MOLECULAR CHARACTERIZATION OF INTESTINAL BACTERIA IN HEALTHY CATS AND A COMPARISON OF THE FECAL BACTERIAL FLORA BETWEEN HEALTHY CATS AND CATS WITH INFLAMMATORY BOWEL DISEASE (IBD)

A Thesis

by

LAUREN ELIZABETH RITCHIE

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

MASTER OF SCIENCE

August 2008

Major Subject: Veterinary Medical Sciences

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Approved by:

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August 2008

Major Subject: Veterinary Medical Sciences

ABSTRACT

Molecular Characterization of Intestinal Bacteria in Healthy Cats and a Comparison of the Fecal Bacterial Flora between Healthy Cats and Cats with Inflammatory Bowel Disease (IBD). (August 2008)

Lauren Elizabeth Ritchie, B.S., Texas A&M University

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Past studies characterizing the feline intestinal microflora have used traditional bacterial culture techniques. However, in recent years it has been recognized that the majority of intestinal bacteria are non cultivable. Therefore, the aim of this study was to describe the microflora along the intestinal tract in healthy cats using comparative 16S ribosomal DNA (16S rDNA) analysis. Intestinal content from the stomach, duodenum, jejunum, ileum, and colon was collected from 4 healthy cats and one specific pathogen free cat (SPF) and the bacterial composition was identified by direct sequencing of bacterial 16S rDNA amplicons. A predominant anaerobic microflora was observed in all evaluated segments of the intestine. Fourteen different bacterial orders were identified with the majority of all sequences classified in the class *Clostridiales*. Six different *Clostridium* clusters were identified with the majority of sequences affiliated with *Clostridium* cluster I. Comparative 16S rDNA analysis was also used to evaluate differences in the fecal microflora between healthy cats (n=6), cats with histopathologically confirmed inflammatory bowel disease (IBD; n=6), and cats with

intestinal neoplasia (n=3). Compared to the IBD group, cats in the control group showed a significantly higher number of sequences classified as *Firmicutes, Bacteroidetes,* and *Actinobacteria* ($p<0.0001$). The control group had a significantly higher proportion of clones affiliated with *Clostridium* cluster XI, and a significantly lower proportion affiliated with cluster I (both $p<0.0001$). In the neoplasia group, the majority of sequences were classified in the phylum *Firmicutes* (97.9%) and clones were predominately affiliated with *Clostridium* clusters I and XI. These data indicate that the feline intestinal microflora is highly diverse and is comprised predominantly of anaerobic bacteria. Further studies are warranted to evaluate the clinical significance of the observed differences in intestinal microflora between healthy cats and cats with gastrointestinal disease.

DEDICATION

To Craig and my family

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I would especially like to thank my committee co-chair Dr. Jan Suchodolski for his patience and guidance throughout this project and to my co-chair Dr. Jörg Steiner and my committee member Dr. Melissa Libal for their support during this research. Thanks also to the entire GI lab team for their help and making my time here a great experience.

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CHAPTER I

INTRODUCTION

THE EFFECT OF THE MICROFLORA ON GASTROINTESTINAL HEALTH

The intestinal bacterial microflora plays a significant role in gastrointestinal health of both humans and animals (Itoh *et al.*, 1984; Greetham *et al.*, 2002). The microbial metabolic activity in the gut has an influence on absorption of nutrients, such as vitamins, lactate, oxygen, and short chain fatty acids (Hold *et al.*, 2002). In addition, the bacterial microflora also provides a natural defense mechanism against invading pathogens. Such mechanisms include competition for adhesion sites in the gastrointestinal tract and production of anti-microbial substances that are harmful to non-resident bacteria (Kanauchi *et al.*, 2005).

CHARACTERIZATION OF THE INTESTINAL MICROFLORA

Culture-based methods. Past studies characterizing the feline intestinal microflora have used traditional microbiological culture techniques, in which samples are plated on selective culture media and are grown in an aerobic or anaerobic environment (Smith, 1965; Osbaldiston & Stowe, 1971; Itoh *et al.,* 1984; Johnston *et al.*, 1993; Papasouliotis *et al.*, 1998; Johnston *et al.*, 1999). Identification of the microorganisms is then achieved by performing various morphological and biochemical tests. More recently, it has been suggested that microbiological culture techniques fail to accurately

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

characterize bacterial communities in complex environmental samples (e.g., the gastrointestinal tract) (Itoh *et al.*, 1984; Amann *et al.*, 1995; Langendijk *et al.,* 1995; Greetham *et al.*, 2002). This is due to the fact that only an estimated 10% - 50% of bacteria present in the mammalian gut are cultivable (Langendijk *et al.,* 1995). One reason for this is that the growth requirements are unknown for many intestinal bacterial species, precluding successful cultivation. Furthermore, culture-based techniques for studying complex biological samples such as intestinal content are laborious and time-consuming. In addition, studies have shown that some commonly used culture media have poor selectivity, and that microorganisms can exhibit metabolic plasticity, which can cause misclassification of related bacterial species (Greetham *et al.*, 2002). For example, in one study a combination of culture-based and molecular-based methods were used to characterize the bacterial microflora in fecal samples (Greetham *et al.*, 2002). Fecal samples were plated on traditional culture media that targeted all aerobic and anaerobic bacteria. In addition, fecal samples were plated on selective media which specifically targeted *Bifidobacterium* spp., *Lactobacillus* spp., *Clostridium* spp., and *Bacteroides* spp. Bacterial DNA was then extracted from individual isolates and the 16S rDNA was amplified by PCR and sequenced for genotypic identification. The authors demonstrated that the majority of culture media used were not selective because sequence analysis of the 16S rDNA showed that the media recovered bacterial groups other than their published target organism (Greetham *et al.*, 2002).

Molecular-based methods. The most recent studies aiming to characterize the intestinal microflora in many mammalian species have used molecular-based methods based on the amplification of the 16S ribosomal RNA gene (16S rDNA) (Greetham *et al.*,

2002; Hold *et al.*, 2002; Wang *et al.*, 2003; Delgado *et al.*, 2006). Bacterial 16S rRNA genes generally contain nine hypervariable regions that demonstrate considerable sequence diversity among different bacterial species and can be used for species identification (Van de Peer *et al.*, 1996). Since these hypervariable regions are flanked by conserved regions, universal bacterial primers can be designed that enable PCR amplification of target sequences. Theoretically, such an approach using universal primers will result in the amplification of all bacteria present in a given sample. Therefore, molecular-based methods allow identification of both cultivable and non-cultivable bacteria. In many studies, molecular techniques have resulted in the observation of an increased bacterial diversity in the intestine compared to strictly culture-based methods (Langendijk *et al.*, 1995; Wilson, 1996; Greetham *et al.*, 2002; Delgado *et al.*, 2006; Inness *et al.*, 2006).

Studies that used a comparative 16S rDNA analysis found a higher bacterial diversity in canine intestinal samples than previously reported using culture-based techniques (Greetham *et al.*, 2002; Suchodolski, 2005). One study amplified 16S rDNA extracted from canine duodenal juice. Using denaturing gradient gel electrophoresis (DGGE), the investigators showed a highly diverse bacterial population within the canine small intestine and this molecular approach can be used to assess bacterial diversity and show variation between samples that can not be achieved using culture-based methods (Suchodolski *et al.*, 2004).

Studies in humans have found similar results when comparing molecular and culture-based techniques. Delgado *et al.* (2006) observed a lower bacterial diversity in human colonic mucosal and fecal samples when analyzed by culture-based techniques

compared to 16S rDNA sequence analysis. Similarly, another study reported that molecular-based methods are able to identify a higher bacterial diversity than standard culture-based techniques (Langendijk *et al.*, 1995). Based on these studies, it is likely that a molecular-based approach may identify greater bacterial diversity in the intestinal tract of cats than previously reported.

THE FELINE INTESTINAL MICROFLORA

The bacterial composition of the feline intestinal microflora remains poorly characterized. Also, no study is available describing the bacterial microflora along the entire feline gastrointestinal tract. Studies in the past have used traditional culture-based methods to analyze samples from the proximal portion of the small intestine (duodenum and jejunum), colon, or fecal material, but have not sampled other compartments of the intestinal tract (Smith, 1965; Johnston *et al.*, 1993; Papasouliotis *et al.*, 1998; Johnston *et al.*, 1999).

Three different studies have analyzed the bacterial microflora of duodenal juice aspirates collected by gastroduodendoscopy (Johnston *et al.*, 1993; Papasouliotis *et al.*, 1998; Johnston *et al.*, 2001). These studies all reported high total bacterial counts ($> 10^5$ colony forming units (cfu) mL^{-1}) in healthy feline duodenal samples with a predominance of facultative and obligate anaerobic bacterial species. In one study, anaerobic bacterial counts in undiluted duodenal juice ranged from 10^4 to 10^8 cfu mL^{-1}. The most commonly observed anaerobic bacterial species were *Bacteroides* spp., *Fusobacteria* spp., and *Eubacteria* spp. (Johnston *et al.*, 1993). Two other studies reported similar anaerobic bacterial counts in the proximal small intestine with a mean of $10^{5.7}$ cfu mL^{-1}

(Papasouliotis *et al.*, 1998), and a median of > 10^4 cfu mL^{-1} (Johnston *et al.*, 2001), respectively. Both studies found *Clostridia* spp. to be the most common bacterial group in the feline duodenum (occurring in over 90% of cats), followed by *Bacteroides* spp. and *Fusobacterium* spp., which were found in about 40% each of evaluated cats (Papasouliotis *et al.*, 1998; Johnston *et al.*, 2001). One study also reported *Escherichia coli* to be present in the duodenum of 40% of evaluated cats (Papasouliotis *et al.*, 1998).

The findings by Johnston *et al.* (1993, 2001) and Papasouliotis *et al.* (1998) are contradicted, however, by a study that reported a predominantly facultative anaerobic microflora in the proximal small intestine (i.e., jejunum) of healthy cats (Osbaldiston & Stowe, 1971). The predominant bacterial species identified were *Enterococcus* spp. (identified in all six cats), *Streptococcus* spp. (identified in five of the six cats), and *Lactobacillus* spp. (identified in four of the six cats). No *Bacteroides* spp. were identified in any of the cats, while *Clostridia* spp. were found in only one cat (Osbaldiston & Stowe, 1971). Bacterial counts exceeded 10^5 cfu gm^{-1} in 80% of cats.

The bacterial composition in the colon of cats has also been investigated (Osbaldiston & Stowe, 1971). One study reported bacterial counts in colonic samples to exceed 10^7 cfu gm^{-1} in 60% of evaluated cats (Osbaldiston & Stowe, 1971). Bacterial groups most commonly identified were *Enterococcus* spp. and *Escherichia* spp., which were identified in all six cats sampled. *Lactobacillus* spp. were identified in the colon of five of those six cats (Osbaldiston & Stowe, 1971). The findings of this study contrasts the results of two other studies that analyzed the bacterial composition of fecal samples obtained from healthy cats. One study analyzing the fecal flora in healthy cats reported total bacterial counts to exceed 10^{10} cfu g^{-1} feces. Anaerobic bacterial counts (i.e.,

Lactobacillus spp., *Bacteroides* spp., and *Clostridia* spp.) exceeded 10^9 cfu g^{-1} (Itoh *et al.*, 1984). Two other studies observed *Bacteroides* spp. and *Bifidobacterium* spp. to be the predominant bacterial groups present in feline fecal samples. In addition, *Lactobacillus* spp. were found in the majority of these samples (Itoh *et al.*, 1984; Inness *et al.*, 2006).

It is difficult to directly compare the results from all these studies because samples were obtained from different segments of the feline intestine, and the collection methods were not consistent between the different studies. Also, the authors of these studies used different types of selective media and differences in the biochemical methods used for identification of the bacterial species may have further contributed to these different results. In addition, all of these studies used different housing environments and diets (e.g. commercial, canned, dry, other), which may have affected the composition of the feline microflora.

INTESTINAL MICROFLORA IN DISEASE

Within the last decade, much research has been done in humans and animals to characterize the normal intestinal bacterial flora and to compare the intestinal microflora of healthy individuals with that of patients with gastrointestinal (GI) disease (Johnston *et al.*, 2001; Linskens *et al.*, 2001; Inness *et al.*, 2006; Janeczko *et al.*, 2007). One commonly studied intestinal disease is small intestinal bacterial overgrowth (SIBO). SIBO is a syndrome resulting in clinical signs such as vomiting, diarrhea, and poor body condition in humans and dogs (Johnston *et al.*, 1993). One study in dogs suggested that bacterial counts in dogs with SIBO exceed 10^5 cfu mL^{-1} in the duodenum (Batt & McLean, 1987). However, more recent studies have shown that the proximal small

intestine of healthy dogs harbors bacterial counts often exceeding the proposed cut-off for SIBO (German *et al.*, 2003). In addition, some dogs with suspected SIBO often have bacterial counts below 10^5 cfu mL^{-1} (Johnston, 1999). Therefore, in dogs, the proposed cut-off value for SIBO is currently under discussion.

Johnston *et al.* (2001) compared the duodenal bacterial composition between healthy cats and cats suspected of having SIBO using culture-based methods and found no difference in bacterial counts between both groups (range for both $10^{3.3}$-$10^{8.21}$ cfu mL^{-1}). Also, no significant differences were found in anaerobic bacterial counts between both groups (Johnston *et al.*, 2001). Diseased cats had lower numbers of *Bacteroides* spp. and *Lactobacillus* spp. than healthy cats. This is particularly interesting because *Bacteroides* spp. are often found in the small intestine of humans with bacterial overgrowth (Welkos *et al.*, 1981). For those reasons it was concluded that SIBO does not appear to be a clinical syndrome in cats (Johnston *et al.*, 2001).

Inflammatory bowel disease (IBD) is a common chronic intestinal disease in humans as well as in cats. The etiology of IBD is unknown, but many factors such as parasites, diet, genetic susceptibility, and the intestinal bacterial microflora have been considered to play a major role in the pathogenesis of IBD (Inness *et al.*, 2006). Studies performed in animal models observed that IBD does not occur in mice that are housed in germ-free conditions, suggesting that the bacterial microflora plays a major role in the pathogenesis of IBD (Sadlack *et al.*, 1993). In humans there is evidence that factors such as a loss of tolerance to the residential intestinal microflora, or an ineffective clearance of enteric pathogens are possible causes of IBD. Additionally, it has been shown in human

IBD patients that inflammation of the intestine is present in areas of high bacterial counts (Linskens *et al.*, 2001).

Very few studies have been performed to study the role of the bacterial microflora in feline IBD. A recent study used fluorescence *in situ* hybridization (FISH) to compare the fecal microflora between healthy cats and cats with IBD (Inness *et al.*, 2006). Healthy cats had significantly higher numbers of *Bacteroides* spp. and *Bifidobacterium* spp. in their fecal samples. In addition, numbers of *Desulfovibrio* spp. were significantly higher in fecal samples from cats with IBD (Inness *et al.*, 2006). Another study investigated the bacterial composition in duodenal biopsy samples from cats with signs of IBD and compared it to healthy control cats using FISH analysis (Janeczko *et al.*, 2007). In this study, it was found that intestinal biopsy samples from cats with IBD had a significantly higher number of mucosa associated organisms belonging to the *Enterobacteriaceae* family than healthy cats. In addition, the composition of the mucosal microflora was distinctly different in intestinal biopsies from cats with IBD. FISH analysis showed that *Enterobacteriaceae*, *Streptococcus* spp., *Clostridium* spp., and *Bacteroides* spp. accounted for 91% of mucosal bacteria in cats with signs of IBD. In biopsies from healthy cats, the same FISH probes were used to show that these bacterial species only accounted for 6% of the mucosal bacteria (Janeczko *et al.*, 2007), suggesting a reduced bacterial diversity in the IBD group.

These studies suggest that the bacterial composition of the feline intestinal tract may play a role in feline IBD, warranting further characterization of the intestinal microflora in healthy cats and cats with IBD.

HYPOTHESIS AND SPECIFIC OBJECTIVES

The hypotheses of this study are that 1) a molecular-based approach can be used to characterize the bacterial microflora along the entire feline intestinal tract, and 2) that the same molecular-based approach can be used to compare the fecal microflora of healthy cats and cats with IBD.

The objectives of this study are 1) to develop a molecular-based protocol to amplify 16S rDNA for the qualitative assessment of the bacterial microflora in the feline intestinal tract, 2) to characterize the bacterial microflora along the entire feline intestinal tract using sequence analysis of amplified 16S rDNA, and 3) to compare the fecal microflora of healthy cats and cats with IBD using sequence analysis of amplified 16S rDNA.

CHAPTER II

MOLECULAR IDENTIFICATION OF INTESTINAL BACTERIA IN HEALTHY CATS

OVERVIEW

The study aim was to describe the microflora along the intestinal tract in healthy cats based on analysis of the 16S ribosomal rRNA gene (16S rDNA). Intestinal content was collected from four healthy research cats (i.e., duodenum, jejunum, ileum, colon) and one healthy specific pathogen free cat (SPF) (i.e., stomach, duodenum, jejunum, ileum, colon, rectum). Bacterial 16S rDNA was amplified using universal bacterial primers. Amplicons were ligated into cloning vectors and 16S rDNA inserts were sequenced. A total of 1,008 clones were analyzed and 109 non-redundant 16S rDNA sequences were identified. A predominant anaerobic microflora was observed in all cats. In the four healthy research cats, sequences were predominantly classified in the phylum *Firmicutes* (68%). The majority of clones fell within the order *Clostridiales* (54 %), followed by *Lactobacillales*, *Bacteroidales, Campylobacterales,* and *Fusobacteriales* (14%, 11%, 10%, and 6%, respectively). *Clostridiales* were predominantly affiliated with *Clostridium* clusters I and XIVa. Clones affiliated with cluster I increased in complexity along the intestinal tract with the highest number of clones isolated from the colon. Sequences affiliated with *Clostridium* cluster XIVa were predominantly isolated from the colon. In the healthy SPF cat, 98% of clones were classified in the phylum *Firmicutes* and were predominantly affiliated with *Clostridium* cluster I.

INTRODUCTION

It has been recognized that the intestinal microflora plays an important role in gastrointestinal health. There is evidence that the resident bacterial flora has an effect on intestinal motility, the physiology of the intestinal epithelium, and its interaction with the immune system (Falk *et al.*, 1998; Hooper *et al.*, 2001). The microflora also influences nutrition by supplying nutrients such as vitamins, lactate, and short chain fatty acids to host tissues (Macfarlane & Macfarlane, 2003). Additionally, it has been shown that residential bacteria provide a natural defense mechanism against invading pathogens (Gibson & Roberfroid, 1995).

