach during the temporary reduction in intragastric acidity. It is also possible that some of these bacteria possess a direct antagonist effect against *Helicobacter* spp., as suggested by a recent study of the effect of two strains of *Lactobacillus* on *H. pylori* (Cui *et al.*, 2010). However, it is not clear whether the bacteria that were found more abundantly during omeprazole administration were native to the stomach or foreign, e.g., from the mouth

and esophagus. One study suggested that the human stomach could contain its own distinct microbial ecosystem (Bik *et al.*, 2006), but the authors warned that this observation was based on a comparison of gastric, oral, and esophageal bacterial communities from different subjects with different clinical syndromes.

In the duodenum, omeprazole led to an increased abundance in *Lactobacillus* and *Enterococcus* in the male dogs, which likely caused the observed higher abundance of all bacteria suggested by FISH analysis. In the past, an abnormal accumulation of bacteria in the small bowel of dogs was termed as small intestinal bacterial overgrowth (SIBO; Johnston, 1999), but the understanding of this phenomenon has undergone several advances (Hall, 2011), in part because of the complex microbial composition discovered in the canine small intestine (Mentula *et al.*, 2005, Suchodolski *et al.*, 2008, Xenoulis *et al.*, 2008, Suchodolski *et al.*, 2009, Suchodolski *et al.*, 2010). In small animal veterinary medicine, small intestinal dysbiosis is a currently used term to define a clinical syndrome caused by an alteration, either qualitative, quantitative, or both, of one or more groups of the small intestinal microbiota. Although the observed changes in the composition of the duodenal microbiota during omeprazole administration may be considered abnormal (from its baseline composition), its clinical significance remains to be determined.

In addition to the changes in the stomach and duodenum, our results also suggest that omeprazole can alter the composition of the fecal microbiota. Similarly, one recent study showed that orally administered omeprazole can lead to changes in fecal microbial communities of mice in a dose-dependent manner (Kanno *et al.*, 2009). However, unlike

the current study that showed a higher abundance of some fecal bacteria (e.g. *Lactobacillus*) accompanied by a lower abundance of other bacteria (e.g. *Faecalibacterium* and *Bacteroides*) during omeprazole administration, Kanno et al. showed that all groups of fecal bacteria (with the exception of *Bifidobacterium*) increased during omeprazole administration in mice (Kanno *et al.*, 2009). Since omeprazole is metabolized by the hepatic cytochrome P450 system after absorption from the small intestine and about 80% of the metabolites are excreted in urine (Petersen, 1995), it is unlikely that any omeprazole reach the large intestine, at least in its native form. Thus, our results and the results reported by Kanno et al. suggest that it is the increase in the bacterial load entering the intestinal tract that is responsible for the changes observed in the fecal microbiota. Another factor affecting the fecal microbiota during inhibition of gastric acid could be the change in the composition of dietary protein reaching the large intestine (Zentek *et al.*, 2003), as gastric acid plays a key role in the initial stages of protein digestion. It seems likely that both mechanisms contribute to the changes observed in the fecal microbiota. The decrease in *Faecalibacterium* during omeprazole administration in the male dogs is especially interesting, as these bacteria possess anti-inflammatory properties (Sokol *et al.*, 2008) and have been found to be depleted during episodes of colitis in humans (Sokol *et al.*, 2009) .

Finally, the interaction between the effect of omeprazole on the GI bacterial microbiota and gender suggested in this study may deserve scrutiny in future studies. Interestingly, Zhang et al. showed that higher endogenous progesterone concentrations in women could have a stimulatory effect on the P450 3A (CYP3A) activity (Zhang *et*

al., 2006), which plays an essential role in the metabolism of omeprazole in the liver (Andersson *et al.*, 1993, Andersson *et al.*, 1994). However, all the females in the current study did not have their first heat season until months after the last sample collection, and it has been shown that bitches have undetectable serum concentrations of progesterone during anestrous (Hase *et al.*, 1999).

In summary, this study suggests that orally administered omeprazole can alter the quantitative abundance of several bacterial communities throughout the GI tract of healthy dogs. Particularly, in this study omeprazole administration was associated with a decrease in *Helicobacter* spp. and an increase of other bacteria in the stomach. Also, omeprazole administration was associated with higher numbers of total bacteria and an increase in *Lactobacillus* in the duodenum of the male dogs. Lastly, omeprazole led to an increase in fecal *Lactobacillus*, which was accompanied by a decrease in *Faecalibacterium* and the *Bacteroides-Prevotella-Porphyromonas* group in the male dogs. However, omeprazole administration was not associated with major qualitative changes in the phylogenetic composition of the stomach and the duodenum, as evaluated by Unifrac analysis of pyrosequencing results. Further studies are warranted to investigate the clinical significance of these findings.

5. CHARACTERIZATION OF THE FECAL MICROBIOTA IN HEALTHY DOGS

5.1 Overview

This study evaluated the abundance of the *Clostridium coccoides- Eubacterium rectale* (Erec) group using fluorescence *in situ* hybridization in feces of healthy dogs (n=6) at two time points (15 days apart). Dogs harbored a median of $1.8x10^{10}$ total bacteria (range: $1.1x10^{10}$ to $2.9x10^{10}$, as assessed by DAPI staining) and $3.7x10^{9}$ organisms belonging to the Erec group (range: $1.2x10^{9}$ to $6.7x10^{9}$) per gram of wet feces. The Erec group comprised a median of 22% of total bacteria (range: 10-42%). The difference in median Erec/DAPI ratios between the two evaluated time points ranged from 1 to 21%. The intra-individual coefficient of variation (%CV) between the two evaluated time points ranged from 27 to 41% for total bacteria (as assessed by DAPI staining), 30 to 53% for Erec, and 28 to 81% for the ratio of Erec/total bacteria. However, when transformed to the $\log_{10}$ scale, the highest difference in median abundance of bacteria between the two evaluated time points was only 0.23 for total bacteria and 0.45 for Erec. The median inter-individual %CV was 43% for total bacteria, 48% for Erec, and 53% for the Erec/total bacteria ratio. This study shows that the Erec group is abundant (22% of all fecal microbiota) and varies little (<0.5 in the $\log_{10}$ scale) within a period of 15 days in the feces of healthy dogs.

5.2 Introduction

Fluorescence *in situ* hybridization (FISH) is a culture-independent molecular technique that allows for the analysis of the composition and dynamics of the intestinal microbiota (Amann & Fuchs, 2008). The principles of the FISH technique have been described elsewhere (Moter & Gobel, 2000).

The oligonucleotide FISH probe Erec482 was designed to detect the *Clostridium coccoides-Eubacterium rectale* (Erec) group (Franks *et al.*, 1998), which at that time included most of the Clostridia and Eubacteria belonging to *Clostridium* clusters XIVa and XIVb. The Erec group (as evaluated by FISH using the Erec482 probe) is one of the most predominant bacterial groups in human feces. For example, several studies have shown that bacteria belonging to the Erec group comprises from 10 to 29% of all fecal bacteria (Franks *et al.*, 1998, Tannock *et al.*, 2000, Marteau *et al.*, 2001, Matsuki *et al.*, 2004, Mueller *et al.*, 2006, Swidsinski *et al.*, 2008). Aside their importance as one of the most predominant bacterial groups in feces (Sekelja *et al.*, 2011), some bacteria belonging to the Erec group have been linked to some forms of inflammatory bowel disease in humans (Sokol *et al.*, 2006).

Despite the relevance of the Erec group as a major component of the fecal microbiota in humans, only one study has used FISH to evaluate the *in vivo* abundance of bacteria of the Erec group in feces of dogs (Jia *et al.*, 2010). Therefore, the objective of this study was to evaluate the abundance and short-term temporal variability of bacteria of the Erec group using FISH.

5.3 Materials and methods

Naturally passed fecal samples from a total of six dogs (median age: 4 years, range: 9 months to 10 years) were used in this study. All subjects were privately owned pets with no clinical signs of gastrointestinal disease, such as diarrhea or vomiting, that consumed their routine diet during the study period. Two separate fecal samples (15 days apart) were collected and stored at 4°C for less than 24 hours before processing.

5.3.1 Fluorescence *in situ* hybridization

Paraffin embedded fecal blocks (PEFB) were prepared for FISH analysis. Briefly, 100 mg (wet weight) of each fecal sample was mixed with 500 µl of paraformaldehyde in a microcentrifuge tube and incubated for 12 h at 4˚C. The tubes were then centrifuged, the supernatant transferred into another tube, and the pellet washed with phosphate buffered saline (pH 7.2). After centrifugation, the supernatant from these two previous steps was mixed and partially dehydrated using a Vacufuge™ (Eppendorf) at 45°C for 2 hours. Both the washed fecal pellet and the dehydrated supernatant were then mixed with 1 ml of HistoGel™ (LabStorage Systems Inc.) using a FastPrep®-24 (MP Biomedicals). The agar containing the fecal specimen was poured into a histology cassette (standard, 25x20x5 mm, Tissue-Tek®) and allowed to solidify for 5 minutes at room temperature. At this stage (i.e., before paraffinization) the fecal blocks can be stored at 4°C for up to two weeks, but longer storage might favor fungal growth. Also, the blocks should not be stored in 70% EtOH before paraffinization (as usually done with tissue samples) because this could have a dilution effect on the fecal specimen with potential loss of bacterial

organisms. After paraffinization, the fecal blocks are rectangular cubes of ~25x20x3 mm (Fig. 12).

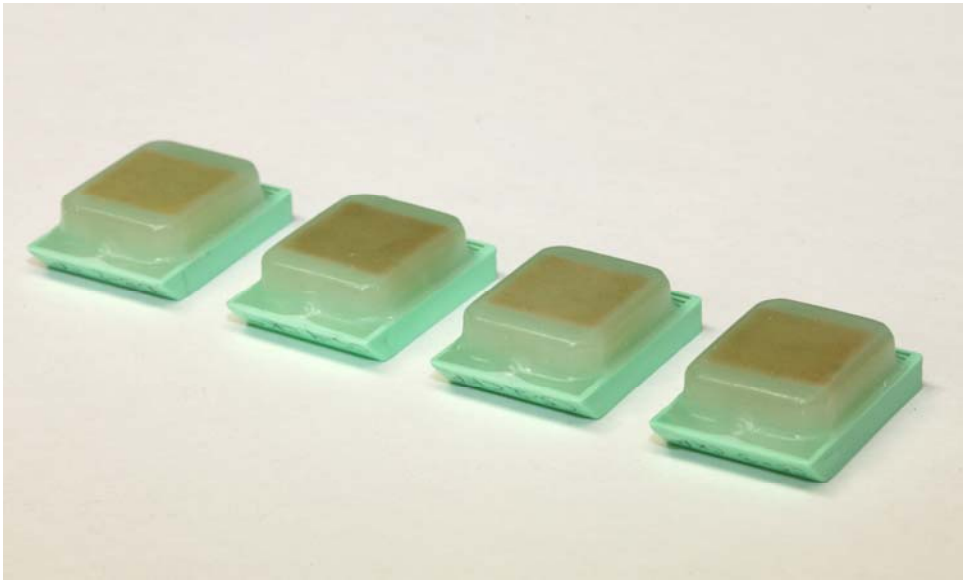

Fig. 12. Paraffin embedded fecal blocks.

