Effect of a multi-species synbiotic formulation on fecal bacterial microbiota of healthy cats and dogs as evaluated by pyrosequencing

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Abstract
The effect of a multi-species synbiotic on the fecal microbiota of healthy cats \((n = 12)\) and dogs \((n = 12)\) was evaluated. The synbiotic (containing \(5 \times 10^9\) CFU of a mixture of seven probiotic strains, and a blend of fructooligosaccharides and arabinogalactans) was administered daily for 21 days. Fecal and serum samples were collected before, during, and up to 3 weeks after administration. Changes in the fecal microbiota were analyzed using denaturing gradient gel electrophoresis, 16S rRNA gene libraries, quantitative real-time PCR, and 16S rRNA gene 454-pyrosequencing. Probiotic species were detectable in 10/12 dogs and 11/12 cats during product administration. Abundances of Enterococcus and Streptococcus spp. were significantly increased in at least one time point during administration, and returned to baseline abundance after treatment was discontinued. No changes in the major bacterial phyla were identified on 454-pyrosequencing. No adverse gastrointestinal effects were recorded and no significant changes in gastrointestinal function or immune markers were observed during the study period. This study shows that while the ingestion of probiotics and prebiotics does not appear to alter the predominant bacterial phyla present in feces, supplementation with the investigated synbiotic leads to an increased abundance of probiotic bacteria in the feces of healthy cats and dogs.

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

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

The increased abundance of probiotics in the intestinal tract after oral ingestion has traditionally been thought to
modify the composition of the intestinal microbiota (Fuller, 1989). However, to date most investigations have only studied the effect of probiotics on select intestinal bacterial groups (Sauter et al., 2006; Biagi et al., 2007; Saulnier et al., 2008), in part due to the challenges associated with the characterization of highly complex microbial ecosystems. Recently developed cost-effective high throughput technologies (e.g. microarray based methods or massive parallel pyrosequencing techniques) allow a deeper phylogenetic coverage of the intestinal microbiota (Zoetendal et al., 2008; Suchodolski et al., 2009; Middelbos et al., 2010; Handl et al., 2011; Swanson et al., 2011) and, therefore, may be useful to assess the effect of probiotics and/or prebiotics on the overall composition of the intestinal microbiota (Middelbos et al., 2010).

The effect of probiotics on intestinal and overall health has been studied in humans (Culligan et al., 2009), but much more limited data are available for veterinary species (Callaway et al., 2008). Although probiotics and prebiotics are administered to dogs and cats with increasing frequency, only few investigations have evaluated the effect of these preparations on intestinal microbial composition and immune function of these animal species (Baillon et al., 2004; Marshall-Jones et al., 2006; Kelley et al., 2009; Ogue-Bon et al., 2010). Because extrapolations of the in vivo effect of probiotics among animal species are inherently weak, the effect of probiotic preparations on the intestinal microbiota of the target animal population deserves investigation. The objective of this study was to evaluate the effect of a multi-species synbiotic preparation designed for use in cats and dogs on the fecal bacterial microbiota of these animal species. Changes in fecal bacterial groups were evaluated using denaturing gradient gel electrophoresis (DGGE), comparative 16S rRNA gene analysis, quantitative real-time PCR (qPCR) assays, as well as massive parallel 16S rRNA gene 454-pyrosequencing. Selected markers of gastrointestinal and immune function were also evaluated to investigate potentially beneficial effects due to the consumption of the synbiotic.

**Materials and methods**

**Synbiotic description**

Proviable®-DC (Nutramax Laboratories, Inc., Edgewood, MD) is a commercially available multi-species synbiotic formulation designed for use in cats and dogs that contains a blend of fructooligosaccharides, arabinogalactans, and a total of five billion (5 × 10^9) CFU of a mixture of seven bacterial species per capsule (Table 1). The exact proportions of each component in the formulation (bacterial strains and prebiotics) are proprietary.

### Table 1. Probiotic bacterial strains in Proviable®-DC

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterococcus faecium</td>
<td>NCIMB 30183</td>
</tr>
<tr>
<td>Streptococcus salivarius ssp. thermophilus</td>
<td>NCIMB 30189</td>
</tr>
<tr>
<td>Bifidobacterium longum</td>
<td>NCIMB 30179</td>
</tr>
<tr>
<td>Lactobacillus acidophilus</td>
<td>NCIMB 30184</td>
</tr>
<tr>
<td>Lactobacillus casei ssp. rhamnosus</td>
<td>NCIMB 30188</td>
</tr>
<tr>
<td>Lactobacillus plantarum</td>
<td>NCIMB 30187</td>
</tr>
<tr>
<td>Lactobacillus delbrueckii ssp. bulgaricus</td>
<td>NCIMB 30186</td>
</tr>
</tbody>
</table>

The exact proportions of each probiotic strain are proprietary.