In contrast, alterations in the commensal intestinal microflora have been implicated in gastrointestinal disease in humans and many animal species including cats (Johnston *et al.*, 2001; Linskens *et al.*, 2001; Janeczko *et al.*, 2007). For example, it has been suggested that an ineffective clearance of enteric pathogens or a loss of tolerance to the residential intestinal microflora maybe be an important factor in the pathogenesis of inflammatory bowel disease (IBD) in humans (Linskens *et al.*, 2001). In cats, a study analyzed intestinal biopsies from the duodenum of healthy cats and cats with IBD using florescence *in situ* hybridization (Janeczko *et al.*, 2007). The composition of the mucosa-associated microflora was distinctly different between healthy cats and cats with IBD, and IBD cats had a significantly higher number of mucosa-associated organisms belonging to the *Enterobacteriaceae* family than healthy cats (Janeczko *et al.*, 2007). Therefore, given the suggested impact of the microflora on gastrointestinal health, knowledge about the composition of the microflora within the digestive tract of healthy

cats is important for future studies exploring differences in the intestinal microflora between healthy and diseased cats.

Past studies characterizing the feline intestinal microflora have used traditional microbiological culture techniques (Smith, 1965; Osbaldiston & Stowe, 1971; Itoh *et al.,* 1984; Johnston *et al.*, 1993; Papasouliotis *et al.*, 1998; Johnston *et al.*, 1999). These studies reported that, in contrast to other mammalian species, the feline intestinal microflora harbors predominantly facultative and obligate anaerobic bacterial species (Johnston *et al.*, 1993; Papasouliotis *et al.*, 1998; Johnston *et al.*, 2001). Also, these studies have described bacterial populations present in samples obtained either from the proximal parts of the small intestine (i.e., duodenum and jejunum) or from the large intestines (i.e., colon or feces) (Osbaldiston & Stowe, 1971; Johnston *et al.*, 1993; Papasouliotis *et al.*, 1998; Johnston *et al.*, 1999). However, to our knowledge, no study has reported the composition of the microflora of the ileum in cats. Additionally, no comparative study has evaluated the bacterial diversity along the entire gastrointestinal tract of cats.

Today it is well recognized that traditional culture-based techniques, as used in previous studies, fail to accurately characterize bacterial communities and underestimate biodiversity in complex biological ecosystems such as the intestine. Reasons for this include difficulties in anaerobic collection of intestinal samples and a lack of knowledge about the growth requirements of many intestinal bacterial species (Itoh *et al.*, 1984; Amann *et al.*, 1995; Langendijk *et al.,* 1995; Greetham *et al.*, 2002). It has also been shown that commonly used culture media allow growth of bacteria other than their published target organisms, and selective agars particularly designed for strict anaerobes

show poor recovery efficiencies (Greetham *et al.*, 2002). In addition, related bacterial species can often be misclassified because microorganisms can exhibit metabolic plasticity (Greetham *et al.*, 2002).

Recent studies aiming to characterize the intestinal microflora in many mammalian species have used molecular methods based on the amplification of the 16S ribosomal RNA gene (16S rDNA) (Greetham *et al.*, 2002; Hold *et al.*, 2002; Wang *et al.*, 2003; Delgado *et al.*, 2006). This approach has greatly enhanced our understanding of microbial communities, and many studies have observed an increased bacterial diversity in the intestine compared to studies using strictly culture-based methods (Langendijk *et al.*, 1995; Wilson, 1996; Greetham *et al.*, 2002; Delgado *et al.*, 2006; Inness *et al.*, 2006). The disadvantages of culture-based analysis warrant characterization of the feline intestinal microflora using molecular-based methods, and it is likely that a molecular-based approach may identify greater bacterial diversity in the intestinal tract of cats than previously reported. Therefore, in the present study we aimed to characterize the residential bacteria found in all segments in the intestine of healthy cats using analysis of 16S rDNA sequences.

MATERIALS AND METHODS

Sample collection. The protocol for sample collection was approved by the University Laboratory Animal Care Committee at Texas A&M University (AUP# 2001-0088).

Five healthy cats, euthanized for a previous, unrelated project, were used for molecular identification of the intestinal bacterial microflora. One of the five healthy cats

was a specific pathogen free (SPF) cat. At the conclusion of the unrelated project, luminal intestinal content was collected by needle aspiration. Samples were transferred into sterile cryotubes, snap-frozen in liquid nitrogen, and stored at -80ºC until further analysis.

It was attempted to collect samples from several segments along the gastrointestinal tract. However, in some cats not all segments could be sampled. For cats 1 and 2, samples from the jejunum, ileum, and colon were available for analysis. For cat 3 only a sample from the colon was available for analysis. For cat 4, samples from the duodenum, ileum, and colon were available for analysis. Finally, for the SPF cat samples from the stomach, duodenum, jejunum, ileum, colon, and rectum were available for analysis.

Extraction of DNA. DNA extraction was carried out as described by Suchodolski *et al.* (2004) using a bead beating method followed by phenol:chloroform:iso-amylalcohol extraction. Each sample was extracted independently in a separate, sterile cryotube. Briefly, 500 µl of cell lysis solution (Puregene cell lysis solution, Gentra Systems, Minneapolis, MN), 200 µl of buffer-saturated phenol-chloroform-isoamyl alcohol (25:24:1, pH 7.2), and 300 µl of 0.1-mm zirconia beads (BioSpec Products Inc., Bartlesville, OK) were added to each sample. The tubes were positioned horizontally on a vortex adapter mounted on a standard vortexer, and the mixture was vortexed for 5 min at maximum speed. The tubes were centrifuged for 7 min at 12,000 x *g* and 4°C, and the supernatant was transferred to a new sterile cryotube. Then 700 µl of phenol-chloroform-isoamyl alcohol was added, and the tube was vortexed for 30 s and centrifuged for 20 min at 12,000 x *g* and 4°C. The aqueous phase was transferred into a new sterile cryotube. To increase the DNA yield, 200 µl of a 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 resulting aqueous phases were combined. To remove RNA, 5.2 U of RNase (Puregene RNase, Gentra Systems) were added to the solution and incubated at 37°C for 30 min. The RNase was removed by phenol-chloroform-isoamyl alcohol extraction as described above. The aqueous phase containing the DNA was mixed with 0.5 volume 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 then stored at -20°C until further use. A negative control containing H_2O instead of sample was purified in parallel to each extraction batch to screen for contamination of extraction reagents.

16S rDNA amplification by PCR. Extracted DNA was used as a template for PCR amplification of approximately 450 bp of 16S rDNA with universal bacterial primers F341 (5'-CCTACGGGAGGCAGCAG-3') and 786R (5'-GACTACCAGGGTATCTAATC -3'). Each reaction mixture (25 µl) consisted of reaction buffer (GeneAmp 10x PCR Gold buffer, Applied Biosystems, Foster City, CA.) (final concentrations: 15 mM Tris-HCl, 50 mM KCl, 3 mM $MgCl_2$ [pH 8.0]), 1.25 U of *Taq* DNA polymerase (Amplitaq Gold LD, Applied Biosystems, Foster City, CA.), 250 µM each of the deoxynucleoside triphosphates, 0.24 µM each primer, and 100 ng of DNA template. To screen for potential contamination of PCR reagents, a negative PCR control using H_2O instead of DNA template was used. The samples were amplified in a thermocycler (Mastercycler Gradient, Eppendorf AG, Hamburg, Germany) using the following PCR protocol: initial denaturing at 95ºC for 3 min; 30 cycles of denaturation at

95ºC for 30 s, annealing at 54ºC for 30 s, extension at 72ºC for 1 min; final extension at 72ºC for 10 min. Two samples (one duodenal and one ileal sample each) collected from cat 4 did not have enough amplified DNA after 30 PCR cycles for a successful ligation. Therefore, these samples were re-amplified using the same PCR protocol with 35 cycles. For jejunal and ileal samples from cats 1 and 2, 8 independent PCR reactions each were performed. For the colonic samples from the same cats 4 independent PCR reactions were performed. For cat 3, 8 independent PCR reactions were performed for the colonic sample. Four independent PCR reactions were performed for the duodenal and ileal samples and 8 independent PCRs for the colonic sample from cat 4. Eight independent PCR reactions were performed for the gastric sample from the SPF cat, 7 for the jejunal sample, 8 for the ileal sample, 4 for the colonic sample, and 5 for the rectal sample. PCR products belonging to the same sample were pooled and concentrated using the DNA Clean & Concentrator-5™ (Zymo Research, Orange, CA.) following the manufacturer's instructions. The purity of the PCR amplicons were assessed on 1% agarose electrophoresis gels stained with Gel Red™ (Biotium Inc., Hayward, CA).

Cloning of bacterial 16S rDNA amplicons. Amplified PCR products were ligated into pCR®4-TOPO® linearized cloning vectors (TOPO TA, Invitrogen, Carlsbad, CA.) as specified by the manufacturer. After ligation, the products were transformed into chemically competent DH5α™-T1^{R} *E. coli* by heat shock following manufacturer's instructions. Transformed products were grown overnight on Luria-Bertani (LB) medium with ampicillin (50 μg ml^{-1}) at 37ºC. The pCR®4-TOPO® vector allows direct selection of recombinant cells via disruption of the lethal *E. coli* gene *ccd*B. Up to 96 colonies for each sample were randomly selected and clones were grown for 24 hours in 1.4 ml LB

broth treated with ampicillin (50 μg ml^{-1}) in 96-well blocks (Perfectprep® BAC 96, Eppendorf, North America, Inc., Westbury, NY) sealed with Gas Permeable Adhesive Seals (ABgene, Surrey, UK.).

Plasmid extraction and sequencing of 16S rDNA. Plasmid DNA was purified in a 96- well format using the Perfectprep® BAC 96 plasmid purification kit (Eppendorf) and a single vacuum manifold (Eppendorf). Plasmid DNA was then eluted using 50 μl of DNA grade water and the products were stored at -30ºC until further use. The amplified 16S rDNA inserts were sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and products were analyzed with an automated sequence analyzer (ABI 3100 Capillary Sequencer, Applied Biosystems).

Sequence analysis. Each sequence was edited to exclude the PCR primer binding sites, and then tested for possible chimeric structures using the Check_Chimera and Pintail software available online through the Ribosomal Database Project (RDP). Identified chimeras were excluded from further analysis.

Cloned sequences were compared with existing 16S rDNA sequences using GenBank and RDP (release 9.59, approximately 489,840 16S rRNA sequences) and the closest neighbor for each sequence was downloaded. Sequences were aligned using the multiple sequence alignment program CLUSTAL_W included in the BioEdit software package. Sequence distances were analyzed by the DNADIST program and the Jukes-Cantor model for inferring evolutionary distances (Van de & Wachter, 1993). Phylogenetic trees were generated with distance matrices and drawn based on the neighbor-joining algorithm using the TREECON software package (version 1.3b). Branch stability was assessed by bootstrap analysis (100 replicates) using the algorithms

available in the TREECON package. *Aquifex pyrophilus* was used as an outgroup for all phylogenetic trees generated. Groups of sequences with less than 2% sequence divergence (98% similarity) to each other were defined as an Operational Taxonomic Unit (OTU).

Statistical analysis. The coverage of the individual clone libraries (i.e., the probability that any additional analyzed clone is different from any previously obtained single clone) was calculated according to Good using the formula $[1-(n/N)] \times 100$, where n is the number of molecular species represented by one clone and N is the total number of sequences (Good, 1953). Bacterial diversity indices were calculated for the analysis of intestinal samples using the Shannon-Weaver diversity index (Atlas et al., 1998). The Shannon-Weaver index (Hs) was defined as $-\sum p_i \ln(p_i)$, where p_i is the proportion of individual bacteria found in a certain species (Atlas et al., 1998).

RESULTS

A total of 1,332 clones were randomly selected. Of these, a total of 1,071 clones contained an insert with a sequence of adequate quality. Sixty-three of these clones were identified as possible chimeras and were excluded from further analysis. A total of 109 operative taxonomical units (OTUs), representing a total of 1,008 clones, were used for subsequent phylogenetic analysis. Table 1 summarizes the number of analyzed samples, analyzed clones, and identified OTUs and the coverage, and bacterial diversity index for each intestinal segment.

Twenty-one OTUs (19%) showed less than 98% similarity to existing 16S rDNA sequences in the NCBI database, and may represent as of yet uncharacterized bacterial

species. Table 2 shows only the OTUs with at least 98% similarity to known isolates in the GenBank database and their representative clones. Five different bacterial phyla were identified with the majority of OTUs being classified as *Firmicutes*, followed by *Proteobacteria, Bacteroidetes*, *Fusobacteria*, and *Actinobacteria*, respectively. A complete phylogenetic analysis of all OTUs is illustrated in Table 3 and Figures 1-8.

Actinobacteria. A total of 21 clones representing 7 OTUs were identified within the phylum *Actinobacteria* (Fig. 1). Two clones were isolated from the jejunum (10%), 11 clones were isolated from the ileum (52%), and 8 clones were isolated from the colon (38%).

Bacteroidetes. A total of 95 clones were classified within the phylum *Bacteroidetes* representing 17 OTUs (Fig. 2). The majority of clones were isolated from the ileum and colon (43% and 50%, respectively), followed by the rectum (5%) and the jejunum (<2%). Four different bacterial families were identified: *Bacteroidaceae*, *Porphyromonadaceae*, *Prevotellaceae*, and *Rikenellaceae*. A total of 54 clones representing 10 OTUs were identified within the *Bacteroidaceae* family. A total of 35 clones representing 4 OTUs were classified within the *Prevotellaceae* family. The *Rikenellaceae* and *Porphyromonadaceae* families were represented by 1 and 2 OTUs, respectively.

Firmicutes. The majority of all clones analyzed (82%) were classified within the phylum *Firmicutes* representing 67 OTUs. Figures 3 and 4 show the OTUs classified within the bacterial class *Clostridiales* and Figure 5 illustrates the OTUs identified within the *Bacilli* and *Mollicutes* class.

A total of 754 clones were classified in the bacterial class *Clostridiales* representing 56 different OTUs. Clones belonging to this bacterial class were affiliated with six different *Clostridium* clusters: Cluster I, III, IV, XI, XIVa, and XIVb. The percentages of the 16S rDNA sequences affiliated with these clusters are illustrated in Figure 6. A total of 541 clones representing 13 OTUs were affiliated with *Clostridium* cluster I. Four OTUs were affiliated with the *Clostridium perfringens* subgroup and one OTU was affiliated with *Sarcina ventriculi* (*Clostridium* subcluster Ia). One OTU associated with *Clostridium* cluster I was represented by clones obtained from all compartments and the number of clones obtained was highest in the stomach and proximal intestine (i.e., duodenum, jejunum) and lowest in the distal intestine (i.e., colon, rectum). One OTU was affiliated with *Clostridium* cluster III. Six OTUs were affiliated with *Clostridium* cluster IV and 2 OTUs were affiliated with *Clostridium* cluster XI. A total of 54 clones were affiliated with *Clostridium* cluster XIVa representing 20 OTUs. The majority of these clones were obtained from the colon (50%). Three OTUs were affiliated with *Clostridium* cluster XIVb.

A total of 70 clones representing 10 different OTUs were classified within the *Bacilli* class. Thirty-four clones were isolated from the jejunum (48%), 9 from the ileum (13%), and 27 from the colon (39%). Further classification showed that 65 clones (92%) representing 8 OTUs were classified in the bacterial order *Lactobacillales*. One OTU consisting of one clone from the colon was classified in the class *Mollicutes*.

Fusobacteria. A total of 26 clones representing 4 OTUs were classified within the phylum *Fusobacteria* (Fig. 7). Four clones were isolated from the jejunum (15%), 4 from the ileum (15%), and 18 from the colon (69%).

Proteobacteria. A total of 78 clones representing 14 OTUs were classified within the phylum *Proteobacteria* (Fig. 8). The majority of clones were isolated from the duodenum (41%), followed by the ileum, colon, jejunum and rectum.

Table 1. Number of analyzed samples and clones, identified OTUs and coverage and bacterial diversity index (H) constructed from samples obtained from various segments of the feline intestinal tract.

	No. of samples	No. of clones	No. of OTUs[a]	Coverage[b]	H[c]
Stomach	1	91	5	97.8	0.9
Duodenum	2	93	7	96.8	1.4
Jejunum	2	172	25	93.1	2.2
Ileum	3	261	49	92.8	3.0
Colon	4	322	84	87.1	3.0
Rectum	1	75	16	92.0	2.0

[a]OTU= operative taxonomical unit
[b]According to Good (Good, 1953)
[c]Shannon-Weaver diversity index

Table 2. OTUs and number of representative clones with at least 98% similarity to known isolates in the GenBank database.

OTUs[a]	GenBank isolate[b]	Percent similarity[c]	No. of clones[d]					
			S	D	J	I	C	R
2FJ-44	*Actinomyces hyovaginalis*	98%	0	0	2	6	2	0
FC-15	*Bacteroides uniformis*	100%	0	0	0	12	12	16
FI-38	*Parabacteroides merdae*	100%	0	0	0	3	1	0
3FD-05	*Clostridium colicanis*	99%	0	1	0	0	0	0
FC-18	*Ruminococcus schinkii*	98%	0	0	0	1	3	0
5FC-14	*Lactobacillus murinus*	99%	0	0	0	4	2	0
FC-104	*Staphylococcus felis*	100%	0	0	0	0	5	0
2FJ-08	*Fusobacterium equinum*	99%	0	0	3	2	10	0
2FJ-10	*Fusobacterium russii*	99%	0	0	1	1	1	0
fFC-08	*Anaerobiospirillum succiniciproducens*	98%	0	0	0	0	4	0
2fFD-07	*Candidatus* Helicobacter heilmannii	100%	0	9	0	0	0	0
FI-109	*Desulfovibrio piger*	99%	0	0	0	3	0	0

[a]OTU= operative taxonomical unit
[b]Isolate classified by NCBI Blast
[c]Similarity to closest known isolate in the Genbank database
[d]S=stomach, D=duodenum, J=jejunum, I=ileum, C=colon, R=rectum

Table 3. Phylogenetic classification of the 109 operative taxonomical units obtained from the feline GI tract. Classification is based on the taxonomical hierarchy proposed in Bergey's Manual of Systematic Bacteriology (Ribosomal Database Project classifier).

