ASSESSMENT OF THE CANINE INTESTINAL MICROFLORA USING MOLECULAR METHODS AND SERUM MARKERS

A Dissertation

by

JAN S. SUCHODOLSKI

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 2005

Major Subject: Veterinary Microbiology

ASSESSMENT OF THE CANINE INTESTINAL MICROFLORA USING MOLECULAR METHODS AND SERUM MARKERS

A Dissertation

by

JAN S. SUCHODOLSKI

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

Approved by:

Co-Chairs of Committee,	Gerald G. Wagner
	David A. Williams
Committee Members,	Jörg M. Steiner
	Thomas A. Ficht
	Randal K. Buddington
Head of Department,	Gerald R. Bratton

December 2005

Major Subject: Veterinary Microbiology

ABSTRACT

Assessment of the Canine Intestinal Microflora using

Molecular Methods and Serum Markers. (December 2005)

Jan S. Suchodolski, med. vet.; Dr. med. vet., University of Veterinary Medicine, Vienna, Austria

Co-Chairs of Advisory Committee: Dr. Gerald G. Wagner
Dr. David A. Williams

Previous studies examining the canine intestinal microflora have focused on cultivation of bacteria from intestinal content. Recently, it has been recognized that the majority of bacteria cannot be identified using standard culture techniques. The aim of this study was to describe the composition and dynamics of the canine intestinal microflora using molecular methods based on identification of the 16S ribosomal DNA (16S rDNA) and to evaluate the clinical use of a ^{13}C-glycocholic acid blood test (^{13}C-GCBT) as a serum marker for small intestinal bacterial biomass. Intestinal content was obtained from healthy dogs and the microflora was characterized in different compartments of each dog by denaturing gradient gel electrophoresis (DGGE) and comparative 16S rDNA analysis. A ^{13}C-glycocholic acid blood test (^{13}C-GCBT) was developed as a marker for small intestinal bacterial biomass and the influence of tylosin administration on the ^{13}C-GCBT, serum concentrations of cobalamin, folate, and unconjugated cholic acid (SUCA) was evaluated. There was marked variation in DGGE profiles between individual dogs and also between different intestinal compartments within dogs. DGGE profiles from duodenal juice samples collected endoscopically at different time-points varied within individuals, possibly due to variations over time or a slight variation in sampling location. Direct sequencing revealed 106 individual 16S rDNA sequences. Forty-two sequences showed less than 98% similarity to described sequences in public databases and may constitute previously uncharacterized bacterial

species. Serum folate concentrations, SUCA, and the cumulative percent dose/min of ^{13}C administered as ^{13}C-glycocholic acid (CUMPCD) increased significantly following tylosin administration ($p<0.01$). The results indicate that dogs have a complex intestinal microflora with marked differences between individual dogs. Different intestinal compartments appear to host a unique microflora and the assessment of a fecal sample does not yield accurate information about the composition of the microflora in proximal compartments of the gut. The intestine harbors many previously uncharacterized bacterial species. The clinical significance of these uncharacterized intestinal bacterial species needs to be further investigated in dogs with gastrointestinal disease. Increased serum folate, SUCA, and CUMPCD in the ^{13}C-GCBT suggest that, in the dogs described here, tylosin administration increased the biomass of organisms carrying out these metabolic functions.

DEDICATION

For Paulette and my family

ACKNOWLEDGMENTS

I would like to thank my committee members Dr. Gale Wagner, Dr. David Williams, Dr. Jörg Steiner, Dr. Randal Buddington, and Dr. Thomas Ficht for their guidance and expertise.

My special thanks go to Dr. Craig Ruaux for his never ending repertoire of ideas and his support in this undertaking. Thanks to Jennifer Camacho and Ladonna Wyatt for their help with the sequence analysis. Finally, thanks to the GI Lab team for all the individual contributions that made this project possible.

TABLE OF CONTENTS

CHAPTER Page

LIST OF FIGURES

FIGURE Page

LIST OF TABLES

CHAPTER I

INTRODUCTION

THE CANINE INTESTINAL MICROFLORA

The canine intestinal microflora has been mainly evaluated by direct culture techniques. It has been reported that the normal canine duodenal microflora harbors a total bacterial count below 10^5 colony forming units per ml of duodenal juice (cfu/ml) (77). Higher duodenal bacterial counts were considered to contribute to intestinal disease and lead to a clinical syndrome termed small intestinal bacterial overgrowth (SIBO) (77). Significantly higher counts, however, have subsequently been documented in dogs with no signs of intestinal disease (41), leading to controversy over the true quantitative composition of the healthy canine duodenal microflora. The jejunal microflora is quantitatively similar to the duodenal microflora (6) whereas the ileal microflora increases in bacterial counts to approximately 10^7 cfu/ml (6, 10, 17). The total bacterial count in the colon ranges between approximately 10^9 and 10^{11} cfu/ml (6, 17). Benno et al. described the canine intestinal microflora in different compartments of the gastrointestinal tract and in two different age groups using bacterial culture techniques (6). In dogs, less than 12 months of age the mean (±SD) aerobic bacterial count in the duodenum was $\log_{10}$ 5.2 (±1.9) cfu/ml, and the total anaerobic count was $\log_{10}$ 4.8 (±1.4) cfu/ml. These bacterial counts in the duodenum were higher than cut-off value previously recommended for SIBO. *Bacteroides, Clostridium, Lactobacillus, Bifidobacterium,* and *Enterobacteriaceae* spp. were the predominant bacterial groups cultivable from the canine intestine. Also, in one study the small intestine of older dogs (more than 11 years old) showed no qualitative or quantitative differences in bacteria compared to a group of younger dogs.

This dissertation follows the style and format of Journal of Clinical Microbiology.

However, there was a qualitative change in the bacterial microflora of the large intestine between these two groups. Older dogs had an increase in *Bacteroides, Clostridium,* and *Streptococcus* spp. with a decrease in *Bifidobacterium,* and *Lactobacillus* spp.

Another study evaluated postnatal changes of the small intestinal microflora in puppies (10). Bacterial counts in the small intestine were highest in 1 day old puppies and decreased significantly thereafter but remaining stable after day 42 with aerobic bacterial count of $\log_{10}$ 7 cfu/g and anaerobic count of approximately $\log_{10}$ 8 cfu/g, respectively.

Differences exist in bacterial counts between the intestinal lumen and the duodenal mucosa as fewer of the bacteria adherent to the mucosa have been isolated (18).

MECHANISMS REGULATING THE INTESTINAL MICROFLORA IN HEALTHY DOGS

Bacteria are constantly ingested with food. There are several physiological mechanisms that regulate bacterial colonization in the intestine including secretion of gastric acid and antibacterial factors (i.e., pancreatic and biliary secretions) in the small intestine, and most importantly, intestinal motility. Most ingested bacteria are inactivated by gastric acid. Humans with atrophic gastritis or who undergo acid suppressant therapy (e.g., omeprazole treatment) show an increase in bacterial counts in the small intestine (11, 22). In one case report a single dog was evaluated for chronic gastrointestinal disease, causing chronic diarrhea and weight loss (95). Based on quantitative and qualitative bacterial culture this dog was diagnosed with small intestinal bacterial overgrowth. The pH of fasted gastric juice was 7.0 (normal < 3.0). The authors concluded that achlorhydria was responsible for development of SIBO in this dog. In another study high gastric pH, despite stimulation with histamine, was observed in a dog with suspected bacterial overgrowth due to intestinal malabsorption (34).

The pancreatic juice contains antimicrobial substances that suppress excessive bacterial growth in the proximal small intestine (108). Dogs with spontaneous exocrine pancreatic insufficiency (EPI) appear to have a higher incidence of duodenal bacterial overgrowth. In one study 8 out of 11 dogs with EPI showed elevated bacterial counts

based on duodenal culture results (109). In another study, six dogs with EPI that received no treatment of any kind had > 10^6 colony forming units per ml duodenal juice (cfu/ml). Similar to this untreated group, six additional dogs that received only pancreatic enzyme supplementation also had elevated bacterial counts. Dogs that received both, pancreatic enzyme supplementation and antibiotic therapy, had significantly lower duodenal bacterial counts when compared to the first two groups. In another study 6 dogs underwent pancreatic duct ligation as a model for experimentally induced EPI (91). Total numbers of aerobic and anaerobic bacteria present in the duodenum increased significantly after pancreatic duct ligation. There was also a qualitative change in the microflora, with an increase in *Lactobacillus* spp. and *Streptococcus* spp. in 3 of 6 dogs, and an increase in *Bacteroides* spp. and *Clostridium* spp. in the other three dogs. Treatment with pancreatic enzyme supplementation (bovine pancreatic extract) led to a reduction in bacterial numbers to pre-surgical levels (91).

The ileocolic valve is believed to serve as a natural barrier between the small and large intestine in dogs (31). This barrier, together with intestinal motility, is believed to prevent retrograde translocation of bacteria from the large into the small intestine. Dysfunction of the ileocolic valve has been suggested as one possible cause of bacterial dysregulation, such as small intestinal bacterial overgrowth (31, 45). Normal intestinal motility is believed to be the most important factor to maintain the physiologic balance of the intestinal microflora. Blind and stagnant loops are a common site of bacterial overgrowth in humans and motility changes after surgery predispose to bacterial overgrowth in humans (29). In a case report a dog diagnosed with duodenal bacterial overgrowth was shown to have two blind intestinal loops on necropsy (110).

INTESTINAL MICROFLORA AND HOST HEALTH

The resident intestinal microflora offers nutritional benefit to the host by production of short chain fatty acids (e.g., butyrate, propionate, and acetate), which stimulate mucosal growth and epithelial cell proliferation. Lactate produced by microbial fermentation also serves as an energy source for the host. In addition, the normal intestinal microflora plays a major role in protection of the host from invasion by

harmful bacteria through competitive exclusion of potentially pathogenic organisms. The implicated defense mechanisms include competition for oxygen and nutrient substrates, competition for mucosal adhesion sites, creation of a physiologically restrictive environment for non-resident bacterial species (e.g., production of metabolic substances that are toxic to other bacteria, changes in pH and redox potential, hydrogen sulfide production), and secretion of antimicrobial substances (e.g., bacteriocins) (43). In animal models *Lactobacillus plantarum* significantly reduced bacterial translocation into mesenteric lymphnodes and the pancreas in rats with experimentally induced pancreatitis (53). *Lactobacillus johnsonii*, a bacterium that is used as a probiotic and is native to the small intestine is able to compete with enteropathogens for the same carbohydrate receptors in vitro, thus potentially preventing adhesion of pathogenic bacteria in the gut (65).

Anecdotal evidence existed for many years that feeding of yoghurt, containing lactic acid bacteria, exhibits a beneficial effect on the host. However, only recently, well designed double blinded prospective studies have been published that have evaluated the contributions of probiotics and prebiotics to the prevention and treatment of intestinal disease by modulating the intestinal microflora in order to increase the number of "protective" bacteria (79). Probiotics are defined as viable microorganisms, that when ingested, promote prevention and treatment of specific pathological conditions (79). Probiotics include *Lactobacillus* spp., *Bifidobacterium* spp., *Streptococcus* spp., *Enterococcus faecium,* non-pathogenic *Escherichia coli* strains, and *Saccharomyces boulardii*. Prebiotics are non digestible food ingredients that beneficially affect the host by stimulating the growth and/or activity of beneficial bacteria in the colon. Most commonly used prebiotics are inulin, fructooligosaccharides (FOS), and oligofructose.

In order for a probiotic to be effective it must to have several properties; it must be: 1) acid resistant in order to survive passage through the stomach, 2) resistant to bile acids, and 3) able to colonize the intestine for an extended period of time. Several recent studies have evaluated these properties for various probiotic strains *in vitro* and *in vivo* in order to select potential probiotic strains for future clinical use. Results of these

studies indicate that most probiotics can be recovered in fecal samples, indicating that they are viable in the intestinal tract, but most probiotics are quickly eliminated when therapy is discontinued (79). Thus, most present probiotics do not appear to colonize the intestinal tract and, in order to exhibit a beneficial effect in the host, prolonged administration of high doses (usually $>10^{10}$ cfu/g) is necessary. Only few prospective double-blinded placebo controlled studies have been published that evaluate the effect of probiotics in humans with intestinal disease. Probiotic strains, including *Lactobacillus* spp., *Enterococcus faecium,* and *Saccharomyces boulardii*, have been demonstrated to be useful in prevention of antibiotic-induced diarrhea and traveler's diarrhea (7). However, it is important to note that probiotic therapy, while significantly reducing the incidence of diarrhea, did not prevent disease in all tested subjects. Thus probiotic therapeutics containing only selected microorganisms might be useful only for a subpopulation of patients and it is possible that different patients require different strains of probiotics in order to have a protective effect.

THE INTESTINAL MICROFLORA IN DISEASE

In a study published in 1983 the duodenal microflora was compared between German Shepherd dogs with chronic gastrointestinal disease and healthy Beagle dogs (5). In this study bacterial counts in dogs with diarrhea were significantly higher than in healthy dogs. The authors concluded that intestinal disease was caused by bacterial overgrowth in the small intestine and proposed that total bacterial counts higher than 10^5 colony forming units per ml of duodenal fluid (cfu/ml) or an anaerobic count higher than 10^4 cfu/ml was diagnostic for small intestinal bacterial overgrowth (SIBO). These cut-off values are similar to those established for diagnosis of SIBO in humans (4).

Proposed effects of an abnormal bacterial flora include damage of carrier proteins with subsequent decrease in absorption of amino acids and monosaccharides, increased deconjugation of bile acids resulting in disturbed fat malabsorption, secretion of toxins that are toxic to enterocytes, hydroxylation of fatty acids contributing to diarrhea, and competition for nutrients und vitamins (e.g., cobalamin) (41).

Subsequent studies performed in dogs with intestinal disease used the above criteria for diagnosing SIBO in dogs (107). Significantly higher counts, however, have subsequently been documented in dogs with no signs of intestinal disease (41), leading to controversy over the true quantitative composition of the normal canine duodenal microflora. Recent studies performed in dogs with chronic enteropathies found no correlation between the number of bacterial counts in the duodenum and clinical signs (25). Some dogs with suspected SIBO had numbers that were substantially lower than 10^5 cfu/ml. Since this condition usually responds to antibiotic treatment, some authors propose the term "antibiotic responsive diarrhea" rather than SIBO (25). However, it should be pointed out that both terms can not be used synonymously as some dogs diagnosed with SIBO based on findings traditionally associated with SIBO do not respond to antibiotic therapy and some dogs that respond to antibiotic therapy do not have findings typically associated with SIBO.

Inflammatory bowel disease (IBD) is one of the most common chronic enteropathies in humans as well as dogs (39). It has been hypothesized that IBD is caused by a loss of tolerance to commensal bacteria or due to bacterial dysregulation (51). The commensal intestinal microflora may contribute or maintain intestinal inflammation in patients with IBD. A study in human patients with active inflammatory bowel disease has shown a reduced bacterial diversity in the colon compared to healthy controls as demonstrated by molecular fingerprinting (67). From 3 individuals bacteria were also identified by direct 16S rDNA sequence analysis. The majority of identified bacteria belonged to the normal anaerobic microflora of the human gut. However, 30% of obtained 16S rDNA sequences represented not yet characterized bacterial species and their role in the pathogenesis of human IBD needs to be further investigated. A study evaluating bacterial diversity of colonic mucosal samples from patients with ulcerative colitis using quantitative dot blot hybridization have found an increase in *Enterobacteriacae* and a decrease in "protective" bacteria such as *Lactobacillus* and *Bifidobacteria* spp. (83).

CHARACTERIZATION OF THE INTESTINAL MICROFLORA

Previous studies characterizing the intestinal bacterial flora in humans and various other species have focused on the identification and enumeration of bacterial species through direct cultivation of intestinal content (6, 17). Recently, limitations of bacterial culture have been recognized. Bacterial culture is technically complex and expensive. Anaerobic bacteria are potentially more prone to damage during sampling, shipping, and storage. Different laboratories use different protocols and not all selective media are completely selective. For example, a study characterizing the fecal microflora from a Labrador Retriever dog demonstrated that despite using Beerens agar, a medium designed for the isolation of *Bifidobacterium* spp., a mixture of various organism other than *Bifidobacterium* spp. were isolated (30). Today it is generally recognized that the majority of microbial species present in intestinal samples can not be cultured. This is believed to be due our lack of knowledge of microbial growth requirements. In contrast, studies using molecular methods have identified a greater number of bacterial species present in the intestine of various species (37, 70, 96). It is important to note that these initial studies have focused almost exclusively on the fecal microflora. Also, samples from only few individuals have been analyzed. Based on a 98% similarity criterion (which is commonly used in these types of studies), 82 operative taxonomical units (OTU) were described in a fecal sample of one healthy man (96). Only 24% of these OTUs belonged to previously characterized bacterial species. Based on phylogenetic analysis the majority of obtained OTUs belonged to the orders *Bacteroidales* and *Clostridiales*. Hayashi and coworkers evaluated the fecal microflora from 3 healthy individuals (33). They identified 130 unique OTUs, and only 25% of these had greater than 98% similarity to previously characterized bacterial species. In this study the majority of bacteria were *Clostridium, Bacteroides, Bifidobacterium,* and *Streptococcus* spp. Interestingly, in contrast to Hayashi et al., the study performed by Suau et al., failed to identify members of the *Bifidobacterium* group, believed to be well established members of the human colonic microflora (19). The authors contributed this finding to potential mismatches between the nucleotide sequences of commonly used universal

primers and nucleotide sequences specific for *Bifidobacterium* spp., indicating a potential shortcoming of direct 16S rDNA sequence analysis.

In humans most studies evaluating the intestinal microflora have some limitations: due to the ease of collection only fecal samples have been analyzed in most studies and only a small number of subjects have been evaluated. Studies in pigs and chickens using molecular fingerprinting and direct 16S rDNA analysis have demonstrated that the intestinal microflora varies in different compartments of the intestinal tract (72, 102). Recent studies have evaluated the microflora, using direct 16S rDNA analysis and molecular fingerprinting, of both luminal samples and mucosal biopsies obtained from the ileum and colon of humans (97, 104, 114). These studies have demonstrated qualitative variations in the intestinal microflora between fecal samples and samples obtained from more proximal parts of the intestine. Also mucosa adherent populations were different from luminal bacteria (114). Fewer members of *Bacteroides* were found in the gut-adherent microflora when compared to that of the intestinal lumen (114). Recent studies have also demonstrated remarkable inter-individual variation in human fecal samples (111). Also, it has been reported that monozygotic twins have a significantly higher similarity of fecal molecular fingerprinting patterns than unrelated individuals, suggesting the host's genotype may have an influence on the intestinal microflora (111).

MOLECULAR FINGERPRINTING TECHNIQUES

Molecular fingerprinting techniques, e.g. denaturing gradient gel electrophoresis (DGGE) allow rapid assessment of the predominant bacterial species present in a sample. Molecular fingerprinting techniques are commonly used to assess genetic diversity in a complex microbial community (49, 90). Amplification of 16S rDNA with subsequent separation of amplicons by denaturing gradient gel electrophoresis (DGGE) has been used for evaluation of bacterial diversity in environmental samples (63). The principle of DGGE is based on the decreasing electrophoretic mobility of double-stranded DNA as it is partially melted (64). Briefly, DNA is extracted from biological samples, and 16S rDNA amplified using universal primers that target conserved regions

(located up- and downstream of variable regions) within the gene encoding 16S rRNA. This approach allows amplification of unknown bacterial species. The mixture of PCR products, all approximately of the same length, are subsequently separated on a polyacrylamide gel containing a linear gradient of DNA denaturants (63). Sequence differences in the double stranded DNA influence the melting behavior of PCR amplicons. Therefore, PCR amplicons with a differing sequence will stop migrating at different positions in the gel. This results in the separation of amplicons, and a pattern of separated bands that illustrates the bacterial diversity in a sample. The DGGE profile typically represents up to 99% of the total bacterial community present in a biological sample (63). While molecular fingerprinting does not allow for immediate discrimination between bacterial species, it does allow for simultaneous analysis of multiple samples and, thus, direct comparison of microbial communities from different samples (63). Molecular fingerprinting further allows the study of changes in individual microbial communities over time and in response to treatment. Also, molecular fingerprinting has been used to evaluate differences in bacterial diversity between healthy and diseased individuals. For example, a reduction in bacterial diversity has been found in the colonic microflora of humans with active inflammatory bowel disease, warranting similar bacterial diversity studies in dogs with intestinal disease (67).

SERUM MARKERS FOR ASSESSMENT OF THE SMALL INTESTINAL MICROFLORA

While molecular techniques allow for accurate identification and characterization of intestinal bacteria they are impractical for clinical application as sampling of intestinal content requires invasive procedures such as endoscopy or laparoscopy. Several indirect serum markers are commonly used for assessment of small intestinal bacterial biomass. These include serum concentrations of vitamins, cobalamin and folate, and measurement of serum unconjugated cholic acid (SUCA).

Serum cobalamin and folate concentrations. Cobalamin (vitamin B12) and folate uptake from the small intestine can be affected by several factors and can therefore be utilized as an indirect marker of gastrointestinal disease. Small intestinal

inflammation, exocrine pancreatic insufficiency (EPI), and small intestinal bacterial overgrowth (SIBO) all can lead to changes in serum cobalamin and/or folate concentrations (108). In addition, measurement of serum cobalamin and folate may yield information on the site and cause of intestinal disease. Cobalamin is a water-soluble vitamin and an important co-factor for a variety of biochemical reactions. Cobalamin is usually abundant in commercial canine and feline diets, making a dietary insufficiency unlikely. The mechanisms involved in cobalamin absorption are complex and depend on intact gastrointestinal function. In the diet, cobalamin is tightly bound to dietary protein. After digestion of these dietary proteins in the stomach by pepsin and hydrochloric acid, cobalamin is released and immediately bound to R-protein, a protein secreted in saliva and gastric juice. Pancreatic enzymes (i.e., trypsin and chymotrypsin) digest R-protein, again releasing cobalamin. Intrinsic factor, produced in the stomach and pancreas binds to cobalamin and serves as a transporter to the distal small intestine (i.e., ileum) where the cobalamin/intrinsic factor complexes are absorbed by specific receptors located in the ileal mucosa.

Folate is also a water soluble vitamin that, similarly to cobalamin, is abundant in commercial canine and feline diets, again making a nutritional deficiency unlikely. Dietary folate is usually present in the poorly absorbable polyglutamate form. Folate deconjugase, a brush border enzyme secreted in the jejunum, removes all but one glutamate residue from the molecule. Specific carriers for folate monoglutamate in the proximal small intestine will promote folate uptake.

Assessment of serum cobalamin and folate concentrations is the most clinically useful aid in the diagnosis of SIBO although they have poor sensitivity and specificity (25). Serum cobalamin may be decreased and serum folate may be increased in animals with SIBO. If both serum vitamin concentrations are altered this is highly suggestive of SIBO. Aberrations in the small intestinal microflora may lead to increased competition for cobalamin resulting in decreased absorption. *Bacteroides* spp. are the principle organisms involved in cobalamin competition since they can utilize cobalamin-intrinsic factor complexes, while other bacteria can only bind free cobalamin, which is present in

lower concentrations in the gut. The reported sensitivity of serum cobalamin concentration for the diagnosis of SIBO ranges between 25 to 55% (25). Bacteria present in the distal small intestine and large intestine produce large quantities of folate, which is excreted in feces. Folate carriers responsible for folate uptake are located exclusively in the proximal small intestine and thus folate produced in distal sections of the intestine will not be absorbed. If folate producing bacteria migrate upwards into the proximal small intestine, folate of bacterial origin can be absorbed by the host resulting in elevated serum folate concentrations. The reported sensitivity of serum folate concentration for the diagnosis of SIBO in dogs ranges from 50 to 66% (25). Dogs with exocrine pancreatic insufficiency (EPI) have a decreased secretion of antibacterial products with subsequent small intestinal bacterial overgrowth. As a consequence, dogs with EPI often have increased serum folate concentrations.

Serum unconjugated cholic acid concentration (SUCA). Measurement of SUCA allows indirect assessment of the metabolic activity of the small intestinal microflora (57). After feeding, gallbladder contractions leads to release of conjugated bile acids, the principal ones in dogs being cholic acids, into the intestinal lumen where they facilitate fat absorption. Because of their relatively hydrophilic nature, conjugated bile acids undergo only minimal passive intestinal reabsorption and more than 90% of conjugated bile acids are absorbed in the distal ileum through specific transporters and subsequently undergo an enterohepatic circulation. Only a small portion of conjugated bile acids will reach the colon and will be deconjugated by the resident colonic bacteria. Bacteria present in the lumen of the small intestine can deconjugate bile acids through the action of deconjugases, a feature unique to bacteria (66). Unconjugated bile acids are readily absorbed by passive diffusion and transported to the liver (81). Hepatic clearance of unconjugated bile acids during “first-pass” circulation is less efficient when compared to that of conjugated bile acids, so that 50% of the unconjugated bile acids remain in serum where they can be measured by gas chromatography and mass spectrometry. Because deconjugation of bile acids in the small intestine is unique to bacteria, measurement of unconjugated bile acid and more specifically cholic acid concentration

in serum is considered an index of bacterial activity in the small intestine (57). The SUCA test has been validated for the use in dogs with a reported initial sensitivity of 80% (57). In recent studies, however, the sensitivity and specificity of SUCA for dogs suspected of having SIBO was reported to be much lower and further studies will be required in order to conclusively determine clinical utility of SUCA for dogs with suspected SIBO (25).

Other tests that are based on the deconjugation of bile acids are the ^{14}C- and the ^{13}C-glycocholic acid test that have been used to diagnose small intestinal bacterial overgrowth in humans (71, 93). The ^{13}C-glycocholic acid test is based on deconjugation of the naturally occurring bile acid glycocholic acid by intestinal bacteria. ^{13}C-glycocholic acid (GCA) is the glycine conjugate of cholic acid. One carbon of the glycine portion is labeled with ^{13}C, a stable carbon isotope. After oral administration, GCA reaches the small intestine where it enters the endogenous bile acid pool and undergoes enterohepatic recirculation. Bacteria present in the small intestine are capable of deconjugating the ^{13}C-glycine portion from the core bile acid. The ^{13}C-glycine is metabolized by intestinal bacteria and ^{13}C is released as $^{13}CO_2$. The ^{13}C diffuses into the blood where it may be transported in three different forms, dissolved in the blood, bound to hemoglobin, and as bicarbonate. The $^{13}CO_2$ can be quantified in blood samples or is eventually exhaled and can then be quantified in breath samples by fractional mass spectrometry (16, 61, 71).

HYPOTHESES AND SPECIFIC OBJECTIVES

The hypotheses of this study are 1) that molecular methods may be used to evaluate bacterial diversity in the canine intestinal tract and 2) that indirect serum markers of canine intestinal microbial biomass and metabolic activity are sensitive enough to detect changes in the composition of the canine intestinal microflora.

The objectives of the proposed research project in order to prove or disprove these hypotheses were 1) to develop a molecular fingerprinting technique for the qualitative assessment of bacterial diversity in the canine intestinal tract, 2) to evaluate the variation of the small intestinal microflora during repeated sampling using molecular

fingerprinting, 3) to evaluate qualitative differences in intestinal bacterial diversity between individual dogs and between different compartments of the intestinal tract within individual dogs using molecular fingerprinting, 4) to identify bacterial species present in the canine intestinal tract by direct sequencing of the 16S rRNA gene, 5) to develop a ^{13}C-glycocholic acid blood test as a minimally invasive marker for assessment of bacterial biomass and it's metabolic activity in the canine small intestine, and 6) to evaluate changes in indirect markers of the intestinal microflora after antibiotic therapy.