FISH was performed as described previously (Thiel & Blaut, 2005), with modifications. Two serial paraffin sections from each paraffin embedded fecal block were cut (5 μm) and placed onto coated slides (ProbeOn Plus, Fisher Scientific). The oligonucleotide probe GCTTCTTAGTCARGTACCG (Erec482, targeting the 16S rRNA of the *Clostridium coccoides- Eubacterium rectale* (Erec) group as described by Franks

et al., 1998) was labeled at the 5'-end with Cy-5 (Integrated DNA technologies), reconstituted with sterile water, and diluted to a concentration of 30 ng/μL with a hybridization buffer (20 mM Tris, 0.9 M NaCl, 0.1% SDS, pH 7.2). Paraffin-embedded biopsy specimens were deparaffinized by passage through xylene (3 x 10 min), 100% ethanol (2 x 5 min), 95% ethanol (5 min), and 70% ethanol (5 min). After the slides were air-dried, the sections were allowed to hybridize with 10 μL of the probe (30 ng/μL) in a hybridization chamber for 4 hours at 50°C. After this, slides were rinsed with water and washed with an appropriate wash buffer (hybridization buffer without SDS) for 30 minutes at 52°C. The slides were rinsed with sterile water and mounted with ProLong® Gold Antifade reagent Gold (Invitrogen) containing 4,6-diamidino-2-phenylindole (DAPI). The number of bacteria per gram of wet feces was calculated using the following formula:

Bacterial cells g^{-1} wet feces = (number of bacteria per microscopic field) X (33,859) X (600) X (10),

where: 33,859 is the number of microscopic fields (area of one microscopic field: 14,767 μm^2) in one paraffin section (area of one paraffin section: 500 mm^2); 600 is the number of 5 μm paraffin sections per paraffin block (height of one paraffin block: 3 mm); and 10 is the factor to multiply by to obtain the number of bacteria per gram of wet feces (100 mg of feces were used to make each paraffin block).

To our knowledge, the use of ImageJ (image analysis software, NIH, USA) to quantify fluorescently-labeled fecal bacteria has not been described. Therefore, it was important to first determine the most suitable method to quantify fecal bacteria. For this

purpose, four serial paraffin sections (5 μm) from one canine PEFB were cut, placed onto a glass slide, and coated. FISH was performed as described above.

5.4 Results

To first identify the most suitable method to quantify the bacteria, a total of 60 microscopic fields (4 paraffin sections, 15 fields each) were analyzed. The Erec group was quantified in all of the 60 microscopic fields using three different approaches. First, the operator counted the bacteria manually using the *cell counter* feature in ImageJ (version 1.44p). Second, the bacteria were quantified by adjusting the threshold signal to match exactly the number of bacteria counted by the operator (JG) in one random microscopic field. After the threshold was adjusted, the *analyze particles* feature in ImageJ was utilized to automatically count all bacteria in each set of 15 microscopic fields. Thirdly, the bacteria were quantified by adjusting the threshold signal until all bacteria were labeled and then by decreasing the threshold signal until the numbers matched the manual quantification of the first paraffin section. After the threshold was adjusted, the *analyze particles* feature in ImageJ was utilized to automatically count all bacteria in each set of 15 microscopic fields. Based on the obtained results, the modified automatic approach (threshold adjusted to all labeled bacteria minus 40 fluorescent threshold units) was used to quantify bacteria belonging to the Erec group. After a similar analysis of images for the Erec group (results not shown), we decided to also use a modified automatic approach (threshold adjusted to all labeled bacteria minus 45 fluorescent threshold units) to quantify all bacteria (DAPI staining).

5.4.1 Quantification of bacteria

To evaluate the abundance and variation of the predominant fecal microbiota, a total of 240 microscopic fields were analyzed (6 dogs, two time points, two paraffin sections each, 10 microscopic fields per section). Dogs harbored a median of $1.8x10^{10}$ total bacteria per gram wet feces (range: $1.1x10^{10}$ to $2.9x10^{10}$), as estimated by DAPI staining, and $3.7x10^{9}$ bacteria of the Erec group per gram wet feces (range: $1.2x10^{9}$ to $6.7x10^{9}$) (Fig. 13). The Erec group comprised an overall median of 22% of all fecal bacteria (range: 10-42%) (Fig. 14). The difference in the median Erec/DAPI ratios between the two evaluated time points ranged from 1 to 21% (Fig. 14). The intra-individual %CV ranged from 27 to 41% for total bacteria (as estimated by DAPI), 30 to 53% for Erec bacteria, and 28 to 81% for the Erec/DAPI ratio (Figs. 13 and 14). When transformed to the $\log_{10}$ scale, the highest difference in median abundance of bacteria between the two evaluated time points was 0.45 for Erec bacteria and 0.23 for total bacteria (DAPI) (Fig. 15). The median inter-individual %CV was 43% for total bacteria, 48% for Erec bacteria, and 53% for the Erec/DAPI ratio (Figs. 13 and 14). The Erec FISH counts correlated positively with the total bacteria as estimated by DAPI staining (R^2=0.10, $p<0.0001$) (Fig. 16). The Erec counts also correlated positively with the Erec/DAPI ratios (R^2=0.32, $p<0.0001$) (Fig. 17). In contrast, total bacterial FISH counts correlated negatively with the Erec/DAPI ratios (R^2=0.23, $p<0.0001$) (Fig. 17).

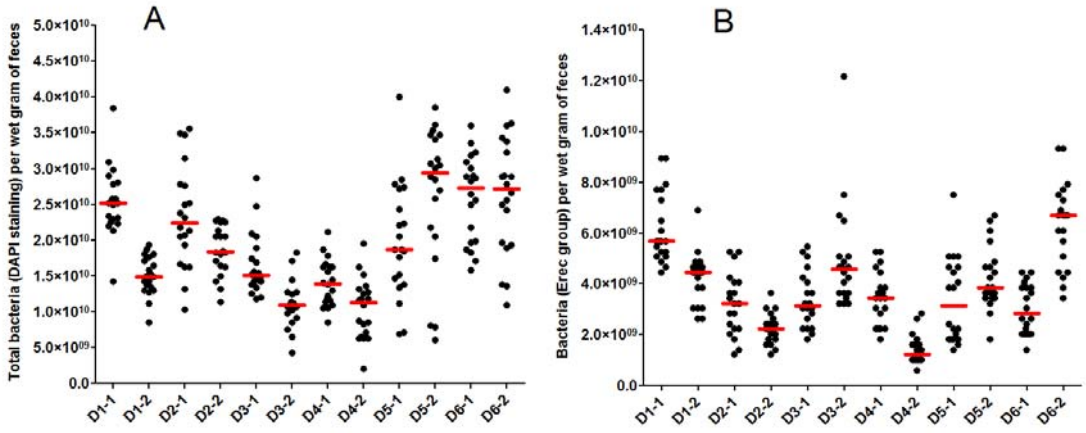

Fig. 13. Total fecal bacteria as estimated by DAPI staining (A) and bacteria of the Erec group (B) per wet gram of feces.

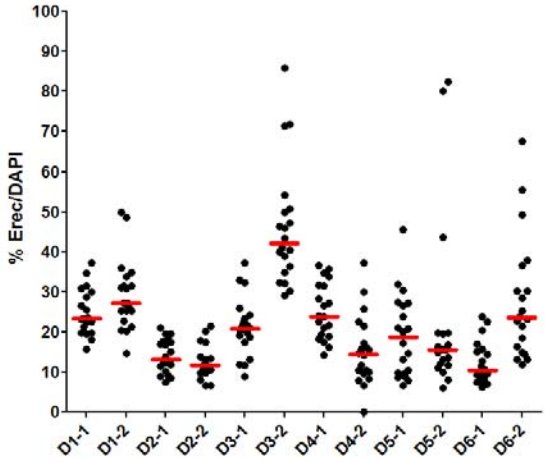

Fig. 14. Percentage of Erec FISH counts to total fecal bacteria (as estimated by DAPI staining).

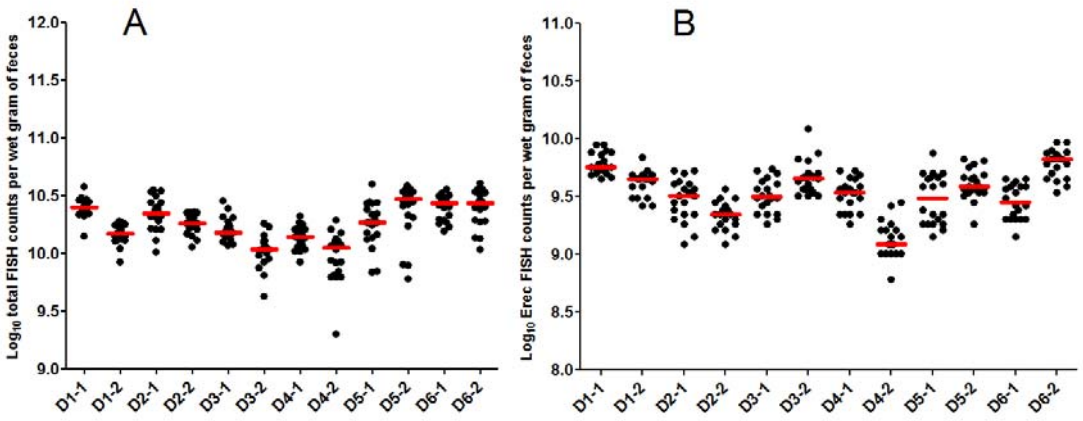

Fig. 15. Log_{10} total fecal bacteria as estimated by DAPI staining (A) and log_{10} bacteria of the Erec group (B) per gram of wet feces.