A. Phylum Firmicutes (67)
- class Mollicutes (1)
  - order Incertae sedis 8 (1)
    - family Erysipelotrichaceae (1)
      - unclassified_Erysipelotrichaceae (1)
- class Bacilli (11)
  - order Bacillales (2)
    - family Staphylococcaceae (2)
      - genus Gemella (1)
      - genus Staphylococcus (1)
  - order Lactobacillales (9)
    - family Incertae sedis 9 (1)
      - genus Oscillospira (1)
    - family Carnobacteriaceae (1)
      - genus Granulicatella (1)
      - genus Lactobacillus (1)
    - family Enterococcaceae (3)
      - genus Enterococcus (2)
    - unclassified_Enterococcaceae (1)
    - family Streptococcaceae (2)
      - genus Streptococcus (2)
  - unclassified_Lactobacillales (1)
- class Clostridia (52)
  - order Clostridiales (52)
    - family Eubacteriaceae (1)
      - unclassified_Eubacteriaceae (1)
    - family Peptococcaceae (1)
      - genus Peptococcus (1)
    - family Acidaminococcaceae (2)
      - genus Allisonella (1)
      - genus Dialister (1)
    - family Peptostreptococcaceae (1)
      - genus Peptostreptococcus (1)
    - family Lachnospiraceae (12)
      - genus Roseburia (1)
      - genus Ruminococcus (6)
      - unclassified_Lachnospiraceae (5)
    - family Clostridiaceae (21)
      - genus Acetanaerobacterium (1)
      - genus Anaerotruncus (1)
      - genus Subdoligranulum (1)
      - genus Faecalibacterium (1)
      - genus Clostridium (2)
      - unclassified_Clostridiaceae (15)
  - unclassified_Clostridiales (14)

unclassified_Firmicutes (3)

B. Phylum Fusobacteria (4)
- class Fusobacteria (4)
  - order Fusobacteriales (4)
    - family Incertae sedis 11 (1)
      - genus Cetobacterium (1)
    - family Fusobacteriaceae (2)
      - genus Fusobacterium (2)
  - unclassified_Fusobacteriales (1)

C. Phylum Actinobacteria (7)
- class Actinobacteria (7)
  - order Coriobacteriales (4)
    - family Coriobacteriaceae (4)
      - genus Slackia (1)
      - genus Collinsella (3)
  - order Actinomycetales (3)
    - family Corynebacteriaceae (1)
      - genus Corynebacterium (1)
    - family Microbacteriaceae (1)
      - unclassified_Microbacteriaceae (1)
    - family Actinomycetaceae (1)
      - genus Actinomyces (1)

D. Phylum Bacteroidetes (17)
- class Bacteroidetes (17)
  - order Bacteroidales (17)
    - family Rikenellaceae (1)
      - genus Alistipes (1)
    - family Porphyromonadaceae (2)
      - genus Parabacteroides (2)
    - family Prevotellaceae (4)
      - genus Prevotella (4)
    - family Bacteroidaceae (10)
      - genus Bacteroides (10)

E. Phylum Proteobacteria (14)
- class Deltaproteobacteria (1)
  - order Desulfovibrionales (1)
    - family Desulfovibrionaceae (1)
      - genus Desulfovibrio (1)
- class Betaproteobacteria (2)
  - order Burkholderiales (2)
    - family Alcaligenaceae (1)
      - genus Sutterella (1)
  - unclassified_Burkholderiales (1)
- class Gammaproteobacteria (7)
  - order Pseudomonadales (1)
    - family Moraxellaceae (1)
      - genus Moraxella (1)
  - order Aeromonadales (2)
    - family Succinivibrionaceae (2)
      - genus Anaerobiospirillum (2)
  - order Enterobacteriales (1)
    - family Enterobacteriaceae (1)
      - genus Shigella (1)
  - order Pasteurellales (3)
    - family Pasteurellaceae (3)
      - genus Actinobacillus (1)
    - unclassified_Pasteurellaceae (2)
- class Epsilonproteobacteria (4)
  - order Campylobacterales (4)
    - family Helicobacteraceae (4)
      - genus Helicobacter (4)

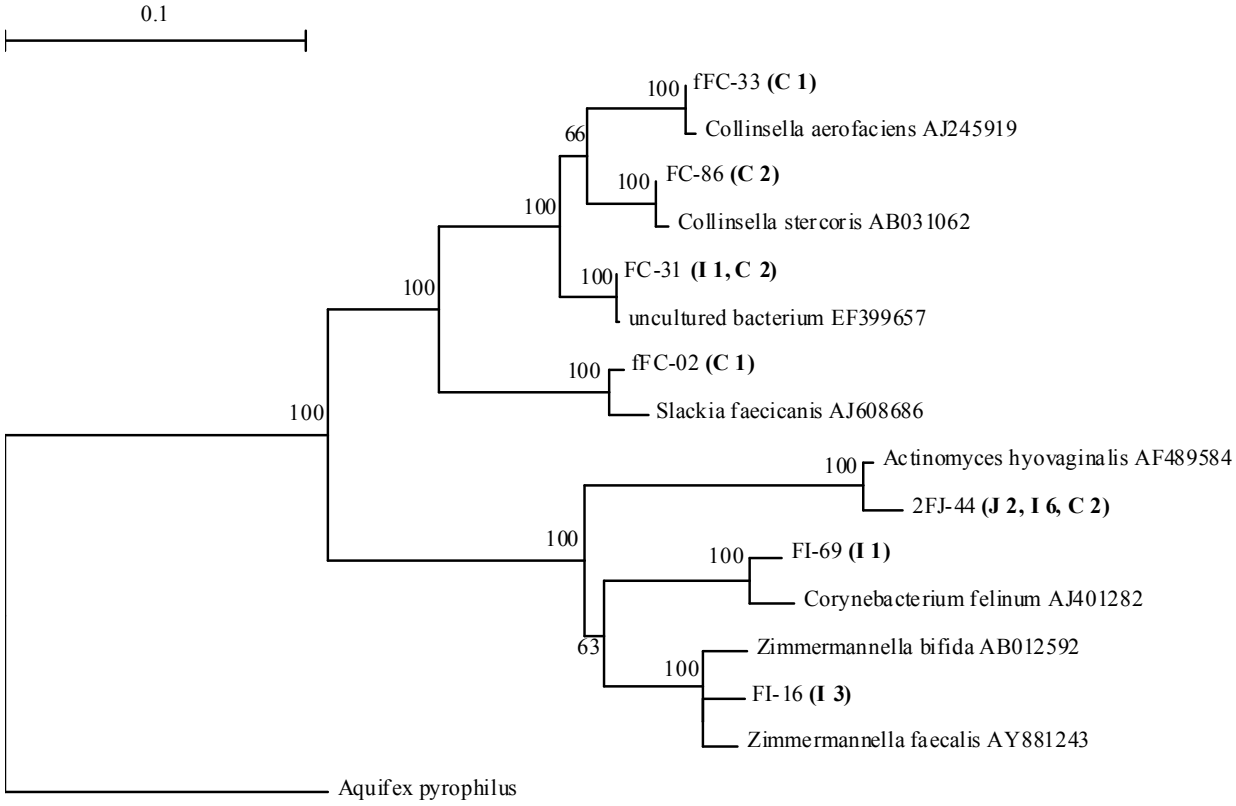

Figure 1. Phylogenetic tree showing the affiliation of OTUs isolated from the feline GI tract for *Actinobacteria*. The bar represents 10% sequence divergence. 16S rDNA sequences were aligned to their closest neighbor in the RDP database. The tree was inferred based on the neighbor-joining algorithm. *Aquifex pyrophilus* was used as an outgroup in order to infer the root of the tree. Bootstrap values shown at the branches are based on 100 replicates; values below 50% are not shown. For each OTU the numbers in parenthesis indicate the number of observed clones in the respective intestinal segments. (S=stomach, D=duodenum, J=jejunum, I=ileum, C=colon, R=rectum).

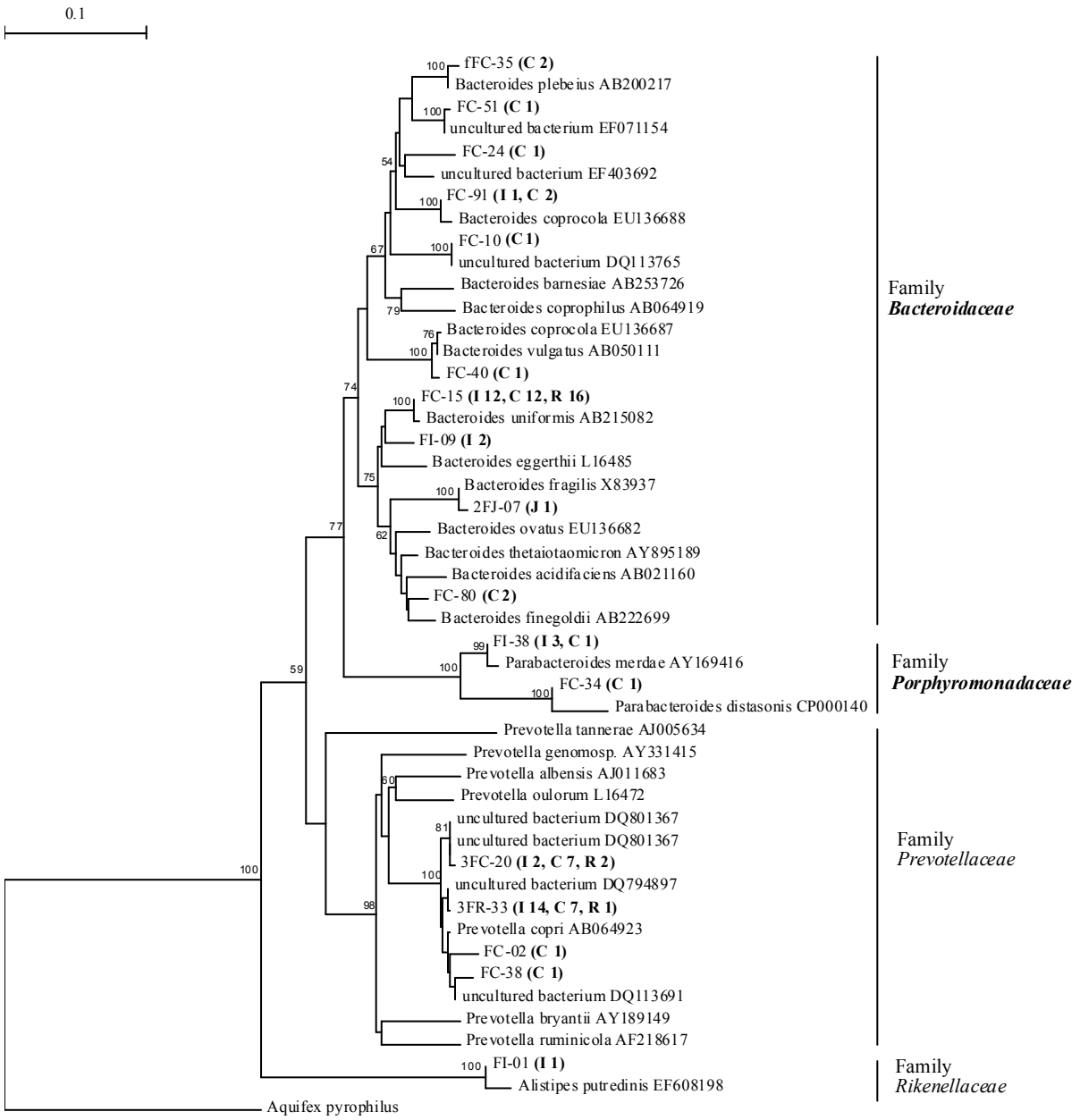

Figure 2. Phylogenetic tree showing the affiliation of OTUs isolated from the feline GI tract for *Bacteroidetes*. The bar represents 10% sequence divergence. 16S rDNA sequences were aligned to their closest neighbor in the RDP database. The tree was inferred based on the neighbor-joining algorithm. *Aquifex pyrophilus* was used as an outgroup in order to infer the root of the tree. Bootstrap values shown at the branches are based on 100 replicates; values below 50% are not shown. For each OTU the numbers in parenthesis indicate the number of observed clones in the respective intestinal segments. (S=stomach, D=duodenum, J=jejunum, I=ileum, C=colon, R=rectum).

Figure 3. Phylogenetic tree showing the affiliation of OTUs isolated from the feline GI tract for the class *Clostridiales*. The bar represents 10% sequence divergence. 16S rDNA sequences were aligned to their closest neighbor in the RDP database. The tree was inferred based on the neighbor-joining algorithm. *Aquifex pyrophilus* was used as an outgroup in order to infer the root of the tree. Bootstrap values shown at the branches are based on 100 replicates; values below 50% are not shown. For each OTU the numbers in parenthesis indicate the number of observed clones in the respective intestinal segments. (S=stomach, D=duodenum, J=jejunum, I=ileum, C=colon, R=rectum).

Figure 3 continued

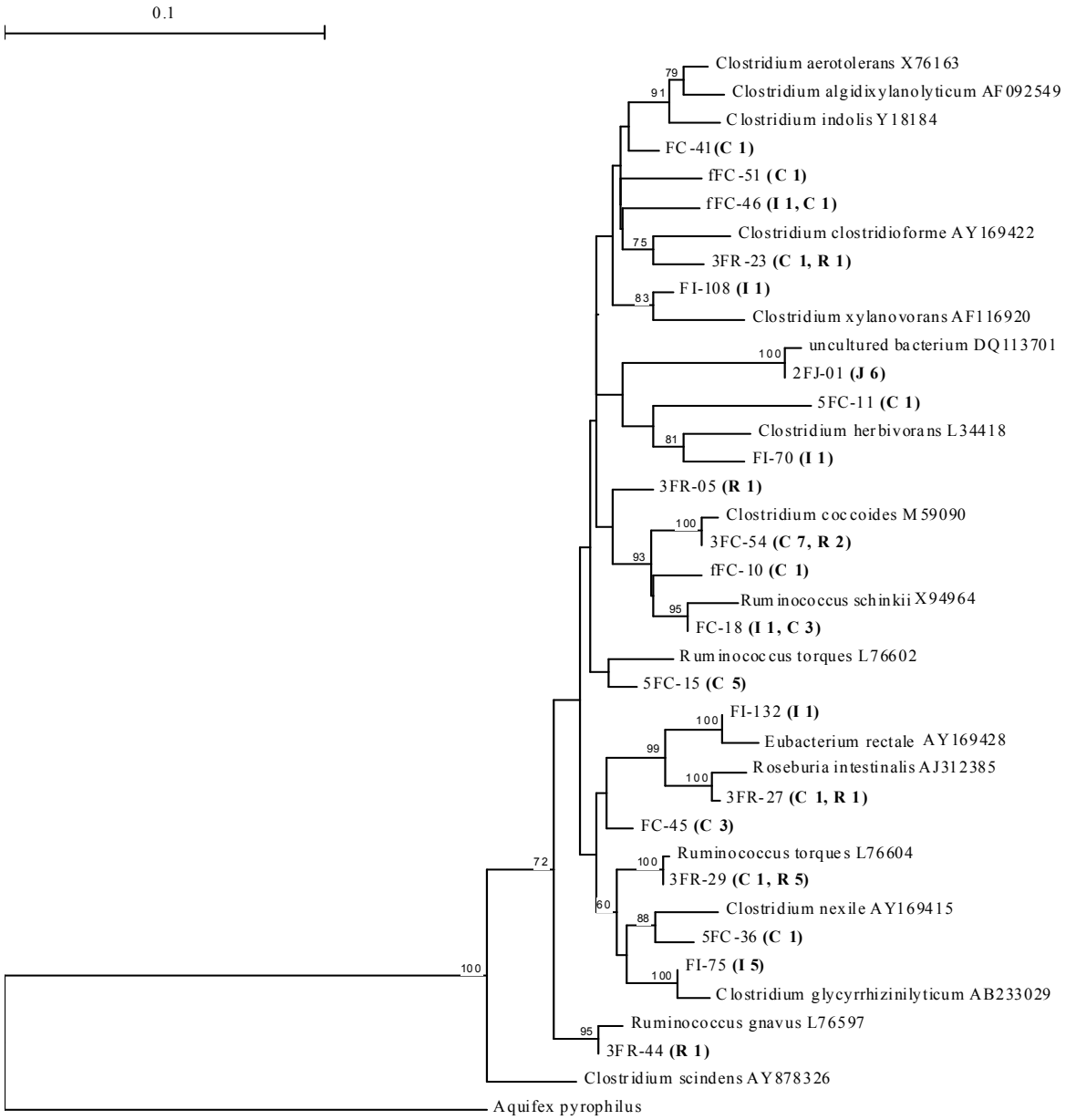

Figure 4. Phylogenetic tree showing the affiliation of OTUs isolated from the feline GI tract for *Clostridium* cluster XIVa. The bar represents 10% sequence divergence. 16S rDNA sequences were aligned to their closest neighbor in the RDP database. The tree was inferred based on the neighbor-joining algorithm. *Aquifex pyrophilus* was used as an outgroup in order to infer the root of the tree. Bootstrap values shown at the branches are based on 100 replicates; values below 50% are not shown. For each OTU the numbers in parenthesis indicate the number of observed clones in the respective intestinal segments. (S=stomach, D=duodenum, J=jejunum, I=ileum, C=colon, R=rectum).