CHAPTER II

APPLICATION OF MOLECULAR FINGERPRINTING FOR QUALITATIVE ASSESSMENT OF SMALL INTESTINAL BACTERIAL DIVERSITY IN DOGS*

OVERVIEW

The aims of this study were to evaluate the use of molecular fingerprinting for assessment of bacterial diversity in canine duodenal juice and to evaluate the variation in small intestinal microflora at repeated sampling. Two groups of dogs (n = 14) were used. Duodenal juice was collected from 8 dogs euthanatized for an unrelated project (group 1). Duodenal juice was also collected endoscopically from 6 dogs at weekly intervals for a total of 3 weeks (group 2). The variable V6-V8 region of bacterial 16S ribosomal DNA was amplified and PCR amplicons separated by denaturing gradient gel electrophoresis (DGGE). The reproducibility of DGGE profiles, and variations in bacterial diversity between dogs, was evaluated by comparing similarity indices (Dice's coefficient, 100% represents complete identity) of DGGE profiles from group 1 dogs. Weekly variations in small intestinal flora were evaluated by comparison of DGGE profiles from different time-points within the same individuals in group 2. Mean±SD similarity of DGGE profiles of duodenal juice between the dogs in group 1 was 38.3 ± 15.7% (range: 12.5-76.65%). There was a significantly higher variation in DGGE profiles between different dogs than between duplicates obtained from the same dog ($p<0.0001$).

---

*Reprinted with permission from **Suchodolski JS, Ruaux CG, Steiner JM, Fetz K, and Williams DA**. Application of molecular fingerprinting for qualitative assessment of small intestinal bacterial diversity in dogs. 2004. J. Clin. Microbiol. **42**:4702-4708.

DGGE profiles from samples collected at different time-points varied within individuals, possibly due to variation over time or slight variation in sampling location. DGGE profiles indicate that dogs have a highly diverse small intestinal microflora, with marked differences between individual dogs.

INTRODUCTION

The domestic dog plays several important roles in modern human society. Dogs are commonly used as a model species for biomedical research, as well as being commonly kept as pets. The normal canine small intestinal microflora is not well characterized. Previous studies aiming to characterize the canine small intestinal bacterial flora have focused on the enumeration and identification of bacterial species from direct cultivation of duodenal juice (5, 78, 106). This technique is considered to be the gold standard for the diagnosis of small intestinal bacterial overgrowth (SIBO) in dogs. Bacterial culture, however, has limitations for assessing bacterial diversity in the gut. Samples of duodenal juice must be processed immediately in order to accurately represent the aerobic and anaerobic microbial flora of the small intestine. Thus, an on-site microbiology laboratory is required in order to obtain reliable bacterial counts when culturing duodenal juice. It is increasingly recognized that the majority of microbial species present in biological samples escape identification by use of standard culture techniques alone (1, 9, 48, 49, 70, 96). Studies using a molecular biological approach, based on identification of 16S ribosomal RNA (rRNA) or 16S ribosomal DNA (DNA encoding the16S rRNA), have identified a greater number of bacterial species when compared to standard culture techniques, indicating that only a small proportion of bacterial species are cultivable with standard bacterial culture techniques (37, 70, 96). It has been estimated, for example, that 60 to 80% of bacterial organisms present in the gastrointestinal tract of humans and pigs have not yet been cultivated (48, 49). Based on these studies, it is very likely that a culture-dependent approach underestimates the bacterial diversity found in the small intestinal fluid of dogs, and an approach based on molecular fingerprinting techniques may identify greater small intestinal bacterial diversity in the domestic dog than previously reported.

Molecular fingerprinting techniques are commonly used to illustrate the genetic diversity in a complex microbial community (49, 90). Amplification of 16S rDNA with subsequent separation of amplicons by denaturing gradient gel electrophoresis (DGGE) has been used for assessment of bacterial diversity in environmental samples (63). The principle of DGGE is based on the decreasing electrophoretic mobility of double-stranded DNA molecules when they are partially melted (64). Briefly, DNA is extracted from biological samples, and 16S rDNA amplified using primers that target conserved regions (located up- and downstream of variable regions) within the gene encoding 16S rRNA. This approach allows amplification of unknown bacterial species. The mixture of PCR products, all approximately of the same length, are subsequently separated on a polyacrylamide gel containing a linear gradient of DNA denaturants (63). Sequence differences in the double stranded DNA influence the melting behavior of the PCR amplicons and, therefore, PCR amplicons with different sequences will stop migrating at different positions in the gel. This results in separation of amplicons, and the pattern of separated bands illustrates the bacterial diversity in the sample.

The aims of this study were to evaluate the utility of DGGE fingerprinting for the qualitative assessment of bacterial diversity of the canine small intestinal microflora, and to evaluate weekly variations in small intestinal bacterial diversity.

MATERIALS AND METHODS

Sample material. Duodenal juice samples were collected from 2 groups of research dogs, all with no clinical signs of gastrointestinal disease. The protocol for sample collection was approved by the University Laboratory Animal care committee at Texas A&M University (AUP #2002-103). **Group 1** consisted of 8 Hound dogs (4 male, 4 female) that were euthanatized as part of an unrelated research project. The mean age was 3.9 years (range: 2.5–6.0 years). All dogs were housed in the same environment and fed the same regular canine maintenance diet. Food was withheld for 24 hours before euthanasia. Samples were collected in duplicate from each dog and treated as independent samples to evaluate the reproducibility of the DNA extraction, amplification of bacterial DNA by polymerase chain reaction (PCR), and the separation of amplicons

by DGGE. Immediately after euthanasia the abdominal cavity was opened, the duodenum isolated, and two samples, each of approximately 0.5 ml of duodenal juice, were collected from approximately the same collection site by needle aspiration using a sterile 16-gauge needle attached to a sterile 3-ml syringe. **Group 2** consisted of 6 healthy research Beagles enrolled in an unrelated research project that required weekly anesthesia. Mean age of these dogs was 5.2 years (range: 2-6.8 years). These dogs were housed in the same environment and fed a regular canine maintenance once daily. Food was withheld for 24 hours before induction of anesthesia on each study day. On the first day of the study serum was obtained and serum cobalamin, serum folate, serum trypsin-like immunoreactivity (TLI), and serum unconjugated cholic acid (SUCA) concentrations were evaluated to screen for gastrointestinal disease. On each study day, dogs were anesthetized and approximately 0.5 ml of duodenal juice was aspirated using a sterile syringe attached to a sterile, 1 mm wide, 150 cm tube introduced through the working channel of an endoscope (Flexible endoscope, Olympus, Melville, NY). The collection site was approximately 10 cm distal to the major duodenal papilla. Between each dog, the endoscope was cleaned (Megazyme, PEA Products Inc., Hunt Valley, Mass.) and disinfected (Cidex, Advanced Sterilization Products, Irvine, Calif.) according to the manufacturer's instructions. This sampling procedure was repeated once a week for a total of 3 weeks (samples 1, 2, and 3). In week 3, in addition to sample 3 a second sample of duodenal content was collected using a disposable sterile cytology brush (Disposable Cytology Brushes, 1.7 mm x 160 cm, Horizons International Corp., Puerto Rico) introduced through the working channel of the endoscope (sample 3A).

Storage of samples. All samples were immediately transferred to sterile cryotubes (Cryule 2 ml, Wheaton, Millville, NJ), snap-frozen in liquid nitrogen, and stored at -80ºC until further analysis.

Extraction of DNA. To each sample of duodenal juice 500 µl of cell lysis solution (Puregene® cell lysis solution, Gentra Systems, Minneapolis, Minn.), 200 µl of buffer saturated phenol:chloroform:iso-amylalcohol (ratio 25:24:1, pH 7.2), and 300 µl of 0.1 mm zirconia beads (BioSpec Products Inc., Bartlesville, Okla.) were added. The

tubes were positioned horizontally on a vortex adapter (Ambion Inc., Austin, Tex.) mounted on a standard vortexer and the mixture was vortexed for 5 minutes at maximum speed. The tubes were centrifuged for 7 min at 12,000 X *g* at 4ºC and the supernatant transferred to a new, sterile, cryotube. Then 700 µl of phenol:chloroform:iso-amylalcohol was added, the tube vortexed for 30 sec and centrifuged for 20 min at 12,000 X *g* at 4ºC. The aqueous phase was transferred into a new sterile cryotube. To increase the DNA yield, 200 µl of buffer (10 mM Tris-HCL, 1 mM EDTA, [pH 8.5]) was added to the remaining phenol and organic phase, the above described extraction procedure repeated, and both aqueous phases so obtained were combined. To remove RNA, 5.2 U of RNAse (Puregene® RNAse, Gentra Systems) was added to the solution and incubated at 37ºC for 30 minutes. The RNAse was removed by phenol:chloroform:iso-amylalcohol extraction as described above. The aqueous phase containing DNA was mixed with 0.5 volumes of 100% ethanol and applied to commercially available spin columns (GenElute Bacterial Genomic DNA Kit, Sigma Chemicals, St. Louis, Mo.). Bound DNA was washed and eluted according to the manufacturer's instructions. Purified DNA was stored at -20ºC until further use. A negative control, containing H_2O instead of sample, was purified parallel to each extraction batch to screen for contamination of extraction reagents.

PCR amplification of the V6-V8 region of 16S rDNA. Isolated DNA was subsequently used as a template to amplify the variable V6 to V8 region of 16S rDNA with universal bacterial primers F-968-GC (5'-GC-clamp+GAACGCGAAGAACCTTAC-3') and R-1401 (5'-GGTGTGTACAAGACCC-3') (100). The GC-clamp (CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACG GGG), incorporated into the forward primer, prevents complete dissociation of the DNA double strand during the following DGGE analysis (64). The amount of DNA was quantified by measuring the absorbance at 260 nm. The reaction mixture (25 µl) consisted of reaction buffer (GeneAmp 10xPCR Gold buffer, Applied Biosystems, Foster City, Calif.) (final concentrations 15 mM Tris-HCl, 50 mM KCl, 3mM $MgCl_2$, [pH 8.0]), 1.25 U *Taq* DNA polymerase (Amplitaq Gold® LD, Applied Biosystems),

250 µM of each deoxynucleoside triphosphate (dNTP), 0.24 µM of both primers, and 100 ng of DNA template. A negative PCR control, containing H_2O instead of the DNA template, was included to screen for contamination of PCR reagents. The samples were amplified in a thermocycler (Mastercycler Gradient, Eppendorf AG, Hamburg, Germany) using a touchdown PCR protocol: initial denaturation step at 94ºC for 3 min; 9 touchdown cycles (denaturation at 94ºC for 30 sec, annealing for 30 sec, extension at 68ºC for 1 min) with annealing temperature decreasing 1ºC per cycle from 62ºC to 54ºC; 25 cycles at 54ºC annealing temperature (denaturation at 94ºC for 30 sec, annealing for 30 sec, extension at 68ºC for 1 min), and final elongation step at 72ºC for 10 min. The purity and correct size of resulting PCR amplicons (approx. 450 bp) were assessed on 1% agarose electrophoresis gels, stained with ethidium bromide (staining for 15 min and destaining in H_2O for 60 min) and visualized under UV light.

DGGE analysis of PCR amplicons. DGGE analysis was performed on a DGGE system (DCode™, Biorad Laboratories, Hercules, Calif.) according to the manufacturer's instructions. PCR amplicons were applied to 16x16 cm, 8% (wt/vol) polyacrylamide gels (acrylamide-bisacrylamide, 37.5:1) in TAE buffer (40 mM Tris-acetate, 1 mM Na_2EDTA, [pH 7.4]) with a linear denaturing gradient of 35% to 70% (100% of denaturant was defined as 7 M Urea and 40% (vol/vol) deionized formamide). To standardize DGGE gels a commercially available DNA ladder (DNA ladder (log2), New England Biolabs Inc., Beverly, Mass.) was loaded as a marker. Electrophoresis was performed in TAE buffer at 60ºC for 16 hours at 70 V. Gels were stained with ethidium bromide for 12 min and subsequently destained in H_2O two times for 30 minutes. Gels were scanned (AlphaImager, Alpha Innotech Corporation, San Leandro, Calif.) and banding patterns were analyzed using gel analysis software (Bionumerics 3.0, Applied Maths, Austin, Tex.). Similarity indices between the banding patterns were calculated using Dice's similarity coefficient (D_{sc} = [2j/(a+b)]x100; where a = number of DGGE bands in lane 1, b = number of DGGE bands in lane 2, and j = number of common DGGE bands; D_{sc} = 100% demonstrates complete identity) (56). Dendrograms, showing clustering according to the similarity of banding patterns of individual samples, were

constructed by the unweighted pair group method using arithmetic averages (UPGMA) (23).

RESULTS

Group 1. The variable V6 to V8 region of 16S rDNA could be successfully amplified from all 16 samples. DGGE profiles illustrating the bacterial diversity in duodenal juice and the variation in bacterial diversity between duplicates and between dogs are illustrated in figure 1. Similarity indices of DGGE profiles between duplicates collected from the same collection site from each dog were 66.7, 76.9, 80.0, 92.3, 94.7, 96.3, 100.0, and 100.0 (mean ± SD: 88.4 ± 12.3%). The mean ± SD similarity index of DGGE profiles of duodenal juice between the 8 dogs was 38.3 ± 15.7% (range: 12.5-76.6%). Student's *t*-test revealed that there was a significantly higher variation in DGGE profiles between different dogs than between duplicates obtained from the same dog ($p<0.0001$; Fig. 2).

Group 2. From 18 potential time-points (3 sampling periods in six dogs) 17 samples of duodenal content were obtained by aspiration. As no duodenal juice could be aspirated in one dog at time-point 3 (sample 3) the corresponding sample of duodenal content collected at the same time-point using the cytology brush (sample 3A) was excluded from further statistical analysis. In another dog no 16S rDNA could be amplified from the DNA extracted at time-point 1. DNA extracted from all other samples was successfully amplified. DGGE analysis revealed a sizeable variation in similarity indices between the different time-points within the same individuals (Fig. 3). The mean ± SD similarity between the different time-points (samples 1, 2, and 3) within individual dogs was 38.3 ± 13.3%. The mean similarity between samples taken in week 3 (sample 3) and their corresponding samples taken using the cytology brush (sample 3A) was 62.6 ± 13.4% (Fig. 4). There was a significantly higher variation between the different time-points in individual dogs than between samples taken at the same time-point 3 (sample 3 and sample 3A; $p = 0.003$; Fig. 5).

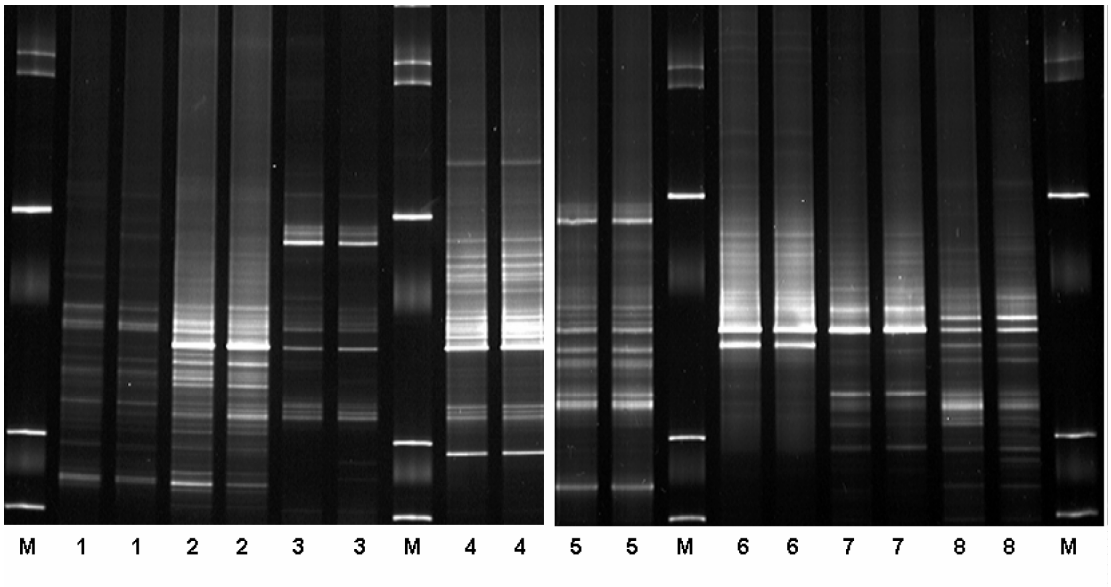


FIG 1. DGGE profiles illustrating small intestinal bacterial diversity in 8 dogs (1-8, in duplicate). Samples were collected in duplicate from approximately the same collection site in each dog and treated as independent samples to evaluate the reproducibility of DGGE profiles. (M = marker)

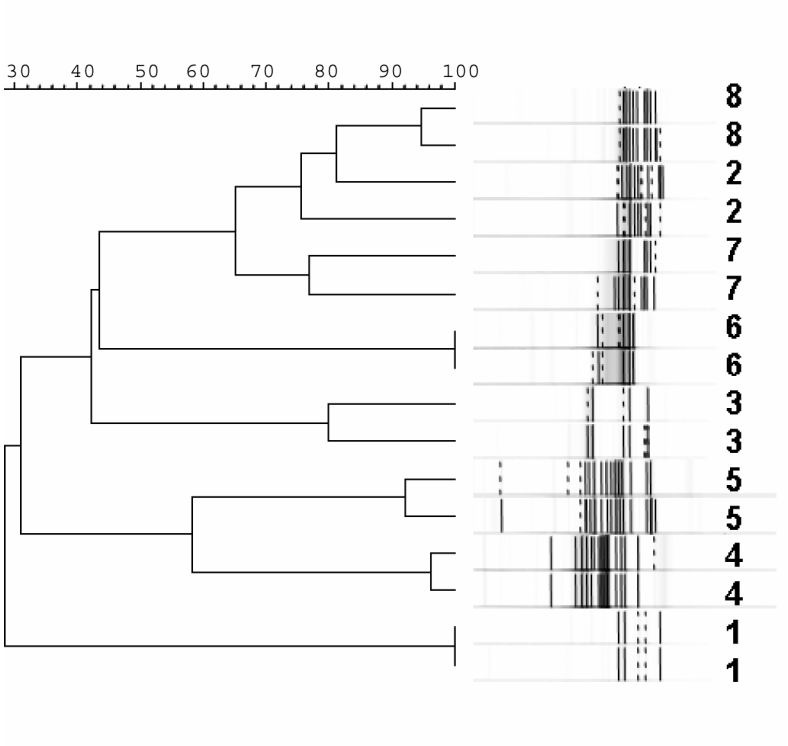


FIG 2**.** Dendrogram (UPMGA) generated from DGGE profiles obtained from canine duodenal juice from 8 dogs (dogs 1-8 of group 1, in duplicate) representing similarities in banding pattern between duplicates obtained from each dog and between different dogs. Mean±SD similarity index between the 8 dogs was 38.3±15.7% (range: 12.5-76.65%). Mean±SD similarity index between the duplicates from each dog was 88.4±12.3%. There was a significantly higher variation in DGGE profiles between dogs than between duplicates obtained from the same dog ($p<0.0001$).

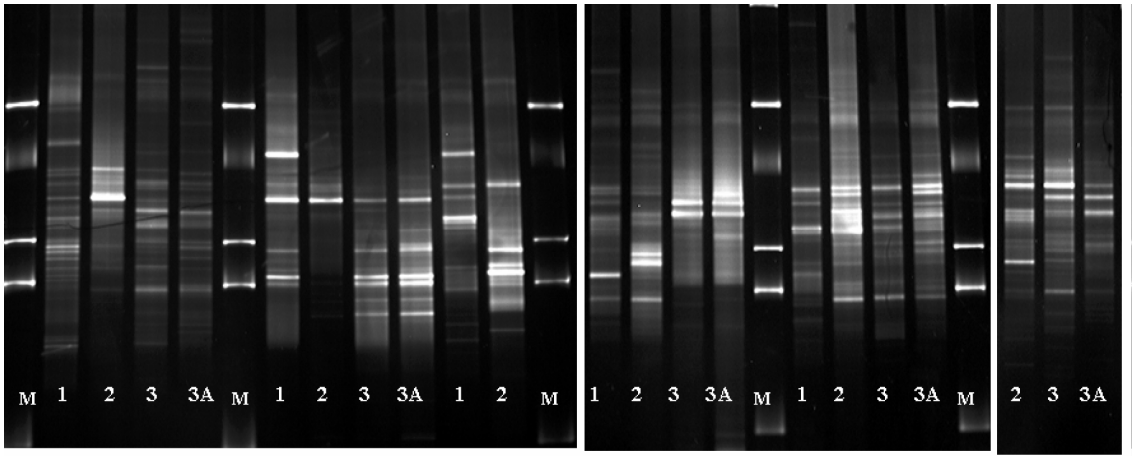


FIG 3**.** DGGE profiles of duodenal juice collected endoscopically in 6 dogs (dogs A-F) illustrating a sizeable variation in duodenal microflora between the different time-points within the same individual. Samples were collected by aspiration once weekly (samples 1, 2, and 3). In week 3 a second sample was collected using a cytology brush (3A). Samples taken at the same time-point from each dog (sample 3 and 3A) showed the highest similarity. No duodenal juice could be aspirated from dog C on week 3. No PCR amplicon could be generated from dog F on week 1. (M=marker)

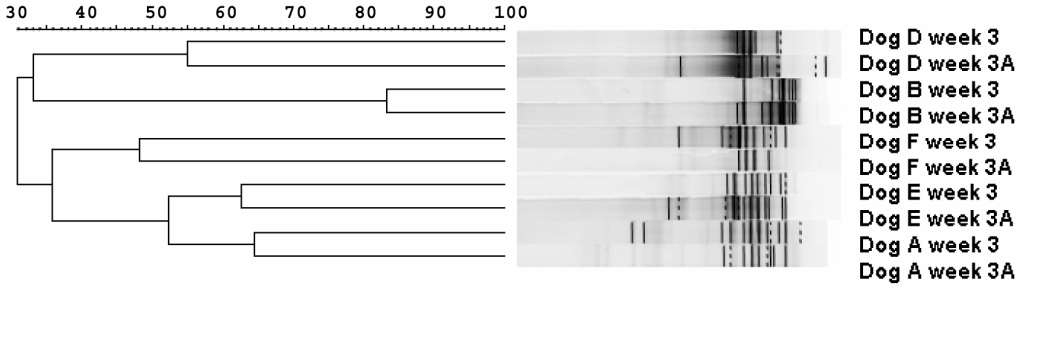


FIG 4. Dendrogram (UPGMA) generated from DGGE profiles representing similarities in banding patterns between the two samples obtained by different techniques in week 3. The mean similarity between aspirated samples (sample 3) and their corresponding samples taken using the cytology brush (sample 3A) was 62.6±13.4%.

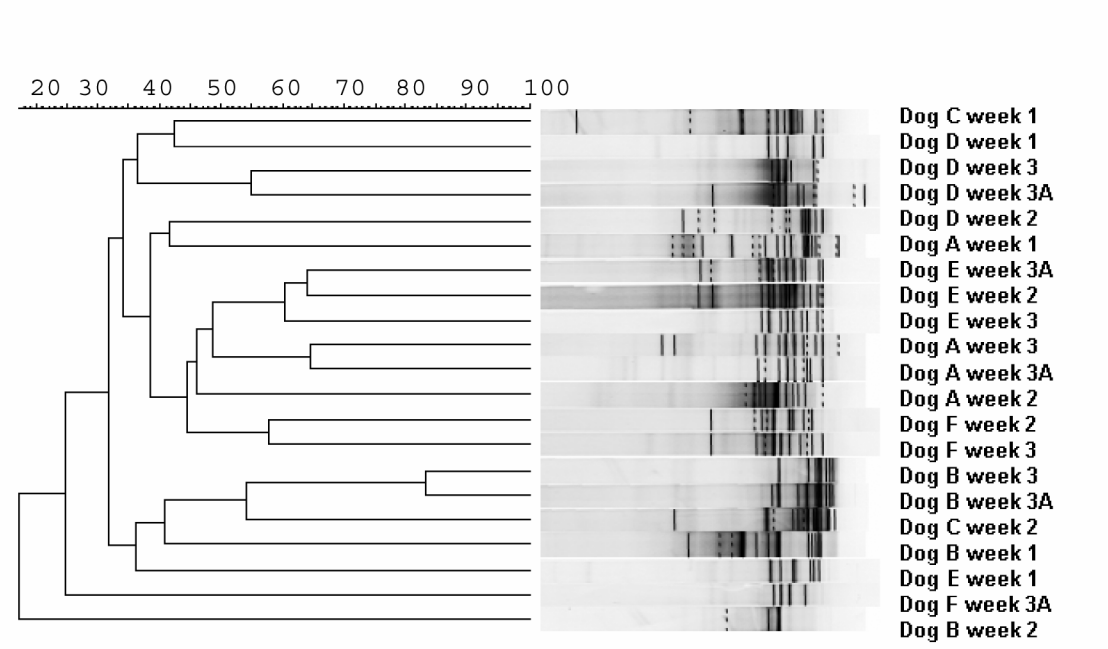


FIG 5. Dendrogram (UPGMA) generated from DGGE profiles representing similarities in banding patterns in samples from all time-points collected endoscopically in 6 dogs (dog A-F). The mean±SD similarity between the different time-points (samples 1, 2, and 3) within individual dogs was 38.3±13.3%. There was a significantly higher variation between the different time-points in individual dogs than between samples taken at the same time-point by two different techniques (sample 3 and sample 3A; p=0.003).

DISCUSSION

Traditionally, assessment of the canine small intestinal microflora has been based on identification and enumeration of intestinal bacteria by bacterial culture (5, 78, 106). It is becoming increasingly apparent, however, that only a proportion of bacterial species present in the gut can be identified using traditional bacterial culture techniques (1, 9, 48, 49, 70, 96). Reasons for this inability to culture many bacterial species include non-viable or stressed microorganisms, obligate requirements for coexisting flora or host-derived products, bias due to selectivity of culture media, and our lack of knowledge regarding essential nutrients for some bacterial species (15).

Molecular approaches, based on assessment of human fecal samples, have revealed that the gastrointestinal microflora is more complex than previously documented (96). Based on sequence analysis of PCR products new bacterial species, not previously detected by cultivation have been identified in fecal samples of humans and the gastrointestinal tract in pigs (48, 49). Sequencing of single PCR clones, however, is a laborious and expensive procedure and not well suited to studying complex microflora or microbial dynamics due to environmental changes. Molecular fingerprinting techniques (eg, DGGE) allow rapid assessment of the predominant bacterial species present in a sample. The DGGE profile typically represents up to 99% of the total bacterial community present in a biological sample (63). While molecular fingerprinting does not allow immediate discrimination between bacterial species, is does allow simultaneous analysis of multiple samples and, thus, direct comparison of microbial communities from different samples (63). Molecular fingerprinting also allows the study of changes in individual microbial communities over time.