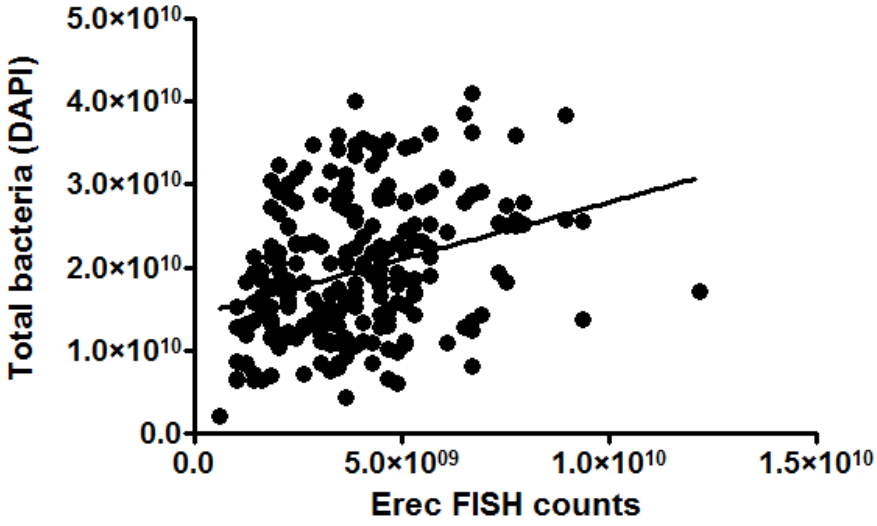

Fig. 16. Linear relationship (R^2: 0.10) between the Erec FISH counts (x axis) and total fecal bacteria (as estimated by DAPI staining, y axis).

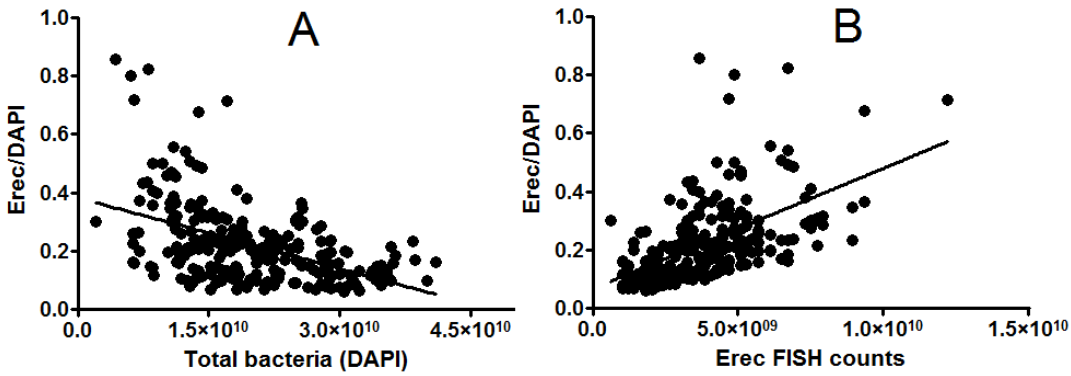

Fig. 17. Linear relationship between total FISH bacterial counts (as estimated by DAPI staining) and the Erec/DAPI ratios (A), and between Erec FISH counts and the Erec/DAPI ratios (B). A: R^2: 0.23; B: R^2: 0.32.

5.5 Discussion

The FISH probe Erec482 was designed to detect the *Clostridium coccoides-Eubacterium rectale* (Erec) group (Franks *et al.*, 1998). Because of the high abundance of bacteria of the Erec group in human feces and its potential involvement in the pathogenesis of inflammatory bowel disease, the goal of this study was to investigate the abundance of bacteria of the Erec group using FISH in feces of healthy dogs at two time points (15 days apart).

The probe Erec482 has been used to evaluate the abundance of bacteria of the Erec group in multiple human studies (Marteau *et al.*, 2001, Matsuki *et al.*, 2004, Sokol *et al.*, 2006). Also, one recent study showed that dogs harbor on average 9.2 to 9.6 $\log_{10}$ cells per gram of wet feces (~10% of total bacteria as estimated by DAPI staining) of the

Erec group (Jia *et al.*, 2010). Similarly, the current study showed an overall median of $3.7x10^9$ (9.6 $\log_{10}$) cells per gram of wet feces of bacteria of the Erec group (overall median: 22 % of all fecal bacteria as estimated by DAPI staining). The difference in the proportions of the Erec group between the study by Jia et al. (10%) and the current study (22%) is likely due to inter-individual differences in total fecal bacterial counts as well as to variations in the method of quantification of bacteria (manual versus automatic quantification in the current study).

The probe Erec482 was designed to detect bacteria of the Erec group (Franks *et al.*, 1998), which at that time included several species of the genera *Butyrivibrio, Clostridium, Eubacterium, Roseburia,* and *Ruminococcus*. However, some of these bacterial groups have recently been reclassified (Wiegel *et al.*, 2006, Liu *et al.*, 2008). Based on the current Ribosomal Database Project (RDP), Erec482 matches 16S rRNA gene sequences mostly within the phylum Firmicutes (>99% of all the 16S rRNA gene sequences detected by the Erec482 probe fall within this phylum), especially within the genus *Blautia* (89% of all sequences fall within the genus) and the family Lachnospiracea (67% of all sequences fall within the family, especially the genera *Butyrivibrio*, *Coprococcus*, *Roseburia*, *Dorea*, *Anaerostipes*, and *Pseudobutyrivibrio*). In contrast, Erec482 does not match many sequences within the families Ruminococcaceae or Eubacteriaceae (<0.1% of all sequences within the families). Studies are needed to evaluate the true specificity of Erec482 for fecal bacteria in dogs.

In summary, this study shows that the *Clostridium coccoides-Eubacterium rectale* (Erec) group (as estimated by FISH using the Erec482 probe) comprises about

22% of all fecal bacteria in healthy dogs. Despite some variation in the actual fecal bacterial numbers between the two sampled time points, when transformed to the $\log_{10}$ scale, the highest difference in median abundance of bacteria between the two time points evaluated was 0.45 for Erec bacteria and 0.23 for all bacteria. More studies are needed and ongoing to evaluate the abundance of more bacterial groups with potential clinical relevance.

6. CONCLUSIONS

The GI microbiota is the collection of all microorganisms inhabiting the GI tract and has been characterized using both culture and molecular methods. While culture methods allow for detailed metabolic and biochemical studies, these methods are not suitable for an in-depth characterization of the GI microbiota because the growth requirements for most GI microorganisms are unknown or poorly understood. The study and understanding of the GI microbiota is important because GI microorganisms serve as a defense mechanism against pathogens, harvest energy from nutrients that were not assimilated by the host, and provide substances that support the growth of intestinal epithelial cells.

Probiotics are defined as live microorganisms that when consumed in adequate quantities confer a health benefit to the host. While probiotics are administered increasingly frequent to cats and dogs in an effort to increase the number of beneficial bacteria in the intestinal tract, most studies have focused on the effect of probiotics on specific bacterial groups. Therefore, very little is known about the effect of probiotics on the overall composition of the GI microbiota. The results of this study suggest that the ingestion of probiotics leads to an increase abundance of the administered bacterial groups in feces. However, this increase does not lead to significant changes in the overall composition of the fecal microbiota, as suggested by pyrosequencing. It is therefore likely that probiotics may exert their effect by inducing the production of beneficial substances without modifying the overall composition of the intestinal

microbiota. More studies are needed to evaluate the functional (metabolic) effect of probiotics on the intestinal microbiota.

This study also demonstrated a highly individualized response to probiotic colonization. This would suggest that baseline characteristics of each individual animal could affect the extent of intestinal colonization by the ingested probiotic. Specifically, quantitative real-time PCR analyses showed big differences in the abundance of probiotic bacteria over time (during and after the administration of the probiotic formulation) among different subjects. Therefore, we investigated the effect of age, baseline abundance of probiotic bacteria, and body weight as potential predictors of fecal colonization by the ingested probiotics (defined as an increase in the abundance of the probiotic groups in feces, as assessed by qPCR). In dogs, neither age nor baseline fecal abundance of probiotic bacteria were significant predictors of fecal colonization. Interestingly, body weight was also not a significant predictor of fecal colonization in the dogs, nor was body weight linearly related with the abundance of any of the evaluated fecal bacterial groups. This observation could reflect a lack of an association between the body weight of the dogs and the number of bacteria excreted in feces. In contrast, cats that had a lower fecal abundance of probiotic bacteria before consuming the probiotic formulation had a higher abundance of the probiotic bacteria during the consumption of the probiotic when compared with the period after discontinuation of probiotic administration. In addition, we found a significant interaction between the age of the cats and period of probiotic administration, suggesting that the age in cats could also be related to the extent of fecal colonization by the probiotics ingested. As in dogs,

body weight was not a significant predictor of fecal colonization in cats, although this finding was not surprising because all the cats enrolled had very similar body weights.

The secretion of gastric acid is one of the most proximal mechanisms to avoid the introduction of potentially harmful infectious agents into the intestinal tract. Suppressors of gastric acid secretion are used extensively in both human and veterinary medicine to treat gastric acid-related disorders of the upper GI tract. The consumption of these therapeutic agents has been linked to GI disorders, such as an increased risk of diarrhea and a higher prevalence of intestinal infections, a phenomenon potentially associated with intestinal microbial dysbiosis. However, the effect of inhibitors of gastric acid secretion on the GI microbiota of dogs has not been investigated. These results showed that the proton-pump inhibitor omeprazole led to a decrease in gastric *Helicobacter* spp. organisms. However, in this study we only obtained samples from the gastric body/antrum and therefore we could not confirm an overall decrease in gastric *Helicobacter* spp. organisms. This is important because studies in humans suggest that one species of *Helicobacter* (*H. pylori*) can relocate from the antrum to the fundus in response to omeprazole administration. In the duodenum, omeprazole administration led to an increase in *Lactobacillus* and *Enteroccous* spp. in male dogs only. Interestingly, in this study we also found evidence that omeprazole could alter the abundance of different bacterial groups in the feces, which is likely due to an increased load of bacteria in the small intestine or to a change on the composition of dietary protein, as gastric acid plays an important role in the digestion of proteins. In spite of the observed increased

abundance of several GI bacterial groups, omeprazole administration was not associated with major changes in predominant bacterial phyla in the stomach or the duodenum.

The studies using 454-pyrosequencing revealed that the GI tract of cats and dogs contain a heterogeneous group of microorganisms, as members of at least five different phyla were identified in different segments of the GI tract. However, the exact quantification of the GI microorganisms remains an important task to fully characterize the composition of the GI microbiota. These results showed that about 22% of all fecal microbiota in healthy dogs are composed by one group of phylogenetically related bacteria within the phylum Firmicutes, the *Clostridium coccoides-Eubacterium rectale* group (*Clostridium* cluster XIVa and XIVb) as described by Franks *et al.* (1998). The results also showed that the abundance of this bacterial group in feces of healthy dogs varies little (less than 0.5 on a $\log_{10}$ scale) within a period of 15 days. The knowledge of this variation may help future studies to define a biologically significant effect of probiotics and other agents on the abundance of the intestinal microbiota.