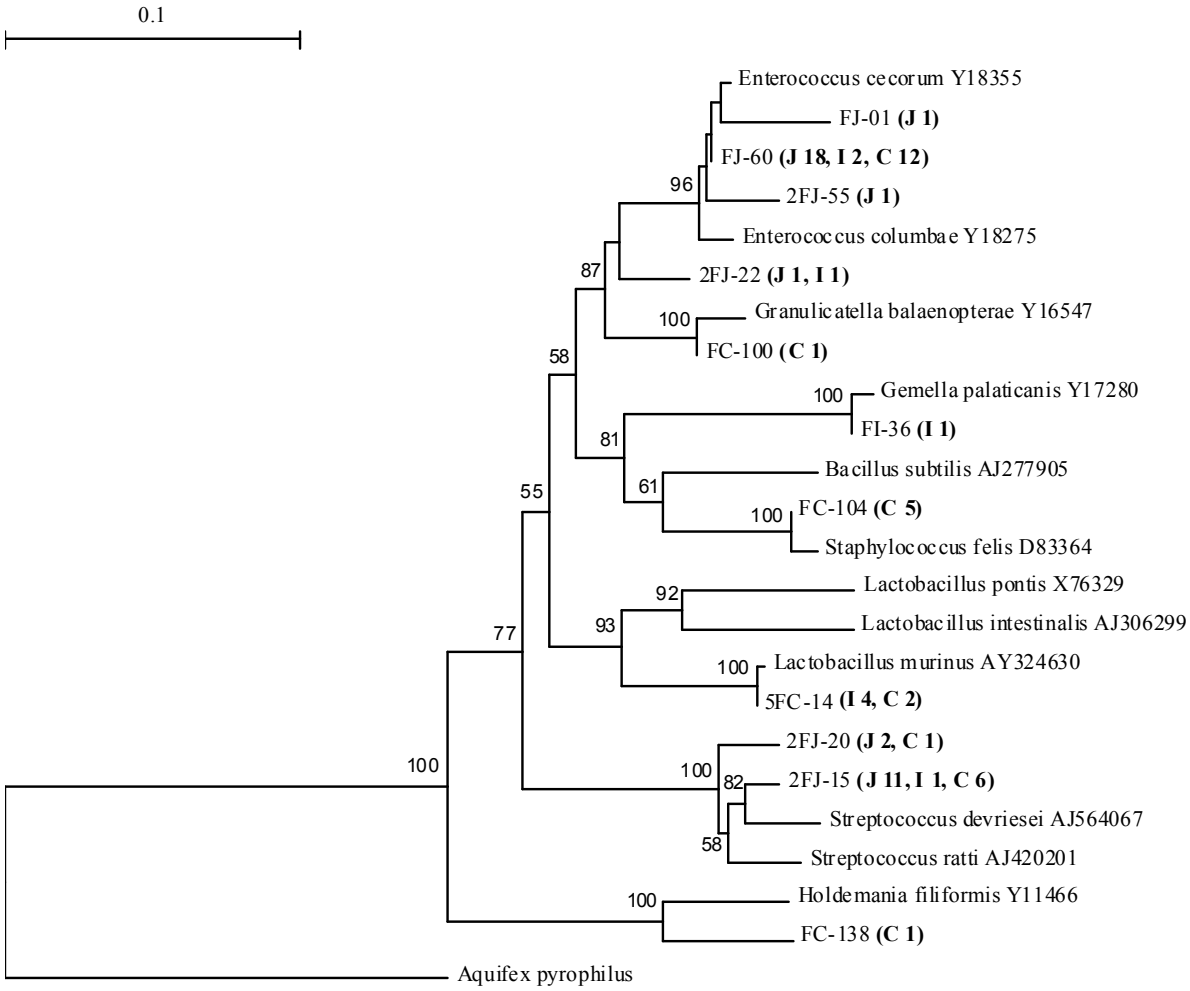

Figure 5. Phylogenetic tree showing the affiliation of OTUs isolated from the feline GI tract for the classes *Bacilli* and *Mollicutes*. The bar represents 10% sequence divergence. 16S rDNA sequences were aligned to their closest neighbor in the RDP database. The tree was inferred based on the neighbor-joining algorithm. *Aquifex pyrophilus* was used as an outgroup in order to infer the root of the tree. Bootstrap values shown at the branches are based on 100 replicates; values below 50% are not shown. For each OTU the numbers in parenthesis indicate the number of observed clones in the respective intestinal segments. (S=stomach, D=duodenum, J=jejunum, I=ileum, C=colon, R=rectum).

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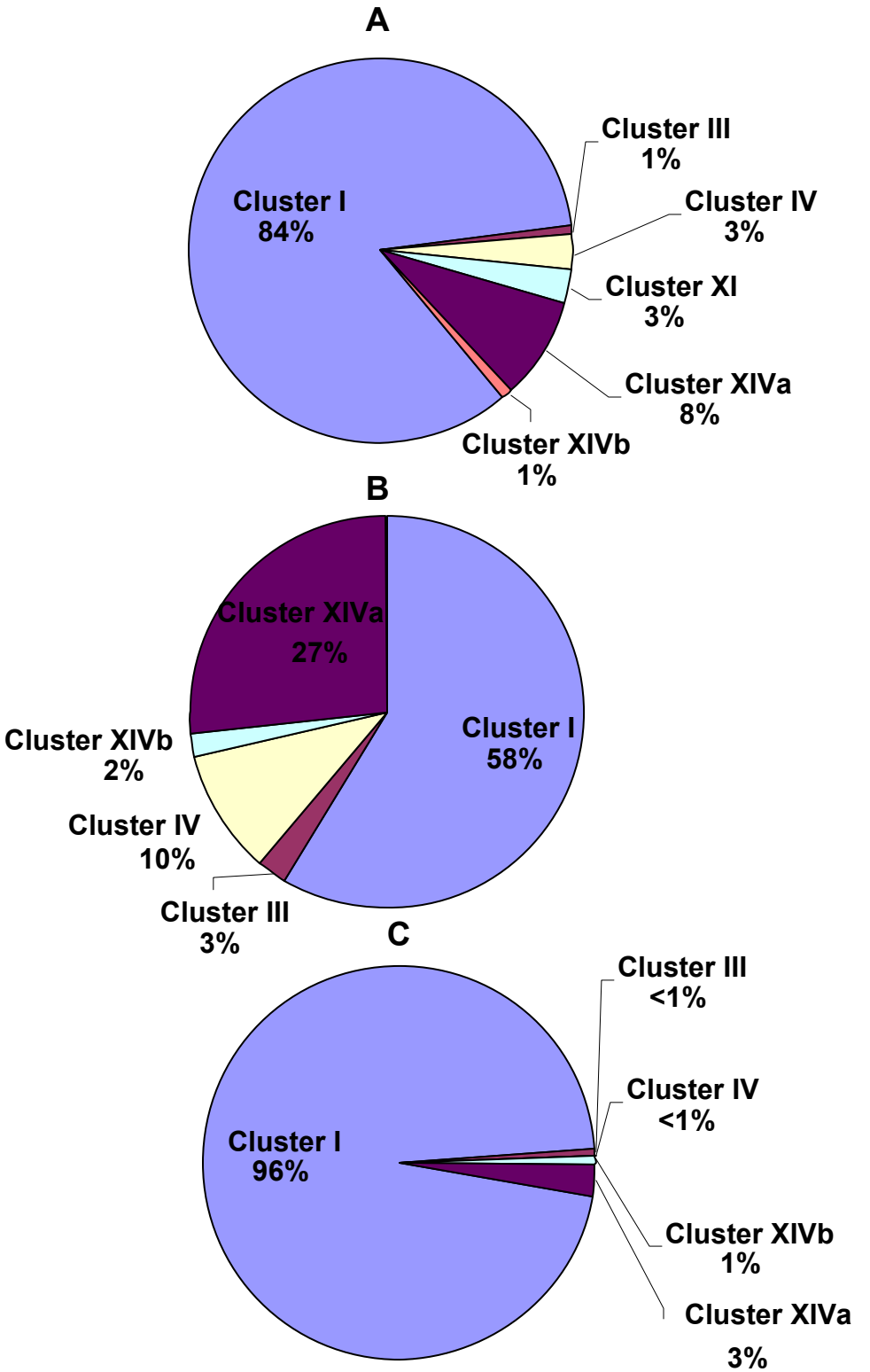

Figure 6. Classification of 16S rDNA sequences belonging to the different *Clostridium* clusters in all 5 cats (A), the four healthy non-SPF cats (B), and the healthy SPF cat (C).

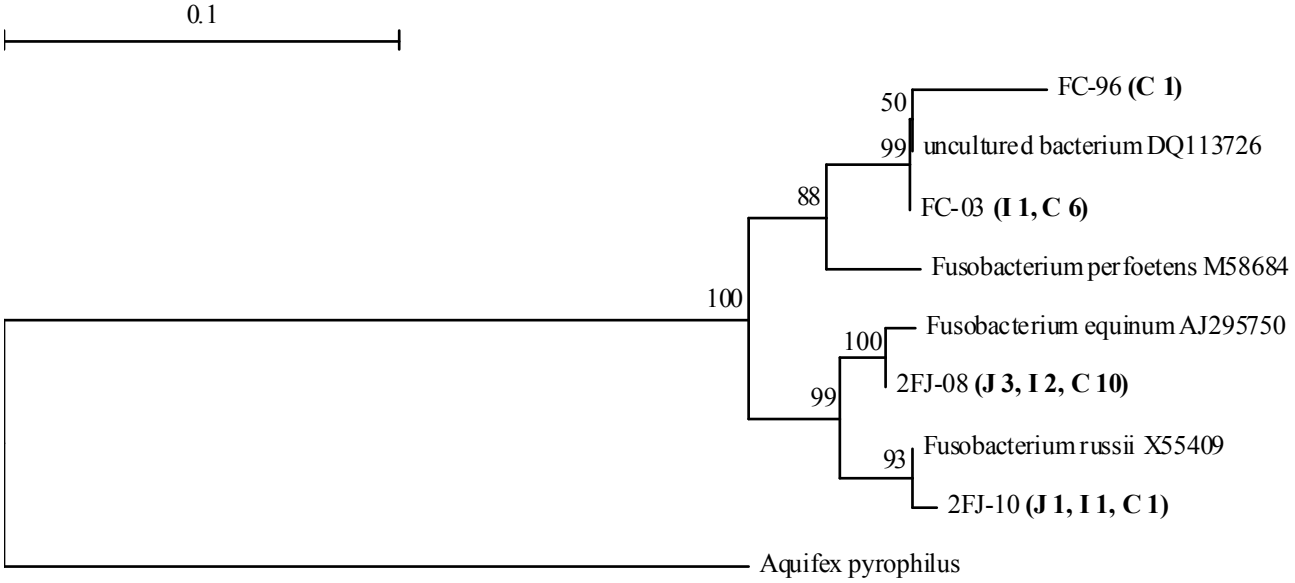

Figure 7. Phylogenetic tree showing the affiliation of OTUs isolated from the feline GI tract for *Fusobacteria.* The bar represents 10% sequence divergence. 16S rDNA sequences were aligned to their closest neighbor in the RDP database. The tree was inferred based on the neighbor-joining algorithm. *Aquifex pyrophilus* was used as an outgroup in order to infer the root of the tree. Bootstrap values shown at the branches are based on 100 replicates; values below 50% are not shown. For each OTU the numbers in parenthesis indicate the number of observed clones in the respective intestinal segments. (S=stomach, D=duodenum, J=jejunum, I=ileum, C=colon, R=rectum).

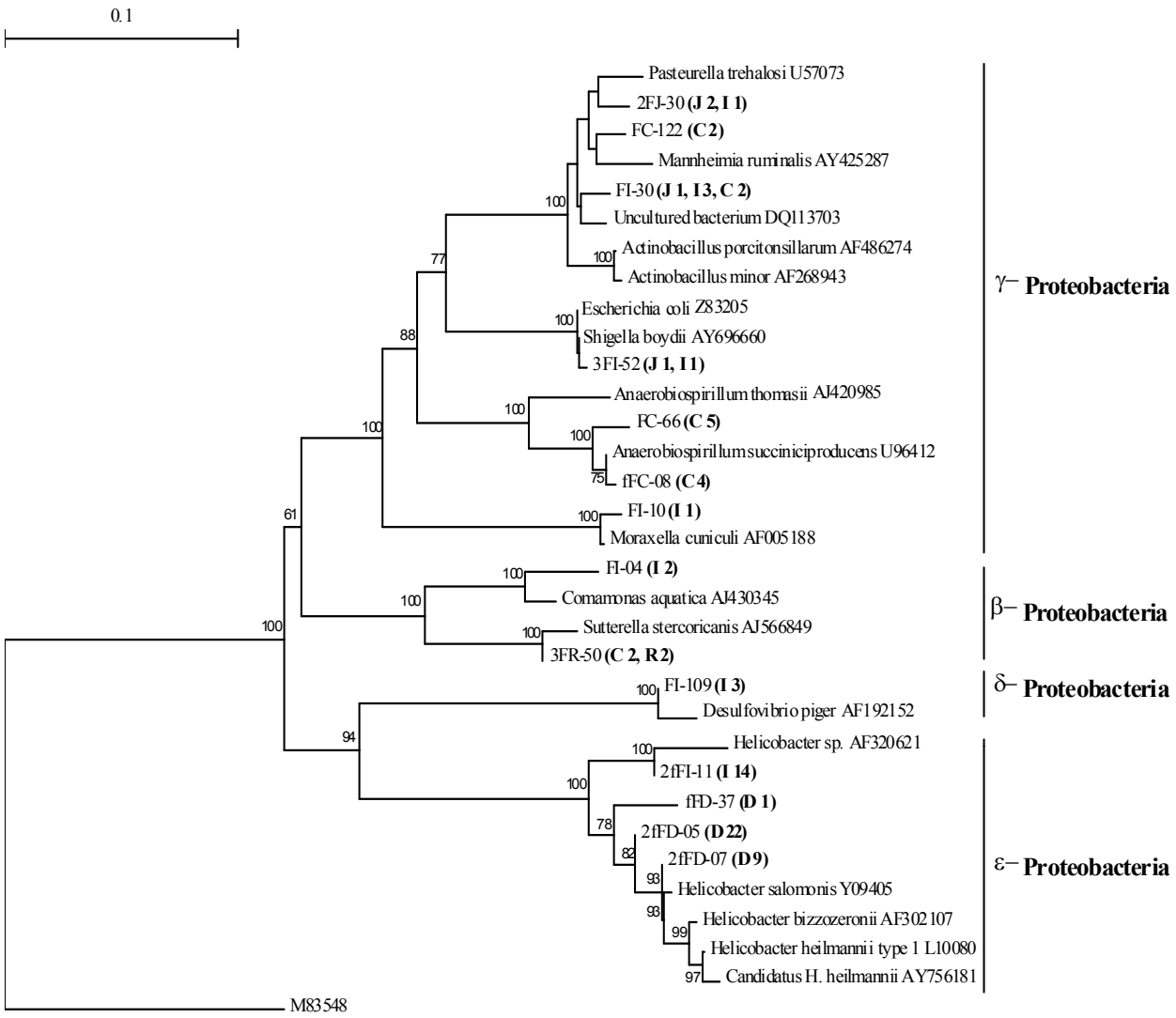

Figure 8. Phylogenetic tree showing the affiliation of OTUs isolated from the feline GI tract for *Proteobacteria*. The bar represents 10% sequence divergence. 16S rDNA sequences were aligned to their closest neighbor in the RDP database. The tree was inferred based on the neighbor-joining algorithm. *Aquifex pyrophilus* was used as an outgroup in order to infer the root of the tree. Bootstrap values shown at the branches are based on 100 replicates; values below 50% are not shown. For each OTU the numbers in parenthesis indicate the number of observed clones in the respective intestinal segments. (S=stomach, D=duodenum, J=jejunum, I=ileum, C=colon, R=rectum).

(32%, 19%, 5%, and 3%, respectively). Four bacterial classes were identified: β-Proteobacteria, δ-*Proteobacteria*, ε-*Proteobacteria,* and γ-*Proteobacteria.* The two predominant classes were γ-*Proteobacteria* and ε-*Proteobacteria*. Within the class γ-*Proteobacteria*, 6 OTUs were identified consisting of 13 clones from the colon, 5 from the ileum, 2 from the jejunum. The ε-*Proteobacteria* class was comprised of 4 OTUs representing 32 clones isolated from the duodenum and 14 clones from the ileum. OTUs from the duodenum were only classified in the ε-*Proteobacteria* class. Two OTUs were classified as β-*Proteobacteria*. Only one OTU from the ileum was classified as a δ-*Proteobacteria* with 99% similarity to *Desulfovibrio piger*.

Phylogenetic analysis of intestinal samples obtained from the SPF cat. The SPF cat was the only cat where samples could be obtained from all intestinal segments. Separate analysis was performed to compare the intestinal microflora between the healthy SPF cat and the other four healthy non-SPF cats. The bacterial classification of clones at the phylum level from the SPF cat and the other four healthy research cats are illustrated in Figure 9. The majority of clones from the SPF cat were classified within the *Firmicutes* phylum (98%). Further classification showed exclusively clones belonging to the class *Clostridiales*. Figure 10 shows the distribution of clones from the SPF cat affiliated with *Clostridium* cluster I. Sequences affiliated with this cluster were isolated from all compartments.

In contrast to the SPF cat, 67% of clones obtained from the four healthy research cats were classified in the phylum *Firmicutes*. Further classification showed clones in three bacterial classes, *Clostridiales, Bacilli*, and *Mollicutes*. Clones isolated from the four healthy research cats were predominantly affiliated with *Clostridium* clusters I and

XIVa. The distributions of clones affiliated with these clusters are illustrated in Figure 11. Sequences affiliated with *Clostridium* cluster I could be isolated from three compartments (i.e., jejunum, ileum, and colon). The number of clones affiliated with cluster I increased in complexity along the intestinal tract with the highest number of clones isolated from the colon. Sequences affiliated with *Clostridium* cluster XIVa were predominantly isolated from the colon.

Phylogenetic analysis of intestinal samples obtained from 4 healthy non-SPF cats. For this analysis, the SPF cat was excluded. As mentioned previously, not all segments could be sampled from all four healthy non-SPF cats. Therefore, a separate analysis was performed describing only the intestinal segments in which samples were obtained from at least 2 individual cats (i.e., jejunum, ileum, and colon). Fourteen different bacterial orders were identified in these three intestinal segments. The proportions of the predominant bacterial orders within the selected compartments are shown in Figure 12. The majority of clones were classified within the order *Clostridiales*. The second most predominant order was *Lactobacillales* in the jejunum and *Bacteroidales* in the ileum and colon. Clones classified within the order *Bacteroidales* were predominantly isolated from the distal intestine (i.e., ileum and colon) with only one clone isolated from the proximal portion of the intestine (i.e., jejunum).