In this study we have demonstrated that DGGE profiles can serve as a rapid and reproducible tool for qualitative assessment of small intestinal bacterial diversity in dogs. Results were typically obtained within 48 hours of sample collection. Duplicates of duodenal juice collected by laparotomy and needle aspiration from the same dog showed a mean similarity of 88%. This reproducibility is similar to previously reported DGGE profiles obtained from fecal samples, showing a reproducibility of 91% (89). The mean similarity of DGGE profiles from duplicates collected from the same dog was significantly higher than the mean similarity of DGGE profiles between dogs. The mean similarity of DGGE profiles between all 8 dogs in group 1 was 38%, indicating that dogs have a highly diverse duodenal microflora with marked differences between individual dogs. This is consistent with previous reports suggesting, based on bacterial culture, that the canine small intestinal microflora appears to be very variable between dogs (41). To our knowledge, molecular fingerprinting illustrating small intestinal bacterial diversity in dogs has not been reported in the literature previously. One study examined the influence of age, breed, and dietary fiber on bacterial diversity in fecal samples of dogs

using DGGE profiles (88) and reported that individual dogs have stable and unique fecal flora.

While DGGE profiles of duodenal juice collected by laparotomy and needle aspiration showed high reproducibility, the invasiveness of this sampling technique has obvious disadvantages. Studies of temporal variation in small intestinal bacterial diversity, and collection of clinical case material, would be difficult using this method of collection. Therefore, a less invasive collection technique such as endoscopic collection of duodenal juice is preferable. Laparotomy with needle aspiration and endoscopic collection of duodenal juice showed significant correlation, based on qualitative and quantitative bacterial culture (42, 69). We evaluated two different endoscopic collection methods, both utilizing the working channel of an endoscope: aspiration of duodenal juice through a sterile plastic tube and the collection of duodenal content using a cytology brush. We also evaluated weekly variations in the DGGE profiles. One problem we encountered in evaluating reproducibility of endoscopic sampling is the limited amount of duodenal juice that can be collected using the aspiration technique. At one time-point no duodenal juice could be collected at all in one of the dogs. While it was possible to collect approximately 0.5 ml of duodenal juice in all other dogs at all other time-points, not enough duodenal juice was available to collect a duplicate sample at the same time-point. Therefore, we were unable to evaluate the reproducibility of DGGE profiles from duodenal juice collected at the same time-point by endoscopic aspiration. In addition, in order to collect 0.5 ml of duodenal juice, a considerable amount of time (approximately 10-20 min) had to be invested per dog, making the collection of duodenal juice by endoscopic aspiration rather impractical for routine clinical application. Additionally, no PCR amplicon could be amplified from one sample obtained by endoscopic aspiration. This inability to amplify DNA is more likely due to carry-over of inhibitory substances during DNA extraction rather than a causal effect of the collection technique. Exocrine pancreatic secretions contain considerable amounts of ribonucleases that might have not been sufficiently eliminated during DNA extraction, these substances might have interfered in the subsequent PCR reaction (44). Based on

our experience during the development of the protocol for the DNA extraction from duodenal juice, it is crucial to obtain highly pure DNA.

The use of a cytology brush introduced through the working channel of an endoscope may be a superior method of sampling the duodenal contents for several reasons: it would make collection of samples easier, faster, and, if taken in the same fashion, also more reproducible, as a more constant amount of sample would be collected. Also, in this study a PCR amplicon could be generated from DNA extracted from all cytology brushes. However, relatively few samples obtained by cytology brush were analyzed and further samples collected using this collection method need to be evaluated.

Samples collected in the same dog at the same time-point either by aspiration or cytology brush showed 62% similarity. While this similarity was significantly higher than the similarity between different time-points in individual dogs, it was significantly lower than the similarity of duplicate duodenal juice collections using a syringe in the group 1 dogs. This could be partially explained by the differences in bacterial populations present in the intestinal lumen vs. adherent to the intestinal mucosa. Quantitative and qualitative differences in microbial species between intestinal lumen and intestinal biopsy samples have been found based on bacterial culture (18). However, we speculate that collection using a cytology brush differs from an intestinal biopsy sample, since the collected sample consists mostly of intestinal fluid and some superficial mucosal cells. Therefore, this collection technique would represent a mixture of both microbial populations and proper standardization of the sampling technique would allow comparison of the microbial community between healthy and diseased individuals.

The fecal microflora has been reported to be stable over time when analyzed by DGGE (88). In contrast, results of studies using bacterial culture sampling at different time-points suggest significant qualitative and quantitative fluctuations in the small intestinal microflora (18, 32, 107). These studies are hampered by the fact that the repeated collection occurred either at long intervals (18, 107), or has been performed in

dogs with IgA deficiency (107) or dogs that had undergone surgery that might have influenced the small intestinal microflora (32). The authors of these studies also do not exclude the possibility that either culture techniques or ingested bacteria might have led to the observed variation.

In this study, samples of duodenal juice collected at weekly intervals in individual dogs showed sizeable variation in their banding patterns. Since, for reasons described above, no duplicate samples could be obtained at the same time-point by aspiration it is difficult to conclude if this variation was due to sampling technique or an inherent variation of the small intestinal microflora over time. However, in dogs from group 2 approximately the same volume of duodenal juice was analyzed as in the dogs from group 1, where the duplicates showed 88% reproducibility. This would suggest that the variation in similarity between the different time-points is less likely due to sampling method variation, instead reflecting a genuine variation over time. The observation that "duplicate" samples collected at the same time-points (sample 3 and 3A), albeit using two different collection techniques, showed significantly less variation than the weekly variation further suggests a genuine variation in the small intestinal microflora over time. However, it can not be definitively concluded from this study that the observed variation at different time-points is solely due to changes in the small intestinal microflora over time. We attempted to collect samples from approximately the same sampling site on each study day using the distance markings located on the outside of the endoscope. However, it is possible that the bacterial composition differs between samples that are taken from locations that are only a short distance apart from each other. This variation of the small intestinal microflora obtained during repeated sampling, regardless of the underlying cause, needs to be taken into consideration when collecting clinical samples.

Using DGGE profiles, we have shown that dogs have a highly diverse bacterial microflora in the small intestine. However, important limitations of this PCR based approach that might interfere with the interpretation of our results need to be acknowledged. Molecular detection techniques do not generally have the ability to determine whether an organism is dead or alive, and the DNA collected may at least in

part be due to orally ingested microbial material (15). No information is available about the persistence of DNA in the canine intestine. Pancreatic juice contains considerable amounts of DNAse, which may degrade DNA present in the small intestinal lumen (44). Studies in mice have also shown that unfragmented aliquots of ingested DNA could be recovered from the small intestine for only up to 30 min after feeding, 18 hours after feeding no foreign DNA could be detected in the lumen of any gut compartment by Southern hybridization (68). Since food was withheld for 24 hours in all dogs in this study, it appears unlikely that ingested DNA would have interfered with our results, assuming that all dogs had normal intestinal motility. It is also assumed that DNA is equally extracted from all bacterial species. PCR may exhibit bias, by targeting only predominant species that constitute more than 1% of the microflora (63). PCR, especially at higher cycle numbers, can introduce mutations, chimeras, and heteroduplexes (74). Some bacterial species are known to have multiple copies of the 16S rRNA gene, making a quantitative interpretation questionable. It has also been shown that DGGE has a limited resolving power for some PCR amplicons as amplicons with closely related sequences may co-migrate and denature at the same time. Thus, bands on DGGE may be comprised of several amplicons, underestimating bacterial diversity. Due to the exponential nature of PCR amplification, PCR is extraordinarily prone to iatrogenic contamination, the use negative PCR controls is crucial to monitor for contamination. Despite these limitations, DGGE profiles have been shown to be a powerful tool for assessment of bacterial diversity in environmental samples (64).

The molecular approach described in our study can facilitate identification of bacterial species not previously cultured from the canine small intestine. Further elucidation of the complexity of the small intestinal microflora will potentially allow us to understand the host-bacteria interactions leading to disease. The clinical significance of the diverse small intestinal microflora in dogs, and the alterations in bacterial diversity that may be present with gastrointestinal disease needs to be further explored.

CHAPTER III

ASSESSMENT OF QUALITATIVE VARIATION IN INTESTINAL MICROFLORA IN DIFFERING COMPARTMENTS OF THE CANINE INTESTINAL TRACT USING A MOLECULAR FINGERPRINTING TECHNIQUE*

OVERVIEW

The aim of this study was to evaluate qualitative variation in bacterial microflora in different compartments of the canine intestinal tract using a molecular fingerprinting technique. Intestinal content was collected from the duodenum, jejunum, ileum, colon, and rectum of 14 adult Hound dogs housed under identical conditions and fed identical diets. Bacterial DNA was extracted, the variable V6-V8 region of 16S rDNA (gene coding for 16S ribosomal RNA) was amplified using universal bacterial primers, and PCR amplicons were separated by denaturing gradient gel electrophoresis (DGGE). Variation in the intestinal microflora between different compartments of the intestine within individual dogs, and between all dogs, was assessed based on similarity indices of DGGE banding patterns. Bacterial diversity was assessed by calculating Simpson's diversity index, the Shannon-Weaver diversity index, and evenness. DGGE profiles indicated marked differences when individual compartments were compared between dogs (range 25.6-36.6%). There was also a sizeable variation in the microflora between different intestinal compartments within individual dogs (range: 36.7–57.9%). Neighboring compartments showed significantly higher similarity than non neighboring compartments ($p<0.0001$). Diversity indices were significantly higher in the large intestine than the small intestine ($p<0.01$).

---

*Reprinted with permission from **Suchodolski J.S., Ruaux C.G., Steiner J.M., Fetz K., and Williams D.A.** 2005. Assessment of qualitative variation in intestinal microflora in differing compartments of the canine intestinal tract using a molecular fingerprinting technique. Am. J. Vet. Res. **66:**1556-1562.

The results indicate that dogs have a complex intestinal microflora with marked differences between individual dogs. Differing intestinal compartments within individual dogs appear to host unique microflora. Assessment of the microflora present in a fecal sample may not yield accurate information about the composition of the intestinal microflora in other compartments of the canine gut.

INTRODUCTION

Dogs are commonly used as a model species for biomedical research, as well as being commonly kept as pets. The intestinal bacterial flora has an important influence on the health of the individual animal, while alterations in this flora have been associated with intestinal disease. Previous studies aiming to characterize the canine intestinal bacterial flora have focused on the enumeration and identification of bacterial species from direct cultivation of either duodenal juice or fecal samples (5, 78, 106). Relatively little is known about the composition of the bacterial microflora in other compartments of the canine intestine, and limited data, based on bacterial culture, are available that evaluate differences in the microflora between dogs or between intestinal compartments within dogs (10, 17).

Bacterial culture has limitations for assessing bacterial diversity in the gut. Samples must be processed immediately in order to accurately represent the aerobic and anaerobic microbial flora present in the gastrointestinal tract. The intestinal tract harbors many anaerobic bacteria, which are more prone to damage during handling of samples. Also, identification of bacteria is based on phenotypic identification systems, this may lead to limitations in accurately characterizing all microorganisms in a given sample. It is also increasingly recognized that the majority of microbial species present in biological samples escape identification by use of standard culture techniques alone (1, 9, 48, 49, 70, 96). It has been estimated that approximately 60-80% of organisms present in the gastrointestinal tract of various species have not yet been cultivated (48, 49). Studies using a molecular approach based on identification of 16S ribosomal RNA (rRNA) or 16S rDNA (DNA coding 16S rRNA) have identified a greater number of bacterial species, when compared to standard culture techniques (37, 70, 96).

Sequencing of single PCR clones is a laborious and expensive procedure and thus not well suited to study complex microflora or microbial dynamics due to environmental changes. Molecular fingerprint techniques are commonly used to illustrate the genetic diversity in a complex microbial community (49, 90). Polymerase chain reaction of 16S rDNA, with subsequent separation of amplicons by denaturing gradient gel electrophoresis (DGGE) based on sequence differences, has been shown to be a useful tool for assessment of bacterial diversity in environmental samples as well as in a mixture of known bacterial species (63, 100). While molecular fingerprints do not allow direct identification of bacterial species, they allow simultaneous analysis of multiple samples and, thus, direct comparison of microbial communities from different samples (63). In a previous study our group has demonstrated that endoscopic collection of duodenal juice with subsequent assessment of small intestinal bacterial diversity by use of a molecular fingerprinting technique is a useful and reproducible tool and warrants further investigation in dogs with small intestinal disease (98).

In a clinical setting the intestinal microflora is typically evaluated by analysis of intestinal samples obtained either by endoscopic collection of duodenal juice or by collection of fecal samples. For future clinical studies evaluating the intestinal microflora using molecular techniques it is necessary to evaluate if the assessment of duodenal or fecal samples is representative of the microflora present in other compartments of the canine intestinal tract. The objective of this study was to evaluate the variation in bacterial microflora in different compartments of the intestinal tract, within and between individual dogs, using a molecular fingerprinting technique.

MATERIALS AND METHODS

Sample material. Intestinal content was collected from the duodenum, jejunum, ileum, colon, and rectum from 14 adult Hound dogs (8 male and 6 female). The mean (±SD) age was 3.9 (±1.3) years (range 2.2-6.2 years). All dogs were raised and housed in the same environment and fed the same regular canine maintenance diet. These dogs were euthanized as part of an unrelated project. The protocol for sample collection was approved by the University Laboratory Animal Care Committee at Texas A&M

University. In all dogs food was withheld for 24 hours before euthanasia. Immediately after euthanasia the abdominal cavity was opened, and the intestines were isolated. Intestinal content was collected by needle aspiration of duplicate samples from approximately the same collection site in each dog. Approximately 0.5 ml of intestinal fluid or, in distal parts of the intestine, solid intestinal content was collected from each collection site using a sterile 16 gauge needle attached to a 3-ml syringe or a fecal collection tube, respectively. To evaluate the reproducibility of DNA extraction and PCR-DGGE, intestinal content obtained from 2 different dogs (referred to as sample A and sample B) was homogenized by rigorous vortexing for 20 minutes. Sample A and sample B were then subdivided into 4 aliquots.

Handling of samples. All samples were immediately transferred into sterile cryotubes (Wheaton, Millville, NJ), snap-frozen in liquid nitrogen, and stored at -80ºC until further analysis.

Extraction of DNA. DNA was extracted using a modified bead beating method. Briefly, 500 µl of cell lysis solution (Puregene® cell lysis solution, Gentra Systems, Minneapolis, Minn.), 200 µl of buffer saturated phenol:chloroform:iso-amylalcohol (25:24:1), and 300 µl of 0.1 mm zirconia beads (BioSpec Products Inc., Bartlesville, Okla.) were added to each sample. The tubes were positioned horizontally on a vortex adapter (Ambion Inc., Austin, Tex.) mounted on a standard vortexer (Vortex Genie-2, VWR, West Chester, Pa.) and the mixture was vortexed for 5 minutes at maximum speed. Tubes were centrifuged for 7 min at 12,000 x *g* and the supernatant transferred into a new sterile tube. A second phenol:chloroform:iso-amylalcohol extraction was performed, and the aqueous phase was transferred into a new sterile tube. RNA was removed by 30 min incubation at 37ºC with 5 U of RNAse (Puregene® RNAse, Gentra Systems, Minneapolis, Minn.). RNAse was removed by phenol:chloroform:iso-amylalcohol extraction as described above. The aqueous phase, containing DNA, was mixed with 0.5 volumes of 100% ethanol and applied onto commercially available spin columns (GenElute Bacterial Genomic DNA Kit, Sigma Chemicals, St. Louis, Mo.). Bound DNA was washed and eluted according to manufacturer's instructions. Purified

DNA was stored at -20ºC until further use. A negative control, containing H_2O instead of intestinal content was purified parallel to each extraction batch to screen for contamination of extraction reagents.

PCR amplification of the V6-V8 region of 16S rDNA. The variable V6 to V8 region of 16S rDNA was amplified using universal bacterial primers F-GC-968 (5'-GC-clamp[CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGG]+GAACGCG AAGAACCTTAC-3') and R-1401 (5'-GGTGTGTACAAGACCC-3') (100). The reaction mixture consisted of 1.25 U *Taq* DNA polymerase (Amplitaq Gold® Low DNA, Applied Biosystems, Foster City, Calif.), 1x reaction buffer (Amplitaq Gold® reaction buffer, Applied Biosystems, Foster City, Calif.) (15 mM Tris-HCl, 50 mM KCl, 3mM $MgCl_2$, [pH 8.0]), 250 µM of each deoxynucleoside triphosphate (dNTP), 0.24 µM of each primer, and 100 ng of DNA template in a total volume of 25 µl. A negative PCR control, containing H_2O instead of DNA template, was evaluated to screen for contamination of PCR reagents. The samples were amplified in a Mastercycler (Mastercycler Gradient, Eppendorf AG, Hamburg, Germany) using a touchdown PCR protocol with an initial denaturation step at 94ºC for 3 min, 9 touchdown cycles (denaturation at 94ºC for 30 sec, annealing for 30 sec, and extension at 68ºC for 1 min) with annealing temperature decreasing by 1ºC per cycle from 62ºC to 54ºC, 20 cycles with an annealing temperature of 54ºC (denaturation at 94ºC for 30 sec, annealing for 30 sec, and extension at 68ºC for 1 min), and a final elongation step at 72ºC for 10 min. The purity of the PCR amplicons were assessed on 1.2% agarose electrophoresis gels stained with ethidium bromide and visualized under UV transillumination. The correct size of PCR amplicons (approx. 450 bp) and the amount of PCR product were evaluated by comparing the band size and the intensity of the bands to commercially available DNA markers (DNA ladder (log2), New England Biolabs Inc., Beverly, Mass.).

DGGE analysis of PCR amplicons. DGGE analysis was performed with the Bio-Rad Universal Mutation Detection System (DCode™, Biorad Laboratories, Hercules, Calif.). PCR products were applied onto 8% (wt/vol) polyacrylamide gels (acrylamide-bisacrylamide, 37.5:1) in buffer (1x TAE buffer, 40 mM Tris-acetate, 1

mM Na_2EDTA, pH 7.4) with a linear denaturing gradient of 35% to 70% (100% of denaturant is defined as 7 M Urea and 40% (vol/vol) deionized formamide). Samples from each individual dog (each compartment in duplicate) were analyzed on one gel. For comparison of samples between gels, a commercially available DNA ladder (DNA ladder (log2), New England Biolabs Inc., Beverly, Mass.) was loaded as a marker (3 lanes per gel). Electrophoresis was performed in 1x TAE buffer at 60ºC for 16 hours at 70 V. Bands were visualized by staining gels with ethidium bromide and viewed by UV transillumination.

Sample analysis. Gel images were digitally captured (AlphaImager, Alpha Innotech Corporation, San Leandro, Calif.) and DGGE profiles analyzed using gel analysis software (Bionumerics 3.0, Applied Maths, Austin, Tex.). The bands in each profile were converted into peak profiles in densitometric curves. The data were used to calculate bacterial diversity indices which yield information about species diversity in a bacterial community: Simpson's reciprocal diversity index, the Shannon-Weaver diversity index, evenness were calculated (3). Simpson's reciprocal diversity index (D) was defined as $D = 1/\sum(n/N)^2$, where n = the total number of organisms of a particular species, and N = the total number of organisms of all species. The Shannon-Weaver index (Hs) was defined as $Hs = [-\sum p_i \ln(p_i)]$, where p_i is the proportion of individual bacteria found in a certain species. A higher value for D and Hs indicates higher bacterial diversity in the sample. Evenness (e) describes how uniformly individual bacterial species are divided between all species present, and was defined as $e = Hs/\ln(S)$, where S is the number of total species. Evenness values range between 0 and 1, with 1 being complete evenness (i.e. the relative abundance of all species is equal). D and Hs were compared between different gut compartments using repeated measures ANOVA followed by Tukey's multiple comparison test. Evenness was compared between different gut compartments using the Friedman test followed by Dunn's multiple comparison test. DGGE banding patterns were compared between all samples by calculating Dice's similarity coefficient ($Cs=[2j/(a+b)]x100$; where a = number of DGGE bands in lane 1, b = number of DGGE bands in lane 2, and j = number of

common DGGE bands). A Cs of 100% demonstrates complete identity between two DGGE banding patterns (56).

The reproducibility of PCR-DGGE was evaluated by comparing the mean similarity (Cs) of banding patterns between the aliquots obtained from homogeneous intestinal content from sample A and B, respectively. The variation in banding patterns between different intestinal compartments within and between dogs was calculated by comparing the Cs between individual samples. Dendrograms, showing clustering according to the similarity of banding patterns of individual samples, were constructed by the unweighted pair group method using arithmetic averages (UPGMA) (23). Samples belonging to the same cluster were determined by a cluster cutoff algorithm based on the Point-Biserial correlation in the Bionumerics software (40). The cophenetic correlation coefficient was calculated using Bionumerics software. This coefficient estimates the goodness of fit for each subcluster within a dendrogram, a coefficient > 0.8 suggests a good fit of the cluster analysis to the data (54).

RESULTS

Ileal content could not be obtained from one dog, resulting in a total of 138 collected samples (14 dogs x 5 collection sites/per dog x 2 replicates). From 2 of these 138 samples the V6 to V8 region of the 16S rDNA could not be amplified (1.4%): both replicates of duodenal content in one dog. The V6 to V8 region of the 16S rDNA was successfully amplified in the remaining 136 samples. The mean±SEM similarity (Cs) between the banding patterns of the aliquots from homogeneous samples A and sample B was 96.2±0.8% and 93.1±1.0%, respectively. There was a sizeable variation in banding patterns when individual compartments were compared between individual dogs, with Cs ranging from 25.6% (ileum) to 36.6% (colon) (Table 1). There was also sizeable variation in similarity of banding patterns between different compartments within individual dogs (Table 2). Neighboring compartments had a significantly higher similarity than non-neighboring compartments ($p<0.0001$), with the colon and rectum showing the highest similarity (mean±SEM: 57.9±3.0%).

The bacterial diversity indices increased gradually down the length of the gut (Fig. 6, Table 3) indicating an increase in bacterial diversity and evenness. Bacterial diversity indices were significantly higher in both compartments of the large intestine compared to the three compartments in the small intestine (Table 4).

The cluster cutoff method in the Bionumerics software suggested 4 major clusters in the dendrogram (Fig. 7). The cophenetic correlation, however, revealed poor fit (r=0.65) of the data for these major clusters indicating that these results need to be interpreted with caution. There was, however, an obvious trend demonstrating that individual dogs tend to have unique bacterial microflora, especially in the large intestine. In all dogs (14/14) the similarity between the colonic and rectal banding pattern was higher within the individual than when compared to other dogs (cophenetic correlation r=1.0 for these samples; Fig. 7). A similar, but less extensive, trend was noticeable in the proximal small intestine (duodenum and jejunum), where 8/14 dogs clustered at least with one neighboring compartment (Fig. 7). In contrast, the microflora in the ileum was very variable. In a subset of dogs (5/14) the ileal microflora clustered with at least one compartment of the small intestine. In another subset (3/14) the ileal microflora clustered together with the large intestine, while in 5/14 dogs the ileal microflora did not cluster with any compartment from the same dogs (Fig. 7).

TABLE 1. Mean similarity (%) of DGGE profiles within each compartment obtained by pair-wise comparison between individual dogs.

	mean Cs[a]	**SEM[b]**	**min**	**max**
Duodenum	28.0	2.7	3.8	51.0
Jejunum	26.8	2.2	7.7	46.3
Ileum	25.6	2.2	5.7	47.0
Colon	36.6	2.2	19.1	54.4
Rectum	36.2	2.3	17.4	51.9

[a]Dice's similarity coefficient
[b]SEM= Standard error of the mean

TABLE 2. Mean similarity (%) of DGGE profiles between different compartments within individual dogs.

	mean Cs[a]	SEM[b]	min	max
Duodenum-Jejunum*	**43.0**	**5.0**	**16.4**	**74.6**
Duodenum-Ileum	32.5	3.3	12.3	57.1
Duodenum-Colon	29.0	3.7	10.0	51.9
Duodenum-Rectum	29.9	3.2	10.6	45.3
Jejunum-Ileum	**36.7**	**4.4**	**16.4**	**66.6**
Jejunum-Colon	28.0	1.9	12.9	40.7
Jejunum-Rectum	26.8	2.7	9.9	46.0
Ileum-colon	**38.8**	**4.2**	**10.0**	**64.3**
Ileum-Rectum	32.8	3.8	8.4	55.9
Colon-Rectum	**57.9**	**3.0**	**37.3**	**72.8**

*Rows in bold indicate neighboring compartments
[a]Dice's similarity coefficient
[b]SEM= Standard error of the mean

TABLE 3. Bacterial diversity indices (mean±SEM) in different intestinal compartments in 14 dogs as indicated by the Simpson diversity index, the Shannon-Weaver diversity index, and evenness.

	Simpson diversity index	**Shannon-Weaver diversity index**	**Evenness**
Duodenum	3.94±0.57	1.44±0.15	0.81±0.03
Jejunum	4.22±0.45	1.52±0.12	0.85±0.03
Ileum	6.17±1.00	1.79±0.18	0.86±0.03
Colon	9.33±0.83	2.32±0.11	0.91±0.02
Rectum	8.49±0.62	2.27±0.07	0.90±0.01

TABLE 4. P-values for differences in the mean number of bacterial diversity indices, between intestinal compartments in the dog.