In summary, the GI tract of cats and dogs as well as other mammals is colonized by different types of microorganisms. Dietary and therapeutic interventions are associated with specific changes in the abundance and/or composition of certain bacterial groups in the GI tract of healthy cats and dogs, as assessed by culture-independent molecular techniques targeting the 16S rRNA and the 16S rRNA gene. However, the effect of these agents on the GI microbiome may also include changes at the metabolic (functional) level. Therefore, future studies should aim to complement a

phylogenetic characterization of the intestinal microbiota with functional assays to evaluate the effect of these and other factors on GI and overall health.

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APPENDIX A

Supplementary Table A1. Medians and interquartile ranges of bacterial tags (percentages of all sequences) obtained by pyrosequencing before synbiotic administration at baseline (BL), after five days of administration of the synbiotic formulation (Day 5), and two days after discontinuation of administration of the synbiotic formulation (Day 23) in the cats enrolled. *P* values are either for a comparison of means (ANOVA) or ranks (non-parametric Friedman's test)[a].

Phylum	BL	DAY 5	DAY 23	*P*
Firmicutes	95.1 (91.1/97.0)	94.5 (92.2/98.7)	97.2 (95.9/98.3)	0.3679
Actinobacteria	4.0 (2.5/8.6)	3.1 (1.1/7.1)	1.6 (1.0/2.6)	0.0458†
Bacteroidetes	0.1 (0.0/0.8)	0.1 (0.0/1.0)	0.5 (0.3/0.9)	0.2028
Proteobacteria	0.0 (0.0/0.2)	0.0 (0.0/0.2)	0.0 (0.0/0.2)	1.0000
Fusobacteria	0.0 (0.0/0.1)	0.0 (0.0/0.1)	0.0 (0.0/0.0)	0.6969
Class				
Clostridia	66.0 (51.1/87.7)	66.7 (46.9/87.1)	78.7 (38.8/88.0)	0.7165
Erysipelotrichi	11.8 (4.4/18.8)	9.3 (2.4/16.0)	14.2 (3.2/25.3)	0.1738
Actinobacteria	4.0 (2.5/8.6)	3.1 (1.1/7.1)	1.6 (1.0/2.6)	0.0458†
Bacilli	3.4 (0.6/20.9)	8.2 (2.8/43.0)	0.6 (0.1/10.0)	0.0755
Bacteroidetes	0.1 (0.0/0.8)	0.1 (0.0/1.0)	0.5 (0.3/0.9)	0.2028
Fusobacteria	0.0 (0.0/0.1)	0.0 (0.0/0.1)	0.0 (0.0/0.0)	0.6969
Order				
Clostridiales	66.0 (51.1/87.7)	66.7 (46.9/87.1)	78.7 (38.8/88.0)	0.7165
Erysipelotrichales	11.8 (4.4/18.8)	9.3 (2.4/16.0)	14.2 (3.2/25.3)	0.1738
Coriobacteriales	4.0 (2.5/8.6)	3.1 (1.1/7.1)	1.6 (1.0/2.6)	0.0458†
Lactobacillales	3.4 (0.6/20.8)	8.2 (2.8/43.0)	0.4 (0.1/10.0)	0.1245
Bacteroidales	0.1 (0.0/0.8)	0.1 (0.0/1.0)	0.5 (0.3/0.9)	0.2028
Fusobacteriales	0.0 (0.0/0.1)	0.0 (0.0/0.1)	0.0 (0.0/0.0)	0.6969
Family				
Clostridiaceae	23.2 (8.6/26.4)	19.0 (8.0/26.4)	24.2 (16.6/39.7)	0.1834
Lachnospiraceae	21.5 (18.5/33.0)	21.9 (10.0/29.2)	17.0 (6.7/26.6)	0.3385
Ruminococcaceae	18.1 (10.1/21.5)	17.9 (8.9/36.7)	13.6 (9.4/21.5)	0.7788
Erysipelotrichaceae	11.8 (4.4/18.8)	9.3 (2.4/16.0)	14.2 (3.2/25.3)	0.3259
Coriobacteriaceae	4.0 (2.5/8.6)	3.1 (1.1/7.1)	1.6 (1.0/2.6)	0.0458†
Eubacteriaceae	1.2 (0.4/2.4)	0.5 (0.2/1.9)	1.1 (0.4/2.5)	0.4724
Enterococcaceae	0.5 (0.0/5.8)	5.4 (1.1/8.2)	0.1 (0.0/0.6)	0.1214
Veillonellaceae	0.1 (0.0/1.2)	0.1 (0.0/0.5)	0.2 (0.0/0.3)	0.9048
Bacteroidaceae	0.0 (0.0/0.1)	0.0 (0.0/0.4)	0.1 (0.0/0.4)	0.1822
Prevotellaceae	0.0 (0.0/0.2)	0.0 (0.0/0.5)	0.3 (0.1/0.5)	0.0793
Lactobacillaceae	0.0 (0.0/1.6)	0.4 (0.2/7.0)	0.0 (0.0/0.9)	0.0390†
Fusobacteriaceae	0.0 (0.0/0.1)	0.0 (0.0/0.1)	0.0 (0.0/0.0)	0.6969
Streptococcaceae	0.0 (0.0/0.2)	0.0 (0.0/0.1)	0.1 (0.0/0.1)	0.5292
Genus[b]				
Clostridium	22.5 (8.6/25.6)	18.9 (7.7/26.4)	24.1 (16.5/29.5)	0.2388
Roseburia	16.1 (11.1/27.0)	14.5 (5.9/18.4)	8.1 (5.0/21.9)	0.1464
Ruminococcus	11.7 (6.5/19.2)	11.4 (5.2/27.7)	9.4 (5.0/14.2)	0.2238
Turicibacter	5.5 (3.1/10.2)	5.8 (0.4/8.3)	9.4 (2.1/11.5)	0.1738
Dorea	5.1 (3.6/8.4)	4.0 (2.3/9.8)	4.2 (1.1/7.5)	0.3385
Catenibacterium	3.4 (0.8/7.7)	1.3 (0.1/6.6)	0.9 (0.2/9.6)	0.4412

Collinsella	2.7 (2.0/7.3)	1.7 (0.8/3.8)*	1.2 (0.8/2.1)*	0.0062
Sporobacter	1.8 (1.0/5.6)	2.7 (0.8/5.0)	2.4 (0.8/5.6)	0.7165
Eubacterium	1.2 (0.4/2.4)	0.5 (0.2/1.9)	1.1 (0.4/2.5)	0.4724
Enterococcus	0.5 (0.0/5.8)	5.4 (1.1/8.2)	0.1 (0.0/0.6)	0.1214
Faecalibacterium	0.4 (0.2/0.9)	0.3 (0.0/1.9)	0.8 (0.2/1.3)	0.7584
Peptococcus	0.2 (0.1/0.6)	0.1 (0.1/0.3)	0.3 (0.0/0.5)	0.7165
Acetanaerobacterium	0.1 (0.0/0.3)	0.0 (0.0/0.1)	0.2 (0.1/0.5)	0.1637
Slackia	0.1 (0.1/0.2)	0.1 (0.1/0.3)	0.2 (0.0/0.3)	0.9200
Coprococcus	0.1 (0.0/0.2)	0.1 (0.0/0.2)	0.0 (0.0/0.1)	0.2053
Olsenella	0.1 (0.0/2.1)	0.1 (0.0/0.8)	0.0 (0.0/0.2)	0.0164†
Bulleidia	0.1 (0.0/0.8)	0.0 (0.0/1.1)	0.0 (0.0/0.8)	0.1801
Anaerovorax	0.1 (0.0/0.2)	0.0 (0.0/0.3)	0.1 (0.0/0.3)	0.8187
Lactobacillus	0.0 (0.0/1.6)	0.4 (0.2/7.0)	0.0 (0.0/0.9)	0.0390†
Eggerthella	0.0 (0.0/0.1)	0.0 (0.0/0.1)	0.0 (0.0/0.1)	0.8588
Anaerotruncus	0.0 (0.0/0.0)	0.0 (0.0/0.1)	0.1 (0.0/0.1)	0.0558
Anaerostipes	0.0 (0.0/0.4)	0.0 (/0.0/0.2)	0.0 (0.0/0.1)	0.1496
Dialister	0.0 (0.0/0.1)	0.0 (0.0/0.1)	0.1 (0.0/0.1)	0.8276
Syntrophococcus	0.0 (0.0/0.1)	0.0 (0.0/0.1)	0.0 (0.0/0.1)	0.0764
Mogibacterium	0.0 (0.00.4)	0.0 (0.0/0.4)	0.0 (0.0/0.1)	0.0970
Desulfotomaculum	0.0 (0.0/0.1)	0.0 (0.0/0.1)	0.1 (0.0/0.1)	0.6291
Prevotella	0.0 (0.0/0.2)	0.0 (0.0/0.5)	0.3 (0.1/0.5)	0.0793
Streptococcus	0.0 (0.0/0.2)	0.0 (0.0/0.1)	0.0 (0.0/0.1)	0.4966
Bacteroides	0.0 (0.0/0.1)	0.0 (0.0/0.4)	0.1 (0.0/0.4)	0.1822

A total of 75,350 pyrosequencing tags were evaluated at baseline (BL), 60,355 on day 5 of administering the synbiotic (Day 5), and 51,691 two days after discontinuation of administration of the synbiotic formulation (Day 23).

[a] *P* values in this table are not adjusted for falsely rejected null hypotheses. After adjustment, none of these *P* values were found to be lower than the Benjamin-Hochberg critical values, suggesting that the significant *P* values ($P<0.05$) are rejecting the null hypothesis that all three time points are equal when this is true (Type I error).

[b] *Clostridium*, *Ruminococcus*, *Collinsella*, and *Sporobacter* were the only bacterial genera that were detected at all time points in all of the subjects. The rest of the genera in this table were found in samples from at least 2 time points in at least half of the subjects. The remaining 58 genera found were only detected sporadically across subjects and therefore are not included in this table.

* Significantly lower than baseline ($P<0.05$).

† Multiple comparisons across the three time points did not reach statistical significance.

Supplementary Table A2. Median and interquartile ranges of bacterial tags (percentages of all sequences) obtained by pyrosequencing before synbiotic administration at baseline (BL), after five days of administration of the synbiotic formulation (Day 5), and two days after discontinuation of administration of the synbiotic formulation (Day 23) in the enrolled dogs. *P* values are either for a comparison of means (ANOVA) or ranks (non-parametric Friedman's test)[a].