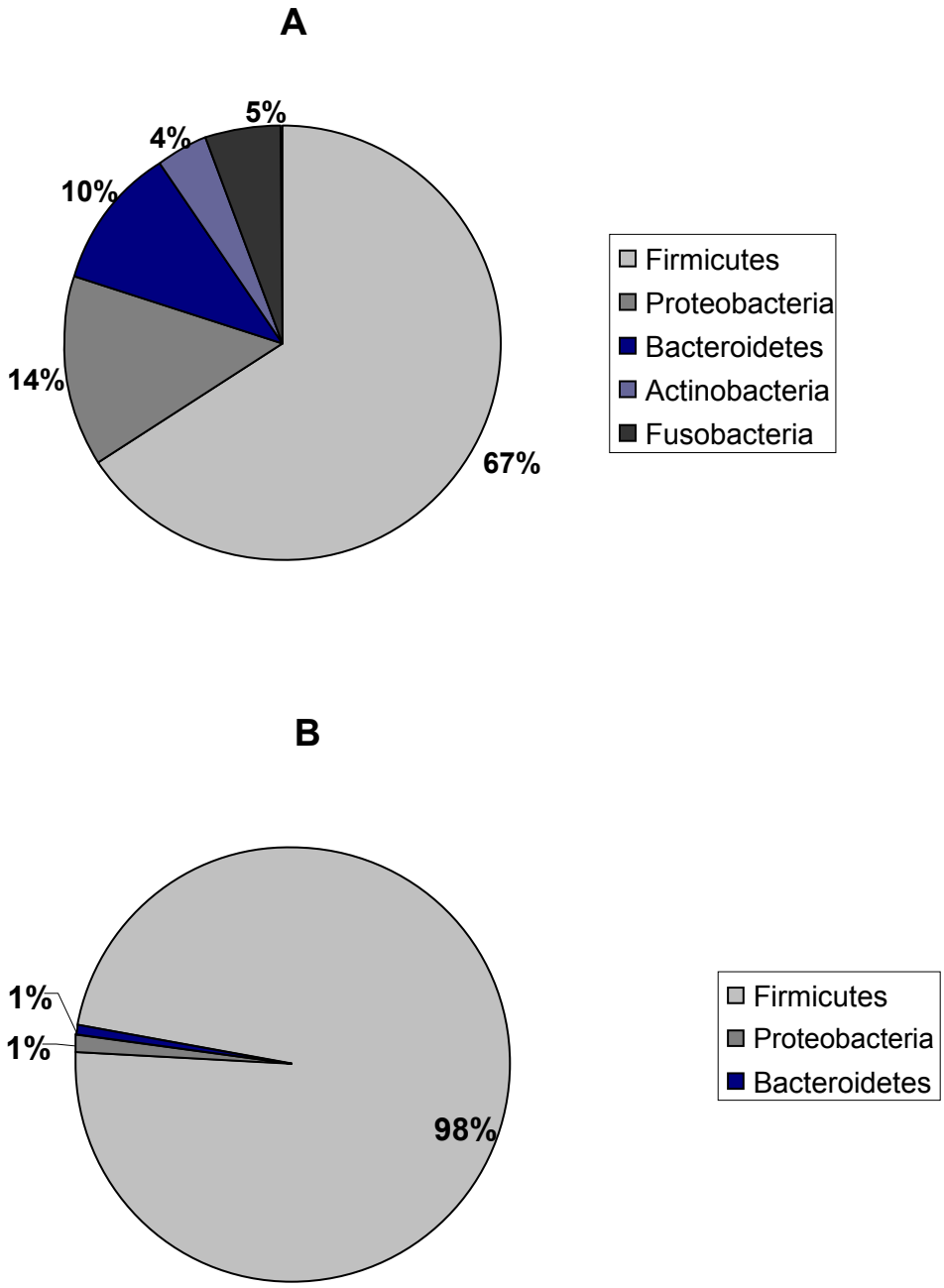

Figure 9. Classification of 16S rDNA sequences belonging to the four healthy non-SPF cats (A) and the healthy SPF cat (B).

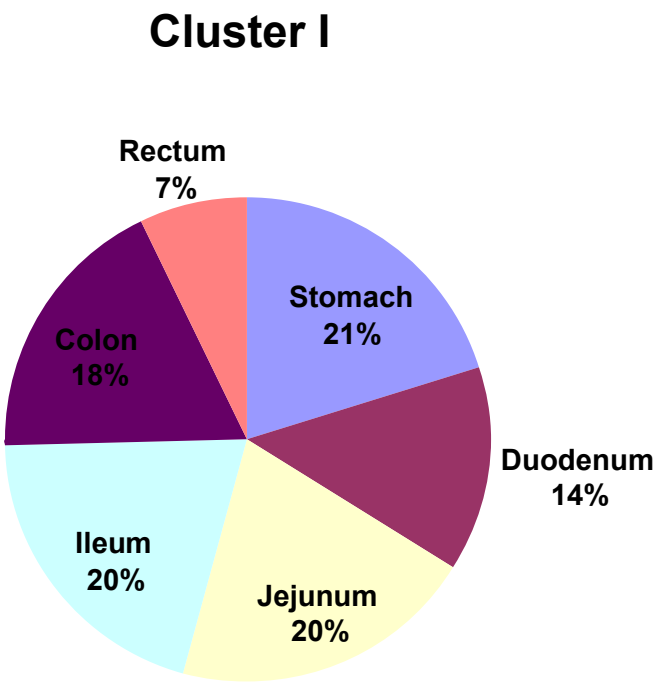

Figure 10. Proportions of 16S rDNA sequences from each compartment of the SPF cat affiliated with *Clostridium* cluster I.

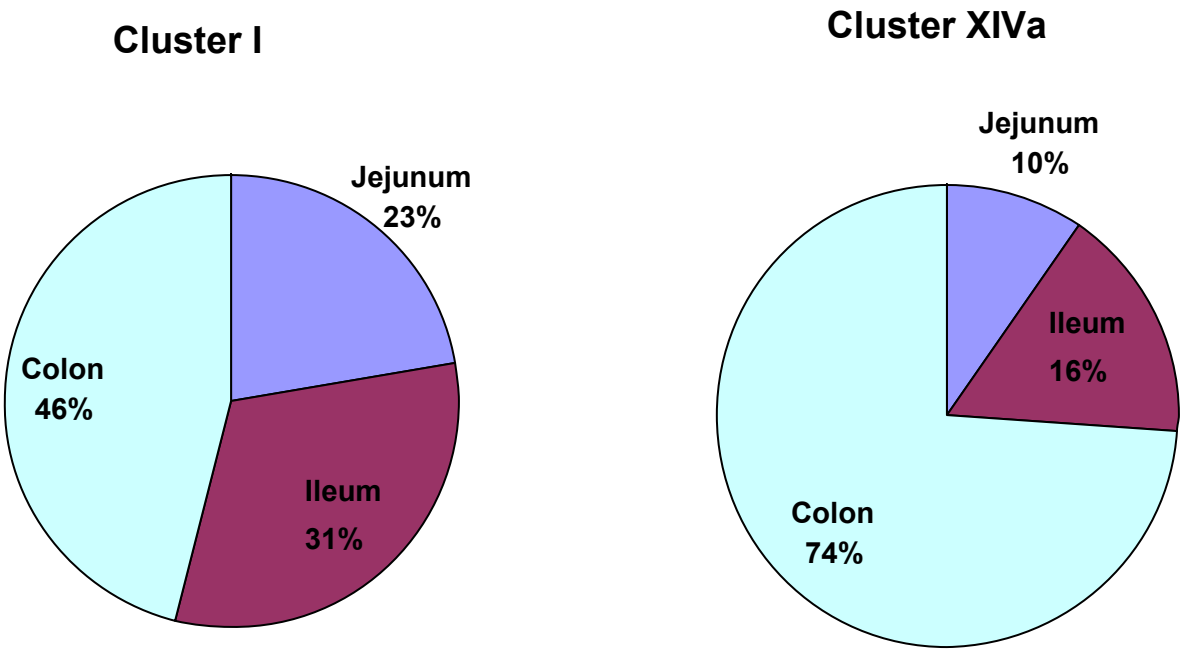

Figure 11. Distribution of the two predominant *Clostridium* clusters I and XIVa from the four healthy non-SPF cats.

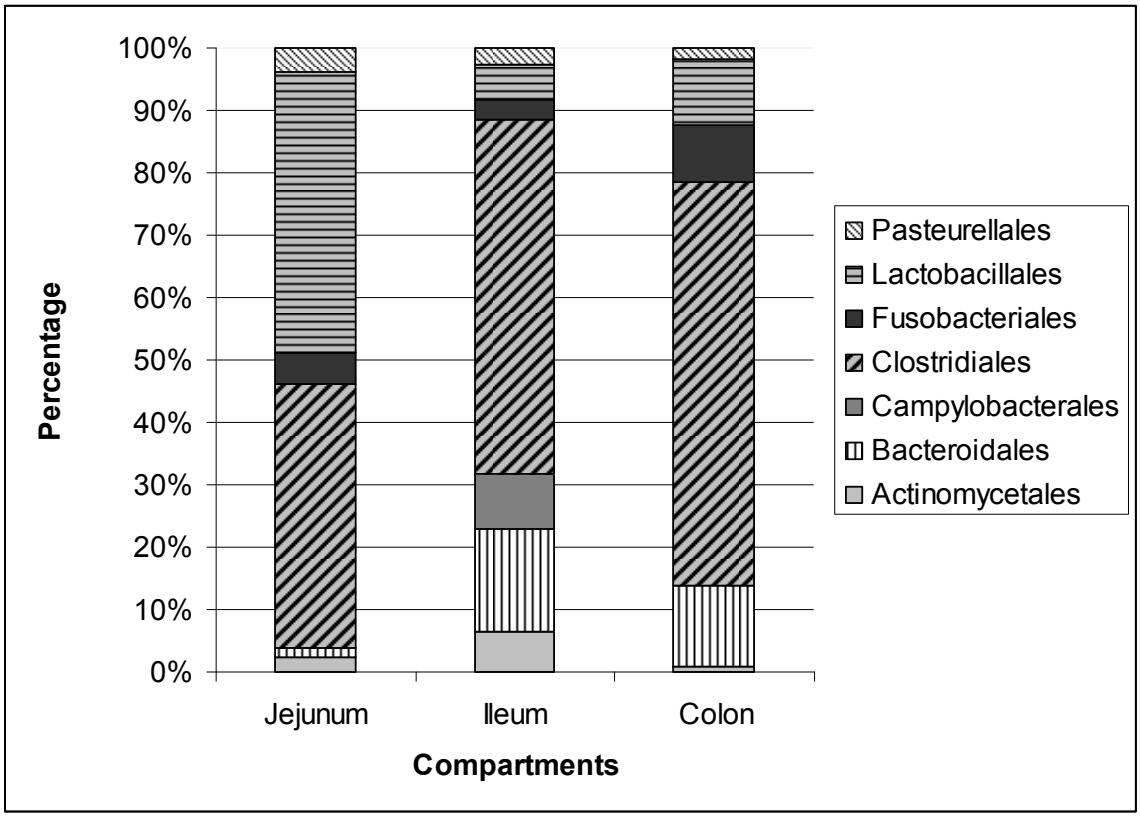

Figure 12. Percentages of 16S rDNA clones belonging to the major phylogenetic lineages in the compartments in which samples were obtained from at least 2 individual cats.

DISCUSSION

The intestinal bacterial microflora of the feline gastrointestinal tract was characterized using comparative 16S rDNA analysis. Our findings are consistent with previous studies, which reported that the feline intestinal microflora harbors predominantly facultative and obligate anaerobic bacterial species (Osbaldiston & Stowe, 1971; Johnston *et al.*, 1993; Papasouliotis *et al.*, 1998; Johnston *et al.*, 2001). The current study revealed the presence of five different bacterial phyla: *Firmicutes*, *Proteobacteria, Bacteroidetes*, *Fusobacteria*, and *Actinobacteria*.

Firmicutes were the most abundant phylum in the feline intestinal tract (825 clones) and the most diverse (67 OTUs). This finding is also consistent with previous studies that used microbiological culture techniques to analyze the bacterial content in the proximal portion of the small intestine in healthy cats (i.e., duodenum, jejunum). Two studies reported *Clostridia* spp. to be the most common bacterial group identified in duodenal aspirates, occurring in over 90% of cats (Papasouliotis *et al.*, 1998; Johnston *et al.*, 2001). The predominant bacterial groups in the jejunum were *Enterococcus* spp., *Streptococcus* spp., and *Lactobacillus* spp., which all belong to the phylum *Firmicutes* (Osbaldiston & Stowe, 1971). Additionally, our findings are consistent with previous studies that analyzed the feline bacterial microflora of colonic and fecal samples using traditional culture techniques. The most common bacterial groups isolated in those studies were *Enterococcus* spp. and *Lactobacillus* spp. (Osbaldiston & Stowe, 1971; Itoh *et al.*, 1984).

Within the *Firmicutes* phylum, *Clostridiales* was the most abundant bacterial class (754 clones) representing 56 OTUs. These OTUs were affiliated with 6 different

Clostridium clusters of which clostridial clusters I and XIVa were the predominant ones. Clones affiliated with *Clostridium* cluster I were identified in all intestinal segments. However, there was a gradual increase in the number of clones along the intestinal tract, with the highest number of clones identified in the colon. This finding differs from studies in humans that reported that very few clones obtained from the colon were affiliated with *Clostridium* cluster I (Hold *et al*., 2002; Wang *et al.*, 2003; Delgado *et al.*, 2006). *Clostridium* cluster XIVa was the most diverse cluster (20 OTUs) in the feline intestinal tract with clones isolated predominantly in the distal intestine (i.e., ileum, colon, and rectum). Interestingly, no clones affiliated with this cluster could be identified in the stomach (SPF cat) or duodenum (one healthy non-SPF cat and one SPF cat). Similar to our results, the majority of isolated clones from the colon of humans and horses were also affiliated with *Clostridium* cluster XIVa (Daly *et al.*, 2001; Wang *et al.*, 2003). Clones affiliated with *Clostridium* cluster IV could only be obtained from the colon and were not isolated in any other segment of the feline intestinal tract. Similar to the results of our study, sequences affiliated with *Clostridium* cluster IV are also abundant in the colon of humans and horses (Daly *et al.*, 2001; Hold *et al*., 2002; Wang *et al.*, 2003; Delgado *et al.*, 2006).

Another major constituent of the *Firmicutes* were clones belonging to the order *Lactobacillales*. These clones were predominantly isolated from the jejunum and colon. This finding is in close agreement with a previous study that reported *Lactobacillus* spp. to be a predominant bacterial group in the jejunum and colon of cats when analyzed by culture-based methods (Osbaldiston & Stowe, 1971).

Clones belonging to the anaerobic orders *Bacteroidales* and *Fusobacteriales* were isolated mainly from the ileum and colon and were only sporadically found in the jejunum and not at all in the duodenum. *Bacteroides* spp. have been reported to be a common bacterial group in the feline large intestine (Osbaldiston & Stowe, 1971; Itoh *et al.*, 1984). However, using bacterial culture methods, *Bacteroidetes* and *Fusobacteria* were routinely isolated from the feline duodenum. One study reported *Bacteroides* spp. and *Fusobacteria* spp. as the most commonly observed anaerobic bacterial species in duodenal juice aspirates from healthy cats (Johnston *et al.*, 1993), and two other studies isolated these bacterial groups in approximately 40% of evaluated cats (Papasouliotis *et al.*, 1998; Johnston *et al.*, 2001). In our study, duodenal samples were analyzed only from two cats, and this may explain why these bacterial groups were not represented in our clone libraries.

Clones classified in the phylum *Proteobacteria* were more commonly isolated in the small intestine compared to the large intestine, and 32% of all clones obtained from the duodenum were classified as ε- *Proteobacteria.* In previous studies, *Escherichia* spp. were isolated from the colon of all six cats evaluated (Osbaldiston & Stowe, 1971). In contrast, only one clone classified as an *Escherichia coli*-like organism was isolated from the ileum in our study.

Clones classified in the phylum *Actinobacteria* were predominantly isolated from the ileum and colon. No *Bifidobacterium* spp. were isolated from the feline intestinal tract. In the past, studies have reported *Bifidobacterium* spp. to be a predominant bacterial group in feline feces (Itoh *et al.*, 1984; Inness *et al.*, 2007). One study used culture-based techniques and reported that the majority of feline fecal samples collected harbored

Bifidobacterium spp. and 44 different *Bifidobacterium* strains were isolated (Itoh *et al.*, 1984). Using fluorescent in situ hybridization (FISH), another study identified *Bifidobacterium* spp. in 91.7% of fecal samples of healthy cats (Inness *et al.*, 2007). It is difficult to explain these differences between these studies. One potential explanation is a bias by the methods employed. For example, it is possible that the PCR primers and amplification protocols used here may be less efficient for the detection of *Bifidobacterium* spp. as *Bifidobacterium* sequences that are uncommonly detected in 16S rDNA libraries (Wilson & Blitchington, 1996; Greetham *et al.*, 2002; Hold *et al*., 2002; Wang *et al.*, 2003; Suchodolski, 2005). Therefore, a comparative 16S rDNA approach as used in this study may underestimate the presence of *Bifidobacterium* spp. In contrast, traditional bacterial culture techniques as used in other studies may have led to an overestimation of the diversity of *Bifidobacterium* spp. 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 organisms were isolated (Greetham *et al.*, 2002).

In this study we analyzed samples obtained from the stomach, duodenum, jejunum, ileum, colon, and rectum of a specific pathogen free (SPF) cat. Characterization of the bacterial microflora in these samples revealed differences when compared to the intestinal microflora of the other four healthy cats (all non-SPF cats). Clones obtained from the SPF cat were predominantly classified within the *Firmicutes* phylum (98%) and this proportion was markedly higher compared to the other cats (67%). Further classification showed that clones obtained from the SPF cat only belonged to the class *Clostridiales*. In contrast, clones belonging to the classes *Clostridiales*, *Bacilli*, and

Mollicutes were identified in the healthy non-SPF cats. Additionally, 93% of clones obtained from the SPF cat were affiliated with *Clostridium* cluster I, and all clones obtained from the stomach and duodenum were affiliated with this cluster. In contrast, only 18% of clones obtained from the four healthy non-SPF cats were affiliated with *Clostridium* cluster I. In addition, 12% of clones obtained from the healthy non-SPF cats were affiliated with *Clostridium* cluster XIVa, compared with 3% of clones obtained from the SPF cat. Unfortunately, only one SPF cat was analyzed in this study, which makes it difficult to conclude if the intestinal microflora of SPF cats is generally different from conventionally raised cats. However, this finding warrants further studies to evaluate differences between SPF and non-SPF cats.

A total of 109 individual sequences were identified in the present study from the intestine of all healthy cats. Of these, 21 OTUs showed less than 98% similarity to available 16S rDNA sequences in the GenBank database, suggesting that these sequences represent previously uncharacterized bacteria. However, due to some limitations of bacterial identification based on comparative 16S rDNA analysis, it is likely that these

findings still underestimate the total diversity of the feline intestinal tract. The average coverage of the clone library was 89.8%, indicating that the probability that the next selected clone was a single non-redundant clone was 10.2%. Therefore, further sampling of clones might have revealed additional non-redundant species, but this was not carried out due to cost. Also, bacteria with low abundance might have escaped identification because a molecular approach using universal primers targets predominant bacterial groups in the intestine. The use of reduced PCR cycle numbers and pooling of PCR products of several PCR reactions minimizes the bias of clone libraries; however, less abundant sequences might have escaped detection. Despite the limitations to molecular methods, this study represents the first report of a comprehensive characterization of the bacterial microflora of the entire feline intestinal tract.