	Simpson diversity index	**Shannon-Weaver diversity index**	**Evenness**
Duodenum-Jejunum	$p > 0.05$	$p > 0.05$	$p > 0.05$
Duodenum-Ileum	$p > 0.05$	$p > 0.05$	$p > 0.05$
Duodenum-Colon	$p < 0.001$	$p < 0.001$	$p < 0.001$
Duodenum-Rectum	$p < 0.001$	$p < 0.001$	$p < 0.01$
Jejunum-Ileum	$p > 0.05$	$p > 0.05$	$p > 0.05$
Jejunum-Colon	$p < 0.001$	$p < 0.001$	$p > 0.05$
Jejunum-Rectum	$p < 0.001$	$p < 0.001$	$p > 0.05$
Ileum-colon	$p < 0.01$	$p < 0.01$	$p > 0.05$
Ileum-Rectum	$p > 0.05$	$p < 0.01$	$p > 0.05$
Colon-Rectum	$p > 0.05$	$p > 0.05$	$p > 0.05$

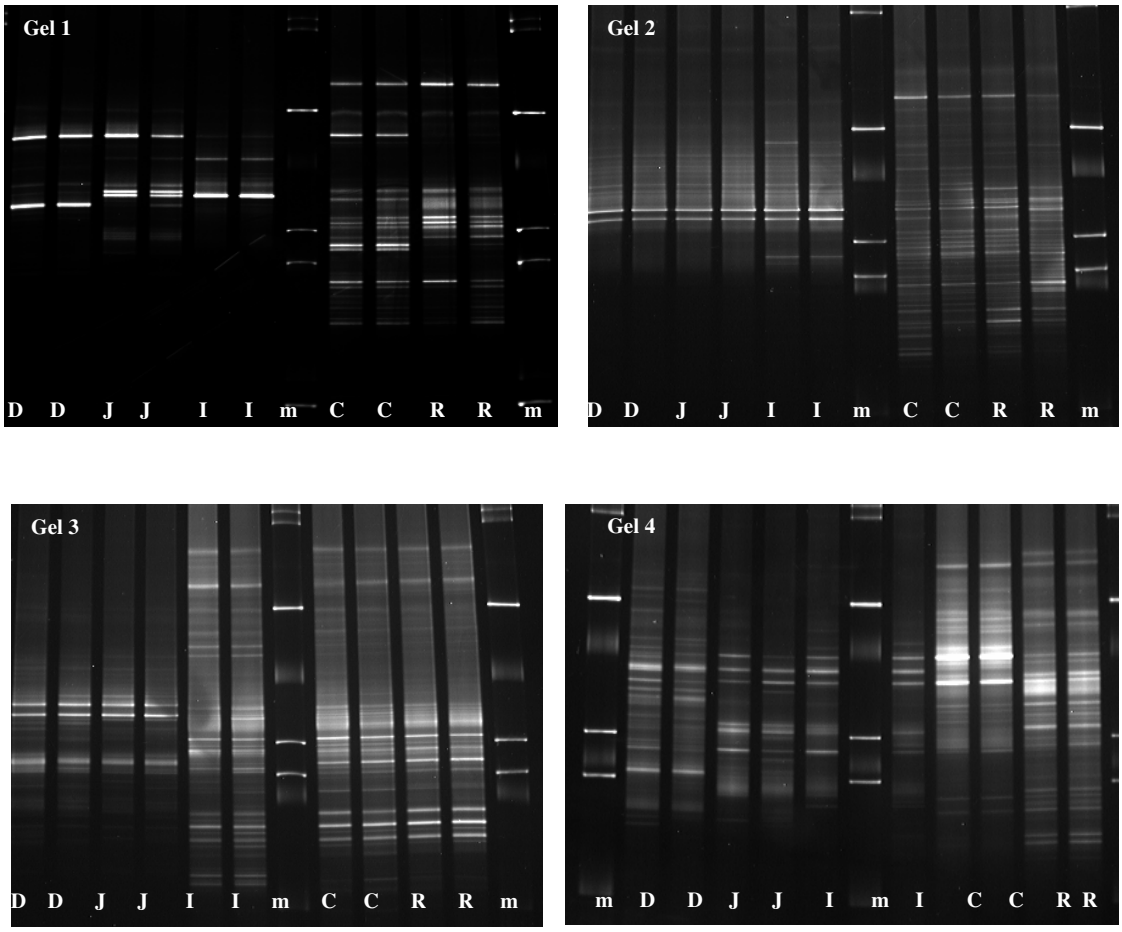
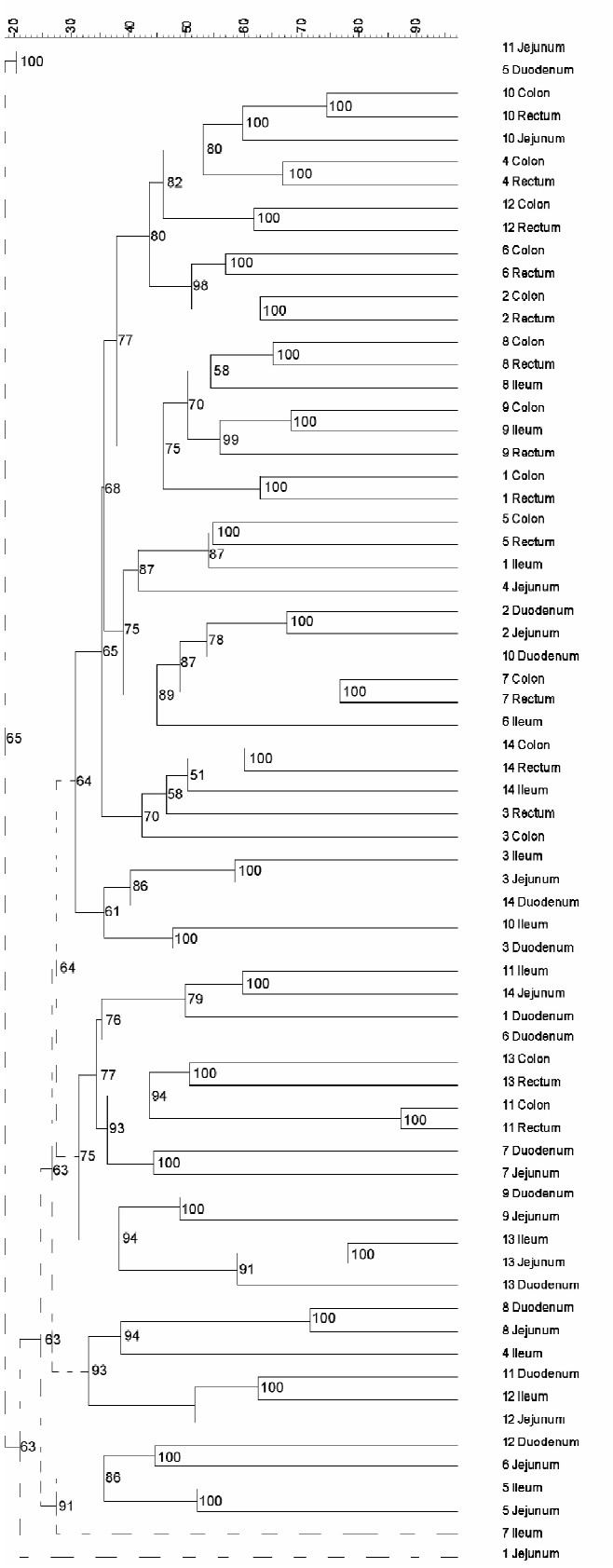


FIG 6. DGGE profiles illustrating bacterial diversity in different intestinal compartments in 4 dogs (gel 1-4). (D=duodenum, J=jejunum, I=ileum, C=colon, R=rectum; m=marker; samples loaded in duplicates). Dogs have marked differences in bacterial microflora between different intestinal compartments within individual dogs and between dogs.

FIG 7-*next page.* Dendrogram (UPGMA) based on the Dice coefficient of similarity between differing intestinal compartments in dogs. The number next to the intestinal compartment indicates the identity of the dog. Branches below the cluster cutoff value are shown with dashed lines. Cophenetic correlation coefficients are shown in each branch of the dendrogram.


20
30
40
50
60
70
80
90
11 Jejunum
5 Duodenum
10 Colon
10 Rectum
10 Jejunum
4 Colon
4 Rectum
12 Colon
12 Rectum
6 Colon
6 Rectum
2 Colon
2 Rectum
8 Colon
8 Rectum
8 Ileum
9 Colon
9 Ileum
9 Rectum
1 Colon
1 Rectum
5 Colon
5 Rectum
1 Ileum
4 Jejunum
2 Duodenum
2 Jejunum
10 Duodenum
7 Colon
7 Rectum
6 Ileum
14 Colon
14 Rectum
14 Ileum
3 Rectum
3 Colon
3 Ileum
3 Jejunum
14 Duodenum
10 Ileum
3 Duodenum
11 Ileum
14 Jejunum
1 Duodenum
6 Duodenum
13 Colon
13 Rectum
11 Colon
11 Rectum
7 Duodenum
7 Jejunum
9 Duodenum
9 Jejunum
13 Ileum
13 Jejunum
13 Duodenum
8 Duodenum
8 Jejunum
4 Ileum
11 Duodenum
12 Ileum
12 Jejunum
12 Duodenum
6 Jejunum
5 Ileum
5 Jejunum
7 Ileum
1 Jejunum

DISCUSSION

In this study we evaluated variation of the intestinal microflora in differing compartments of the canine intestinal tract within individuals and between dogs. Environmental influences were minimized by housing all dogs in an identical environment and feeding the same diet. The results of DGGE analysis suggest marked variation in bacterial microflora between individual dogs, and in differing intestinal compartments within individual dogs.

Inter-individual variation of the intestinal microflora has been suggested, based on bacterial culture, in the ileum, cecum, and colon of dogs (17). Davis et al. have also shown that dogs housed for years in the same environment and fed the same diet have marked differences in their microflora (17). While molecular fingerprinting techniques have been used to evaluate inter-individual variation in the intestinal microflora in various species such as humans (112), dogs (88), and pigs (89), these studies have focused predominantly on the microflora present in fecal samples (88, 89, 112). Marked differences in the fecal microflora between humans have been observed using temperature gradient gel electrophoresis (112). Simpson et al. examined the influence of age, breed, and dietary fiber on bacterial diversity in fecal samples from dogs using DGGE profiles, reporting that individual dogs have a stable, unique fecal flora (88). Also individually unique fecal flora have been observed in piglets living in the same environment and fed the same diet (89). These differences between individuals have been attributed to hitherto unknown host specific factors (111). In a recent study it has been reported that monozygotic twins have significantly higher similarity of fecal DGGE banding patterns than unrelated individuals, suggesting the host's genotype may have an influence on the intestinal microflora (111).

The canine intestinal microflora has almost exclusively been evaluated by traditional bacterial culture techniques (6, 10, 17). It has been reported that the normal canine duodenal microflora harbors a total bacterial count below 10^5 colony forming units per ml of duodenal juice (cfu/ml) (77). Higher duodenal bacterial counts were considered to contribute to intestinal disease and lead to a clinical syndrome called small

intestinal bacterial overgrowth (SIBO) (77). Significantly higher counts, however, have subsequently been documented in dogs with no signs of intestinal disease (41), leading to controversy over the true quantitative composition of the healthy canine duodenal microflora. The jejunal microflora has been reported to be quantitatively similar to the duodenum (6), whereas the ileal microflora increases in absolute bacterial counts to approximately 10^7 cfu/ml (6, 10, 17). The number of bacteria in the colon increases to a total bacterial count of approximately 10^9 to 10^{11} cfu/ml (6, 17). Very little is known about the qualitative variation in intestinal microflora between differing compartments within individual dogs. To our knowledge no study has evaluated differences between differing compartments of the canine intestinal tract using a molecular fingerprinting technique. In the study reported here we observed a remarkable variation in similarity of banding patterns between differing intestinal compartments within individual dogs. There was a gradual increase in band numbers down the length of the gut. Bacterial diversity indices were significantly higher in the large intestine than in the proximal small intestine. Bacterial diversity indices take into account the relative abundance of each individual species within a given community and, thus, provide information about the dominance of single species within a community or equal abundance of all species within a community, respectively. The more species that are present and the more evenly the total population is distributed between the species present, the more diverse the bacterial community. A highly diverse microflora is hypothesized to have more interactions within the community (73) and may be more stable and, therefore, more resistant to environmental changes (2). For example, a reduction in bacterial diversity has been found in the colonic microflora in humans with active inflammatory bowel disease, warranting bacterial diversity studies in dogs with intestinal disease (67). Several confounding factors of a molecular fingerprinting approach as described in this study, that may cause an over- or underestimation of bacterial diversity, need to be acknowledged. It has been suggested that DGGE bands represent the predominant bacterial species found in a microbial community (64), however PCR-DGGE analysis may exhibit bias by preferential amplification of bacterial species with DNA sequences

that show higher affinity for the universal primers compared to other bacterial species. The universal primers used in this study are commonly used in PCR-DGGE analysis from intestinal samples (102, 112). At higher cycle numbers PCR may exhibit bias, targeting only predominant species in the sample. For these reasons, PCR cycles should be kept to a minimum. In this study at least 29 cycles were needed to yield sufficient PCR product for DGGE analysis from samples obtained from the proximal small intestine. DGGE may also have limited resolving power, as PCR amplicons with closely related sequences may co-migrate and denature at the same time (64). Thus, bands on DGGE may be comprised of several amplicons, further underestimating bacterial diversity. Also, different strains of the same bacterial species may have resolvable differences in the V6-V8 region, yielding multiple bands. Despite this limitations, molecular fingerprint techniques have been proven to be powerful tools to illustrate the genetic diversity in a complex microbial community (49, 90).

Individual dogs appear to possess unique individual bacterial communities in their large intestines. The colonic and rectal banding patterns obtained from the same dog tended to be more similar than the banding patterns from other dogs. This would suggest that each individual has a specific microflora in the large intestine, rather than the presence of a characteristic colonic or rectal microflora in dogs. While a similar trend was observed in the proximal small intestine, there was considerably more overlap between individual samples. This may be due to the lower bacterial diversity found in the proximal small intestinal microflora. In contrast, the microflora in the ileum was highly variable. In a subset of dogs the ileal microflora showed higher similarity with the proximal small intestine, while in another subset of dogs it showed higher similarity with the large intestine. This is surprising as the ileocolic valve is believed to be a natural barrier between the small and large intestine in dogs (31). This barrier, together with intestinal motility, is believed to prevent retrograde translocation of bacteria from the large into the small intestine. Dysfunction of the ileocolic valve has been suggested as a possible cause of intestinal disease, such as small intestinal bacterial overgrowth (SIBO) (31, 45). At this point it remains speculative that the variation in bacterial microflora in

the ileum between different dogs is caused by dysfunction in the ileocolic valve, and if so, whether this has any clinical significance. Clearly, further studies evaluating changes in the ileal microflora in a large group of dogs with intestinal disease are warranted.

Our results are in agreement with a suggested qualitative variation in the canine intestinal microflora between compartments using bacterial culture (17). Few previous studies have examined this variation in the intestinal microflora in other species using molecular fingerprinting techniques (90, 102, 114). For example, in humans it has been shown that the colonic microflora differs from the rectal microflora (114). In chickens and pigs, similar to the results in our study, neighboring compartments showed significantly higher similarity values than non-neighboring compartments (90, 102). These studies, together with data presented here, suggest that unknown, host-related factors, contribute to the development of a unique microflora in an individual, and that differing compartments of the intestinal tract should be seen as unique ecosystems (89, 112). Differences in nutrient composition and concentration, differences in pH, and host secretions between various compartments of the intestine may contribute to this effect (88). Based on those findings, assessment of a duodenal or a fecal sample may not yield accurate information about the diversity of the microflora in other intestinal compartments.

CHAPTER IV

ASSESSMENT OF THE CANINE INTESTINAL MICROFLORA BY COMPARATIVE 16S rDNA ANALYSIS

OVERVIEW

The normal intestinal bacterial flora in dogs has not been well defined. Previous studies have focused on identification and enumeration of bacterial species by direct culture of intestinal contents. However, recently, it has been recognized that the majority of microbial species cannot be identified using standard culture techniques. Reasons for this inability to culture many bacterial species include non-viable or stressed microorganisms, obligate requirements for coexisting flora or host-derived products, bias due to selectivity of culture media, and a lack of knowledge regarding essential nutrients for some bacterial species. Thus, a culture-dependent approach may underestimate the bacterial diversity of complex microbial communities such as those found in the intestinal tract. The aim of this study was to describe the intestinal microflora in healthy dogs by direct sequence analysis of the 16S ribosomal DNA (16S rDNA; gene encoding 16S ribosomal RNA). Six healthy dogs, euthanatized for an unrelated study, were used. Immediately after euthanasia intestinal content was collected from the duodenum, jejunum, ileum, and colon. Bacterial DNA was purified by phenol:chloroform:iso-amylalcohol extraction, and the 16S rDNA was amplified with universal bacterial primers at low PCR cycle numbers. Amplicons were ligated into linearized cloning vectors and chemically competent *Escherichia coli* organisms were transformed. Colonies were randomly selected, the plasmid DNA purified, and the 16S rDNA insert identified by bidirectional automated cycle sequencing. All non-redundant sequences were tested for possible chimeric structures and putative chimeras were excluded from further analysis. The cloned sequences were compared to existing 16S rDNA sequences in GenBank and the Ribosomal Database Project (RDP). From a total of 864 clones analyzed, 106 non-redundant bacterial 16S rDNA sequences were identified, reaching coverage of 87.4%. Forty two (40%) of these sequences showed less than 98% sequence

similarity to 16S rDNA sequences listed in GenBank and RDP, and may represent as of yet uncharacterized bacterial species. Four major phylogenetic lineages were identified, with the majority of 16S rDNA sequences belonging to the *Clostridium*, *Bacteroides, Lactobacillus, Enterobacteriaceae* and *Fusobacterium* groups. These data indicate that the canine intestinal microflora is very complex, and that the molecular approach described in this study can facilitate identification of bacterial species in the canine intestinal tract that have not previously been characterized. The clinical significance of the diverse intestinal microflora in dogs, and alterations in bacterial diversity that may occur with gastrointestinal disease, need to be further investigated.

INTRODUCTION

The normal canine intestinal microflora is not well characterized. Previous studies aiming to characterize the intestinal bacterial flora in dogs have focused on the identification and enumeration of bacterial species through cultivation of intestinal content (5, 6, 10, 17, 78, 106). Bacterial culture, however, has limitations for assessing bacterial diversity in the gut. Samples of intestinal fluid must be processed immediately in order to preserve both aerobic and anaerobic species. Thus, an on-site microbiology laboratory is required in order to obtain a reliable assessment of bacterial species present as well as bacterial counts when culturing intestinal content. The intestinal tract harbors many anaerobic bacteria, which are prone to damage during handling of samples. Identification of bacteria is based on phenotypic identification systems. This may lead to limitations in accurately characterizing all microorganisms in a given sample. It is also increasingly recognized that the majority of microbial species present in biological samples escape identification when standard culture techniques are used alone (1, 9, 48, 49, 70, 96). Studies using a molecular biological approach, e.g. based on identification of 16S ribosomal RNA (rRNA) or 16S ribosomal DNA (DNA encoding the16S rRNA), have identified a greater number of bacterial species when compared to standard culture techniques, indicating that only a small proportion of bacterial species are cultivable using standard bacterial culture techniques (37, 70, 96). Based on sequence analysis of PCR products new bacterial species, not previously identified by cultivation have been

demonstrated in fecal samples of humans and in the gastrointestinal tract of pigs (48, 49).

It has been estimated that the human gastrointestinal tract harbors 300-500 different bacterial species and that 60 to 80% of these bacterial species have not yet been cultivated (48, 49). Reasons for this inability to culture many bacterial species include non-viable or stressed microorganisms, obligate requirements for coexisting flora or host-derived products, bias due to selectivity of culture media, and our lack of knowledge regarding essential nutrients for some bacterial species (15). Based on these studies, it is very likely that a culture-dependent approach underestimates the bacterial diversity found in the intestine of dogs, and an approach based on molecular methods may identify greater bacterial diversity in the intestinal tract of the domestic dog than previously reported. The aim of this study was to define the bacterial microflora in a group of healthy dogs by comparative 16S rDNA analysis.

MATERIALS AND METHODS

Sample material. Six healthy dogs, euthanatized for an unrelated study were used. No dog received any treatment (e.g., antibiotic therapy) that would be expected to have an impact on the composition of the intestinal microflora. Immediately after euthanasia intestinal content was collected from the duodenum, jejunum, ileum, and colon as described above.

Extraction of DNA and 16S rDNA amplification. Genomic DNA was extracted from intestinal samples using a bead beating method followed by phenol:chloroform:iso-amylalcohol extraction as described previously (98). The 16S rDNA was amplified using primers Bact-0008F (5' AGAGTTTGATCMTGGCTCAG 3') and Univ-1492R (5' GGTTACCTTGTTACGACTT 3') (49). Both primers were purchased from Gene Technologies Lab, College Station, Tex. DNA was amplified using the following reaction conditions: 20 mM Tris-HCl (pH 8.8), 2 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton® X-100, 0.1 mg/ml bovine serum albumin, 150 µM deoxynucleoside triphosphate, 1 mM $MgCl_2$, 0.25 µM of each primer, 2.5 U *Pfu* DNA Polymerase (proofreading capacity) with exonuclease activity (Strategene, La

Jolla, Calif.), and 2 µl DNA template (approximately 100 ng of DNA) in a 50µl reaction volume. The samples were amplified in a thermocycler (Mastercycler Gradient, Eppendorf AG, Hamburg, Germany) using the following PCR protocol: an initial denaturation step at 94ºC for 3 min 15 sec; 15 cycles (denaturation at 94ºC for 45 sec, annealing at 54ºC for 45 sec, extension at 72ºC for 3 min 30 sec), and a final elongation step at 72ºC for 30 min. For samples that were obtained from the colon and ileum 5 independent PCR reactions were performed, for samples that were obtained from the duodenum and jejunum 10 independent PCR reactions were performed. PCR products belonging to the same sample were pooled and concentrated using the QIAquick® PCR Purification Kit (Qiagen, Valencia, Calif.) following the manufacturer's instructions. The purity and correct size of resulting PCR amplicons (approx. 1,450 bp) were assessed on 1.2% agarose electrophoresis gels, stained with ethidium bromide (staining for 15 min and destaining in H_2O for 60 min), and visualized under UV light.

Cloning of 16S rDNA amplicons. Blunt end PCR products were ligated into linearized pCR-Blunt vectors (pCR®4Blunt-TOPO, Invitrogen, Carlsbad, Calif.) as specified by the manufacturer. Competent One Shot TOP10 *Escherichia Coli* organisms (Invitrogen) were transformed with ligation products by heat shock following the manufacturer's instructions. Recombinant organisms were grown on Luria-Bertani medium with ampicillin (50 µg ml^{-1}) at 37°C overnight. The pCR®4Blunt vector allows direct selection of recombinant cells via disruption of the lethal *E. coli* gene *ccd*B. Up to 96 colonies per sample were picked randomly and transferred to 1.5 ml Luria-Bertani broth and grown at 37°C for 24 hours in 2 ml well 96-well blocks (Perfectprep® BAC 96, Eppendorf) sealed with AirPore film (Eppendorf).

Plasmid extraction and sequencing of 16S rDNA. Plasmid extraction was performed in a 96-well format using the Perfectprep® BAC 96 plasmid purification kit (Eppendorf) and a single vacuum manifold (Eppendorf) following the manufacturer's instructions. Plasmid DNA was eluted with 50 µl of deionized water and the products were stored at -80°C until further use. The 16S rDNA inserts were analyzed by cycle sequencing using the ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied

Biosystems, Perkin-Elmer Corporation, Foster City, Calif.) and products were analyzed with an automated sequence analyzer (ABI PRISM 377 DNA Sequencer, Applied Biosystems). For provisional grouping of clones all clones were re-amplified from the 5'-terminal of the 16S rDNA using a single primer (Bact-8F; 5' AGAGTTTGATCMTGGCTCAG 3'). Obtained sequences with at least 98% similarity to each other were placed in groups using the software program FastGroup (82). One representative of each group was subjected to near-full-length bidirectional sequencing of both strands from positions 27 to 1492 of the 16S rDNA (*E. Coli* numbering) using the following primers: Bact-683R (5' GCATTTCACCGCTACAC 3'), Bact-968F (5' GAACGCGAAGAACCTTAC 3'), Bact-1054R (5' ACGAGCTGACGACAGCCATG 3'), and Univ-1492R (5' GGTTACCTTGTTACGACTT 3').

Sequence analysis. All near-full-length sequences were edited to exclude the PCR primer binding sites and tested for possible chimeric artifacts using the Check-_Chimera program and the Bellerophon software (36), both available through the Ribosomal Database Project (RDP). Putative chimeras were excluded from further analysis.

All newly obtained near-full-length sequences were compared to existing sequences in RDP (release version 9.26; approximately 120,000 16S rRNA sequences) and the closest neighbor for each sequence was downloaded. Sequences from both, the intestinal clone library and public databases, were aligned with the CLUSTAL_W program. The resulting alignment was inspected and manually adjusted using the alignment editor in the BioEdit software package. Phylogenetic trees were inferred and drawn based on the neighbor-joining algorithm using the TREECON software package (version 1.3b) and the Jukes-Cantor model for inferring evolutionary distances (101). The stability of branches was assessed by the bootstrap method (100 replicates) by using the algorithms available in the TREECON package. An Operational Taxonomic Unit (OTU) was defined as a group of sequences with less than 2% sequence divergence (98% similarity) to each other. The coverage of the clone library (i.e., the probability that any additional analyzed clone is different from any previously analyzed clone) was

calculated according to Good (28) using the formula [1-(*n*/*N*)] x 100, where *n* is the number of molecular species represented by one clone and *N* is the total number of sequences. Diversity of the clone library was evaluated by rarefaction analysis (38). Rarefaction curves were produced by using the software program aRarefactWin (available at http://www.uga.edu/~strata/software).

Nucleotide sequence accession numbers. Obtained near-full-length 16S rDNA sequences have been deposited in the GenBank database with accession numbers DQ113666 to DQ113771.

RESULTS

Phylogenetic analysis. A total of 988 clones were randomly selected from all samples. The cloning efficiency was 87.4% and a total of 864 clones contained an insert that could be sequenced. The partial sequence at the 5' end of the 16S rDNA comprising the variable region V1 to V3 of the 16S rDNA gene was used for provisional grouping of sequences based on a 98% similarity criterion. One representative from each of the provisional groups was subjected to near-full-length sequencing yielding 124 16S rDNA sequences. Eighteen (14.5%) of these near-full-length sequences were identified as possible chimeras and were, together with the clones of the group they represented, excluded from further analysis. A total of 106 non-redundant near-full-length 16S rDNA sequences, representing a total of 711 clones, were used for subsequent phylogenetic analysis representing a coverage of 85.1% for the entire clone library (i.e., the probability of the next clone to represent a yet undiscovered OTU was 14.9%). The calculated rarefaction curve approached a horizontal line, indicating that the diversity of the OTUs was almost covered in the clone library (Fig. 8).

Forty two (40%) of the obtained near-full-length sequences showed less than 98% sequence similarity to existing 16S rDNA sequences in the GenBank and RDP databases, and may represent as yet uncharacterized bacterial species. The results of the phylogenetic positioning of the clones are shown in figure 9 and table 5. Four major phylogenetic lineages were identified: the *Firmicutes* (47.7%), *Bacteroidetes* (12.4%), *Proteobacteria* (23.0%), and *Fusobacteria* (16.6%).

***Flexibacter-Cytophaga-Bacteroides* group.** A total of 88 clones were affiliated with the class *Bacteroides* representing 13 individual phylotypes. Of these 38 clones representing 8 phylotypes belonged to the *Bacteroides fragilis* subgroup. Fifty clones representing 5 phylotypes fell into the *Prevotella* subgroup.

***Fusobacterium* and relatives.** A total of 118 clones representing 9 phylotypes belonging to the class *Fusobacteria* were observed. The genus *Fusobacterium* was the most predominant group within this class with 97 clones comprising 8 individual OTUs. One OTU observed in the jejunum, ileum, and colon, which was represented by 36 clones, showed 96% similarity to *Fusobacterium varium* X55413. One OTU from the jejunum represented by 9 clones showed 99% similarity with *Clostridium rectum*. Two jejunal clones showed 98% similarity with *Fusobacterium necrogenes*. The *Cetobacterium* subgroup and *Fusobacterium perfoetens* were represented by 1 and 2 OTUs, respectively.

***Bacillus-Lactobacillus-Streptococcus* subdivision.** Eighty-one clones representing 15 individual phylotypes were affiliated with the order *Lactobacillales*. The genus *Lactobacillus* was the largest subgroup with 41 clones representing 6 individual phylotypes. Several clones from the duodenum, jejunum, and colon showed more than 98% similarity with *Lactobacillus reuteri, Lactobacillus murinus,* and *Lactobacillus johnsonii*, respectively. One OTU observed in the jejunum showed 96% similarity with *Lactobacillus aviarius*.