Phylum	BL	DAY 5	DAY 23	*P*
Firmicutes	96.9 (94.6/98.5)	96.3 (89.3/97.9)	97.5 (86.6/99.1)	0.9200
Actinobacteria	1.6 (0.7/2.7)	2.0 (1.1/5.3)	0.9 (0.6/2.1)	0.1482
Bacteroidetes	0.1 (0.0/1.0)	1.1 (0.0/4.4)	0.3 (0.0/0.7)	0.3872
Proteobacteria	0.1 (0.0/0.2)	0.1 (0.0/0.3)	0.1 (0.1/0.3)	0.5441
Fusobacteria	0.1 (0.0/0.5)[c]	0.8 (0.0/1.9)[c]	0.2 (0.1/1.1)[d]	0.0128
Class				
Clostridia	69.9 (42.9/82.6)	80.7 (66.8/90.9)	75.0 (67.6/92.0)	0.0755
Erysipelotrichi	6.7 (0.8/27.7)	6.7 (2.5/13.8)	7.5 (1.4/11.9)	0.4657
Bacilli	1.5 (0.1/42.4)	0.2 (0.0/1.4)	1.2 (0.0/12.7)	0.7165
Actinobacteria	1.6 (0.7/2.7)	2.0 (1.1/5.3)	0.9 (0.6/2.1)	0.1482
Bacteroidetes	0.1 (0.0/1.0)	1.1 (0.0/4.4)	0.3 (0.0/0.7)	0.3872
Fusobacteria	0.1 (0.0/0.5)[c]	0.8 (0.0/1.9)[c]	0.2 (0.1/1.1)[d]	0.0128
Order				
Clostridiales	69.9 (42.9/82.6)	80.7 (66.8/90.9)	75.0 (67.6/92.0)	0.0755
Erysipelotrichales	6.7 (0.8/27.7)	6.7 (2.5/13.8)	7.5 (1.4/11.9)	0.4657
Lactobacillales	1.5 (0.0/42.4)	0.2 (0.0/1.4)	1.2 (0.0/12.7)	0.7165
Coriobacteriales	1.6 (0.7/2.7)	2.0 (1.1/4.3)	0.9 (0.6/2.1)	0.1482
Bacteroidales	0.1 (0.0/1.0)	1.1 (0.0/4.4)	0.3 (0.0/0.7)	0.3872
Fusobacteriales	0.1 (0.0/0.5)[c]	0.8 (0.0/1.9)[c]	0.2 (0.1/1.1)[d]	0.0128
Family				
Clostridiaceae	22.8 (15.3/43.7)	34.9 (16.8/39.4)	26.8 (17.1/44.3)	0.7624
Ruminococcaceae	19.1 (13.4/27.8)	22.6 (16.5/31.3)	20.5 (6.8/32.7)	0.3892
Lachnospiraceae	13.0 (9.2/20.9)	13.2 (7.4/24.7)	12.0 (8.2/17.7)	0.6976
Erysipelotrichaceae	6.7 (0.8/27.7)	6.7 (2.5/13.8)	7.5 (1.4/11.9)	0.4657
Bacteroidaceae	0.0 (0.0/0.8)	0.1 (0.0/1.1)	0.1 (0.0/0.3)	0.6065
Prevotellaceae	0.0 (0.0/0.4)	0.4 (0.0/3.2)	0.1 (0.0/0.5)	0.1522
Coriobacteriaceae	1.6 (0.7/2.7)	2.0 (1.1/4.3)	0.9 (0.6/2.1)	0.1482
Lactobacillaceae	0.0 (0.0/0.3)	0.0 (0.0/0.1)	0.0 (0.0/0.0)	0.5308
Enterococcaceae	0.0 (0.0/0.1)	0.2 (0.0/0.5)	0.0 (0.0/2.7)	0.2319
Eubacteriaceae	0.0 (0.0/0.5)[c]	0.4 (0.9/1.0)[c]	0.3 (0.0/0.7)[d]	0.0388
Fusobacteriaceae	0.1 (0.0/0.5)[c]	0.8 (0.0/1.9)[c]	0.2 (0.1/1.1)[d]	0.0128
Veillonellaceae	0.1 (0.0/0.9)	0.2 (0.0/13.6)	0.1 (0.0/2.1)	0.0626
Streptococcaceae	0.1 (0.0/18.2)	0.0 (0.0/0.2)	0.0 (0.0/0.2)	0.7148
Enterobacteriaceae	0.0 (0.0/0.0)	0.0 (0.0/0.0)	0.0 (0.0/0.0)	0.5404
Genus[b]				
Clostridium	19.3 (11.0/32.9)	34.9 (16.6/39.3)	23.9 (8.6/39.6)	0.1603
Ruminococcus	17.1 (9.5/23.7)	18.1 (8.7/25.4)	14.8 (5.6/26.4)	0.9139
Dorea	6.8 (4.4/11.8)	5.8 (3.7/17.5)	7.2 (5.3/9.4)	0.9200
Roseburia	5.1 (2.0/6.7)[c]	4.5 (2.6/8.6)[d]	3.3 (1.4/5.7)[c,d]	0.0183
Turicibacter	3.0 (0.2/9.9)	1.9 (0.5/7.2)	1.9 (0.4/9.7)	0.7788
Megamonas	0.0 (0.0/0.7)	0.1 (0.0/12.4)	0.1 (0.0/1.9)	0.1102
Faecalibacterium	0.3 (0.1/5.1)	0.3 (0.0/6.4)	0.3 (0.0/0.5)	0.9770

Papillibacter	0.0 (0.0/0.1)	0.0 (0.0/0.4)	0.0 (0.0/0.1)	0.7979
Prevotella	0.0 (0.0/0.4)	0.4 (0.0/3.2)	0.1 (0.0/0.5)	0.1522
Lactobacillus	0.0 (0.0/0.3)	0.0 (0.0/0.1)	0.0 (0.0/0.0)	0.5308
Sporobacter	0.0 (0.0/0.1)	0.1 (0.0/0.2)	0.0 (0.0/0.1)	0.1324
Fusobacterium	0.1 (0.0/0.5)[c]	0.8 (0.0/1.9)[c]	0.2 (0.1/1.1)[d]	0.0128
Allobaculum	0.6 (0.0/1.6)	0.8 (0.0/1.7)	0.2 (0.0/1.2)	0.3281
Collinsella	1.3 (0.6/2.6)	1.7 (0.9/3.9)	0.7 (0.4/2.0)	0.1482
Coprobacillus	0.1 (0.0/2.0)	0.1 (0.0/1.7)	0.0 (0.0/0.9)	0.5488
Catenibacterium	0.1 (0.0/4.1)	0.5 (0.0/8.9)	0.4 (0.0/3.0)	0.9683
Eubacterium	0.0 (0.0/0.5)[c]	0.4 (0.1/1.0)[c]	0.3 (0.0/0.7)[d]	0.0388
Streptococcus	0.0 (0.0/16.2)	0.0 (0.0/0.2)	0.0 (0.0/0.2)	0.9753
Bacteroides	0.0 (0.0/0.8)	0.1 (0.0/1.1)	0.1 (0.0/0.3)	0.6065
Enterococcus	0.0 (0.0/0.1)	0.2 (0.0/0.5)	0.0 (0.0/2.7)	0.2319

A total of 87,737 pyrosequencing tags were evaluated at baseline (BL), 56,852 at day 5 of feeding the synbiotic (Day 5) and 57,053 after two days of discontinuation of treatment (Day 23).

[a] *P* values in this table are not adjusted for falsely rejected null hypotheses. After adjustment, none of these *P* values were found to be lower than the Benjamin-Hochberg critical values, suggesting that the significant *P* values ($P<0.05$) are rejecting the null hypothesis that all three time points are equal when this is true (Type I error).

[b] *Clostridium*, *Ruminococcus*, *Dorea*, *Roseburia* and *Turicibacter* were the only bacterial genera that were detected at all time points in all the subjects. The rest of the genera in this table were found in at least 2 time points in at least the half of the subjects. The remaining 56 genera found were only detected sporadically across subjects and therefore are not included in this table.

[c,d] Same letter indicates a statistically significant difference.

Supplementary Table A3 Oligonucleotides used for quantitative real-time PCR (qPCR) assays and fluorescent in *situ* hybridization (FISH).

qPCR primers/probe	Sequence (5'- 3')	Target	Annealing (°C)	Reference
UniF	CCTACGGGAGGCAGCAG	All bacteria	59	Muyzer *et al.*, 1993
UniR	ATTACCGCGGCTGCTGG			
EncocF	CCCTTATTGTTAGTTGCCATCATT	*Enterococcus*	61	Malinen *et al.*, 2005
EncocR	ACTCGTTGTACTTCCCATTGT			
LacF	AGCAGTAGGGAATCTTCCA	*Lactobacillus*	58	Malinen *et al.*, 2005
LacR	CACCGCTACACATGGAG			
BactF	GGTGTCGGCTTAAGTGCCAT	*Bacteroides/Prevotella/ Porphyromonas*	55	Malinen *et al.*, 2005
BactR	CGGACGTAAGGGCCGTGC			
Firm350f	GGCAGCAGTRGGGAATCTTC	Firmicutes	60	Muhling *et al.*, 2008
Firm814r	ACACYTAGYACTCATCGTTT			
Gamma395F	CMATGCCGCGTGTGTGAA	γ Proteobacteria	69	Muhling *et al.*, 2008
Gamma871R	ACTCCCCAGGCGGTCDACTTA			
BifF	TCGCGTCYGGTGTGAAAG	*Bifidobacterium*	60	Malinen *et al.*, 2005
BifR	CCACATCCAGCRTCCAC			
FaecaF	GAAGGCGGCCTACTGGGCAC	*Faecalibacterium*	60	This study
FaecaR	GTGCAGGCGAGTTGCAGCCT			
RumiF	ACTGAGAGGTTGAACGGCCA	Family Ruminococcacea	59	This study
RumiR	CCTTTACACCCAGTAAWTCCGGA			
CPerf165F	CGCATAACGTTGAAAGATGG	*C. perfringens*	58	Wise & Siragusa, 2005
CPerf269R	CCTTGGTAGGCCGTTACCC			
CPerf187F (probe)	TCATCATTCAACCAAAGGAGCAATCC			
Forward	TTGAGCGATTTACTTCGGTAAAGA	*C. difficile*	61	Penders *et al.*, 2005
Reverse	TGTACTGGCTCACCTTTGATATTCA			
probe	CCACGCGTTACTCACCCGTCCG			
tcdB-F	GGTATTACCTAATGCTCCAAATAG	*C. difficile* toxin B gene	58	Houser *et al.*, 2010
tcdB-R	TTTGTGCCATCATTTTCTAAGC			
tcdB-P (probe)	ACCTGGTGTCCATCCTGTTTCCCA			
HelF[a]	ACCAAGGCAATGACGGGTATC	*Helicobacter*	60	Huijsdens *et al.*, 2004
HelR	CGGAGTTAGCCGGTGCTTATT			
HelP (probe)	AACCTTCATCCTCCACGCGGC			
FISH probes				
Eub 338	FAM-GCTGCCTCCCGTAGGAGT	All bacteria	46	Amann *et al.*, 1990
Hel 274[b]	Cy5-GGCCGGATACCCGTCATAGCCT	*Helicobacter* spp.	46	Chan *et al.*, 2005
Hel 717[b]	Cy5-AGGTCGCCTTCGCAATGAGTA			

[a]This oligonucleotide does not match *Helicobacter bilis* nor *Flexispira rapinni* based on the Ribosomal Database Project (http://rdp.cme.msu.edu/).
[b]These oligonucleotides may also detect *Wolinella* spp. (Family Helicobacteriacea), based on the Ribosomal Database Project.