CHAPTER III

COMPARISON OF THE FECAL MICROFLORA BETWEEN HEALTHY CATS AND CATS WITH INFLAMMATORY BOWEL DISEASE USING MOLECULAR METHODS

OVERVIEW

The aim of this study was to compare the fecal microflora of healthy cats and cats with gastrointestinal disease based on direct sequence analysis of the 16S ribosomal RNA gene (16S rDNA). Fecal samples were collected from 6 healthy cats, 6 cats with inflammatory bowel disease (IBD), and 3 cats with intestinal neoplasia. Bacterial 16S rDNA was amplified using universal bacterial primers. For identification of bacterial 16S rDNA sequences, a clone library was constructed. A total of 977 clones were analyzed and 133 non-redundant bacterial 16S rDNA sequences were identified. A predominant anaerobic microflora was observed and the majority of sequences from all groups were classified in the phylum *Firmicutes*. Compared to the IBD group, cats in the control group had a significantly higher number of clones belonging to *Firmicutes, Bacteroidetes,* and *Actinobacteria* ($p<0.0001$). The control group also had a significantly higher proportion of clones affiliated with *Clostridium* cluster XI, and a significantly lower proportion affiliated with cluster I (both $p<0.0001$). For the neoplasia group, the majority of clones were also classified as *Firmicutes* (97.9%) and clones were predominately affiliated with *Clostridium* clusters I and XI.

INTRODUCTION

The residential intestinal microflora is known to influence the health of the host by affecting several biochemical, physiological, and immunological parameters. These effects include the supply of nutrients to host tissues, gastrointestinal motility, the development of the gastrointestinal epithelium, and providing a natural defense against invading pathogens (Falk *et al.*, 1998; Hooper *et al.*, 2001; Macfarlane & Macfarlane, 2003). Also, alterations in the commensal intestinal microflora are believed to play a role in the development of intestinal disease (Johnston *et al.*, 2001; Linskens *et al.*, 2001; Janeczko *et al.*, 2007).

Several studies have been performed in humans and animals characterizing the intestinal microflora, but little is known about the gastrointestinal microflora in cats (Amann *et al.*, 1995; Langendijk *et al.,* 1995; Daly *et al.*, 2001; Greetham *et al.*, 2002; Hold *et al*., 2002; Wang *et al.*, 2003). Past studies have reported that the feline intestine harbors predominantly facultative and obligate anaerobic bacterial species (Johnston *et al.*, 1993; Papasouliotis *et al.*, 1998; Johnston *et al.*, 2001). However, these studies utilized traditional microbiological culture techniques to describe the feline intestinal microflora and recent investigations would suggest that these methods do not reflect the true microbial diversity of intestinal samples (Itoh *et al.*, 1984; Amann *et al.*, 1995; Langendijk *et al.,* 1995; Greetham *et al.*, 2002). To overcome the limitations of traditional culture techniques, recent studies have used molecular-based methods based on the amplification of the 16S ribosomal RNA gene (16S rDNA) to characterize the intestinal microflora in many mammalian species (Greetham *et al.*, 2002; Hold *et al.*, 2002; Wang *et al.*, 2003; Delgado *et al.*, 2006). These methods have enhanced our

understanding of complex microbial communities. Also, many authors using these new techniques have observed an increased bacterial diversity in the intestine when compared to results obtained by strictly culture-based methods (Langendijk *et al.*, 1995; Wilson, 1996; Delgado *et al.*, 2006; Inness *et al.*, 2006).

Inflammatory bowel disease (IBD) is a common chronic intestinal disease that affects humans as well as animals. In cats, IBD is associated with inflammation of the intestine and results in clinical signs such as vomiting, diarrhea, and weight loss (Willard, 1999). The etiology of IBD in cats is not well understood, but is likely due, in part to several underlying mechanisms, such as dietary factors, parasites, genetic susceptibility, stress, reduced immune tolerance, and changes in the intestinal microflora (Simpson, 1998). Studies in humans and animal models have implicated the intestinal microflora as an important factor in the development of IBD. These mechanisms include a loss of tolerance to the residential intestinal microflora, an immune response to extra-cellular bacterial substances, or an ineffective clearance of enteric pathogens (Sartor, 1997; Linskens *et al.*, 2001). In human studies there is evidence that enhanced mucosal permeability, as a result of genetic predisposition or direct contact with bacteria, leads to an increased absorption of bacteria or bacterial products, which in turn can act as a possible cause of IBD (Linskens *et al.*, 2001). Additionally, it has been shown in human IBD patients that inflammation of the intestine is present in areas of high bacterial counts (Linskens *et al.*, 2001). Also, it has been reported that IBD does not occur in mice housed under germ-free conditions, further suggesting that the intestinal microflora may play an integral role in the pathogenesis of IBD (Sadlack *et al.*, 1993).

Only few studies have investigated the role of the bacterial microflora in feline IBD. Two recent studies used fluorescence *in situ* hybridization (FISH) to compare the bacterial flora of feline duodenal biopsies and fecal samples from healthy cats and cats with IBD (Inness *et al.*, 2006; Janeczko *et al.*, 2007). Both studies concluded that there were significant differences in the composition of the microflora of duodenal biopsies and fecal samples between healthy cats and cats with IBD. However, phylogenetic information was not provided in either study. Therefore, in the present study we aim to characterize the microflora of fecal samples from healthy cats and cats with IBD using a comparative 16S rDNA molecular approach.

MATERIALS AND METHODS

Sample collection. One gram of feces was collected from 6 healthy cats, 6 cats with histopathologically confirmed mild to severe inflammatory bowel disease (IBD; 3 with mild to moderate IBD, and 3 with severe IBD), and 3 cats with intestinal neoplasia (2 cats with intestinal lymphosarcoma and 1 cat with intestinal adenocarcinoma). Samples were transferred to sterile cryotubes and stored at -80ºC until further analysis.

The 6 healthy cats ranged in age from 1 to 9 years. The age range of cats in the IBD group was 4-15 years. All 3 cats in the intestinal neoplasia group were approximately 3 years old.

Extraction of DNA. DNA extraction was carried out as described by Suchodolski *et al.* (2004) using a bead beating method followed by phenol:chloroform:iso-amylalcohol extraction. Each sample was extracted independently in a separate sterile

cryotube. Briefly, 500 µl of cell lysis solution (Puregene cell lysis solution, Gentra Systems, Minneapolis, MN), 200 µl of buffer-saturated phenol-chloroform-isoamyl alcohol (25:24:1, pH 7.2), and 300 µl of 0.1-mm zirconia beads (BioSpec Products Inc., Bartlesville, OK) were added to each sample. The tubes were positioned horizontally on a vortex adapter mounted on a standard vortexer, and the mixture was vortexed for 5 min at maximum speed. The tubes were centrifuged for 7 min at 12,000 x *g* and 4°C, and the supernatant was transferred to a new sterile cryotube. Then 700 µl of phenol-chloroform-isoamyl alcohol was added, and the tube was vortexed for 30 s and centrifuged for 20 min at 12,000 x *g* and 4°C. 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 were combined. To remove RNA, 5.2 U of RNase (Puregene RNase, Gentra Systems) were added to the solution and incubated at 37°C for 30 min. The RNase was removed by phenol-chloroform-isoamyl alcohol extraction as described above. The aqueous phase containing the DNA was mixed with 0.5 volume 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 then stored at -20°C until further use. A negative control containing H_2O instead of sample was purified in parallel to each extraction batch to screen for contamination of extraction reagents.

16S rDNA amplification by PCR. Extracted DNA was used as a template for PCR amplification of approximately 450 bp of 16S rDNA with universal bacterial

primers F341 (5'-CCTACGGGAGGCAGCAG-3') and 786R (5'-GACTACCAGGGTATCTAATC-3'). Each reaction mixture (25 µl) consisted of reaction buffer (GeneAmp 10x PCR Gold buffer, Applied Biosystems, Foster City, CA; final concentrations: 15 mM Tris-HCl, 50 mM KCl, 3 mM $MgCl_2$ [pH 8.0]), 1.25 U of *Taq* DNA polymerase (Amplitaq Gold LD, Applied Biosystems, Foster City, CA.), 250 µM each of the deoxynucleoside triphosphates, 0.24 µM each primer, and 100 ng of DNA template. To screen for potential contamination of PCR reagents, a negative PCR control using H_2O instead of DNA template was used. The samples were amplified in a thermocycler (Mastercycler Gradient, Eppendorf AG, Hamburg, Germany) using the following PCR protocol: initial denaturation at 95ºC for 3 min; 30 cycles of denaturation at 95ºC for 30 s, annealing at 54ºC for 30 s, extension at 72ºC for 1 min; final extension at 72ºC for 10 min. The purity of the PCR amplicons was assessed on 1% agarose electrophoresis gels stained with Gel Red™ (Biotium Inc., Hayward, CA).

Cloning of bacterial 16S rDNA amplicons. Amplified PCR products were ligated into pCR®4-TOPO® linearized cloning vectors (TOPO TA, Invitrogen, Carlsbad, CA.) as specified by the manufacturer. After ligation, the products were transformed into chemically competent DH5α™-T1^R *E. coli* by heat shock following manufacturer's instructions. Transformed products were grown overnight on Luria-Bertani (LB) medium with ampicillin (50 µg ml^{-1}) at 37ºC. The pCR®4-TOPO® vector allows direct selection of recombinant cells via disruption of the lethal *E. coli* gene *ccd*B. Up to 96 colonies for each sample were randomly selected and clones were grown for 24 hours in 1.4 ml LB broth treated with ampicillin (50 µg ml^{-1}) in 96-well blocks (Perfectprep® BAC 96,

Eppendorf, North America, Inc., Westbury, NY) sealed with Gas Permeable Adhesive Seals (ABgene, Surrey, UK.).

Plasmid extraction and sequencing of 16S rDNA. Plasmid DNA was purified in a 96- well format using the Perfectprep® BAC 96 plasmid purification kit (Eppendorf) and a single vacuum manifold (Eppendorf). Plasmid DNA was then eluted using 50 µl of DNA grade water and the products were stored at -30ºC until further use. The amplified 16S rDNA inserts were sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and products were analyzed with an automated sequence analyzer (ABI 3100 Capillary Sequencer, Applied Biosystems).

Sequence analysis. Each sequence was edited to exclude the PCR primer binding sites, and then tested for possible chimeric structures using the Check_Chimera and Pintail software available online through the Ribosomal Database Project (RDP). Identified chimeras were excluded from further analysis.

Cloned sequences were compared with existing 16S rDNA sequences using GenBank and RDP (release 9.59, approximately 489,840 16S rRNA sequences). Sequences were aligned using the multiple sequence alignment program CLUSTAL_W included in the BioEdit software package. Sequence distances were calculated using the DNAdist program and the Jukes-Cantor model for inferring evolutionary distances (Van de & Wachter, 1993). Groups of sequences with less than 2% sequence divergence (98% similarity) to each other were defined as an Operational Taxonomic Unit (OTU). The RDP Library Compare tool, based on a naïve Bayesian classifier, was used to classify the 16S rDNA sequences into the new higher-order taxonomy proposed in Bergey's Taxonomic Outline of the Prokaryotes.

Statistical analysis. The coverage of the individual clone libraries (i.e., the probability that any additional analyzed clone is different from any previously obtained single clone) was calculated according to Good 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 (Good, 1953). Bacterial diversity was calculated using the Shannon-Weaver index (Hs) and was defined as $-\sum p_i \ln(p_i)$, where p_i is the proportion of individual bacteria found in a certain species (Atlas et al., 1998).

Data were tested for normal distribution using the Kolmogorov-Smirnov test (Prism5, GraphPad Software Inc, San Diego, CA). A Kruskal-Wallis test was used to compare the coverage, species richness (i.e., the number of species identified in each group), and the bacterial diversity between all three groups. Due to the small sample size of the intestinal neoplasia group (n=3), the subsequent statistical analyses were performed only between the healthy and the IBD group. A Mann-Whitney test was used to compare number of clones classified in different phylogenetic taxa between groups. Fisher's exact tests (including odds ratios and 95% confidence intervals (CI)) were used to compare proportions of cats between groups.

RESULTS

A total of 1,327 clones were randomly selected. Of these, a total of 1,028 clones contained a sequence of adequate quality. Fifty-one of these clones were identified as possible chimeras and were excluded from further analysis. A total of 133 non-redundant operative taxonomical units (OTUs), representing a total of 977 clones, were used for subsequent phylogenetic analysis. Table 4 shows the number of cats analyzed, clones

analyzed, and OTUs identified as well as coverage and bacterial diversity index for each group of cats.

The coverage, species richness (i.e., the number of species identified in each group), and Shannon-Weaver diversity index were compared between all three groups using the Kruskal-Wallis test. There was no significant difference in the diversity index or species richness between the three groups ($p= 0.1337$ and $p=0.1456$, respectively). Also, there was no significant difference in the coverage of the clone libraries between all three groups ($p= 0.5247$), permitting meaningful comparison between the groups.

Overall, 9 OTUs showed less than 98% similarity to existing 16S rDNA sequences in the NCBI database, and may represent as of yet uncharacterized bacterial phylotypes. A total of five bacterial phyla were identified across all three groups. Figure 13 shows the percentage of 16S rDNA clones belonging to the predominant bacterial orders from each group of cats. A complete phylogenetic analysis of all groups is shown in Tables 5, 6, and 7.

Phylogenetic analysis of fecal samples obtained from the control group. A total of 447 clones representing 69 OTUs were identified in the six healthy cats. The majority of OTUs were classified in the phylum *Firmicutes* followed by *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, and *Fusobacteria*, respectively (Tables 2-4). OTUs within the *Firmicutes* phylum were further classified in two bacterial classes, *Bacilli* and *Clostridia*. OTUs classified within the *Clostridia* class were affiliated with six *Clostridium* clusters: I, III, IV, XI, XIVa, and XIVb. Figure 14 shows the percentages of clones affiliated with the different *Clostridium* clusters for all three groups. The majority of clones obtained from the control group were affiliated with *Clostridium* cluster XI

followed by XIVa, I, and IV respectively. Less than 2% of all clones isolated from the control group were affiliated with clusters III and XIVb.

Phylogenetic analysis of fecal samples obtained from the IBD group. For the six cats diagnosed with mild to severe IBD, a total of 340 clones representing 38 non-redundant OTUs were used for subsequent phylogenetic analysis. The majority of OTUs were classified within the *Firmicutes* phylum, followed by *Proteobacteria* and *Actinobacteria*, respectively (outlined in tables 5, 6, and 7). OTUs within the *Firmicutes* phylum were further classified within two bacterial classes, *Bacilli* and *Clostridia*. OTUs classified within the *Clostridia* class were affiliated with seven *Clostridium* clusters: I, III, IV, XI, XIVa, XIVb, and XVIII. The majority of clones were affiliated with *Clostridium* cluster XIVa followed by I, XI, and IV respectively (Fig. 14).

Phylogenetic analysis of fecal samples obtained from cats with intestinal neoplasia. For the 3 cats with intestinal neoplasia a total of 490 clones representing 26 non-redundant OTUs were used for subsequent phylogenetic analysis. The majority of sequences from this group were classified in the *Firmicutes* phylum (97.9%). The

remaining sequences were classified in the *Actinobacteria* and *Proteobacteria* phylums (both 1.1%; Tables 5, 6, and 7). Within the phylum *Firmicutes*, the majority of sequences were classified within the class *Clostridia* (88.9%). OTUs in this class were affiliated with 6 *Clostridium* clusters: I, III, IV, XI, XIVa, and XIVb. Clones were predominately affiliated with *Clostridium* clusters I and XI (Fig. 14).

Statistical analysis. Statistical comparisons, based on the phylogenetic analysis of sequences from the control group and IBD group, were performed to asses any differences between the fecal microflora in health and disease. The neoplasia group was not included in this analysis due to its low sample size. Using Fisher's exact tests, no bacterial groups were significantly associated with health or disease status based on the

Table 4. Number of analyzed cats, analyzed clones, and identified OTUs and coverage and bacterial diversity index for the 16S rDNA clone libraries constructed from the three groups.

	No. of cats	No. of clones	No. of OTUs[a]	Coverage[b]	H[c]
Control group	6	447	69	93.1	3.2
IBD group	6	340	38	97.1	2.8
Neoplasia group	3	490	26	93.2	2.3

[a]OTU=operative taxonomical unit
[b]According to Good (Good, 1953)
[c]Shannon-Weaver diversity index

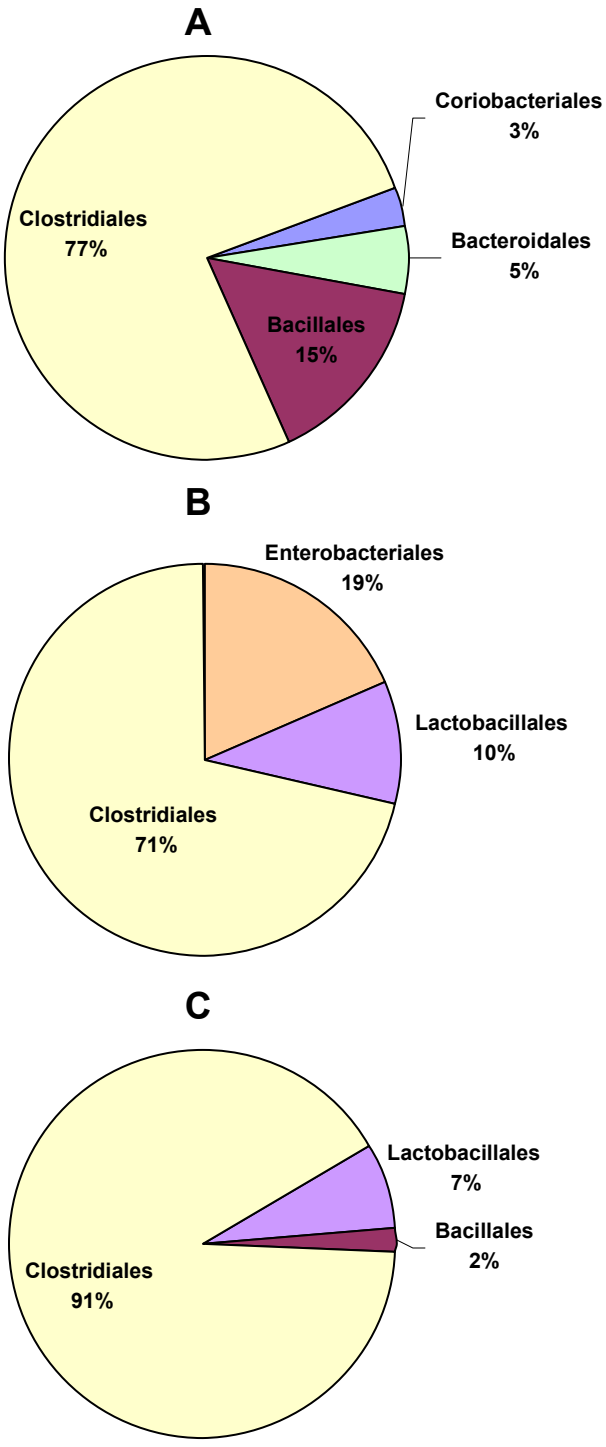

Figure 13. Percentage of 16S rDNA clones belonging to the major bacterial orders in control cats (A), cats with IBD (B), and cats with intestinal neoplasia (C).