Twenty-five clones representing 5 phylotypes were affiliated with the genus *Streptococcaceae*. One OTU observed in the jejunum, ileum, and colon showed 99% similarity with *Streptococcus lutetiensis*. One OTU in the duodenum showed 99% similarity with *Streptococcus alactolyticus* and one OTU from the jejunum showed 99% similarity with *Streptococcus suis*. Two OTUs in the duodenum and jejunum showed 95% similarity with *Streptococcus agalactiae*.

One OTU showed 99% with *Enterococcus cecorum* Y18355. Two OTUs were affiliated with the *Abiotrophia* group.

***Clostridium* and relatives.** A total of 203 clones were affiliated with the class *Clostridia* representing 34 different phylotypes. The majority of observed OTUs were affiliated with the *Clostridium leptum* subgroup and *Clostridium barati* subgroup. Several clones showed more than 98% similarity to *Clostridium perfringens* and were observed in the duodenum, ileum, and colon.

***Proteobacteria*.** A total of 164 clones representing 25 OTUs were affiliated with the phylum *Proteobacteria*. The class *Gammaproteobacteria* was represented by 138 clones representing 22 OTUs. Within this class the family *Enterobacteriaceae* was the predominant subgroup with 126 clones representing 18 individual OTUs. The genus *Escherichia* was the most common representative with 85 clones, followed by the genus *Klebsiella*, which was represented by 20 clones.

***Eubacterium* and relatives.** Twenty-five clones representing 8 individual OTUs were affiliated with the *Clostridium coccoides* subgroup. A total of 117 clones representing 12 OTUs were affiliated with the *Clostridium lituseburense* subgroup. One OTU in the jejunum showed 99% similarity to *Clostridium hiranonis*, a bacterial species that displays bile acid 7-alpha-dehydroxylating activity. One OTU in the jejunum showed 99% similarity with *Clostridium glycolicum.* Finally, one OTU in the jejunum showed 93% similarity with *Clostridium propionicum.*

Spatial differences within the canine intestinal tract. The majority of observed 16S rDNA clones were affiliated with the bacterial orders of *Clostridiales, Enterobacteriales, Lactobacillales, Fusobacteriales*, and *Bacteroidales*. A spatial difference of these bacterial groups within different intestinal compartments was observed (Fig. 10). Table 6 summarizes the percentage of observed 16S rDNA clones belonging to different phylogenetic lineages in the different compartments of the canine intestinal tract.

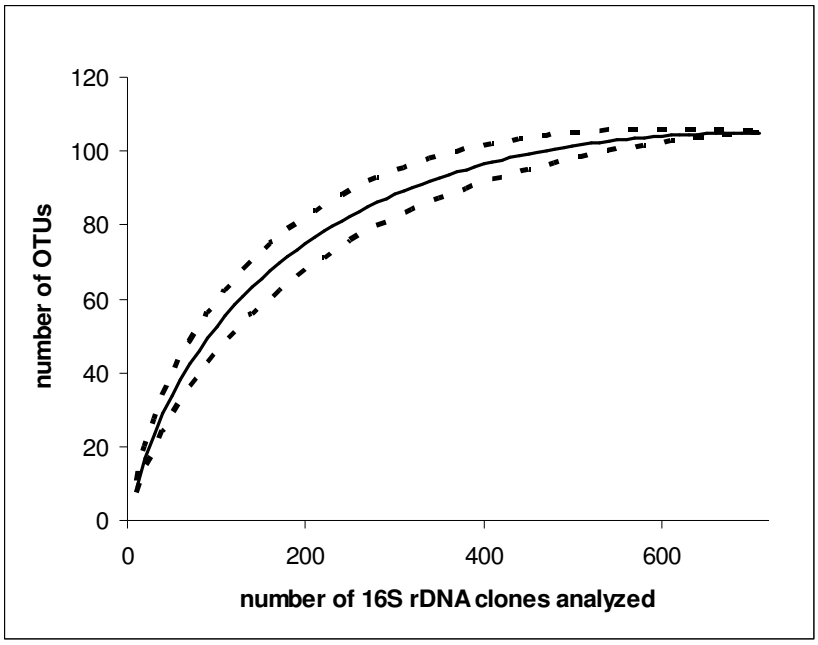
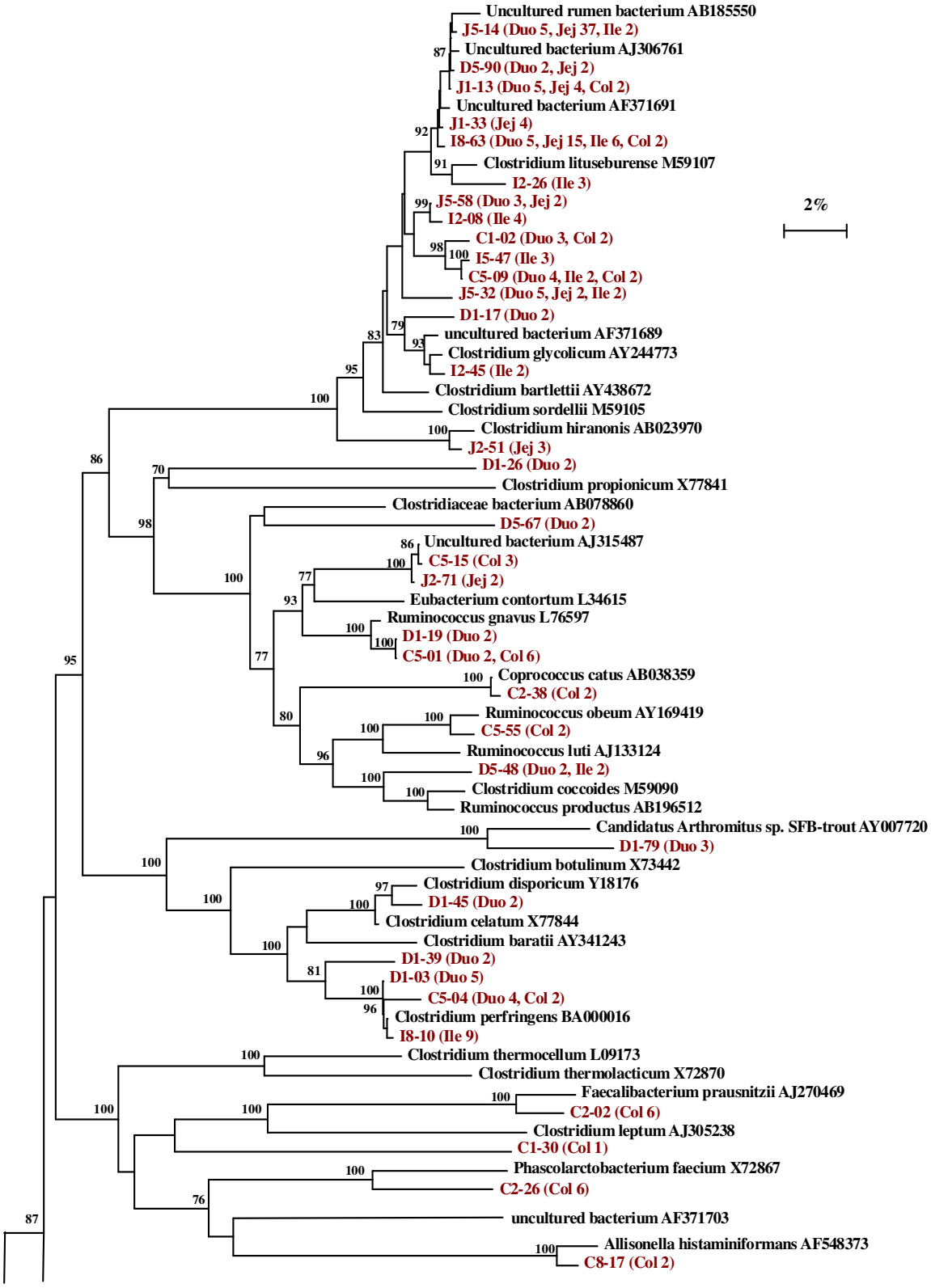


FIG 8. Rarefaction curve for 16S rDNA clones. The observed number of individual OTUs is plotted against the number of analyzed clones. The rarefaction curve approaches a horizontal line indicating that the diversity of the clone library is almost covered. The dotted lines represent the 95% confidence interval for the rarefaction curve.

FIG 9—*Next page*. Dendrogram showing the phylogenetic affiliation of OTUs isolated from the canine GI tract. The bar represents 2% sequence divergence. Near-full-length 16S rDNA sequences were aligned to their closest neighbor in the RDP database. The tree was inferred based on the neighbor-joining algorithm. Bootstrap values shown at the branches are based on 100 replicates. For each OTU the numbers in parenthesis indicate the number of observed clones in different intestinal compartments. (Duo=duodenum, Jej=jejunum, Ile=Ileum, Col=colon).


Uncultured rumen bacterium AB185550
J5-14 (Duo 5, Jej 37, Ile 2)
Uncultured bacterium AJ306761
D5-90 (Duo 2, Jej 2)
J1-13 (Duo 5, Jej 4, Col 2)
Uncultured bacterium AF371691
J1-33 (Jej 4)
I8-63 (Duo 5, Jej 15, Ile 6, Col 2)
Clostridium lituseburense M59107
I2-26 (Ile 3)
J5-58 (Duo 3, Jej 2)
I2-08 (Ile 4)
C1-02 (Duo 3, Col 2)
I5-47 (Ile 3)
C5-09 (Duo 4, Ile 2, Col 2)
J5-32 (Duo 5, Jej 2, Ile 2)
D1-17 (Duo 2)
uncultured bacterium AF371689
Clostridium glycolicum AY244773
I2-45 (Ile 2)
Clostridium bartlettii AY438672
Clostridium sordellii M59105
Clostridium hiranonis AB023970
J2-51 (Jej 3)
D1-26 (Duo 2)
Clostridium propionicum X77841
Clostridiaceae bacterium AB078860
D5-67 (Duo 2)
Uncultured bacterium AJ315487
C5-15 (Col 3)
J2-71 (Jej 2)
Eubacterium contortum L34615
Ruminococcus gnavus L76597
D1-19 (Duo 2)
C5-01 (Duo 2, Col 6)
Coprococcus catus AB038359
C2-38 (Col 2)
Ruminococcus obeum AY169419
C5-55 (Col 2)
Ruminococcus luti AJ133124
D5-48 (Duo 2, Ile 2)
Clostridium coccoides M59090
Ruminococcus productus AB196512
Candidatus Arthromitus sp. SFB-trout AY007720
D1-79 (Duo 3)
Clostridium botulinum X73442
Clostridium disporicum Y18176
D1-45 (Duo 2)
Clostridium celatum X77844
Clostridium baratii AY341243
D1-39 (Duo 2)
D1-03 (Duo 5)
C5-04 (Duo 4, Col 2)
Clostridium perfringens BA000016
I8-10 (Ile 9)
Clostridium thermocellum L09173
Clostridium thermolacticum X72870
Faecalibacterium prausnitzii AJ270469
C2-02 (Col 6)
Clostridium leptum AJ305238
C1-30 (Col 1)
Phascolarctobacterium faecium X72867
C2-26 (Col 6)
uncultured bacterium AF371703
Allisonella histaminiformans AF548373
C8-17 (Col 2)
2%

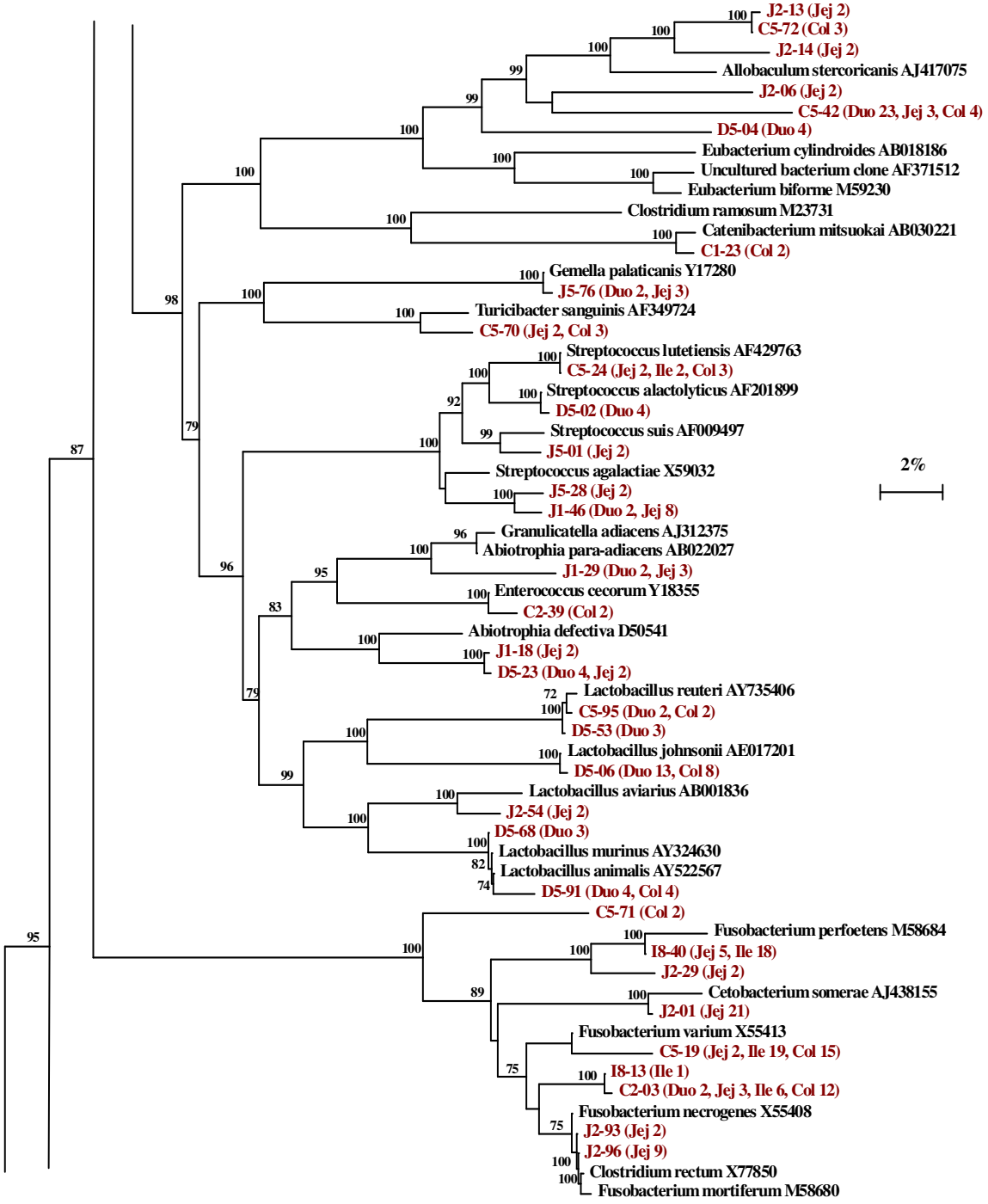


FIG 9—*Continued*.

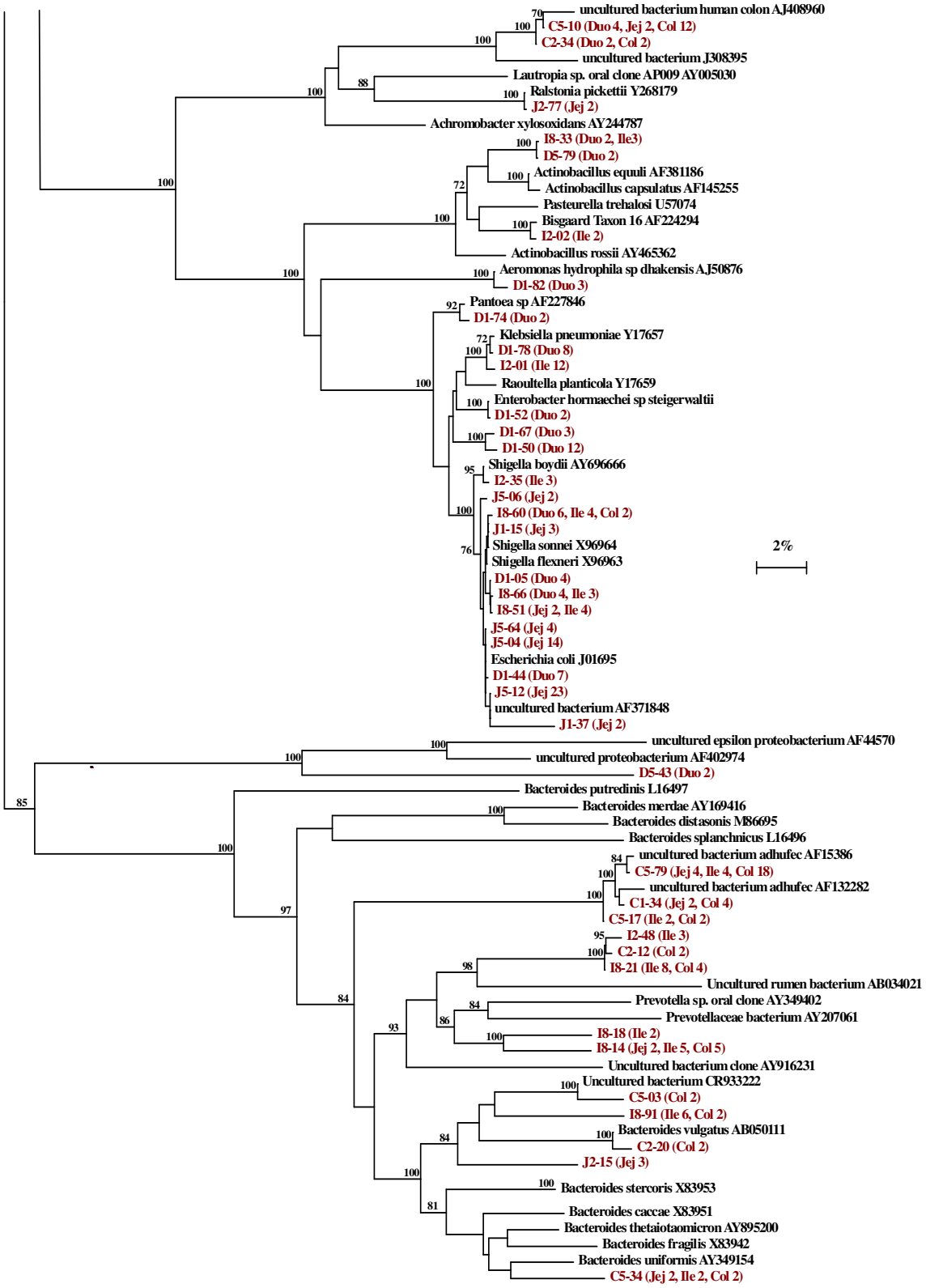


FIG 9—*Continued*.

TABLE 5. Phylogenetic classification of the 106 operative taxonomical units (OTUs) and respective number of clones obtained from the canine GI tract. Classification is based on the taxonomical hierarchy proposed in Bergey's Manual of Systematic Bacteriology (Ribosomal Database Project classifier).

A. Phylum Firmicutes (58 OTUs; 339 clones)
- class Bacilli (17; 91)
  - order Bacillales
    - family Staphylococcaceae
      - genus Gemella (1; 5)
  - order Lactobacillales
    - family Carnobacteriaceae
      - genus Granulicatella (1; 5)
    - family Aerococcaceae
      - genus Facklamia (1; 2)
      - unclassified Aerococcaceae (1; 6)
    - family Lactobacillaceae
      - genus Lactobacillus (6; 41)
    - family Streptococcaceae
      - genus Streptococcus (5; 25)
    - family Enterococcaceae
      - genus Enterococcus (1; 2)
  - unclassified Bacilli (1; 5)
- class Mollicutes (2; 32)
  - order Incertae sedis
    - family Erysipelotrichaceae
      - genus Erysipelothrix (1; 2)
  - unclassified Mollicutes (1; 30)
- class Clostridia (34; 203)
  - order Clostridiales
    - family Peptostreptococcaceae
      - genus Peptostreptococcus (1; 3)
    - family Acidaminococcaceae
      - genus Allisonella (1; 2)
      - genus Phascolarctobacterium (1;6)
    - family Lachnospiraceae
      - genus Ruminococcus (2; 10)
      - genus Anaerofilum (1; 6)
      - unclassified Lachnospiraceae (4;10)
    - family Clostridiaceae
      - genus Dorea (1; 5)
      - genus Clostridium (17; 151)
      - unclassified Clostridiaceae (1; 3)
  - unclassified Clostridiales (5; 13)
- unclassified Firmicutes (5; 13)

B. Phylum Fusobacteria (9 OTUs; 118 clones)
- class Fusobacteria (9; 118)
  - order Fusobacteriales
    - family Incertae sedis
      - genus Cetobacterium (1; 21)
    - family Fusobacteriaceae
      - genus Fusobacterium (8; 97)

C. Phylum Bacteroidetes (13 OTUs; 88 clones)
- class Bacteroidetes (13; 88)
  - order Bacteroidales
    - family Bacteroidaceae
      - genus Bacteroides (8; 38)
    - family Prevotellaceae
      - genus Prevotella (5; 50)

D. Phylum Proteobacteria (25 OTUs; 164 clones)
- class Betaproteobacteria (3; 26)
  - order Burkholderiales
    - family Burkholderiaceae
      - genus Ralstonia (1; 2)
    - unclassified Burkholderiales (2; 24)
- class Gammaproteobacteria (22; 138)
  - order Aeromonadales
    - family Aeromonadaceae
      - genus Aeromonas (1; 3)
  - order Pasteurellales
    - family Pasteurellaceae
      - unclassified Pasteurellaceae (3; 9)
  - order Enterobacteriales
    - family Enterobacteriaceae
      - genus Klebsiella (2; 20)
      - genus Enterobacter (2; 4)
      - genus Escherichia (11; 85)
      - unclassified Enterobacteriaceae (3;17)

E. Unclassified Bacteria (1 OTU)

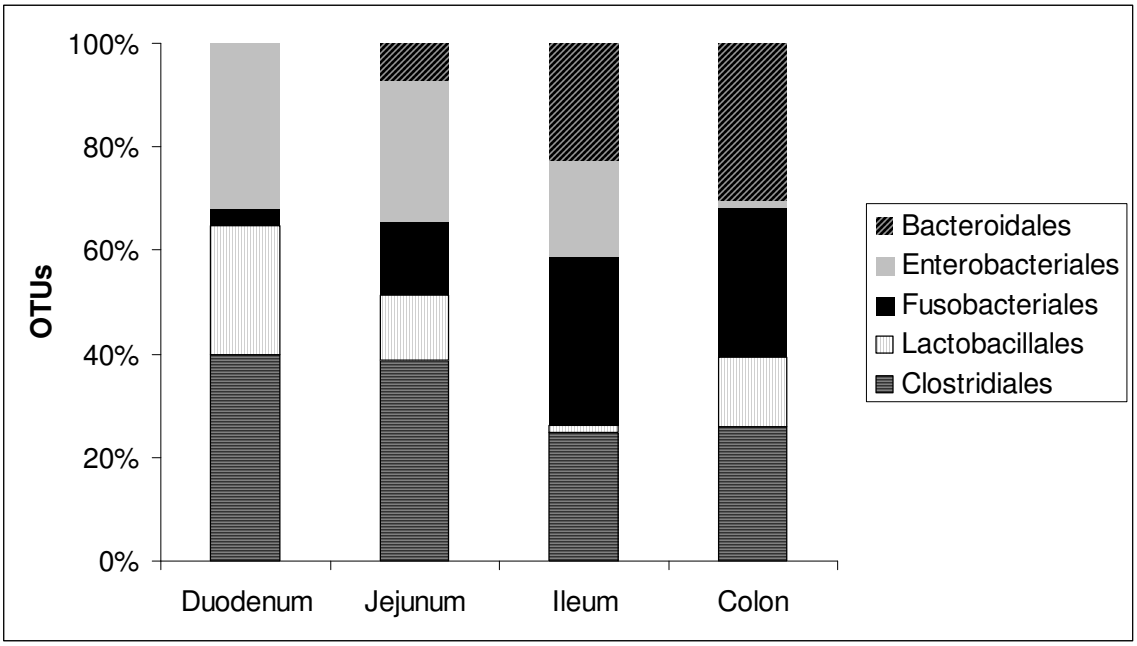


FIG 10. Percentage of 16S rDNA clones belonging to the major phylogenetic lineages in the different compartments of the canine intestinal tract.

TABLE 6. Percentages of 16S rDNA clones belonging to the major phylogenetic lineages in different compartments of the canine intestinal tract.

	Duodenum	Jejunum	Ileum	Colon
Clostridiales	40.0%	38.8%	24.8%	26.1%
Lactobacillales	24.7%	12.6%	1.4%	13.4%
Fusobacteriales	3.3%	14.2%	32.6%	28.9%
Enterobacteriales	32.0%	27.3%	18.4%	1.4%
Bacteroidales	0.0%	7.1%	22.7%	30.3%

DISCUSSION

To date bacterial culture techniques have been used for characterization of the canine intestinal microflora (6, 10, 17). However, recently it has been recognized that a majority of intestinal bacteria is not cultivable using standard bacterial culture techniques and molecular approaches based on the identification of the 16S rDNA have been successful in identifying many new bacterial species present in the intestine of various mammalian species (e.g., humans, pigs, and chickens) (9, 30, 33, 35, 47, 49, 70, 80, 84, 85, 87, 96, 103, 113). However, these molecular studies have focused almost exclusively on the microflora present in the colon or in fecal samples. Only few studies, performed in humans and pigs, have attempted to characterize the microflora in the ileum (49, 104). No published studies are available that characterize the intestinal microflora in the proximal small intestine (i.e., duodenum and jejunum) or along the entire intestinal tract of any mammalian species. A previous study using molecular fingerprinting techniques in healthy dogs has revealed marked qualitative differences in molecular fingerprints between individual intestinal compartments, suggesting that each area of the intestine harbors an unique ecosystem (98). Therefore, accurate characterization of the microflora in all intestinal compartments is warranted. The molecular approach, as described in this study, has revealed the presence of a complex intestinal microflora in the canine intestine. Our findings confirm the presence of several bacterial species that have been recently identified in fecal samples of other mammalian species. For example, one OTU observed in the canine jejunum shared 98% similarity with *Cetobacterium somerae*, a gram-negative, microaerotolerant, non-spore-forming, rod-shaped bacterium belonging to the class *Fusobacteriales* that has recently been isolated from the feces of children (20). Another OTU observed in the colon of one dog shared 99% similarity with *Allisonella histaminiformis*, a histamine producing bacterium belonging to the family *Acidaminococcaceae* (class *Clostridiales*) that has previously been identified in bovine and equine fecal samples (24). One OTU found in the duodenum of a dog shared 97% similarity with the gram-positive bacterium *Candidatus Arthromitus*, a long segmented filamentous bacterium (SFB). To date SFB have not been

successfully cultured but have been identified in the intestine of mice, rats, and chickens using molecular methods (92).