Supplementary Table A4 Median (interquartile range) proportions of pyrosequencing tags on Day 30 (Day -30) and Day 15 (Day -15) before starting omeprazole administration, Day 15 during omeprazole administration, and Day 30 after discontinuation of omeprazole administration in the stomach. P values were calculated by non-parametric Friedman's tests.

	Day -30	Day -15	Day 15	Day 30	p value
Proteobacteria	99.6 (97.2-99.9)	99.5 (96.9-99.8)	97.0 (57.2-98.8)	99.4 (90.9-99.8)	0.0427
Helicobacter	98.6 (96.6-98.9)	98.4 (96.0-99.0)	91.6 (53.6-97.4)	98.0 (90.2-99.4)	0.0336
Curvibacter	0.5 (0.4-0.6)	0.7 (0.4-0.7)	0.5 (0.2-1.1)	0.3 (0.1-1.1)	0.7892
Herbaspirillum	0.1 (0.0-0.2)	0.1 (0.1-0.1)	0.1 (0.0-0.2)	0.1 (0.0-0.1)	0.6823
Neisseria	0.0 (0.0-0.0)	0.0 (0.0-0.1)	0.1 (0.0-0.4)	0.0 (0.0-0.0)	0.0151
Actinobacillus	0.0 (0.0-0.0)	0.0 (0.0-0.2)	0.3 (0.2-3.4)	0.0 (0.0-0.1)	0.0272
Firmicutes	0.3 (0.1-2.8)	0.2 (0.0-3.0)	1.5 (0.5-42.6)	0.4 (0.1-8.8)	0.0803
Clostridium	0.0 (0.0-0.4)	0.0 (0.0-0.2)	0.1 (0.0-0.6)	0.0 (0.0-0.1)	0.1730
Turicibacter	0.0 (0.0-0.7)	0.0 (0.0-0.1)	0.0 (0.0-0.2)	0.0 (0.0-8.7)	0.8243
Lactobacillus	0.0 (0.0-0.1)	0.0 (0.0-1.6)	0.2 (0.0-28.6)	0.1 (0.0-0.3)	0.2140
Prevotella	0.0 (0.0-0.0)	0.0 (0.0-0.1)	0.0 (0.0-0.4)	0.0 (0.0-0.0)	0.2683
Streptococcus	0.0 (0.0-0.0)	0.0 (0.0-0.2)	0.3 (0.2-1.4)[a]	0.0 (0.0-0.0)[a]	0.0028
Bacteroidetes	0.0 (0.0-0.1)	0.1 (0.0-0.2)	0.3 (0.0-1.7)	0.0 (0.0-0.1)	0.1351
Porphyromonas	0.0 (0.0-0.0)	0.0 (0.0-0.1)	0.1 (0.0-0.3)	0.0 (0.0-0.0)	0.0338
Fusobacteria	0.0 (0.0-0.0)[a]	0.0 (0.0-0.0)	0.2 (0.1-0.4)[ab]	0.0 (0.0-0.0)[b]	0.0006
Fusobacterium	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.1 (0.0-0.2)[a]	0.0 (0.0-0.0)[a]	0.0017

This table only shows those bacterial genera (and their respective phyla) that were identified in at least two time points in at least half the subjects. Only the genera *Helicobacter* and *Curvibacter* were detected in all animal subjects at all time points.

[a,b] Same superscripts indicate statistically significant difference ($p<0.05$, Dunn's multiple comparison).

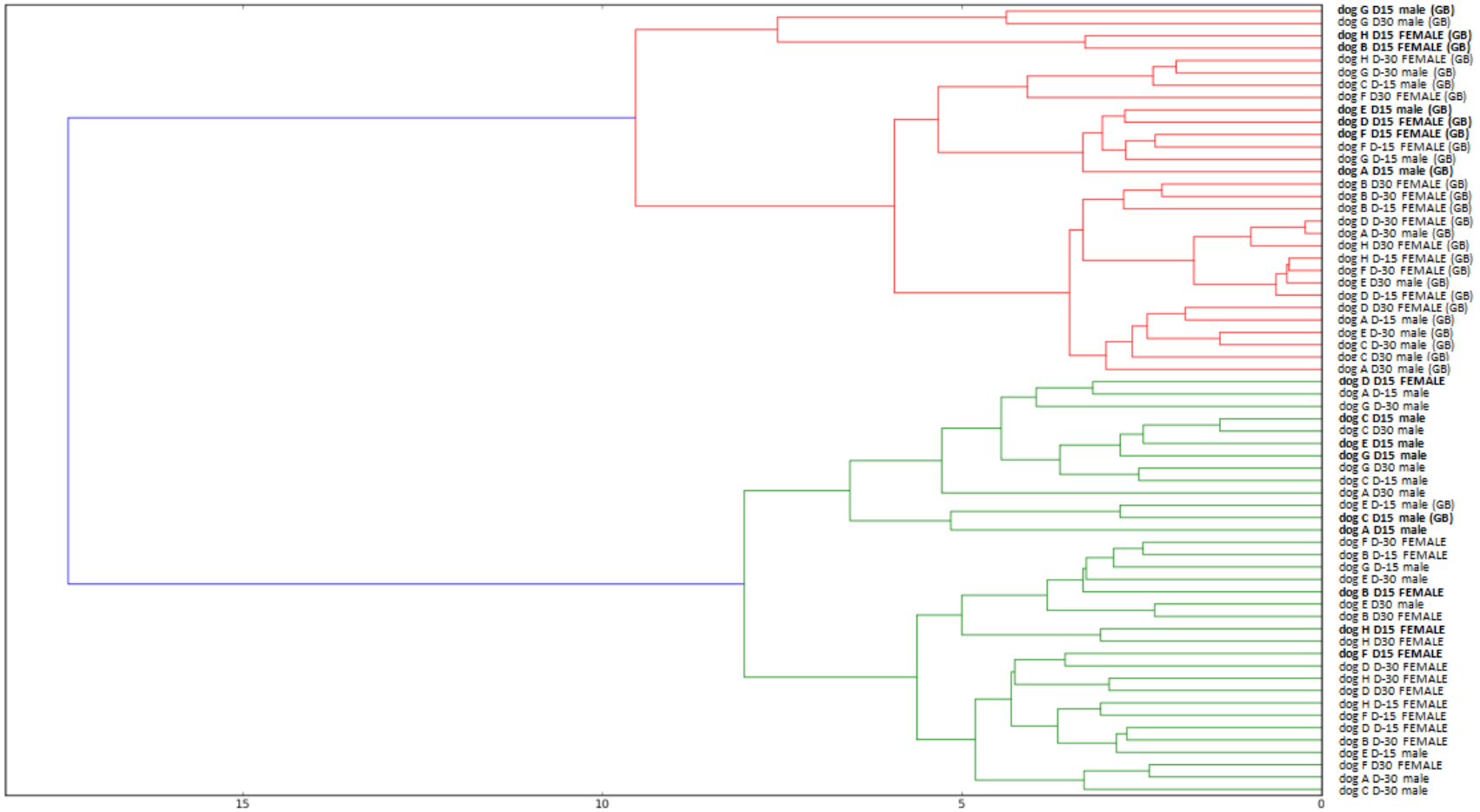
Supplementary Table A5 Number of microscopic fields analyzed for each gender for FISH analyzes in the gastric (stomach) and the duodenal (duodenum) biopsies.

	Stomach			
	Day -30	Day -15	Day 15	Day 30
males	185	155	145	151
females	137	121	145	135
	Duodenum			
males	106	111	136	124
females	114	125	143	130

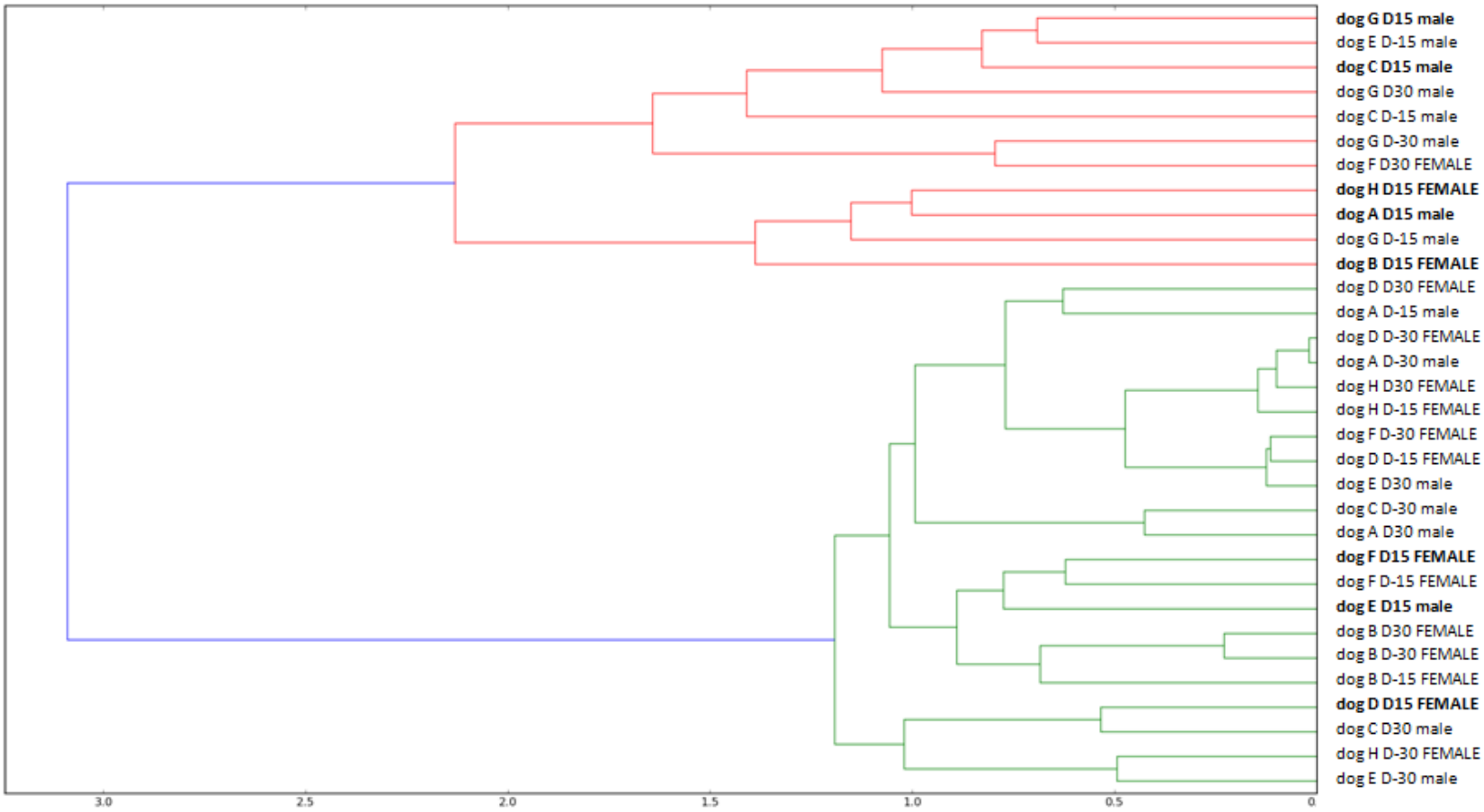
Supplementary Table A6 Median (interquartile range) proportions of pyrosequencing tags on Day 30 (Day -30) and Day 15 (Day -15) before initiation of omeprazole administration, Day 15 during omeprazole administration, and Day 30 after discontinuation of omeprazole treatment in the duodenum. P values come from the non-parametric Friedman's test.