Table 5. Overall phylogenetic distribution of the sequences classified in the phylums *Actinobacteria*, *Bacteroidetes*, and *Fusobacteria* for all three groups of cats. For each group, the number of clones classified for each taxa, the number of cats with clones classified in each taxa, and proportion of clones classified in each taxa are shown. Statistical analysis was performed between the control group and the IBD group.

	Number of clones			Number of cats			Proportions[a]			Statistical analysis[b]		
	Control	IBD	Intestinal Neoplasia	Control	IBD	Intestinal Neoplasia	Healthy cats (n=447)	IBD Cats (n=340)	Neoplasia Cats (n=190)	RDP library compare p-value [c]	Fisher's exact test p-value[d]	Mann-Whitney p-value[e]
Phylum Actinobacteria	18	2	2	2	2	2	4.0	0.6	1.1	0.0020	NS	NS
class Actinobacteria	18	2	2	2	2	2	4.0	0.6	1.1	0.0020	NS	NS
order Coriobacteriales	13	2	1	2	2	1	2.9	0.6	0.5	0.0198	NS	NS
family Coriobacteriaceae	13	2	1	2	2	1	2.9	0.6	0.5	0.0198	NS	NS
genus Slackia	1	0	0	1	0	0	0.2	0.0	0.5	NS	NS	NS
genus Collinsella	9	2	1	2	1	1	2.0	0.6	0.0	NS	NS	NS
genus Olsenella	3	0	0	1	0	0	0.7	0.0	0.0	NS	NS	NS
order Bifidobacteriales	5	0	1	1	0	1	1.1	0.0	0.5	NS	NS	NS
family Bifidobacteriaceae	5	0	1	1	0	1	1.1	0.0	0.5	NS	NS	NS
genus Bifidobacterium	5	0	1	1	0	1	1.1	0.0	0.5	NS	NS	NS
Phylum Bacteroidetes	23	0	0	3	0	0	5.1	0.0	0.0	<0.0001	NS	NS
class Bacteroidetes	23	0	0	3	0	0	5.1	0.0	0.0	<0.0001	NS	NS
order Bacteroidales	23	0	0	3	0	0	5.1	0.0	0.0	<0.0001	NS	NS
family Porphyromonadaceae	5	0	0	1	0	0	1.1	0.0	0.0	NS	NS	NS
genus Parabacteroides	5	0	0	1	0	0	1.1	0.0	0.0	NS	NS	NS
family Bacteroidaceae	4	0	0	2	0	0	0.9	0.0	0.0	NS	NS	NS
genus Bacteroides	4	0	0	2	0	0	0.9	0.0	0.0	NS	NS	NS
family Prevotellaceae	14	0	0	3	0	0	3.1	0.0	0.0	0.0004	NS	NS
genus Prevotella	10	0	0	3	0	0	2.2	0.0	0.0	0.0041	NS	NS
unclassified Prevotellaceae	4	0	0	1	0	0	0.9	0.0	0.0	NS	NS	NS
Phylum Fusobacteria	2	0	0	1	0	0	0.4	0.0	0.0	NS	NS	NS
class Fusobacteria	2	0	0	1	0	0	0.4	0.0	0.0	NS	NS	NS
order Fusobacteriales	2	0	0	1	0	0	0.4	0.0	0.0	NS	NS	NS
unclassified Fusobacteriales	2	0	0	1	0	0	0.4	0.0	0.0	NS	NS	NS

[a] The proportion of clones in each category (i.e., Phylum, Class, Order, Family, Genus) in relation to the total number of clones selected

[b] Statistical comparisons between the control group and the IBD group (NS = not significant)

[c] p-value for the relative proportion of clones

[d] p-value based on the number of cats

[e] p-value based on the relative number of clones per cat

Table 6. Overall phylogenetic distribution of the sequences classified in the phylum *Proteobacteria*. For each group, the number of clones classified for each taxa, the number of cats with clones classified in each taxa, and proportion of clones classified in each taxa are shown. Statistical analysis was performed between the control group and the IBD group.

	Number of clones			Number of cats			Proportions[a]			Statistical analysis[b]		
	Control	IBD	Intestinal Neoplasia	Control	IBD	Intestinal Neoplasia	Healthy cats (n=447)	IBD Cats (n=340)	Neoplasia Cats (n=190)	RDP library compare p-value [c]	Fisher's exact test p-value[d]	Mann-Whitney p-value[e]
Phylum Proteobacteria	13	64	2	4	2	1	2.9	18.8	1.1	<0.0001	NS	NS
class Alphaproteobacteria	2	0	0	1	0	0	0.4	0.0	0.0	NS	NS	NS
order Sphingomonadales	2	0	0	1	0	0	0.4	0.0	0.0	NS	NS	NS
family Sphingomonadaceae	2	0	0	1	0	0	0.4	0.0	0.0	NS	NS	NS
genus Sphingomonas	2	0	0	1	0	0	0.4	0.0	0.0	NS	NS	NS
class Betaproteobacteria	1	1	0	1	1	0	0.2	0.3	0.0	NS	NS	NS
order Burkholderiales	1	1	0	1	1	0	0.2	0.3	0.0	NS	NS	NS
family Alcaligenaceae	1	0	0	1	0	0	0.2	0.0	0.0	NS	NS	NS
genus Sutterella	1	0	0	1	0	0	0.2	0.0	0.0	NS	NS	NS
family Oxalobacteraceae	0	1	0	0	1	0	0.0	0.3	0.0	NS	NS	NS
unclassified Oxalobacteraceae	0	1	0	0	1	0	0.0	0.3	0.0	NS	NS	NS
class Epsilonproteobacteria	2	0	0	1	0	0	0.4	0.0	0.0	NS	NS	NS
order Campylobacterales	2	0	0	1	0	0	0.4	0.0	0.0	NS	NS	NS
family Helicobacteraceae	2	0	0	1	0	0	0.4	0.0	0.0	NS	NS	NS
genus Helicobacter	2	0	0	1	0	0	0.4	0.0	0.0	NS	NS	NS
class Deltaproteobacteria	3	0	0	1	0	0	0.7	0.0	0.0	NS	NS	NS
order Desulfovibrionales	3	0	0	1	0	0	0.7	0.0	0.0	NS	NS	NS
family Desulfovibrionaceae	3	0	0	1	0	0	0.7	0.0	0.0	NS	NS	NS
genus Desulfovibrio	3	0	0	1	0	0	0.7	0.0	0.0	NS	NS	NS
class Gammaproteobacteria	5	63	2	2	1	2	1.1	18.5	1.1	<0.0001	NS	NS
order Pseudomonadales	1	0	0	1	0	0	0.2	0.0	0.0	NS	NS	NS
family Moraxellaceae	1	0	0	1	0	0	0.2	0.0	0.0	NS	NS	NS
genus Acinetobacter	1	0	0	1	0	0	0.2	0.0	0.0	NS	NS	NS
order Aeromonadales	4	0	0	1	0	0	0.9	0.0	0.0	NS	NS	NS
family Succinivibrionaceae	4	0	0	1	0	0	0.9	0.0	0.0	NS	NS	NS
genus Anaerobiospirillum	1	0	0	1	0	0	0.2	0.0	0.0	NS	NS	NS
genus Succinivibrio	3	0	0	1	0	0	0.7	0.0	0.0	NS	NS	NS
order Enterobacteriales	0	63	2	0	1	1	0.0	18.5	1.1	<0.0001	NS	NS
family Enterobacteriaceae	0	63	2	0	1	1	0.0	18.5	1.1	<0.0001	NS	NS
genus Shigella	0	62	2	0	1	1	0.0	18.2	1.1	<0.0001	NS	NS
unclassified Enterobacteriaceae	0	1	0	0	1	0	0.0	0.3	0.0	NS	NS	NS

[a] The proportion of clones in each category (i.e., Phylum, Class, Order, Family, Genus) in relation to the total number of clones selected

[b] Statistical comparisons between the control group and the IBD group (NS = not significant)

[c] p-value for the relative proportion of clones

[d] p-value based on the number of cats

[e] p-value based on the relative number of clones per cat

Table 7. Overall phylogenetic distribution of the sequences classified in the phylum *Firmicutes*. For each group, the number of clones classified for each taxa, the number of cats with clones classified in each taxa, and proportion of clones classified in each taxa are shown. Statistical analysis was performed between the control group and the IBD group.

	Number of clones			Number of cats			Proportions[a]			Statistical analysis[b]		
	Control	IBD	Intestinal Neoplasia	Control	IBD	Intestinal Neoplasia	Healthy cats (n=447)	IBD Cats (n=340)	Neoplasia Cats (n=190)	RDP library compare p-value [c]	Fisher's exact test p-value[d]	Mann-Whitney p-value[e]
Phylum Firmicutes	393	274	186	6	6	3	87.9	80.6	97.9	0.0076	NS	NS
class Bacilli	69	33	17	4	4	3	15.4	9.7	8.9	0.0188	NS	NS
order Lactobacillales	4	33	13	2	4	1	0.9	9.7	6.8	<0.0001	NS	NS
family Enterococcaceae	1	2	0	0	1	0	0.2	0.6	0.0	NS	NS	NS
genus Enterococcus	1	2	0	1	2	0	0.2	0.6	0.0	NS	NS	NS
family Incertae sedis 9	3	0	0	1	0	0	0.7	0.0	0.0	NS	NS	NS
genus Oscillospira	3	0	0	1	0	0	0.7	0.0	0.0	NS	NS	NS
family Lactobacillaceae	0	24	13	0	1	1	0.0	7.1	6.8	<0.0001	NS	NS
genus Lactobacillus	0	24	13	0	1	1	0.0	7.1	6.8	<0.0001	NS	NS
family Streptococcaceae	0	7	0	0	2	0	0.0	2.1	0.0	0.0024	NS	NS
genus Streptococcus	0	7	0	0	2	0	0.0	2.1	0.0	0.0024	NS	NS
order Bacillales	65	0	4	3	0	1	14.5	0.0	2.1	<0.0001	NS	NS
family Turicibacteraceae	65	0	4	3	0	1	14.5	0.0	2.1	<0.0001	NS	NS
genus Turicibacter	65	0	4	3	0	1	14.5	0.0	2.1	<0.0001	NS	NS
class Clostridia	324	241	169	6	6	3	72.5	70.9	88.9	NS	NS	NS
order Clostridiales	324	241	169	6	6	3	72.5	70.9	88.9	NS	NS	NS
family Peptococcaceae	17	1	0	1	1	0	3.8	0.3	0.0	0.0007	NS	NS
genus Peptococcus	17	1	0	1	1	0	3.8	0.3	0.0	0.0007	NS	NS
family Eubacteriaceae	1	0	0	1	0	0	0.2	0.0	0.0	NS	NS	NS
unclassified Eubacteriaceae	1	0	0	1	0	0	0.2	0.0	0.0	NS	NS	NS
family Lachnospiraceae	42	52	7	6	6	2	9.4	15.3	3.7	0.0080	NS	NS
genus Roseburia	1	7	0	2	2	0	0.2	2.1	0.0	0.0132	NS	NS
genus Ruminococcus	27	20	7	6	5	2	6.0	5.9	3.7	NS	NS	NS
genus Anaerostipes	0	16	0	0	2	0	0.0	4.7	0.0	<0.0001	NS	NS
unclassified Lachnospiraceae	14	9	0	6	5	0	3.1	2.6	0.0	NS	NS	NS
family Clostridiaceae	196	153	138	6	6	3	43.8	45.0	72.6	NS	NS	NS
genus Subdoligranulum	3	1	1	2	1	1	0.7	0.3	0.5	NS	NS	NS
genus Acetanaerobacterium	4	0	17	4	0	1	0.9	0.0	8.9	NS	NS	NS
genus Dorea	5	15	0	4	4	0	1.1	4.4	0.0	0.0138	NS	NS
genus Faecalibacterium	18	1	1	4	1	1	4.0	0.3	0.5	0.0004	NS	NS
genus Clostridium	23	50	51	5	6	3	5.1	14.7	26.8	<0.0001	NS	NS
genus Sporacetigenium	4	0	1	4	0	1	0.9	0.0	0.5	NS	NS	NS
genus Coprobacillus	0	5	0	0	1	0	0.0	1.5	0.0	NS	NS	NS
unclassified Clostridiaceae	139	80	67	6	6	3	31.1	23.5	35.3	0.0200	NS	NS
unclassified Clostridiales	68	36	24	6	6	3	15.2	10.6	12.6	0.0257	NS	NS

[a] The proportion of clones in each category (i.e., Phylum, Class, Order, Family, Genus) in relation to the total number of clones selected

[b] Statistical comparisons between the control group and the IBD group (NS = not significant)

[c] p-value for the relative proportion of clones

[d] p-value based on the number of cats

[e] p-value based on the relative number of clones per cat

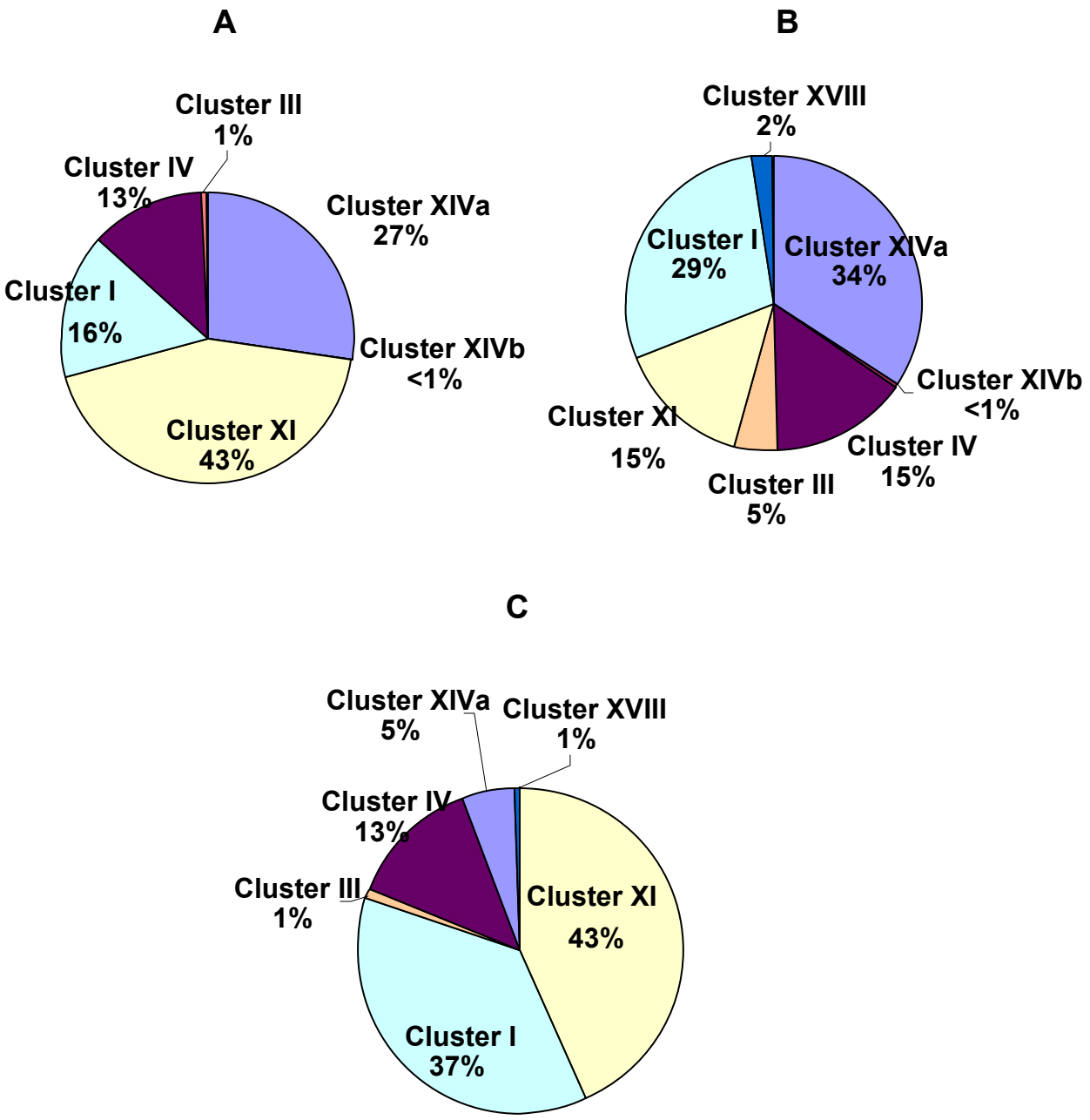

Figure 14. Percentage of 16S rDNA clones affiliated with *Clostridium* clusters in the control group (A), the IBD group (B), and the group with intestinal neoplasia (C).

number of cats they had been identified in. Also, there were no significant differences between the control group or IBD group when the total number of clones representing specific bacterial taxa were compared for each cat. However, based on the relative proportion of clones, several bacterial taxa were significantly more frequently represented in the control group compared to the IBD group (outlined in tables 5, 6, and 7). In addition, significant differences were found for the proportion of clones affiliated with the different *Clostridium* clusters between the control and IBD groups.

Compared to the cats in the IBD group, cats in the control group were significantly enriched in sequences belonging to the phyla *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* ($p=0.0075$, $p<0.0001$, $p<0.0001$, respectively). Sequences within the *Actinobacteria* phylum were predominantly classified in the *Coriobacteriales* family. The proportion of clones classified in this bacterial family was significantly higher in the control group than in the IBD group ($p=0.0198$). Within the *Bacteroidetes* phylum, sequences from the control group were predominantly classified within the *Prevotellaceae* family. The proportion of clones classified in this bacterial family was significantly higher in the control group compared to the IBD group ($p=0.0004$).