It has been estimated that the mammalian intestinal microflora comprises approximately 300-400 different bacterial species (49). In this present study a total of 106 individual near-full-length sequences have been identified in the intestine of six healthy dogs. Of these, 42 near-full-length 16S rDNA sequences showed less than 98% similarity to available 16S rDNA sequences in public databases, suggesting that these sequences represent previously uncharacterized bacteria. However, due to some limitations of bacterial identification based on direct sequencing of the 16S rDNA, it is likely that these findings still underestimate the total diversity of the canine intestinal microflora. Since a molecular approach using universal primers targets the predominant bacterial groups in the intestine (63), bacteria with low abundance might have escaped identification in this study. Further studies using group specific primers may lead to the identification of bacteria present in low numbers in the intestine. Currently, there is no consensus in the scientific literature about the degree of sequence divergence in the 16S rDNA that allows for clear discrimination between two bacterial species. Some closely related species may share a high similarity between their 16S rDNA sequences thus not allowing their discrimination on a species level. While, less than 97% similarity between 16S rDNA sequences is generally an accepted cut-off for differentiation between different bacterial species (94), some bacterial species clearly belonging to different bacterial groups may exhibit more than 97% similarity of their 16S rDNA (21). A 98% similarity cut-off is commonly used if near-full-length 16S rDNA sequences are analyzed (33, 96).

There was an obvious difference in the bacterial microflora between different intestinal compartments. While *Proteobacteria* (including *Escherichia coli*) were a substantial constituent of the duodenal microflora (32%), there was a low abundance of this phylum in the colon (1.4%). This low abundance in the colon is consistent with other studies where it has been shown that facultative anaerobic species represent only approximately 0.1% of bacteria in the strict anaerobic environment of the colon (96).

Anaerobic *Bacteroides* spp. and *Fusobacterium* spp. were only sporadically found in the proximal small intestine (i.e., duodenum and jejunum) but were substantial constituents of the microflora of the ileum and colon, respectively. Based on the relative abundance of 16S rDNA clones, bacteria belonging to the class *Clostridiales* were a substantial constituent of the canine intestinal microflora in all intestinal compartments and a total of 34 individual members of the class *Clostridia* could be identified.

Members of the class *Lactobacillales* were present in high abundance in the duodenum, jejunum, and colon in all dogs. While *Lactobacillus* spp. were also observed in the ileum, they were present only as a minor fraction (1.4%) of all identified clones in the ileum. Similar to a study performed using bacterial culture on jejunal fluid in dogs *Streptococcus alactolyticus*, *Lactobacillus murinus*, and *Lactobacillus reuteri* were observed in the proximal small intestine of dogs (75). Other prominent members of the *Bacillus-Lactobacillus-Streptococcus* subdivision were *Lactobacillus johnsonii* and *Lactobacillus aviarius*. In the present study no *Bifidobacteria* spp. were observed. *Bifidobacteria* spp. are part of the normal human intestinal microflora and are considered beneficial microorganisms. In dogs isolation of *Bifidobacteria* spp. has not been consistently reported. Based on bacterial culture up to 10^{10} colony forming units of *Bifidobacteria* spp. per ml intestinal juice (cfu/ml) have been reported in the large intestine of Beagle dogs (6, 17, 59). A lower abundance of *Bifidobacteria* spp. has also been reported in the proximal small intestine (6, 17, 59). However, other authors have not isolated *Bifidobacteria* spp. from the canine small intestine (18, 107). Also, a study characterizing the fecal microflora from a Labrador Retriever demonstrated that despite using Beerens agar, a medium specifically designed for the isolation of bifidobacteria, a mixture of various organisms other than *Bifidobacteria* spp. was isolated (30).

In conclusion, a molecular approach as described in this study facilitated identification of several previously uncharacterized bacterial 16S rDNA sequences in the intestinal tract of healthy dogs. A molecular approach may further aid in the identification of uncharacterized bacteria in dogs with intestinal disease and warrants future studies about their role in dogs with gastrointestinal disease.

CHAPTER V

DEVELOPMENT OF A ^{13}C-GLYCOCHOLIC ACID BLOOD TEST FOR ASSESSMENT OF SMALL INTESTINAL METABOLIC ACTIVITY IN DOGS*

OVERVIEW

The objectives of this study were to establish optimal doses of ^{13}C-glycocolic acid (GCA) for use in a ^{13}C-glycocolic acid blood test (GCA) as a marker for small intestinal bacterial metabolic activity in dogs. Eight healthy dogs were enrolled in this study. Four doses of GCA, 0.5, 1, 2, and 4 mg/kg body weight, were evaluated. GCA was administered orally. Blood samples were collected at 0, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, 180, 210, 240, 270, 300, 330, 360, 390, 420, 450, and 480 min. Blood samples were immediately transferred into evacuated tubes containing 2 ml of 6 N hydrochloric acid. The percent dose/min of ^{13}C administered as GCA (PCD) and cumulative PCD (CUMPCD) were determined by fractional mass spectrometry. No dog showed any clinically obvious side effects after oral administration of GCA. Doses of 1 and 2 mg/kg led to a significant increase in PCD and CUMPCD over time ($p<0.001$). The time-point for the peak PCD showed a high degree of variation between dogs and doses. The mean CUMPCD was significantly higher for the 1 mg/kg dose compared to the 2 and 4 mg/kg doses ($p<0.05$), suggesting saturation of the capacity of the intestinal microflora to deconjugate administered GCA. Administration of 1 mg/kg of ^{13}C-glycocholic acid led to an increase in CUMPCD over baseline in gas extracted from blood samples in all 8 healthy dogs and thus appears to be the best parameter to evaluate for future clinical studies.

---

*Reprinted with permission from **Suchodolski J.S., Ruaux C.G., Steiner J.M., Fetz K., Berghoff N., and Williams D.A.** 2005. Development of a 13C-glycocholic acid blood test for assessment of the small intestinal microflora in dogs. Can. J. Vet. Res. (in press)

INTRODUCTION

It is generally accepted that some canine patients present with a chronic, relapsing diarrhea that is responsive to antibiotic therapy. This condition has been referred to as "Small Intestinal Bacterial Overgrowth" by many authors (52, 62), although this terminology remains controversial as there is little objective data regarding the normal bacterial flora of the canine small intestine. While quantitative aerobic and anaerobic bacterial culture of the duodenal juice is considered the gold-standard for diagnosis of small intestinal bacterial overgrowth, the technical difficulty of this procedure limits its routine use in clinical practice. Also, it has been recently recognized that the majority of microbial species present in biological samples escapes identification by use of standard culture techniques alone (49, 96). For example, it has been estimated that 60 to 80% of bacterial organisms present in the gastrointestinal tract of humans and pigs have not yet been identified (48, 49). There is a significant need for a diagnostic test that will accurately reflect the bacterial content of the small intestine in dogs. Ideally, such a test would be sensitive and specific, non-invasive, readily available to general practitioners, and not require complicated sample handling.

^{14}C- and the ^{13}C-glycocholic acid breath tests have been used to diagnose small intestinal bacterial overgrowth in humans (71, 93). The ^{13}C-glycocholic acid breath test is based on the deconjugation of the naturally occurring bile acid glycocholic acid by intestinal bacteria. ^{13}C-glycocholic acid (GCA) is the glycine conjugate of cholic acid (Fig. 11). One carbon of the glycine residue is labeled with ^{13}C, a stable carbon isotope, and thus can serve as a tracer. After oral administration, GCA reaches the small intestine where it enters the physiological bile acid pool and undergoes enterohepatic circulation. Bacteria present in the small intestine deconjugate the ^{13}C-glycine portion from the core bile acid. The ^{13}C-glycine is metabolized by intestinal bacteria and ^{13}C is released. The ^{13}C freely diffuses into the blood stream where it may be transported in three different forms, dissolved in the plasma, bound to hemoglobin, and as bicarbonate. Eventually $^{13}CO_2$ is exhaled and can be quantified in breath (71). Alternatively, ^{13}C can be measured

in blood samples (16, 61). Addition of 6 N HCl to a blood sample releases $^{13}CO_2$, which can then be quantified by fractional mass spectrometry.

The presence of increased bacterial numbers in the upper small intestine may lead to an increase in the proportion of the orally administered dose of ^{13}C-glycocholic acid, which undergoes deconjugation. Thus, as has been observed in humans, an increase in small intestinal bacterial numbers may lead to an increase in the fraction of the $^{13}CO_2$ fraction of CO_2 in the circulation in comparison to healthy individuals (71). The aim of this study was to establish an optimal dose of GCA for use in a GCA blood test as a potential marker for small intestinal bacterial biomass and metabolic activity in dogs.

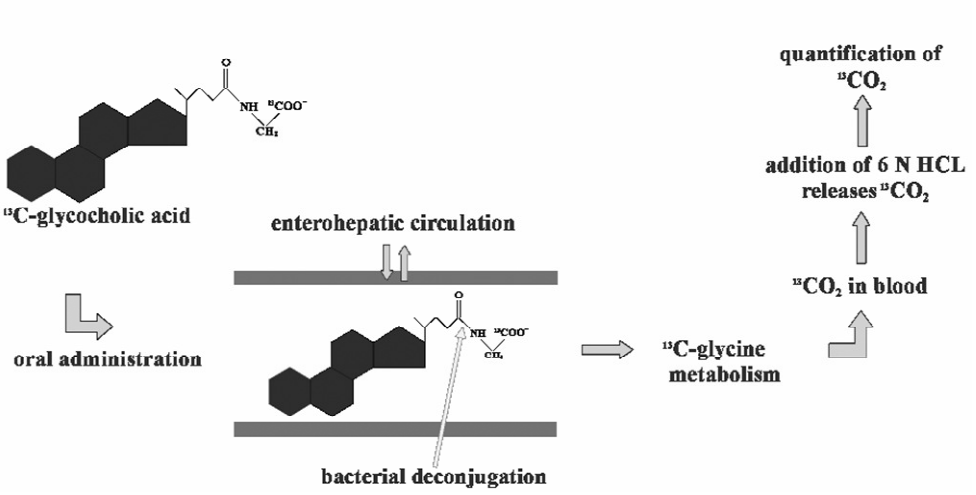


FIG 11. Principles of the ^{13}C- glycocholic acid blood test.

MATERIALS AND METHODS

The protocol was approved by the University Laboratory Animal Care Committee at Texas A&M University. Eight healthy female Hound dogs (median age: 3.0 years; range: 1 to 6.5 years) were enrolled in this study. Before the beginning of the

study serum folate, cobalamin, unconjugated bile acids, and trypsin-like immunoreactivity concentrations were evaluated to screen for gastrointestinal disease.

Four doses of ^{13}C-glycocholic acid (Glycocholic acid-^{13}C [glycyl-1-^{13}C], CDN Isotopes, Point-Claire, Quebec, Canada), 0.5 mg/kg (body weight), 1 mg/kg, 2 mg/kg, and 4 mg/kg body weight, were evaluated in a randomized study design. During each study period all dogs were given the same dose, with at least a 14-day rest period between individual study periods. Dogs were fed at 10 AM on the day prior to each study period. At 3 PM of that day, all food dishes and unconsumed food were removed from the dog runs. Venous access was established in each dog by insertion of a 12-inch-long, 18.5-gauge, indwelling catheter (Venocath, Abbott Laboratories, Abbott Park, Illinois, USA) into a jugular vein. After collection of a 1 ml baseline blood sample, GCA dissolved in 50 ml of deionized H_2O was administered using a gastric feeding tube. Additional 1 ml blood samples were collected at 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, 180, 210, 240, 270, 300, 330, 360, 390, 420, 450, and 480 min after GCA administration. Blood samples were immediately transferred into evacuated tubes (Vacutainer Sodium Lithium 10mL, Becton Dickinson, Franklin Lanes, New Jersey, USA) containing 2 ml of 6 N hydrochloric acid.

The $^{13}CO_2$ released into the gas phase above the blood sample in each tube was determined by gas chromatograph isotope ratio mass spectrometry (Automated Breath ^{13}Carbon Analyzer, Europa Scientific Ltd., Crewe, United Kingdom) and expressed as a relative isotope ratio $^{13}CO_2$:$^{12}CO_2$ (i.e. percentage $^{13}CO_2$ in total CO_2 above background $^{13}CO_2$ concentration in the environment) (13). This relative isotope ratio was then converted into an absolute ratio (R_{Sample}) by comparing the measured $^{13}CO_2$:$^{12}CO_2$ of the sample with the absolute $^{13}CO_2$:$^{12}CO_2$ ratio of the international calcium carbonate standard PDB (Pee Dee belemnite; PDB is a limestone fossil of *Belemnitella americana* from the Cretaceous Pee Dee formation in South Carolina) and calculated from the following equation (8):

$$\delta^{13}C_{Sample}\,(‰) = [(R_{Sample}/R_{PDB}) - 1] \times 1000$$

where

$$R_{Sample} = {}^{13}C/{}^{12}C = [(\delta^{13}C_{Sample}/1000) + 1] \times R_{PDB}$$

The $\delta^{13}C_{Sample}$ is the relative difference of the sample to PDB. PDB has, by international convention, an arbitrarily assigned $\delta^{13}C$ value of 0‰, its absolute ^{13}C:^{12}C (R_{PDB}) has been reported as 0.0112372 (8). A negative or positive $\delta^{13}C$ indicates that the sample contains relatively less or more ^{13}C than the PDB, respectively.

The data was initially expressed as $\delta^{13}C_{Sample}$ over baseline (DOB). The ^{13}C expired (µmol/min) was calculated as DOB x 0.0112372 x CO_2 produced. The CO_2 production was estimated using the following calculation: (RER x RQ)/4.8 L, where RER is the resting energy requirement ($BW^{0.75}$ x 70) and RQ the respiratory quotient (estimated RQ for canine diets was 0.8).

The percent dose/min of ^{13}C administered as GCA (PCD) was calculated using the following formula:

$$\frac{^{13}C \text{ expired (µmol/min)}}{\{\{[(\text{amount of } ^{13}GCA \text{ given (mg) X 1000})/MW] \text{ X (atom\%) X (\# labeled atoms)}\} \text{ X 1000}\}}$$

where MW is the molecular weight of the ^{13}GCA.

The cumulative PCD (CUMPCD) is the cumulative % of the dose recovered, and was calculated as:

$$CUMPCD = \{[(PCD_t + PCD_{t-1}) \text{ X } \Delta t \text{ (min)}]/2\} + CUMPCD_{t-1}$$

PCDs and CUMPCDs were compared for each one of the 4 individual doses using a 1-way ANOVA followed by Tukey's multiple comparison tests. The effect of the differing doses on PCDs and CUMPCDs over time was analyzed by a 2-way ANOVA. Coefficients of variation (CV% = (standard deviation/mean) X 100) were calculated for both PCD and CUMPCD values for each time point and for each dose. The mean %CVs for the 1 mg/kg and 2 mg/kg dose were compared using a student's *t*-test. Data were analyzed using a statistical software package (GraphPad Prism 3.0, GraphPad Software Inc, San Diego, California, USA).

RESULTS

Prior to being enrolled into the study none of the dogs had any history of clinical signs or any changes in evaluated blood parameters that would be consistent with gastrointestinal disease.

None of the dogs showed any clinically obvious side effects after oral administration of GCA. Neither the 0.5 mg/kg nor the 4 mg/kg dose lead to a significant increase in PCD over time ($p=0.083$ and $p=0.107$, respectively), but both doses led to a significant increase in CUMPCD over time ($p<0.0001$ for both doses). Both the 1 and 2 mg/kg doses led to significant increases in PCD ($p<0.0001$ and $p=0.024$, respectively) and CUMPCD over time ($p<0.0001$ for both doses; Fig. 12). The time-points for peak PCDs showed a high degree of variation between dogs and doses (Fig. 13). The mean CUMPCD was significantly higher for the 1 mg/kg dose than the other doses ($p<0.05$; Fig. 12). Administration of 1 mg/kg of GCA led to an increase in CUMPCD over baseline in gas extracted from blood samples in all 8 healthy dogs (Fig. 14), whereas 7/8 dogs had an increase in CUMPCD over baseline after administration of 2 mg/kg GCA. There was no significant difference between the mean %CVs for CUMPCD between the 1 mg/kg and 2 mg/kg dose ($p=0.535$).

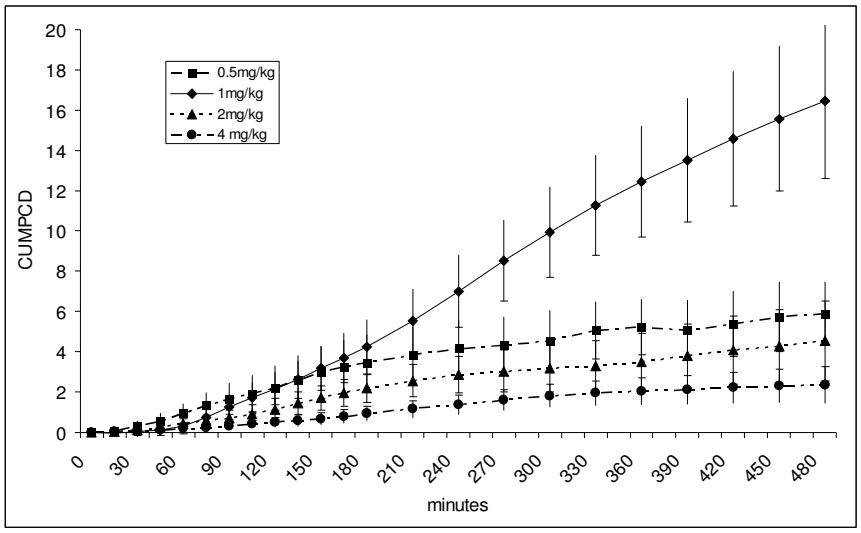


FIG 12. Cumulative percent dose/min of ^{13}C administered as ^{13}C-glycocholic acid (CUMPCD). All 4 doses led to a significant increase of CUMPCD over time ($p<0.001$). The lower CUMPCD of the 0.5 mg/kg dose compared to the 1 mg/kg dose suggests lack of substrate for intestinal bacteria, while the lower CUMPCD of the 2 and 4 mg/kg dose suggests saturation of the deconjugation capacity of the small intestinal microflora.

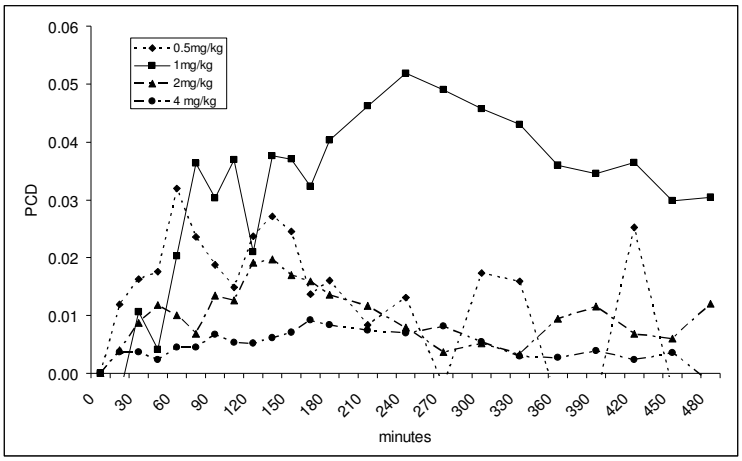


FIG 13. The percent dose/min of ^{13}C administered as GCA (PCD). The 1 and 2 mg/kg doses led to a significant increase in PCD. However, the time-point for the peak PCD showed a high degree of variation between dogs and doses. Therefore, determination of CUMPCD (Figure 2) is suggested for future clinical studies.

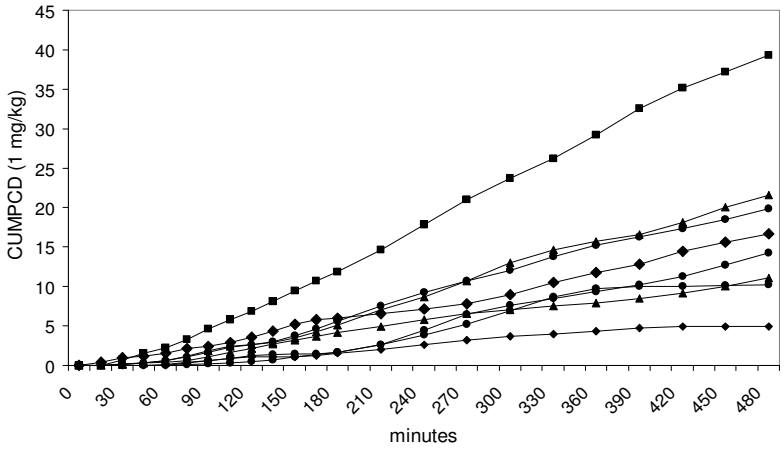


FIG 14. Individual cumulative percent dose/min of ^{13}C administered as ^{13}C-glycocholic acid (CUMPCD) in 8 healthy dogs after administration of 1 mg/kg ^{13}C-glycocholic acid.

DISCUSSION

Previous studies have shown that bacterial deconjugation (i.e. the ability of bacteria to remove a glycine or taurine moiety) of bile acids, and the subsequent appearance of unconjugated bile acids in serum, can serve as an non-invasive marker for small intestinal bacterial metabolic activity in dogs (57). Sample preparation for measurement of serum unconjugated bile acids is technically challenging, time-consuming and, therefore, relatively expensive. Also, it has been speculated that spurious elevations of serum unconjugated bile acid concentrations and hence a false positive diagnosis of small intestinal bacterial overgrowth may also be obtained through entry of small amounts of bile into the small intestine during the fasted state as a result of migrating motor complexes and spontaneous gall bladder contraction (76). The ^{13}C-glycocholic acid blood test (GCA) potentially combines the benefits of the measurement of a bacteria-specific metabolic activity with the minimally invasive nature of a blood test, and has a considerably lower technical difficulty in sample preparation than the unconjugated bile acids test. As the compound being administered is not present in the normal bile acid pool, this test is potentially less dependant upon strict fasting and less susceptible to interference from migrating motor complexes and spontaneous gall bladder contraction. The carbon dioxide is easily extracted from a blood sample by the addition of hydrochloric acid, and no subsequent processing is necessary before measurement of the ^{13}C-labeled carbon dioxide via fractional mass spectrometry. While the measurement of ^{13}C in clinical samples necessitates the use of expensive equipment, i.e. an automated breath ^{13}C analyzer, recent studies have shown that samples can be obtained easily in clinical settings and shipped to an appropriate laboratory which has the necessary equipment for analysis of samples (12). Also, recent studies have demonstrated that samples are stable for up to 3 weeks at room temperature, thus allowing storage and shipment to the laboratory (12).

In this study we evaluated four different doses of GCA. After oral administration of up to 4 mg/kg of ^{13}C-glycocholic acid no gross clinical evidence of adverse effects were observed during the course of the study, suggesting that the GCA test is safe in

healthy dogs. Safety of GCA needs to be further evaluated in dogs with gastrointestinal disease.

In this study only the 1 mg/kg and 2 mg/kg dose led to a significant increase in both PCD and CUMPCD over time. The lower CUMPCD of the 0.5 mg/kg dose compared to the 1 mg/kg dose suggests lack of substrate for intestinal bacteria. Increasing the GCA dose to 2 and 4 mg/kg led to a decrease in CUMPCD, suggesting saturation of the capacity of the intestinal microflora to deconjugate administered GCA. Determination of PCD and their summation (ie, cumulative PCD or CUMPCD) have been traditionally used in humans to measure bacterial metabolic mass (71). Determination of a peak PCD would be preferable compared to CUMPCD as fewer samples, i.e. a baseline sample and a sample taken at the time of the PCD peak, would be required. The peak PCD for the 1 mg/kg and 2 mg/kg dose showed a high degree of variation between individual dogs, preventing the selection of an optimal sampling time point for determination of PCD. This variation might be due to differences in intestinal motility or differences in composition of the intestinal microflora leading to different deconjugation kinetics, between individual dogs. The CUMPCD showed a lower degree of variation between dogs, suggesting that the CUMPCD would be a more reliable parameter than the PCD. Administration of 1 mg/kg of ^{13}C-glycocholic acid led to an increase in CUMPCD over baseline in gas extracted from blood samples in all 8 healthy dogs and thus this dose appears to be the best parameter to evaluate for future clinical studies. However, while a 1mg/kg dose appears to be the best dose to use in healthy dogs, it may be necessary also to evaluate other higher doses in clinically ill dogs with suspected bacterial overgrowth or the lack of substrate effect as noted in the current study at the 0.5 mg/kg dose may be noted. Additional studies that evaluate the clinical utility of the GCA test in dogs with suspected small intestinal bacterial overgrowth are warranted.

CHAPTER VI

EFFECT OF ANTIBIOTIC THERAPY ON SERUM MARKERS FOR ASSESSMENT OF THE SMALL INTESTINAL MICROFLORA

OVERVIEW

The difficulties in culturing canine duodenal juice limit the ability of clinicians to assess the small intestinal microflora in dogs. Serum markers and dynamic tests for assessment of the small intestinal microflora have been described. These tests assess changes in bacterial metabolic mass (serum unconjugated cholic acid (SUCA) and ^{13}C-glycocholic acid blood test (^{13}C-GCBT)), bacterial synthetic activity (serum folate concentration), and bacterial competition for dietary substrates (serum cobalamin concentration). The aim of this study was to determine the effect of antibiotic therapy with oral tylosin of these markers of small intestinal microflora. Ten healthy hound-cross laboratory dogs, all intact females, were selected from an in-house colony. Dogs were relocated into a special laboratory dog ward and acclimated for 1 month prior to starting the study. At day 0, following overnight withholding of food, indwelling jugular catheters were placed and baseline sera for determination of cobalamin, folate, and unconjugated cholic acid concentrations were collected. A ^{13}C-GCBT was then carried out, using 1 mg/kg ^{13}C-glycocholic acid mixed with an egg yolk and baked in a microwave for 1 minute. The dogs then received 25 mg/kg tylosin per os BID for 28 days. On day 28, jugular catheters were once again placed, baseline sera were collected, and the ^{13}C-GCBT was repeated. Serum concentrations of cobalamin, folate, and unconjugated cholic acids were determined also days 28 and 56. The cumulative percentage of the administered dose recovered (CUMPCD) was determined in blood samples by fractional mass spectrometry. Data were analyzed using repeated measures ANOVA, or a Friedman's test if data were not normally distributed.

Tylosin treatment was associated with significant changes in some of the serum markers for assessment of the intestinal microflora in these dogs. Serum cobalamin

concentrations showed no change after tylosin administration. Serum folate concentrations increased significantly after tylosin administration ($p<0.001$). Also, SUCA increased significantly after tylosin administration ($p<0.01$). Overall bacterial bile acid metabolism as assessed by the ^{13}C-GCBT increased significantly ($p<0.001$) following tylosin administration. These data suggest that tylosin administration is associated with alterations in the canine small intestinal flora, reflected in alterations in serum markers and dynamic tests of the small intestinal microflora. Increased serum folate, SUCA, and CUMPCD in the ^{13}C-GCBT suggest that, in the dogs described here, tylosin administration increased the biomass of organisms carrying out these metabolic functions.

INTRODUCTION

Assessment of the numbers and types of bacteria in the small intestine of dogs is challenging, as obtaining a sample requires anesthesia and either endoscopic collection of duodenal juice or an invasive surgical procedure. This complicates both, research concerning the intestinal microflora and also the possibility of diagnosing disorders that involve the small intestinal microflora in dogs. We recently developed a ^{13}C-glycocholic acid blood test (^{13}C-GCBT) as a minimally invasive test for quantification of small intestinal bacterial biomass in dogs, and established optimal dose and sampling times. The principles behind ^{13}C-GCBT are that after oral administration of ^{13}C-labeled glycocholic acid (a naturally occurring bile acid), the glycocholic acid can be broken down by bacteria in the intestine, yielding $^{13}CO_2$ that can be quantified in a blood sample (see Chapter V).