	Day -30	Day -15	Day 15	Day 30	p value
Firmicutes	46.9 (21.2-86.4)	20.2 (9.6-88.2)	87.3 (40.4-98.8)	83.1 (16.0-98.6)	0.3266
Streptococcus	0.4 (0.1-2.2)	0.8 (0.0-1.6)	0.9 (0.5-3.9)	0.3 (0.0-1.2)	0.1331
Lactobacillus	1.7 (1.1-3.3)	0.5 (0.2-5.2)	5.3 (0.6-92.3)	2.6 (0.6-17.3)	0.4539
Enterococcus	0.0 (0.0-0.1)	0.0 (0.0-0.1)	0.5 (0.1-0.9)	0.0 (0.0-0.4)	0.0137
Clostridium	3.9 (0.7-18.1)	0.7 (0.4-2.3)	1.1 (0.1-17.4)	1.3 (0.4-27.5)	0.3691
Eubacterium	0.4 (0.0-1.1)	0.6 (0.2-0.7)	0.1 (0.0-0.5)	0.2 (0.0-0.6)	0.7748
Turicibacter	1.7 (0.0-33.6)	0.2 (0.0-1.6)	0.1 (0.0-0.2)	0.8 (0.4-40.5)	0.1261
Peptostreptococcus	0.2 (0.0-1.6)	0.9 (0.2-1.5)	0.1 (0.0-1.4)	0.2 (0.0-1.8)	0.7876
Gemella	0.4 (0.0-0.6)	0.1 (0.0-0.2)	0.3 (0.0-4.0)	0.2 (0.0-1.2)	0.3208
Granulicatella	0.0 (0.0-1.2)	0.3 (0.1-0.6)	0.0 (0.0-0.3)	0.0 (0.0-0.1)	0.2457
Roseburia	0.2 (0.0-1.9)	0.0 (0.0-0.4)	0.0 (0.0-0.2)	0.7 (0.1-0.8)	0.0529
Ruminococcus	0.1 (0.0-0.4)	0.0 (0.0-0.2)	0.0 (0.0-0.0)	0.2 (0.0-0.2)	0.0576
Allobaculum	0.1 (0.0-0.3)	0.2 (0.0-0.8)	0.1 (0.0-0.3)	0.0 (0.0-2.1)	0.6766
Abiotrophia	0.2 (0.0-3.4)	0.6 (0.2-1.1)	0.6 (0.0-3.3)	0.0 (0.0-2.1)	0.6463
Proteobacteria	21.2 (3.4-41.9)	15.9 (3.3-36.6)	5.6 (0.1-16.3)	11.1 (0.3-69.9)	0.1635
Helicobacter	2.2 (0.1-4.3)	0.3 (0.0-1.0)	0.0 (0.0-0.0)	5.4 (0.0-64.6)	0.0287
Achromobacter	0.5 (0.0-2.2)	1.1 (0.0-2.8)	0.0 (0.0-0.8)	0.1 (0.0-0.8)	0.3127
Moraxella	0.4 (0.1-1.5)	0.3 (0.0-1.3)	0.0 (0.0-0.2)	0.1 (0.0-0.3)	0.1559
Pasteurella	0.1 (0.0-0.4)	0.4 (0.0-0.7)	0.1 (0.0-0.7)	0.0 (0.0-0.1)	0.4180
Neisseria	1.0 (0.0-2.3)	1.2 (0.1-1.5)	0.1 (0.0-0.8)	0.1 (0.0-0.5)	0.2598
Bacteroidetes	6.9 (0.8-36.8)	23.7 (4.3-71.2)	1.6 (0.1-16.3)	2.2 (0.9-8.0)	0.4894
Prevotella	2.4 (0.4-10.4)	7.1 (1.0-12.4)	1.5 (0.0-10.7)	1.1 (0.3-3.5)	0.2830
Bacteroides	0.0 (0.0-1.5)	0.1 (0.0-0.2)	0.0 (0.0-0.1)	0.1 (0.0-0.1)	0.4753
Bergeyella	0.2 (0.0-0.6)	0.3 (0.0-1.0)	0.0 (0.0-0.4)	0.2 (0.0-0.3)	0.6233
Porphyromonas	2.3 (0.2-20.1)	10.1 (2.3-57.5)	0.0 (0.0-4.5)	0.5 (0.0-2.1)	0.0316
Actinobacteria	1.4 (0.4-4.0)	1.2 (0.4-4.5)	0.1 (0.0-2.3)	0.5 (0.1-0.8)	0.6688
Actinobacillus	1.0 (0.0-6.2)	6.1 (1.5-17.5)	2.4 (0.0-8.0)	0.4 (0.0-2.8)	0.0894
Leucobacter	0.1 (0.0-1.0)	0.1 (0.0-0.7)	0.0 (0.0-1.1)	0.0 (0.0-0.2)	0.9098
Actinomyces	0.3 (0.0-0.5)	0.1 (0.0-1.7)	0.0 (0.0-0.0)	0.0 (0.0-0.2)	0.0570
Collinsella	0.3 (0.0-1.1)	0.0 (0.0-0.2)	0.0 (0.0-0.1)	0.0 (0.0-0.2)	0.1086
Fusobacteria	0.2 (0.0-0.5)	0.2 (0.0-0.5)	0.0 (0.0-0.1)	0.0 (0.0-0.3)	0.3023
Fusobacterium	0.2 (0.0-0.5)	0.2 (0.0-0.5)	0.0 (0.0-0.1)	0.0 (0.0-0.2)	0.1584

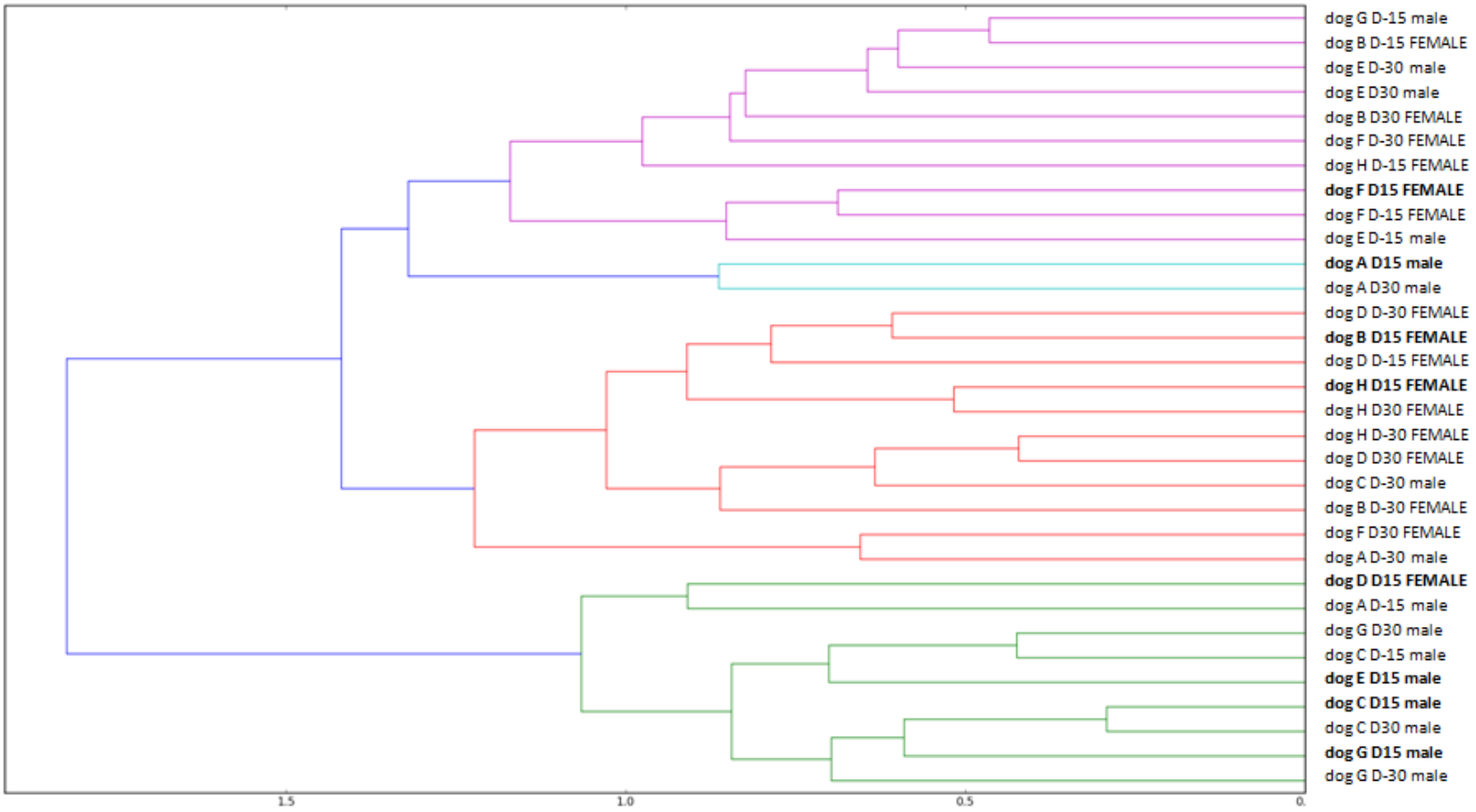
This table only shows those bacterial genera (and their respective phyla) that were identified in at least two time points in at least half the subjects. Firmicutes and Proteobacteria were the only two phyla that were detected in all animal subjects at all time points. Only the bacterial genera *Lactobacillus* and *Clostridium* were detected in all animal subjects at all time points.