The predominant bacterial phylum in both groups was *Firmicutes.* Further classification showed that the control group had a significantly higher proportion of clones classified in the *Turicibacteraceae* family than the IBD group ($p<0.0001$). In contrast, the proportion of clones classified in the family *Lactobacillaceae* was significantly higher in the IBD group than in the control group ($p<0.0001$). All of these clones were classified as *Lactobacillus* spp. but were only obtained from one IBD cat. Within the class *Clostridia*, the control group had a significantly higher proportion of

clones classified in the *Peptococcaceae* family than the IBD group ($p<0.0001$). Based on Fisher's exact test, the control group had a significantly higher proportion of clones affiliated with *Clostridium* cluster XI than the IBD group ($p<0.0001$). In contrast, cats in the IBD group had a significantly higher proportion of clones affiliated with *Clostridium* cluster I (Fisher's exact test, $p<0.0001$).

The proportion of clones classified within the *Proteobacteria* phylum were significantly higher in the IBD group than in the control group ($p<0.0001$). The majority of the clones in the *Proteobacteria* phylum from cats in the IBD group were classified as *Escherichia coli*-like organisms (94%). However, these organisms were only isolated in one IBD cat. No *Escherichia coli*-like organisms were isolated from any cat in the control group.

DISCUSSION

In this study, amplification of the 16S rRNA gene was used to characterize and compare the fecal microflora of healthy cats, cats with histopathologically confirmed mild to severe IBD, and cats with intestinal neoplasia (2 cats with intestinal lymphosarcoma and 1 cat with intestinal adenocarcinoma). *Firmicutes* was the predominant bacterial phylum in the control group, followed by *Bacteroidetes*, *Actinobacteria*, *Proteobacteria,* and *Fusobacteria*, respectively. In general these findings are comparable to previous studies analyzing the bacterial microflora of the feline colon and in fecal samples from cats using culture-based methods. In these previous studies the predominant bacterial groups isolated from fecal samples in healthy cats were classified in the *Bacteroidetes* and *Firmicutes* phylum (i.e., *Bacteroides* spp., *Lactobacillus* spp.,

and *Clostridia* spp.) (Itoh *et al.*, 1984; Inness *et al.*, 2006). Another study reported that *Enterococcus* spp. and *Lactobacillus* spp., both members of the phylum *Firmicutes*, were isolated from the majority of feline colon samples (Osbaldiston & Stowe, 1971). One contrast, however, is that studies in the past have reported *Bifidobacterium* spp. to be a predominant bacterial group in the feces of healthy cats (Itoh *et al.*, 1984; Inness *et al.*, 2006). This is markedly different to this study where only 5 clones were classified as *Bifidobacterium* spp. and all these clones were obtained from a single healthy cat.

To our knowledge only few studies have been performed that compare the bacterial microflora between healthy cats and cats with intestinal disease. In one recent study fluorescent in situ hybridization (FISH) was used to compare the fecal microflora between healthy cats and cats diagnosed with IBD (Inness *et al.*, 2006). This study reported quantitative differences in bacterial groups between healthy and IBD cats. Fecal samples obtained from healthy cats harbored significantly higher numbers of *Bacteroides* spp. and *Bifidobacterium* spp. when compared to cats with IBD ($p=0.048$) (Inness *et al.*, 2006). In addition, the number of *Desulfovibirio* spp. (phylum *Proteobacteria*) were significantly higher in cats with IBD than in healthy cats ($p= 0.002$). Similar to those findings, the proportion of clones classified in the phylum *Bacteroidetes* was also significantly higher in the control group compared to the IBD group ($p<0.001$) in the current study. Additionally, the proportions of clones classified in the phylum *Proteobacteria* were significantly higher in the IBD group ($p<0.001$). However, in contrast to the study by Inness et al., *Bifidobacterium* spp. were not commonly isolated from the control cats in the present study (1.1%). Differences in the results between both studies could be due to the techniques employed. The PCR protocol used in this study

may be less efficient for the detection of *Bifidobacterium* spp. Further studies using group specific *Bifidibacteria* primers and PCR amplification protocols are warranted to investigate the role of *Bifidobacterium* spp. in healthy cats and cats with IBD.

Another study compared the bacterial composition of feline duodenal biopsy samples of healthy cats and cats with IBD using FISH analysis (Janeczko *et al.*, 2007). That study reported that duodenal biopsies obtained from IBD cats showed significantly higher numbers of *Enterobacteriaceae* ($p=0.002$). Further analysis showed that 30% of the bacteria in that group were identified as *E. coli* (Janeczko *et al.*, 2007). Additionally, the bacterial groups analyzed (*Enterobacteriaceae*, *E. coli*, *Streptococcus* spp., *Clostridium* spp., and *Bacteriodes/ Prevotella* spp.) accounted only for 6% of the mucosa-associated bacteria in healthy cats. Thus, the dominant intestinal bacterial species of healthy cats remained undetermined in this study. In contrast, the findings of the present study identified the predominant bacterial group to be *Clostridiales* in both healthy cats and cats with IBD. In addition, *Escherichia coli*-like organisms were only isolated in one of the six IBD cats studied. Again, differences between the techniques used (FISH analysis vs. comparative 16S analysis) could be the reason for the differences in results between these two studies.

The majority of sequences from all three groups evaluated in this study were classified in the *Firmicutes* phylum. An important finding of the present study was the affiliation of sequences from each group with different *Clostridium* clusters. Sequences obtained from the control group were predominantly affiliated with *Clostridium* cluster XI, and a significantly higher proportion of clones was affiliated with this cluster compared to the IBD group ($p<0.0001$). In contrast, sequences obtained from IBD cats

were predominantly affiliated with *Clostridium* cluster XIVa. The IBD cats had also a significantly higher proportion of clones affiliated with *Clostridium* cluster I ($p<0.0001$) than the control group.

The majority of sequences in fecal samples from 3 cats with intestinal neoplasia were classified in the phylum *Firmicutes* (97.9%). No clones obtained in these cats were classified as *Bacteroidetes* or *Fusobacteria*, and less than 2% of clones were classified as *Actinobacteria*. This phylogenetic distribution was generally similar to the phylogenetic distribution in the IBD group. However, the majority of clones classified in the bacterial class *Clostridia* were predominantly affiliated with *Clostridium* cluster XI in the neoplasia group, which was also the predominant *Clostridium* cluster in the control group. Unfortunately, the low sample size of the neoplasia group precluded statistical analysis between all three groups. However, this finding warrants further evaluation of the intestinal microflora in cats with intestinal neoplasia.

To our knowledge, this is the first study comparing the fecal microflora of healthy cats, cats with IBD, and cats with intestinal neoplasia using a comparative 16S rDNA

approach. This study showed some differences in the bacterial composition between groups based on the relative proportion of clones. Significant differences were observed between healthy and cats with IBD based on the proportion of clones classified in different bacterial taxa. However, we did not observe significant difference in bacterial groups based on the number of cats that harbored these taxa. Therefore, these findings need to be interpreted with caution. Some bacterial taxa might have been over-represented in individual cats, potentially leading to confounding results. While these results raise the possibility that differences in the composition of the intestinal microflora may play a role in feline IBD and neoplasia, further studies sampling a larger population of cats belonging to all three groups are warranted.

CHAPTER IV

CONCLUSIONS

It has previously been reported that the feline intestinal microflora harbors predominantly anaerobic bacterial species (Johnston *et al.*, 1993; Papasouliotis *et al.*, 1998; Johnston *et al.*, 2001). Studies to date have characterized the feline intestinal microflora using traditional microbiological culture techniques and described bacterial populations present in samples obtained either from the proximal segments of the small intestine (i.e., duodenum and jejunum) or from the distal segments (i.e., colon or feces) (Smith, 1965; Osbaldiston & Stowe, 1971; Itoh *et al.,* 1984; Johnston *et al.*, 1993; Papasouliotis *et al.*, 1998; Johnston *et al.*, 1999). Recently, it has been shown that standard culture techniques are less efficient for the detection of some bacterial species (Amann *et al.*, 1995; Langendijk *et al.,* 1995; Greetham *et al.*, 2002). Reasons for this include difficulties in anaerobic collection of intestinal samples, a lack of knowledge about the growth requirements of many intestinal bacterial species, and bias due to the employed selective culture media, allowing growth of bacteria other than the published target organisms. Thus, previous studies using standard culture techniques may have underestimated the overall bacterial diversity present in the intestinal tract of cats.

Inflammatory bowel disease (IBD) is a common chronic intestinal disease in cats and is associated with inflammation of the intestine and results in clinical signs such as vomiting, diarrhea, and weight loss (Willard, 1999). Studies in humans and animal models have implicated the intestinal microflora as an important factor in the development of IBD (Sadlack *et al.*, 1993; Sartor, 1997; Linskens *et al.*, 2001). However,

only few studies have investigated the role of the bacterial microflora in feline IBD. These studies revealed significant differences in the bacterial composition of intestinal samples from healthy cats and cats with IBD, suggesting that the intestinal microflora may play an integral role in the pathogenesis of IBD in cats (Inness *et al.*, 2006; Janeczko *et al.*, 2007).

This study was designed to characterize the intestinal microflora in healthy cats and to compare the fecal microflora in healthy cats to cats with IBD and intestinal neoplasia using a comparative 16S rDNA molecular approach.

Comparative 16S rDNA analysis revealed several previously uncharacterized 16S rDNA sequences in the intestinal tract of four healthy non-SPF cats and one healthy SPF cat. Four major bacterial phyla were identified in the four healthy non-SPF cats, with the majority of 16S rDNA sequences belonging to the bacterial orders *Clostridiales*, *Lactobacillales*, *Bacteroidales, Campylobacterales* and *Fusobacteriales*. Sequences classified in the order *Clostridiales* were predominantly affiliated with *Clostridium* clusters I and XIVa. Clones affiliated with cluster I increased in complexity along the intestinal tract with the highest number of clones isolated from the colon. Sequences affiliated with *Clostridium* cluster XIVa were predominantly isolated from the colon. In contrast to the four healthy non-SPF cats, 98% of clones from the healthy SPF cat were classified in the phylum *Firmicutes*. These clones were classified exclusively in the class *Clostridiales* and were predominantly affiliated with *Clostridium* cluster I. Sequences affiliated with this cluster were isolated from all gastrointestinal compartments of the healthy SPF cat. These data indicate that the feline intestinal microflora is highly diverse and is comprised predominantly of anaerobic bacteria. Additionally, the observed

differences between the intestinal microflora of healthy conventionally raised cats and the SPF cat warrants further examinations.

Comparative 16S rDNA analysis also revealed differences in the fecal microflora of healthy cats, cats with histopathologically confirmed IBD, and cats with intestinal neoplasia. Cats in the control group had significantly higher number of clones in the *Bacilli* class and the *Bacteroidetes* and *Actinobacteria* phylums (all $p<0.0001$). Also, cats of the control group had a significantly higher proportion of clones affiliated with *Clostridium* cluster XI, and a significantly lower proportion affiliated with cluster I than cats of the IBD group (both $p<0.0001$). In the neoplasia group, the majority of sequences were classified in the *Firmicutes* phylum (97.9%) and clones were predominately affiliated with *Clostridium* clusters I and XI. However, these findings need to be interpreted with caution, because no significant differences were observed between the number of cats in each group that harbored those bacterial taxa. While the results of the current studies raise the possibility that alterations in bacterial diversity may play a role in feline IBD and intestinal neoplasia, further studies involving larger numbers of cats in each group are indicated.

REFERENCES

Amann R, Ludwig W & Schleifer KH (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev* **59**: 143-169.

Atlas R & Bartha R (1998) Microbial ecology: Fundamentals and applications. Addison-Wesley Publishing Company, Reading, PA.

Batt RM, Needham JR & Carter MW (1983) Bacterial overgrowth associated with a naturally occurring enteropathy in the German shepherd dog. *Res Vet Sci* **35**: 42-46.

Batt RM & McLean L (1987) Comparison of the biochemical changes in the jejunal mucosa of dogs with aerobic and anaerobic bacterial overgrowth. *Gastroenterology* **93**: 986-993.

Daly K, Stewart CS, Flint HJ & Shirazi-Beechey SP (2001) Bacterial diversity within the equine large intestine as revealed by molecular analysis of cloned 16S rRNA genes. *FEMS Microbiol Ecol* **38**: 141-151.

Davis CP, Cleven D, Balish E & Yale CE (1977) Bacterial association in the gastrointestinal tract of Beagle dogs. *Appl Environ Microbiol* **34**: 194-206.

Delgado S, Suárez A & Mayo B (2006) Identification of dominant bacteria in feces and colonic mucosa from healthy Spanish adults by culturing and by 16S rDNA sequence analysis. *Dig Dis Sci* **51**: 744-751.

Falk PG, Hooper LV, Midtvedt T & Gordon JI (1998) Creating and maintaining the gastrointestinal ecosystem: What we know and need to know from Gnotobiology. *Microbiol Mol Biol Rev* **62**: 1157-1170.

German AJ, Day MJ, Ruaux CG, Steiner JM, Williams DA & Hall EJ (2003) Comparison of direct and indirect tests for small intestinal bacterial overgrowth and antibiotic-responsive diarrhea in dogs. *J Vet Intern Med* **17**: 33-43.

Gibson GR & Roberfroid MB (1995) Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. *J Nutr* **125**: 1401-1412.

Good IJ (1953) The population frequencies of species and the estimation of population parameters. *Biometrica* **40**:237-264.

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

Hold GL, Pryde SE, Russell VJ, Furrie E & Flint HJ (2002) Assessment of microbial diversity in human colonic samples by 16S rDNA sequence analysis. *FEMS* **39**: 33-39.

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

Inness VL, McCartney AL, Khoo C, Gross KL & Gibson GR (2006) Molecular characterisation of the gut microflora of healthy and inflammatory bowel disease cats using fluorescence in situ hybridisation with special reference to Desulfovibrio spp. *J Anim Physiol Anim Nutr* **91**: 48-53.

Itoh K, Mitsuoka T, Maejima K, Hiraga C & Nakan K (1984) Comparison of faecal flora of cats based on different housing conditions with special reference to *Bifidobacterium*. *Lab Anim* **18**: 280-284.

Janeczko S, Atwater D, Bogel E, Greiter-Wilke A, Gerold A, Baumgart M, Bender H, McDonough PL, McDonough SP, Goldstein R & Simpson KW (2007) The relationship of mucosal bacteria to duodenal histopathology, cytokine mRNA, and clinical disease activity in cats with inflammatory bowel disease. *Vet Microbiol* **128**:178-193.

Jergens A (1999) Inflammatory bowel disease. Current Perspectives. *Vet Clin North Am Small Anim Pract* **29**: 501-521.

Johnston K, Lamport A & Batt M (1993) An unexpected bacterial flora in the proximal small intestine of normal cats. *Vet Rec* **132**:362-363.

Johnston K (1999) Small intestinal bacterial overgrowth. *Vet Clin North Am Small Anim Pract* **29**: 523-549.

Johnston K, Lamport A, Ballèvre O & Batt RM (1999) 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.

Johnston K, Swift N, Hijfte M, Rutgers C, Lamport A, Ballèvre O & Batt R (2001) Comparison of the bacterial flora of the duodenum in healthy cats and cats with signs of gastrointestinal tract disease. *JAVMA* **218**: 48-51.

Kanauchi O, Matsumoto Y, Matsumura M, Fukuoka M & Bamba T (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.

Landendijk P, Schut F, Jansen G, Raangs G, Kamphuis G, Wilkinson M & Welling G (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.

Linskens RK, Huijsdens XW, Savelkoul PHM, Vandenbroucke-Grauls CMJE & Meuwissen SGM (2001) The bacterial flora in infammatory bowel disease: Current insights in pathogenesis and the infuence of antibiotics and probiotics. *Scand J Gastroenterol* **234**: 29-40.

Macfarlane S & Macfarlane GT (2003) Session: Short-chain fatty acids. Regulation of short-chain fatty acid production. *Proc Nutr Soc* **62**:67-72.

Osbaldiston G & Stowe E (1971) Microflora of alimentary tract of cats. *Am J Vet Res* **32**: 1399-1405.

Papasouliotis K, Sparkes A, Werrett G, Egan K, Gruffydd-Jones E & Gruffydd-Jones T (1998) Assessment of the bacterial flora of the proximal part of the small intestine in healthy cats, and the effect of sample collection method. *AJVR* **59**: 48-51.

Sadlack B, Merz H, Schorle H, Schimpi A, Feller A & Horack I (1993) Ulcerative colitis-like disease in mice with a disrupted Interleukin-2 gene. *Cell* **75**:253-261.

Sartor RB (1997) Pathogenesis and immune mechanisms of chronic inflammatory bowel diseases. *Am J Gastroenterol* **92**: 5S-11S.

Simon GL & Gorbach SL (1984) Intestinal flora in health and disease. *Gastroenterology* **86**: 174-193.

Simpson J (1998) Diet and large intestinal disease in dogs and cats. *J Nutr* **128:** 2717S-2722S.

Smith H (1965) Observation on the flora of the alimentary tract of animals and factors affecting its composition. *J Path Bact* **89**: 95-122.

Suchodolski J, Ruaux D, Steiner J, Fetz J & Williams D (2004) Application of molecular fingerprinting for qualitative assessment of small-intestinal bacterial diversity in dogs. *J Clin Microbiol* **42**: 4702-4708.

Suchodolski J (2005) Assessment of the canine intestinal microflora using molecular methods and serum markers. PhD Dissertation, Texas A&M University College of Veterinary Medicine, USA.

Thadepalli H, Lou MA, Bach V, Matsui T & Mandal A (1979) Microflora of the human small intestine. *Am J Surg* **138**: 845-849.

Van de Peer Y & De Wachter R (1993) TREECON: A software package for the construction and drawing of evolutionary trees. *Comput Appl Biosci* **9**:177-182.

Van de Peer Y, Chapelle S & De Wachter R (1998) A quantitative map of nucleotide substitution rates in bacterial rRNA. *Nucleic Acids Res* **24**: 3381-3391.

Wang X, Heazlewood SP, Krause DO & Florin THJ (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.

Welkos SL, Toskes PP & Baer H (1981) Importance of anaerobic bacteria in the cobalamin malabsorption of the experimental rat blind loop syndrome. *Gastroenterology* **80**: 313-320.

Westermark E, Siltanen R & Maijala R (1993) Small intestinal bacterial overgrowth in seven dogs with gastrointestinal signs. *Acta Vet Scand* **34**; 311-314.

Willard MD (1999) Feline inflammatory bowel disease: A review. *J Feline Med Surg* **1**: 155-164.

Wilson K & Blitchington R (1996) Human colonic biota studied by ribosomal DNA sequence analysis. *Appl Environ Microbiol* **62**: 2273- 2278.

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