The hypotheses for this study were that antibiotic therapy with a broad spectrum antibiotic agent, tylosin, leads to a decrease in the number of bacteria present in the small intestine, that these changes can be subsequently assessed by the ^{13}C-GCBT, and changes in commonly used minimally-invasive serum markers (i.e., serum cobalamin, serum folate, and serum unconjugated cholic acid concentrations) are altered by antibiotic therapy with tylosin. The antibiotic tylosin (Tylan®) was chosen because it is

commonly recommended for treatment of intestinal disorders such as small intestinal bacterial overgrowth or antibiotic-responsive diarrhea in canine patients (105).

MATERIALS AND METHODS

Animals. This part of the project involved the use of research dogs and was approved by the University Laboratory Animal Care Committee at Texas A&M University (AUP #2004-117). Ten healthy hound-cross laboratory dogs, all intact females, were selected from an in-house colony. Dogs were relocated into a special laboratory dog ward and acclimated for 1 month prior to the start of the study.

^{13}C-glycocholic acid blood test (^{13}C-GCBT). On day 0, following withholding of food overnight, indwelling jugular catheters were placed and baseline sera samples were collected. A ^{13}C-GCBT was then carried out, using 1 mg/kg ^{13}C-glycocholic acid (Glycocholic acid-^{13}C [glycyl-1-^{13}C], CDN Isotopes, Point-Claire, Quebec, Canada) mixed with an egg yolk and baked in a microwave for 1 minute. The baked egg yolk was then added to 5 g/kg bodyweight of a canned maintenance dog food (Hill's Science Diet, Hill's Science and Technology Center, Topeka, Kan.). Additional blood samples of 1 ml each were collected at 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, 180, 210, 240, 270, 300, 330, 360, 390, 420, 450, and 480 min after ^{13}C-glycocholic acid administration. Blood samples were immediately transferred into evacuated tubes (Vacutainer Sodium Lithium 10mL, Becton Dickinson, Franklin Lanes, New Jersey, USA) containing 2 ml of 6 N hydrochloric acid. After collection of the last blood samples jugular catheters were removed. The dogs then received 25 mg/kg tylosin (Tylan®) per os BID for 28 days. On day 28, jugular catheters were once again placed, baseline sera were collected, and the ^{13}C-GCBT was repeated. Serum concentrations of cobalamin, folate, and unconjugated cholic acid were measured on days 0, 28, and 56 (4 weeks after cessation of tylosin administration). Serum cobalamin and folate concentrations were measured using a commercially available automated competitive chemiluminescence immunoassay (DPC IMMULITE, Diagnostic Products Corp, Randolph, NJ.). SUCA concentrations were measured using solid-phase extraction followed by gas chromatography-mass spectrometry with selected ion monitoring as

described previously (76). The cumulative percent dose/min of ^{13}C administered as GCA (CUMPCD) was determined in blood samples by fractional mass spectrometry (Automated Breath ^{13}Carbon Analyzer, Europa Scientific Ltd, Crewe, UK). Data were analyzed using repeated measures ANOVA, or Friedman's test if the data sets to be compared failed normality testing using Kolmogorov Smirnov normality test, followed by Bonferroni's multiple comparison test.

RESULTS

Tylosin treatment was associated with significant changes of some of the marker tests for assessment of intestinal microflora in these dogs. Tables 7 to 9 and figures 15 to 17 summarize the changes in serum concentrations of cobalamin, folate, and unconjugated cholic acid, respectively. While serum cobalamin concentrations did not differ significantly between the three measurements (Table 7 and Fig. 15), serum folate concentrations increased significantly after 4 weeks of tylosin administration from a mean±SD of 12.3±1.8 µg/L to 15.0±2.0 µg/L ($p<0.001$; Table 8 and Fig. 16). At day 56, four weeks after withdrawal of tylosin, the mean folate serum concentration was significantly higher compared to baseline (mean±SD: 14.6±2.4 µg/L; $p<0.05$) but not significantly different from day 28 ($p>0.05$). Similarly, SUCA concentration increased significantly after tylosin administration from a median serum concentration of 7.6 nmol/L to 126.9 nmol/L ($p<0.01$; Table 9 and Fig. 17). After withdrawal of tylosin, median SUCA concentration was 23.3 nmol/L and was not significantly different from day 0 ($p>0.05$).

The CUMPCD (determined up to 480 minutes) of the ^{13}C-GCBT increased in 8 out of 10 dogs after tylosin treatment (Figs. 18 and 19). Two-way ANOVA indicated that there was a significant effect of tylosin treatment ($p<0.001$) on the CUMPCD.

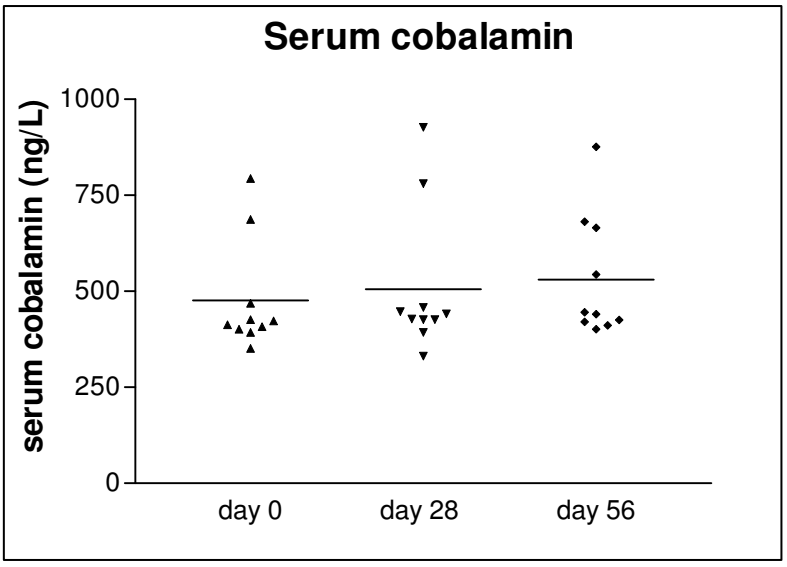


FIG 15. Serum concentrations of cobalamin (Vitamin B12) before (day 0), after 4 weeks of tylosin administration (day 28), and 4 weeks after withdrawal of tylosin (day 56). The bars depict the mean cobalamin concentration for each data set.

TABLE 7. Serum concentrations of cobalamin (Vitamin B12) before (day 0), after 4 weeks of tylosin administration (day 28), and 4 weeks after withdrawal of tylosin (day 56). (SD = standard deviation)

Cobalamin (ng/L)					
	day 0		**day 28**		**day 56**
dog 1	413.0		428.0		445.0
dog 2	423.0		393.0		425.0
dog 3	393.0		447.0		401.0
dog 4	469.0		331.0		411.0
dog 5	401.0		426.0		420.0
dog 6	687.0		780.0		681.0
dog 7	351.0		441.0		544.0
dog 8	426.0		426.0		665.0
dog 9	794.0		927.0		876.0
dog 10	408.0		458.0		440.0
Mean	**476.5**		**505.7**		**530.8**
SD	**144.4**		**189.9**		**159.9**

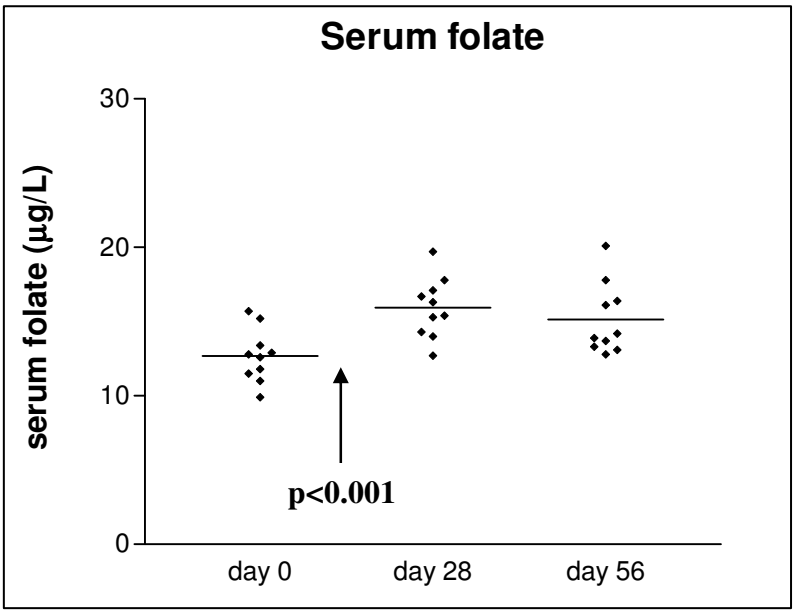


FIG 16. Serum concentrations of folate before (day 0), after 4 weeks of tylosin administration (day 28), and 4 weeks after withdrawal of tylosin (day 56). The bars depict the mean serum folate concentrations for each data set.

TABLE 8. Serum concentrations of folate before (day 0), after 4 weeks of tylosin administration (day 28), and 4 weeks after withdrawal of tylosin (day 56). (SD = standard deviation)

Folate (µg/L)					
	day 0		**day 28**		**day 56**
dog 1	11.5		14.3		12.8
dog 2	15.2		16.7		14.2
dog 3	11.0		19.7		13.3
dog 4	12.8		12.7		13.1
dog 5	9.9		14.0		16.1
dog 6	12.6		15.3		16.4
dog 7	12.9		17.8		13.7
dog 8	11.8		17.1		13.9
dog 9	13.4		15.4		17.8
dog 10	15.7		16.3		20.1
Mean	**12.3**		**15.9**		**14.6**
SD	**1.8**		**2.0**		**2.4**

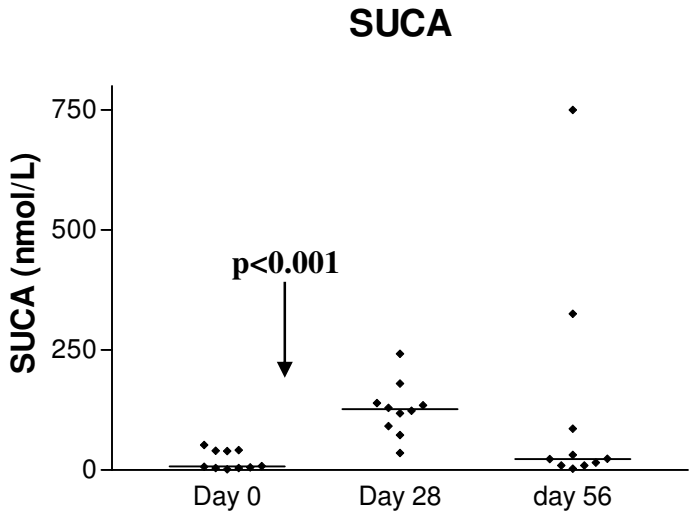


FIG 17. Serum concentrations of unconjugated cholic acid (SUCA) before (day 0), after 4 weeks of tylosin administration (day 28), and 4 weeks after withdrawal of tylosin (day 56). The bars depict the median SUCA concentrations for each data set.

TABLE 9. Serum concentrations of unconjugated cholic acid (SUCA) before (day 0), after 4 weeks of tylosin administration (day 28), and 4 weeks after withdrawal of tylosin (day 56). (SD = standard deviation).

SUCA (nmol/L)					
	day 0		**day 28**		**day 56**
dog 1	4.2		118.0		31.4
dog 2	41.5		139.8		9.5
dog 3	1.9		35.9		22.8
dog 4	5.4		134.7		15.7
dog 5	52.3		242.6		325.4
dog 6	8.2		91.7		86.0
dog 7	4.1		73.0		3.1
dog 8	6.9		123.8		750.2
dog 9	40.3		129.9		9.5
dog 10	39.9		180.0		23.8
Median	**7.6**		**126.9**		**23.3**
SD	**20.2**		**56.7**		**239.3**

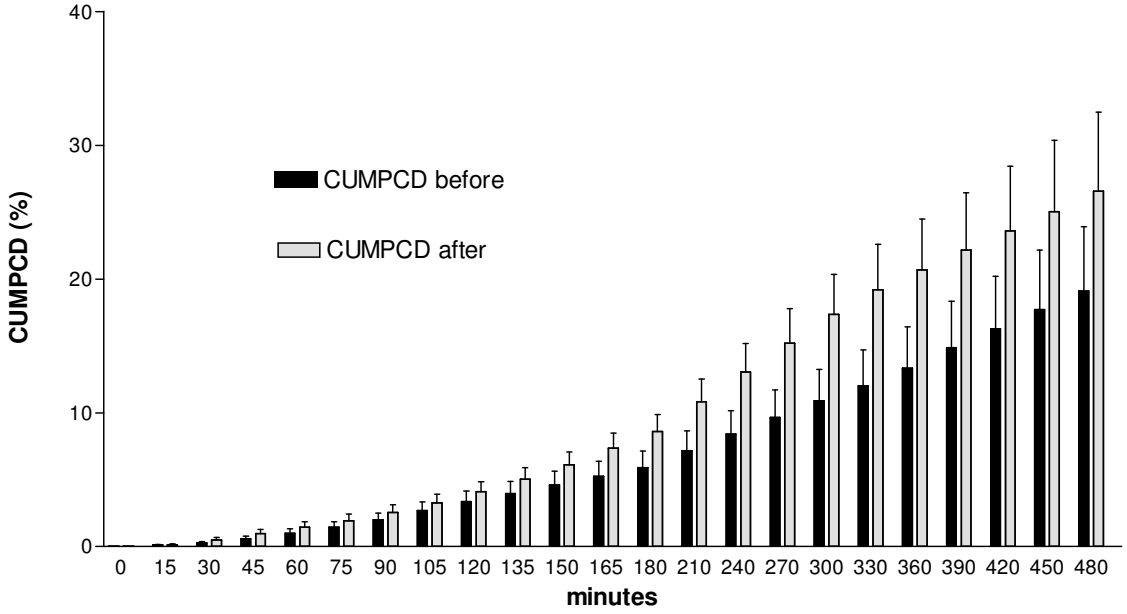


FIG 18. Cumulative percent dose/min of ^{13}C administered as ^{13}C-glycocholic acid (CUMPCD) before and after 4 weeks of tylosin administration.

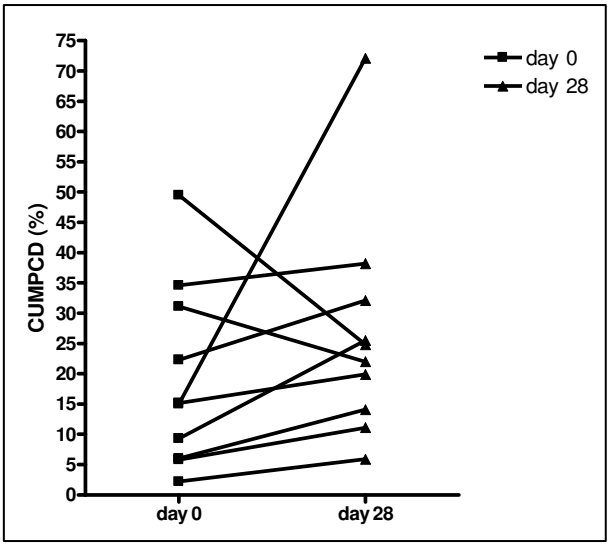


FIG 19. Changes in CUMPCD up to 480 minutes for the ^{13}C-GCBT before (day 0) and after 4 weeks of tylosin administration (day 28) for each one of the 10 dogs enrolled.

DISCUSSION

Tylosin belongs to the macrolide class of antibiotics that is characterized by a multi-membered lactone ring (58). Antibiotics of the macrolide class inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit (86). This inhibits the translocation of peptidyl-tRNA from the acceptor to the donor side on the ribosome as well as the initial steps of assembly of the 50S subunit (86). Tylosin has antibiotic activity predominantly against gram-positive bacteria (e.g., *Stapylococcus* spp., *Streptococcus* spp., and *Clostridium* spp.) and also against some *Mycoplasma* and *Chlamydia* spp. While tylosin also has an effect against some gram-negative bacteria (e.g., *Campylobacter* spp., *Helicobacter pylori, Hemophilus* spp., *Pasteurella* spp., and *Legionella* spp.) it has no effect against members of the *Enterobacteriaceae* group (e.g., *Escherichia coli* and *Salmonella* spp.). In pigs tylosin is an effective drug against the causative agent of proliferative enteropathy, *Lawsonia intracellularis* (86).

Some pathogenic bacteria including *Clostridium perfringens* and *Campylobacter* spp. have been suggested to play a role in the etiopathogenesis of chronic or intermittent diarrhea in dogs, and these bacteria are sensitive to tylosin (55). Tylosin also has been reported to have anti-inflammatory and immunomodulatory properties and may thus be useful in the treatment of canine chronic enteropathies such as inflammatory bowel disease (26, 86, 105).

Tylosin was chosen as an antibiotic agent for this study since it's use is commonly recommended for the treatment of intestinal disorders such as small intestinal bacterial overgrowth or antibiotic-responsive diarrhea in dogs (105). Our hypothesis was that administration of tylosin leads to a reduction in small intestinal bacterial biomass reflected in changes of serum concentrations of indirect markers. More specifically we expected an increase in concentrations of serum cobalamin and a decrease in serum concentrations of folate and serum unconjugated cholic acid, and also a decrease of the cumulative percent dose/min of ^{13}C administered as ^{13}C-glycocholic acid (CUMPCD). In contrast to the hypothesis administration of tylosin was associated with an increase in serum concentrations of folate, serum unconjugated cholic acid (SUCA), and the

CUMPCD of the ^{13}C-GCBT. Four weeks after withdrawal of tylosin (day 56), both mean serum folate concentrations and median SUCA decreased, albeit not significantly. Due to its complexity the ^{13}C-glycocholic acid blood test was not repeated on day 56.

In a previous study evaluating changes in indirect serum markers in dogs with tylosin-responsive diarrhea the authors also reported that median SUCA concentrations increased after tylosin administration and decreased after withdrawal of tylosin. However, these changes in SUCA concentrations did not reach significance (105). Unlike in our study serum folate concentrations remained unchanged in that study (105). However, tylosin was administered for only 2 weeks and at a lower dose as each dog was given the minimum required dose for controlling signs of diarrhea (the mean dose was 11 mg/kg q24 hours). This dose was much lower than the dose of tylosin used in our study (25 mk/kg q12 hours).

At this point it can only be speculated about reasons for the increase in concentrations of serum folate, SUCA, and CUMPCD of the ^{13}C-GCBT. One explanation could be changes in the composition of the small intestinal microflora due to tylosin administration. For example, recent studies performed in pigs have shown that tylosin administration leads to changes in the composition of the ileal microflora in pigs (14). After 14 days of tylosin administration as a food additive, the total bacterial count decreased, but the percentage of *Lactobacillus* spp. increased significantly (14). It has also been shown that some *Lactobacillus* spp. isolated from human feces (including *L. johnsonii, L. acidophilus*, *L. brevis, L. casei, L. fermenti, L. plantarum, L. salivarius*, *L. aviarius,* and *Streptococcus alactolyticus*) are capable of deconjugating glycocholic acid (27, 46, 60). Also, some lactic acid bacteria and *Lactobacillus* spp. (e.g., *L. delbrueckii*, *L. plantarum, L. lactis, Streptococcus thermophilus*) have been shown to synthesize folate (50, 99). Future studies need to directly evaluate qualitative and quantitative changes in the small intestinal microflora using bacterial culture techniques and molecular techniques to elucidate the effect of tylosin on the small intestinal microflora.

CHAPTER VII

CONCLUSIONS

The normal intestinal bacterial flora in dogs has not been well characterized. Previous studies have focused on enumeration and identification of bacterial species by direct culture of intestinal content. However, bacterial culture has limitations for assessment of bacterial diversity in intestinal samples as it is technically complex and expensive. Anaerobic bacteria, which are a substantial component of the intestinal microflora, are potentially more prone to damage during sampling, shipping, and storage and therefore may be underrepresented when assessing the microflora by bacterial culture. It is also now recognized that the majority of microbial species cannot be identified using standard culture techniques. Reasons for this inability to culture many bacterial species include non-viable or stressed microorganisms, obligate requirements for coexisting flora or host-derived products, bias due to selectivity of culture media, and a lack of knowledge regarding essential nutrients for some bacterial species. Thus, a culture-dependent approach may underestimate the bacterial diversity of complex microbial communities such as those found in the intestinal tract. This study was designed to characterize the composition and the dynamics of the canine intestinal microflora in healthy dogs using molecular methods and indirect serum markers.

Molecular fingerprinting, based on amplification of the gene encoding the 16S ribosomal RNA (16S rDNA), has revealed marked differences in the qualitative composition of the intestinal microflora between individual dogs. Also, molecular fingerprinting has shown marked differences in the microflora between different areas of the intestine within individual dogs. Interestingly, the microflora in the ileum was highly variable. In one subset of dogs the ileal microflora showed higher similarity with the proximal small intestine, while in another subset of dogs it showed higher similarity with the large intestine. This is surprising as the ileocolic valve is believed to serve as a natural barrier between the small and large intestine in the dog. This barrier, together

with intestinal motility, is believed to prevent retrograde translocation of bacteria from the large into the small intestine. Dysfunction of the ileocolic valve has been suggested as a possible cause of intestinal disease, such as small intestinal bacterial overgrowth (SIBO). At this point it remains speculative that the variation in bacterial microflora in the ileum between different dogs is caused by dysfunction of the ileocolic valve, and if so, whether this has any clinical significance. Further studies evaluating changes in the ileal microflora in a large group of dogs with intestinal disease are warranted. These findings suggest that unknown, host-related factors, contribute to the development of a unique microflora in an individual, and that different compartments of the intestinal tract should be viewed as unique ecosystems. Differences in nutrient composition and concentration, differences in pH, and host secretions between various compartments of the intestine may contribute to this effect. Based on these findings, assessment of a fecal sample does not yield accurate information about the diversity of the microflora in proximal intestinal compartments. Therefore, for diagnosis of bacteria-associated small intestinal disease, samples need to be obtained from the small intestine rather than fecal samples.

In this study two different collection techniques for obtaining small intestinal fluid for assessment of small intestinal bacterial diversity were evaluated: aspiration of duodenal juice through a sterile tube and the collection using a sterile disposable cytology brush. The aspiration technique proved to be impractical for routine clinical application since a considerable amount of time (approximately 10-20 min) was needed for each dog in order to collect a sufficient amount of duodenal juice. The collection of small intestinal fluid using a sterile cytology brush introduced through the working channel of the endoscope proved to be a rapid and reproducible collection technique and proper standardization should allow comparison of the bacterial diversity between healthy and diseased animals in future clinical studies.

In contrast to the fecal microflora, which has been reported to be stable over time, molecular fingerprinting of samples collected from the duodenum at different time-points varied within individuals, possibly due to variation of the bacterial flora over time

or a slight variation in sampling location. This variation of the small intestinal microflora obtained during repeated sampling, regardless of the underlying cause, needs to be taken into consideration when collecting samples from patients.

Comparative 16S rDNA analysis revealed several previously uncharacterized 16S rDNA sequences. Four major phylogenetic lineages were identified, with the majority of 16S rDNA sequences belonging to the order of *Clostridiales*, *Bacteroidales, Lactobacillales, Enterobacteriales,* and *Fusobacteriales*. These data indicate that the canine intestinal microflora is very complex, and that the molecular approach described in this study can facilitate identification of bacterial species in the canine intestinal tract that have not previously been characterized. Based on relative clone abundance a group of dogs had a predominant anaerobic microflora in the proximal small intestine (i.e., duodenum and jejunum), another group dogs had a predominantly aerobic microflora, whereas a third group of dogs had a mixed microflora. This observed marked inter-individual variation in the qualitative composition of the intestinal microflora makes it difficult to establish parameters for a normal intestinal microflora. The clinical significance of the diverse intestinal microflora in dogs, and alterations in bacterial diversity that may occur with gastrointestinal disease, need to be further investigated. Studies that characterize the intestinal microflora in dogs with gastrointestinal disease are warranted, as potential undiscovered pathogens may be identified. Future studies also may aim to evaluate the diversity of the bacterial community within individuals. Reduction in community diversity may cause susceptibility to certain bacterial products and lead to intestinal disease. Molecular fingerprints could be used in future studies to evaluate community diversity.

In contrast to our expectations, administration of tylosin, a commonly used antibiotic used in small animal gastroenterology, was associated with an increase in serum concentrations of folate, serum unconjugated cholic acid (SUCA), and the CUMPCD of the ^{13}C-GCBT. These data suggest that tylosin administration is associated with alterations in the canine small intestinal flora, reflected in alterations in serum markers and dynamic tests of the small intestinal microflora. Increased serum folate,

SUCA, and CUMPCD in the C^{13}-GCBT suggest that, in the dogs described here, tylosin administration increased the biomass of organisms carrying out these metabolic functions. Future studies are needed to directly assess qualitative and quantitative changes in the small intestinal microflora using bacterial culture and qualitative and quantitative molecular techniques to elucidate the effect of tylosin on the small intestinal microflora. It should also be recommended that tylosin administration should be discontinued before retesting serum concentrations of folate and SUCA and ^{13}GCABT.

REFERENCES

1. **Amann, R. I., W. Ludwig, and K. H. Schleifer**. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59**:143-169.

2. **Atlas, R. M.** 1994. Diversity of microbial communities, p. 1-47. *In* K. C. Marshall (ed.), Advances in microbial ecology. Plenum Press, New York.

3. **Atlas, R. and R. Bartha**. 1998. Microbial ecology: fundamentals and applications. Addison-Wesley Publishing Company, Reading, PA.

4. **Bardhan, P. K., K. Gyr, C. Beglinger, J. Vögtlin, R. Frey, and W. Vischer**. 1992. Diagnosis of bacterial overgrowth after culturing proximal small-bowel aspirate obtained during routine upper gastrointestinal endoscopy. Scand. J. Gastroenterol. **27**:253-256.

5. **Batt, R. M., J. R. Needham, and M. W. Carter**. 1983. Bacterial overgrowth associated with a naturally occurring enteropathy in the German Shepherd dog. Res. Vet. Sci. **35**:42-46.

6. **Benno, Y., H. Nakao, K. Uchida, and T. Mitsuoka**. 1992. Impact of the advances in age on the gastrointestinal microflora of beagle dogs. J. Vet. Med. Sci. **54**:703-706.

7. **Bourlioux, P., B. Koletzko, F. Guarner, and V. Braesco**. 2003. The intestine and its microflora are partners for the protection of the host: report on the Danone Symposium "The Intelligent Intestine," held in Paris, June 14, 2002. Am J Clin Nutr **78**:675-683.

8. **Boutton, T.** 1991. Stable carbon isotope ratios of natural materials: I. sample preparation and mass spectrometric analysis, p. 155-171. *In* D. C. Coleman and B. Fry (eds.), Carbon isotope techniques. Academic Press, Inc., San Diego.

9. **Brooks, S. P., M. McAllister, M. Sandoz, and M. L. Kalmokoff**. 2003. Culture-independent phylogenetic analysis of the faecal flora of the rat. Can. J. Microbiol. **49**:589-601.

10. **Buddington, R. K.** 2003. Postnatal changes in bacterial populations in the gastrointestinal tract of dogs. Am. J. Vet. Res. **64**:646-651.