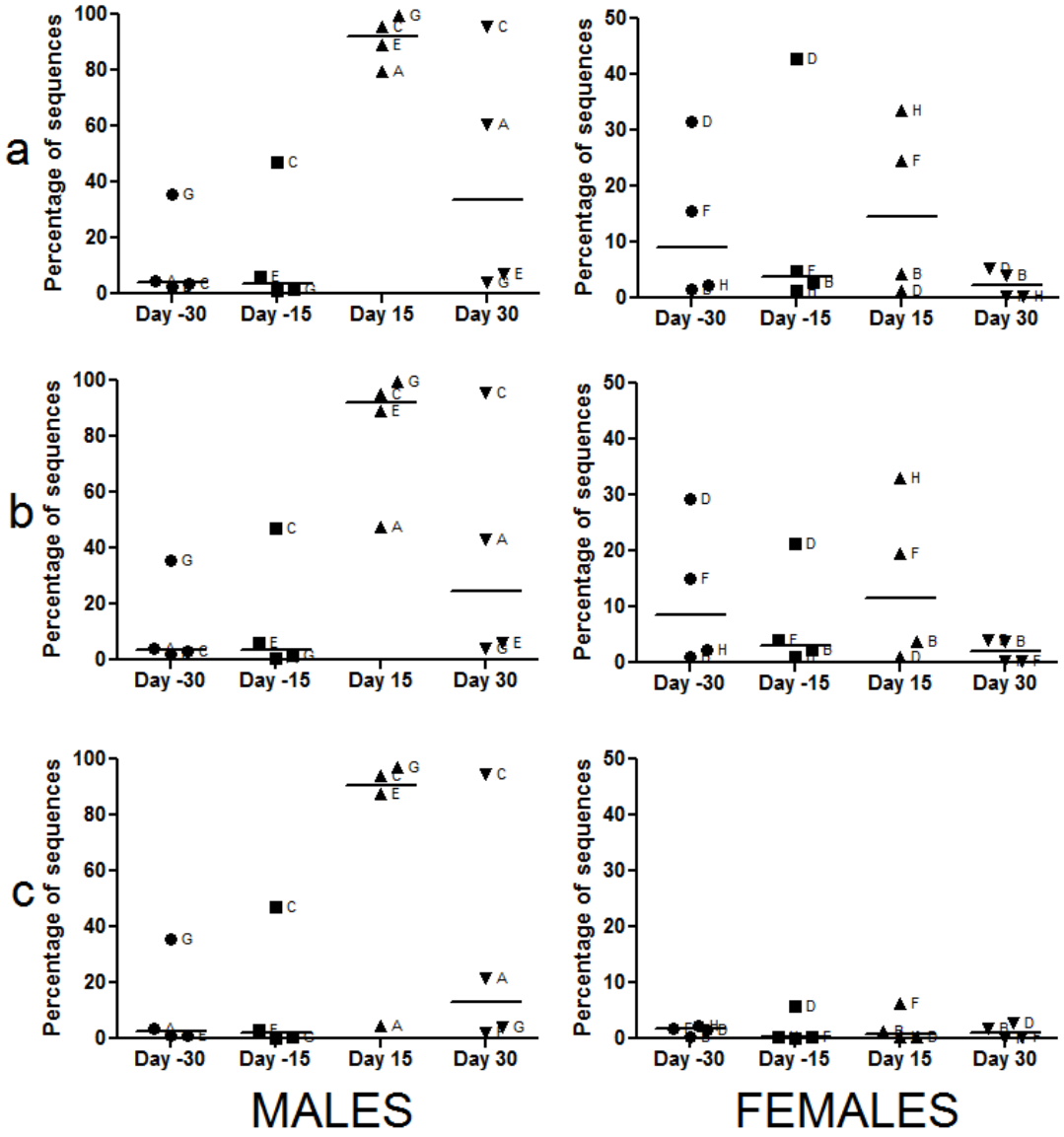
Supplementary Fig. A1. Dendrogram illustrating the phylogenetic clustering of the gastric (GB: gastric biopsy) and duodenal bacterial microbiota. Pyrosequencing was performed on all dogs (labeled A to H) on Day 30 (D-30) and Day 15 (D-15) before omeprazole administration, the last day of omeprazole administration (D15, bold), and 15 days after discontinuation of omeprazole administration (D30).



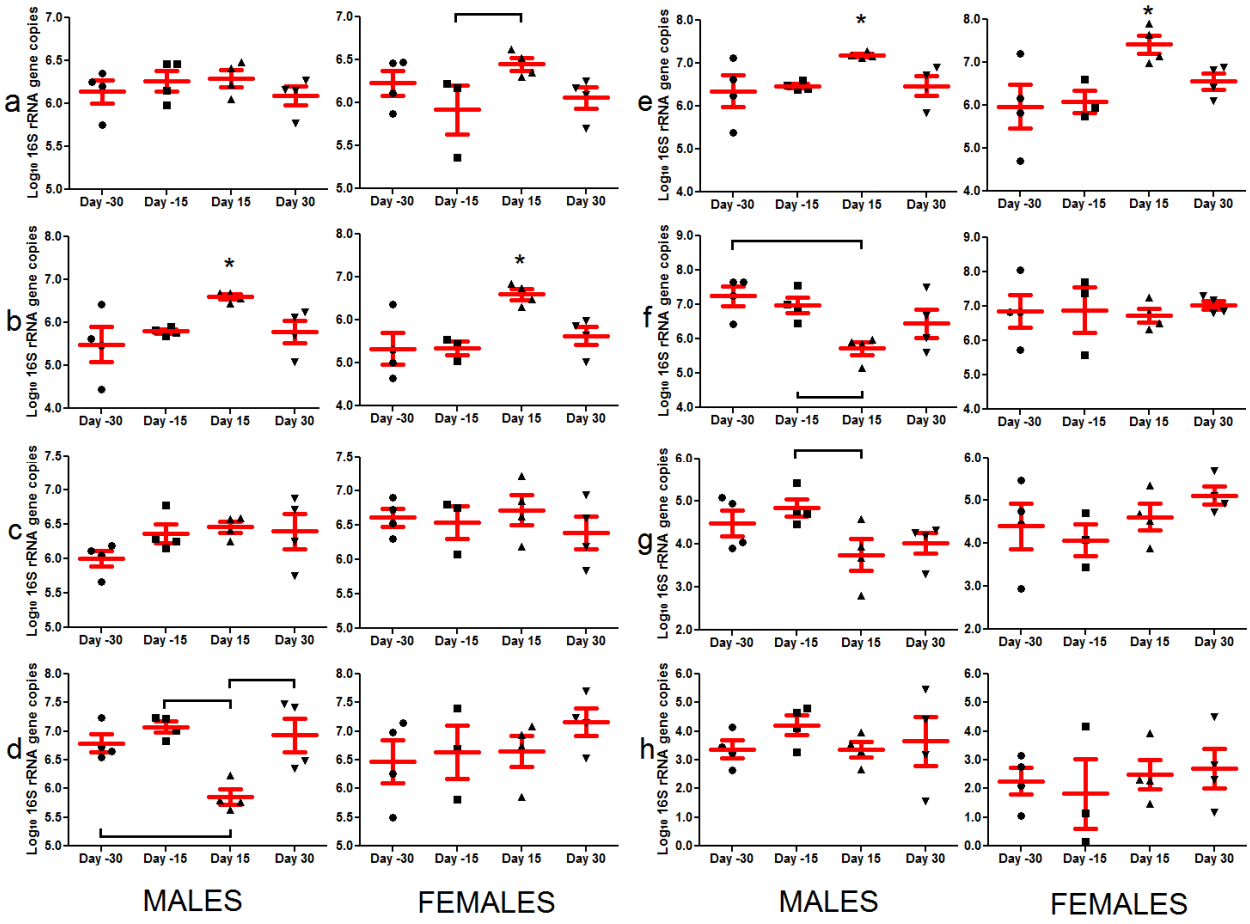
Supplementary Fig. A2. Dendrogram illustrating the phylogenetic clustering of the gastric microbiota. Pyrosequencing was performed on all dogs (labeled A to H) on Day 30 (D-30) and Day 15 (D-15) before omeprazole administration, the last day of omeprazole administration (D15, bold), and 15 days after discontinuation of omeprazole administration (D30).



Supplementary Fig. A3. Dendrogram illustrating the phylogenetic clustering of the duodenal microbiota. Pyrosequencing was performed on all eight dogs (labeled A to H) on Day 30 (D-30) and Day 15 (D-15) before omeprazole administration, the last day of omeprazole administration (D15, bold), and 15 days after discontinuation of omeprazole administration (D30).



Supplementary Fig. A4. Percentage of sequences in the male (left) and the female (right) dogs at the class Bacilli (a), order Lactobacillales (b), and *Lactobacillus* on Day 30 (Day -30) and Day 15 (Day -15) before omeprazole administration, the last day of omeprazole administration (Day 15), and on Day 30 after discontinuation of omeprazole administration. The line represents the median percentage of sequences and the letters identify the dogs. Notice that the y axis (percentage of sequences) is in a different scale for each gender.



Supplementary Fig. A5. Quantitative real-time PCR results for all fecal bacteria (a), Firmicutes (b), Ruminococcaceae (c), *Faecalibacterium* (d), *Lactobacillus* (e), the *Bacteroides-Prevotella-Porphyromonas* group (f), gammaproteobacteria (g), and *Bifidobacterium* (h) on Day 30 (Day -30) and Day 15 (Day -15) before omeprazole administration, during omeprazole administration on Day 15, and 15 days after completion of omeprazole administration (Day 30). Error bars represent the mean and the standard error. Horizontal brackets represent statistical significance ($p<0.01$). There was a significant interaction ($p<0.05$) between omeprazole administration and gender for all fecal bacteria (a), *Faecalibacterium* (d), the *Bacteroides-Prevotella-Porphyromonas* group (f) and gammaproteobacteria (g). * Statistically significantly different ($p<0.0001$) than all other time points regardless of gender. Notice that the y axis is in a different scale for each bacterial group.

VITA

Name: Jose Francisco Garcia Mazcorro

Permanent Address: Violetas 505 Industrias del Vidrio Oriente 3er sector
San Nicolas de los Garza 66470, Nuevo Leon, Mexico

Email Address: josegarcia_mex@hotmail.com

Education: M.V.Z., Degree in Veterinary Medicine, Universidad Autonoma de Nuevo Leon, 2005
Ph.D., Biomedical Sciences, Texas A&M University, 2011

Selected publications:

Garcia-Mazcorro JF, Lanerie D, Dowd S, Paddock C, Grützner N, Steiner JM, Ivanek R, & Suchodolski JS (2011) Effect of a multi-species synbiotic formulation on fecal bacterial microbiota of healthy cats and dogs as evaluated by pyrosequencing. *FEMS Micro Ecol* (in press).

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