11. **Camilo, E., J. Zimmerman, J. B. Mason, B. Golner, R. Russell, J. Selhub, and I. H. Rosenberg**. 1996. Folate synthesized by bacteria in the human upper small intestine is assimilated by the host. Gastroenterol. **110**:991-998.

12. **Chiaramonte, D., J. M. Steiner, J. D. Broussard, K. Baer, S. Gumminger, E. M. Moeller, D. A. Williams, and R. Shumway**. 2003. Use of a ^{13}C-aminopyrine blood test: first clinical impressions. Can. J. Vet. Res. **67**:183-188.

13. **Choi, M. G., M. Camilleri, D. D. Burton, A. R. Zinsmeister, L. A. Forstrom, and K. S. Nair**. 1998. Reproducibility and simplification of ^{13}C-octanoic acid breath test for gastric emptying of solids. Am. J. Gastroenterol. **93**:92-98.

14. **Collier, C. T., M. R. Smiricky-Tjardes, D. M. Albin, J. E. Wubben, V. M. Gabert, B. Deplancke, D. Bane, D. B. Anderson, and H. R. Gaskins**. 2003. Molecular ecological analysis of porcine ileal microbiota responses to antimicrobial growth promoters. J. Anim. Sci. **81**:3035-3045.

15. **Collins, K. and L. O'Mahony**. 2002. The "unculturables", p. 25-33. *In* A. L. Hart, A. J. Stagg, H. Graffner, H. Glise, P. Falk, and M. A. Kamm (eds.), Gut ecology. Martin Dunitz Ltd, London.

16. **Cornetta, A. M., K. W. Simpson, D. Strauss-Ayali, P. L. McDonough, and R. D. Gleed**. 1998. Use of a [^{13}C]urea breath test for detection of gastric infection with *Helicobacter* spp in dogs. Am. J. Vet. Res. **59**:1364-1369.

17. **Davis, C. P., D. Cleven, E. Balish, and C. E. Yale**. 1977. Bacterial association in the gastrointestinal tract of beagle dogs. Appl. Environ. Microbiol. **34**:194-206.

18. **Delles, E. K., M. D. Willard, R. B. Simpson, T. W. Fossum, M. Slater, D. Kolp, G. E. Lees, R. Helman, and G. Reinhart**. 1994. Comparison of species and numbers of bacteria in concurrently cultured samples of proximal small intestinal fluid and endoscopically obtained duodenal mucosa in dogs with intestinal bacterial overgrowth. Am. J. Vet. Res. **55**:957-964.

19. **Drasar, B. S. and M. J. Hill**. 1974. Human intestinal flora. Academic Press, London.

20. **Finegold, S. M., M. L. Vaisanen, D. R. Molitoris, T. J. Tomzynski, Y. Song, C. Liu, M. D. Collins, and P. A. Lawson**. 2003. Cetobacterium somerae sp. nov. from human feces and emended description of the genus Cetobacterium. Syst. Appl. Microbiol. **26**:177-181.

21. **Forney, L. J., X. Zhou, and C. J. Brown**. 2004. Molecular microbial ecology: land of the one-eyed king. Curr. Opin. Microbiol. **7**:210-220.

22. **Fried, M., H. Siegrist, R. Frei, F. Froehlich, P. Duroux, J. Thorens, A. Blum, J. Bille, J. J. Gonvers, and K. Gyr**. 1994. Duodenal bacterial overgrowth during treatment in outpatients with omeprazole. Gut **35**:23-26.

23. **Fromin, N., J. Hamelin, S. Tarnawski, D. Roesti, K. Jourdain-Miserez, N. Forestier, S. Teyssier-Cuvelle, F. Gillet, M. Aragno, and P. Rossi**. 2002. Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. Environ. Microbiol. **4**:634-643.

24. **Garner, M. R., J. F. Flint, and J. B. Russell**. 2002. Allisonella histaminiformans gen. nov., sp. nov. A novel bacterium that produces histamine, utilizes histidine as its sole energy source, and could play a role in bovine and equine laminitis. Syst. Appl. Microbiol. **25**:498-506.

25. **German, A. J., M. J. Day, C. G. Ruaux, J. M. Steiner, D. A. Williams, and E. J. Hall**. 2003. Comparison of direct and indirect tests for small intestinal bacterial overgrowth and antibiotic-responsive diarrhea in dogs. J. Vet. Int. Med. **17**:33-43.

26. **German, A. J., E. J. Hall, and M. J. Day**. 2001. Immune cell populations within the duodenal mucosa of dogs with enteropathies. J. Vet. Int. Med. **15**:14-25.

27. **Gilliland, S. E. and M. L. Speck**. 1977. Deconjugation of bile acids by intestinal lactobacilli. Appl. Environ. Microbiol. **33**:15-18.

28. **Good, I. J.** 1953. The population frequencies of species and the estimation of poulation parameters. Biometrica **40**:237-264.

29. **Greenlee, H. B., S. M. Gelbart, A. J. DeOrio, D. S. Francescatti, J. Paez, and G. F. Reinhardt**. 1977. The influence of gastric surgery on the intestinal flora. Am. J. Clin. Nutr. **30**:1826-1833.

30. **Greetham, H. L., C. Giffard, R. A. Hutson, M. D. Collins, and G. R. Gibson**. 2002. Bacteriology of the Labrador dog gut: a cultural and genotypic approach. J. Appl. Microbiol. **93**:640-646.

31. **Griffen, W. O., Jr., J. D. Richardson, and E. S. Medley**. 1971. Prevention of small bowel contamination by ileocecal valve. South. Med. J. **64**:1056-1058.

32. **Harmoinen, J. A., J. M. Matto, M. L. Rinkinen, M. Wilsson-Rahmberg, and E. Westermarck**. 2001. Permanent jejunal fistula: promising method for obtaining small intestinal chyme without disturbing intestinal function. Comp. Med. **51**:252-256.

33. **Hayashi, H., M. Sakamoto, and Y. Benno**. 2002. Phylogenetic analysis of the human gut microbiota using 16S rDNA clone libraries and strictly anaerobic culture-based methods. Microbiol. Immunol. **46**:535-548.

34. **Hoenig, M.** 1980. Intestinal malabsorption attributed to bacterial overgrowth in a dog. J. Am. Vet. Med. Assoc. **176**:533-535.

35. **Hopkins, M. J., R. Sharp, and G. T. Macfarlane**. 2001. Age and disease related changes in intestinal bacterial populations assessed by cell culture, 16S rRNA abundance, and community cellular fatty acid profiles. Gut **48**:198-205.

36. **Huber, T., G. Faulkner, and P. Hugenholtz**. 2004. Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics. **20**:2317-2319.

37. **Hugenholtz, P., C. Pitulle, K. L. Hershberger, and N. R. Pace**. 1998. Novel division level bacterial diversity in a Yellowstone hot spring. J. Bacteriol. **180**:366-376.

38. **Hurlbert, S. H.** 1971. The nonconcept of species diversity: a critique and alternative parameters. Ecology **52**:577-586.

39. **Jergens, A. E., C. A. Schreiner, D. E. Frank, Y. Niyo, F. E. Ahrens, P. D. Eckersall, T. J. Benson, and R. Evans**. 2003. A scoring index for disease activity in canine inflammatory bowel disease. J. Vet. Int. Med. **17**:291-297.

40. **Jobson, J. D.** 1996. Applied multivariate data analysis: categorical and multivariate methods. Springer-Verlag, Berlin, Germany.

41. **Johnston, K. L.** 1999. Small intestinal bacterial overgrowth. Vet. Clin. North Am. Small Anim. Pract. **29**:523-550.

42. **Johnston, K. L., A. Lamport, O. Ballevre, and R. M. Batt**. 2004. A comparison of endoscopic and surgical collection procedures for the analysis of the bacterial flora in duodenal fluid from cats. Vet. J. **157**:85-89.

43. **Kanauchi, O., Y. Matsumoto, M. Matsumura, M. Fukuoka, and T. Bamba**. 2005. The beneficial effects of microflora, especially obligate anaerobes, and their products on the colonic environment in inflammatory bowel disease. Curr. Pharm. Des. **11**:1047-1053.

44. **Kaneko, Y., H. Takeshita, K. Mogi, T. Nakajima, T. Yasuda, M. Itoi, H. Kuwano, and K. Kishi**. 2003. Molecular, biochemical and immunological analyses of canine pancreatic DNase I. J. Biochem. (Tokyo) **134**:711-718.

45. **King, C. E. and P. P. Toskes**. 1979. Small intestine bacterial overgrowth. Gastroenterol. **76**:1035-1055.

46. **Knarreborg, A., R. M. Engberg, S. K. Jensen, and B. B. Jensen**. 2002. Quantitative determination of bile salt hydrolase activity in bacteria isolated from the small intestine of chickens. Appl. Environ. Microbiol. **68**:6425-6428.

47. **Lan, P. T., H. Hayashi, M. Sakamoto, and Y. Benno**. 2002. Phylogenetic analysis of cecal microbiota in chicken by the use of 16S rDNA clone libraries. Microbiol. Immunol. **46**:371-382.

48. **Langendijk, P. S., F. Schut, G. J. Jansen, G. C. Raangs, G. R. Kamphuis, M. H. Wilkinson, and G. W. Welling**. 1995. Quantitative fluorescence in situ hybridization of Bifidobacterium spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. Appl. Environ. Microbiol. **61**:3069-3075.

49. **Leser, T. D., J. Z. Amenuvor, T. K. Jensen, R. H. Lindecrona, M. Boye, and K. Moller**. 2002. Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. Appl. Environ. Microbiol. **68**:673-690.

50. **Lin, M. Y. and C. M. Young**. 2000. Biosynthesis of folates by *Streptococcus thermophilus* and *Lactobacillus delbruekii* ssp. *bulgaricus*. J. Food Drug Analysis **8**:198-199.

51. **Linskens, R. K., X. W. Huijsdens, P. H. Savelkoul, C. M. Vandenbroucke-Grauls, and S. G. Meuwissen**. 2001. The bacterial flora in inflammatory bowel disease: current insights in pathogenesis and the influence of antibiotics and probiotics. Scand. J. Gastroenterol. Suppl **243**:29-40.

52. **Ludlow, C. L. and D. J. Davenport**. 2000. Small intestinal bacterial overgrowth, p. 637-641. *In* J. D. Bonagura (ed.), Kirk's current veterinary therapy. W.B. Saunders, Philadelphia.

53. **Mangiante, G., G. Colucci, P. Canepari, C. Bassi, N. Nicoli, A. Casaril, P. Marinello, C. Signoretto, and S. Bengmark**. 2001. *Lactobacillus plantarum* reduces infection of pancreatic necrosis in experimental acute pancreatitis. Dig. Surg. **18**:47-50.

54. **Mantel, N. A.** 1967. The detection of disease clustering and a generalized regression approach. Cancer Res. **27**:209-220.

55. **Marks, S. L. and E. J. Kather**. 2003. Antimicrobial susceptibilities of canine Clostridium difficile and Clostridium perfringens isolates to commonly utilized antimicrobial drugs. Vet. Microbiol. **94**:39-45.

56. **McCracken, V. J., J. M. Simpson, R. I. Mackie, and H. R. Gaskins**. 2001. Molecular ecological analysis of dietary and antibiotic-induced alterations of the mouse intestinal microbiota. J. Nutr. **131**:1862-1870.

57. **Melgarejo, T., D. A. Williams, N. C. O'Connell, and K. D. Setchell**. 2000. Serum unconjugated bile acids as a test for intestinal bacterial overgrowth in dogs. Dig. Dis. Sci. **45**:407-414.

58. **Menozzi, A., C. Pozzoli, E. Poli, M. Lazzaretti, A. Cantoni, D. Grandi, E. Giovannini, and G. Coruzzi**. 2005. Effect of the macrolide antibacterial drug, tylosin, on TNBS-induced colitis in the rat. Pharmacol. **74**:135-142.

59. **Mentula, S., J. Harmoinen, M. Heikkila, E. Westermarck, M. Rautio, P. Huovinen, and E. Kononen**. 2005. Comparison between cultured small-intestinal and fecal microbiotas in Beagle dogs. Appl. Environ. Microbiol. **71**:4169-4175.

60. **Midtvedt, T. and A. Norman**. 1967. Bile acid transformations by microbial strains belonging to genera found in intestinal contents. Acta Pathol. Microbiol. Scand. **71**:629-638.

61. **Moeller, E. M., J. M. Steiner, D. A. Williams, and P. D. Klein**. 2001. Preliminary studies of a canine 13C-aminopyrine demethylation blood test. Can. J. Vet. Res. **65**:45-49.

62. **Morris, T. H., S. H. Sorensen, J. Turkington, and R. M. Batt**. 1994. Diarrhoea and increased intestinal permeability in laboratory beagles associated with proximal small intestinal bacterial overgrowth. Lab. Anim. **28**:313-319.

63. **Muyzer, G., E. C. de Waal, and A. G. Uitterlinden**. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59**:695-700.

64. **Muyzer, G. and K. Smalla**. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek **73**:127-141.

65. **Neeser, J. R., D. Granato, M. Rouvet, A. Servin, S. Teneberg, and E. A. Karlsson**. 2000. *Lactobacillus johnsonii* Lal shares carbohydrate-binding specificities with several enteropathogenic bacteria. Glycobiology **10**:1193-1199.

66. **Northfield, T. C., B. S. Drasar, and J. T. Wright**. 1973. Value of small intestinal bile acid analysis in the diagnosis of stagnant loop syndrome. Gut **14**:1815-1825.

67. **Ott, S. J., M. Musfeldt, D. F. Wenderoth, J. Hampe, O. Brant, U. R. Fölsch, K. N. Timmins, and S. Schreiber**. 2004. Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. Gut **53**:685-693.

68. **Palka-Santini, M., B. Schwarz-Herzke, M. Hosel, D. Renz, S. Auerochs, H. Brondke, and W. Doerfler**. 2003. The gastrointestinal tract as the portal of entry for foreign macromolecules: fate of DNA and proteins. Mol. Genet. Genomics **270**:201-215.

69. **Papasouliotis, K., A. H. Sparkes, G. Werrett, K. Egan, E. A. Gruffydd-Jones, and T. J. Gruffydd-Jones**. 1998. Assessment of the bacterial flora of the proximal part of the small intestine in healthy cats, and the effect of sample collection method. Am. J. Vet. Res. **59**:48-51.

70. **Paster, B. J., S. K. Boches, J. L. Galvin, R. E. Ericson, C. N. Lau, V. A. Levanos, A. Sahasrabudhe, and F. E. Dewhirst**. 2001. Bacterial diversity in human subgingival plaque. J. Bacteriol. **183**:3770-3783.

71. **Peled, Y., C. Levy-Gigi, D. Ayalon, and T. Gilat**. 1979. The cholyl glycine-1-14C breath test in various gastrointestinal disorders. Digestion **19**:267-276.

72. **Pryde, S. E., A. J. Richardson, C. S. Stewart, and H. J. Flint**. 1999. Molecular analysis of the microbial diversity present in the colonic wall, colonic lumen, and cecal lumen of a pig. Appl. Environ. Microbiol. **65**:5372-5377.

73. **Putman, R. J.** 1994. Community ecology. Chapman & Hall, London, United Kingdom.

74. **Qiu, X., L. Wu, H. Huang, P. E. McDonel, A. V. Palumbo, J. M. Tiedje, and J. Zhou**. 2001. Evaluation of PCR-generated chimeras, mutations, and heteroduplexes with 16S rRNA gene-based cloning. Appl. Environ. Microbiol. **67**:880-887.

75. **Rinkinen, M. L., J. M. K. Koort, A. C. Ouwehand, E. Westermarck, and K. J. Björkroth**. 2004. *Streptococcus alactolyticus* is the dominating culturable lactic acid bacterium species in canine jejunum and feces of four fistulated dogs. FEMS Microbiol. Lett. **230**:35-39.

76. **Ruaux, C. G., J. M. Steiner, and D. A. Williams**. 2002. Postprandial changes in serum unconjugated bile acid concentrations in healthy beagles. Am. J. Vet. Res. **63**:789-793.

77. **Rutgers, H. C., R. M. Batt, C. M. Elwood, and A. Lamport**. 1995. Small intestinal bacterial overgrowth in dogs with chronic intestinal disease. J. Am. Vet. Med. Assoc. **206**:187-193.

78. **Rutgers, H. C., R. M. Batt, F. J. Proud, S. H. Sorensen, C. M. Elwood, G. Petrie, L. A. Matthewman, H. A. F. Van Hijfte, A. Boswood, M. Entwistle, and R. H. Fensome**. 1996. Intestinal permeability and function in dogs with small intestinal bacterial overgrowth. J. Sm. Anim. Pract. **37**:428-434.

79. **Saavedra, J. M.** 2001. Clinical applications of probiotic agents. Am. J. Clin. Nutr. **73**:1147S-1151S.

80. **Salzman, N. H., H. De Jong, Y. Paterson, H. J. M. Harmsen, G. W. Welling, and N. A. Bos**. 2002. Analysis of 16S libraries of mouse gastrointestinal microflora reveals a large new group of mouse intestinal bacteria. Microbiol. **148**:3651-3660.

81. **Schiff, E. R., N. C. Small, and J. M. Dietschy**. 1972. Characteristics of the kinetics of the passive and active transport mechanisms for bile acid absorption in the small intestine and colon of the rat. J. Clin. Invest. **51**:1351-1362.

82. **Seguritan, V. and F. Rohwer**. 2001. FastGroup: a program to dereplicate libraries of 16S rDNA sequences. BMC. Bioinformatics. **2**:9-10.

83. **Seksik, P., L. Rigottier-Gois, G. Gramet, M. Sutren, P. Pochart, P. Marteau, R. Jian, and J. Doré**. 2003. Alterations of the dominant faecal bacterial groups in patients with Crohn's disease of the colon. Gut **52**:237-242.

84. **Sghir, A., G. Gramet, A. Suau, V. Rochet, P. Pochart, and J. Dore**. 2000. Quantification of bacterial groups within human fecal flora by oligonucleotide probe hybridization. Appl. Environ. Microbiol. **66**:2263-2266.

85. **Shinozaki, J. K., R. K. Sellon, G. H. Cantor, T. E. Besser, K. L. Mealey, and S. L. Vaden**. 2002. Fecal polymerase chain reaction with 16S ribosomal RNA primers can detect the presence of gastrointestinal Helicobacter in dogs. J. Vet. Int. Med. **16**:426-432.

86. **Shryock, T. R., J. E. Mortensen, and M. Baumholtz**. 1998. The effects of macrolides on the expression of bacterial virulence mechanisms. J. Antimicrob. Chemother. **41**:505-512.

87. **Simpson, J. M., S. A. Kocherginskaya, R. I. Aminov, L. T. Skerlos, T. M. Bradley, R. I. Mackie, and B. A. White**. 2002. Comparative microbial diversity in the gastrointestinal tracts of food animal species. Integ. Comp. Biol. **42**:327-331.

88. **Simpson, J. M., B. Martineau, W. E. Jones, J. M. Ballam, and R. I. Mackie**. 2002. Characterization of fecal bacterial populations in canines: effects of age, breed and dietary fiber. Microb. Ecol. **44**:186-197.

89. **Simpson, J. M., V. J. McCracken, H. R. Gaskins, and R. I. Mackie**. 2000. Denaturing gradient gel electrophoresis analysis of 16S ribosomal DNA amplicons to monitor changes in fecal bacterial populations of weaning pigs after introduction of Lactobacillus reuteri strain MM53. Appl. Environ. Microbiol. **66**:4705-4714.

90. **Simpson, J. M., V. J. McCracken, B. A. White, H. R. Gaskins, and R. I. Mackie**. 1999. Application of denaturant gradient gel electrophoresis for the analysis of the porcine gastrointestinal microbiota. J. Microbiol. Methods **36**:167-179.

91. **Simpson, K. W., R. M. Batt, D. Jones, and D. B. Morton**. 1990. Effects of exocrine pancreatic insufficiency and replacement therapy on the bacterial flora of the duodenum in dogs. Am. J. Vet. Res. **51, No. 2**:203-206.

92. **Snel, J., P. P. Heinen, H. J. Blok, R. J. Carman, A. J. Duncan, P. C. Allen, and M. D. Collins**. 1995. Comparison of 16S rRNA sequences of segmented filamentous bacteria isolated from mice, rats, and chickens and proposal of "Candidatus Arthromitus". Int. J. Syst. Bacteriol. **45**:780-782.

93. **Solomons, N. W., F. E. Viteri, and L. H. Hamilton**. 1977. Application of a simple gas chromatographic technique for measuring breath hydrogen. J. Lab. Clin. Med. **90**:856-862.

94. **Stackebrandt, E. and B. M. Goebel**. 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. **44**:846-849.

95. **Strombeck, D. R., M. Doe, and S. Jang**. 1981. Maldigestion and malabsorption in a dog with chronic gastritis. J. Am. Vet. Med. Assoc. **179**:801-805.

96. **Suau, A., R. Bonnet, M. Sutren, J. J. Godon, G. R. Gibson, M. D. Collins, and J. Dore**. 1999. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl. Environ. Microbiol. **65**:4799-4807.

97. **Suau, A., Marteau, P., Dore, J., Collins, M. D., and Pochart, P.** 2003. Differences, in human, between the bacterial communities of the cecum and the feces: a study using direct analysis of 16S rDNA genes. Gastroenterol. **124**:A478-A479.

98. **Suchodolski, J. S., C. G. Ruaux, J. M. Steiner, K. Fetz, and D. A. Williams**. 2005. Variation in bacterial microflora in different compartments of the canine intestinal tract. Am. J. Vet. Res. **66**:1556-1562.

99. **Sybesma, W., M. Starrenburg, L. Tijsseling, M. H. Hoefnagel, and J. Hugenholtz**. 2003. Effects of cultivation conditions on folate production by lactic acid bacteria. Appl. Environ. Microbiol. **69**:4542-4548.

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

101. **Van de, P. Y. and R. De Wachter**. 1993. TREECON: a software package for the construction and drawing of evolutionary trees. Comput. Appl. Biosci. **9**:177-182.

102. **van der Wielen, P. W., D. A. Keuzenkamp, L. J. Lipman, F. van Knapen, and S. Biesterveld**. 2002. Spatial and temporal variation of the intestinal bacterial community in commercially raised broiler chickens during growth. Microb. Ecol. **44**:286-293.

103. **Wang, R. F., W. W. Cao, and C. E. Cerniglia**. 1996. PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. Appl. Environ. Microbiol. **62**:1242-1247.

104. **Wang, X., S. P. Heazlewood, D. O. Krause, and T. H. J. Florin**. 2003. Molecular characterization of the microbial species that colonize human ileal and colonic mucosa by using 16S rDNA sequence analysis. J. Appl. Microbiol. **95**:508-520.

105. **Westermarck, E., T. Skrzypczak, J. Harmoinen, J. M. Steiner, C. G. Ruaux, D. A. Williams, E. Eerola, P. Sundbäck, and M. Rinkinen**. 2005. Tylosin-responsive chronic diarrhea in dogs. J. Vet. Int. Med. **19**:177-186.

106. **Willard, M. D., R. B. Simpson, E. K. Delles, N. D. Cohen, T. W. Fossum, D. Kolp, and G. Reinhart**. 1994. Effects of dietary supplementation of fructo-oligosaccharides on small intestinal bacterial overgrowth in dogs. Am. J. Vet. Res. **55**:654-659.

107. **Willard, M. D., R. B. Simpson, T. W. Fossum, N. D. Cohen, E. K. Delles, D. L. Kolp, D. P. Carey, and G. A. Reinhart**. 1994. Characterization of naturally developing small intestinal bacterial overgrowth in 16 German Shepherd dogs. J. Am. Vet. Med. Assoc. **204**:1201-1206.

108. **Williams, D. A.** 1996. Malabsorption, small intestinal bacterial overgrowth, and protein-losing enteropathy., p. 367-380. *In* D. R. Strombeck, W. G. Guilford, S. A. Center, and D. J. Meyer (eds.), Small animal gastroenterology. W.B. Saunders, Philadelphia.

109. **Williams, D. A., R. M. Batt, and L. McLean**. 1987. Bacterial overgrowth in the duodenum of dogs with exocrine pancreatic insufficiency. J. Am. Vet. Med. Assoc. **191**:201-206.

110. **Zenger, E., W. N. Evering, and M. D. Willard**. 1992. Chronic diarrhea associated with intestinal anomalies in a six-year-old dog. J. Am. Vet. Med. Assoc. **201**:1737-1740.

111. **Zoetendal, E. G., A. D. Akkermans, W. M. Akkermans van-Vliet, J. de Visser, and W. M. de Vos**. 2001. The host genotype affects the bacterial community in the human gastrointestinal tract. Microb. Ecol. Health Disease **13**:129-134.

112. **Zoetendal, E. G., A. D. Akkermans, and W. M. de Vos**. 1998. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl. Environ. Microbiol. **64**:3854-3859.

113. **Zoetendal, E. G., C. T. Collier, S. Koike, R. I. Mackie, and H. R. Gaskins**. 2004. Molecular ecological analysis of the gastrointestinal microbiota: a review. J. Nutr. **134**:465-472.

114. **Zoetendal, E. G., A. Von Wright, T. Vilpponen-Salmela, K. Ben Amor, A. D. Akkermans, and W. M. de Vos**. 2002. Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. Appl. Environ. Microbiol. **68**:3401-3407.

VITA

Name: Jan S. Suchodolski

Permanent Address: 2805 Pueblo Court N.
College Station, Texas, 77845

Education: University of Veterinary Medicine, Vienna, Austria
Veterinary Degree (Mag.med.vet.), 1997
Doctor of Veterinary Medicine, 2003

Texas A&M University, College Station, Texas
Ph.D., 2005, Veterinary Microbiology

Selected publications:

Suchodolski J.S., Ruaux C.G., Steiner J.M., Fetz K., Berghoff N., and Williams D.A. 2005. Development of a ^{13}C-glycocholic acid blood test for assessment of the small intestinal microflora in dogs. Can. J. Vet. Res. (in press)

Suchodolski J.S., Ruaux C.G., Steiner J.M., Fetz K., and Williams D.A. 2005. Assessment of qualitative variation in intestinal microflora in differing compartments of the canine intestinal tract using a molecular fingerprinting technique. Am. J. Vet. Res. **66**:1556-1562.

Suchodolski J.S., Ruaux C.G., Steiner J.M., Fetz K., Williams D.A. 2004. Application of molecular fingerprinting for qualitative assessment of small intestinal bacterial diversity in dogs. J. Clin. Microbiol. **42**.:4702-4708.

Fetz K., Ruaux C.G., Steiner J.M., Suchodolski J.S., and Williams D.A. 2004. Purification and partial characterization of feline alpha$_1$-proteinase inhibitor (fα_1-PI) and the development and validation of a radioimmunoassay for the measurement of fα_1-PI in serum. Biochimie. **86**:67-75.

Suchodolski J.S., Steiner J.M., Ruaux C.G., and Williams D.A. 2003. Serum concentrations of pepsinogen A in healthy dogs after food deprivation and after feeding. Am. J. Vet. Res. **64**:1146-1150.

Suchodolski J.S., Steiner J.M., Ruaux C.G., Boari A., and Williams D.A. 2002. Purification and partial characterization of canine pepsinogen A and B. Am. J. Vet. Res. **63**:1585-1590.