**Animal subjects and study design**

Privately owned healthy cats (n = 12) and dogs (n = 12) of different breeds and ages were enrolled (Table 2). None of the enrolled subjects had a history of antibiotic use or any other medication known to influence the intestinal microbiota for at least 3 months before the beginning of the study. All animal subjects were fed different commercial diets.

Baseline blood and fecal samples were collected before synbiotic administration (day 0). All cats and dogs then received one capsule of the formulation daily for 21 days. Owners were allowed to administer the capsule orally, mix the capsule into food, or open the capsule and mix the preparation contained in the capsule into the food, depending on the particular way of their pets to accept medications (Table 2). Owners (mainly students of Veterinary Medicine at Texas A&M University) were instructed to maintain the usual diet throughout the study period and asked to complete a daily questionnaire during the 21 days of synbiotic administration to record clinical signs of gastrointestinal discomfort such as diarrhea, vomiting, and/or abdominal pain. Additional serum samples were obtained on day 21 (last day of synbiotic administration) and day 42 (3 weeks after cessation of administration of the synbiotic). Additional fecal samples were collected every 3–4 days during and up to 3 weeks after administration of the synbiotic. All subjects were maintained on their typical diet during the study period. The study protocol was approved by the Clinical Research Review Committee of Texas A&M University and written informed consent was obtained from the owners of all enrolled animals.

**Assessment of fecal microbiota**

**Extraction of DNA**

An aliquot of 100 mg (wet weight) of each fecal sample was mixed with 500 μL of lysis buffer (Puregene® cell lysis solution; Gentra Systems, Minneapolis, MN) and
100 μL of 0.1 mm-diameter zirconia beads (BioSpec Products Inc., Bartlesville, OK). This mixture was vortexed for 5 min at maximum speed on a standard vortex. After centrifugation (7 min at 12 000 g), the supernatant was transferred into a sterile tube and mixed with 500 μL of a solution of phenol–chloroform–isoamyl alcohol (Applied Biosystems, Foster City, CA). Further steps of DNA extraction and purification were performed as previously described (Suchodolski et al., 2005).

**Denaturing gel gradient electrophoresis**

To first investigate whether or not the synbiotic led to a noticeable change on the fecal microbiota, qualitative changes in fecal bacterial communities were evaluated by DGGE at baseline, day 21 (last day of synbiotic administration) and day 38 (2 weeks after treatment) with some modifications to a protocol described previously (Suchodolski et al., 2004). Briefly, universal bacterial primers F341 and R518 (Table 3) were used to amplify the variable V3 region of the 16S rRNA gene. A GC clamp (CGCCCGGGGGCGCCGGCCGGGGCCGGGGCGGACG) was incorporated into the forward primer to prevent the complete dissociation of the DNA double strand during the subsequent DGGE analysis. PCR amplicons were loaded on 8% w/v polyacrylamide gels in TAE buffer with a linear denaturing gradient of 40–60%. Electrophoresis was performed in TAE buffer at 60 °C for 16 h at 70 V. Gels were stained with ethidium bromide for 12 min, destained in water two times for 30 min and visualized under UV light.

**16S rRNA gene clone libraries**

In addition to a change in the qualitative composition of the fecal microbiota, it was also of interest to investigate in feces the presence of closely related probiotic genera (see Quantitative real-time PCR below) and species over...
Table 3. Oligonucleotides used for the amplification of bacterial targets in this study

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence (5′−3′)</th>
<th>Annealing temperature (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bifidobacterium spp.</td>
<td>F-TCGCTCCGTTGCTGAG</td>
<td>60</td>
<td>Rinttila et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>R-CCACATCCAGCACTCAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterococcus spp.</td>
<td>F-CCTATTTGATGTTGGCCATCATT</td>
<td>61</td>
<td>Malinen et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>R-ATCCTGTTGAATCCTCCCCATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactobacillus spp.</td>
<td>F-AGCGATAGGGGAAATCTCAA</td>
<td>58</td>
<td>Malinen et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>R-ACGCACTACATTGGGAAATCTCAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptococcus spp.</td>
<td>F-TTATTTGAAAGGGGCAATTGCT</td>
<td>54</td>
<td>Furet et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>R-GTGAACTTTCCACTCTCACAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All bacteria</td>
<td>F-CTACCGGAGGCAGCAGGACG</td>
<td>57</td>
<td>Muyzer et al. (1993)</td>
</tr>
<tr>
<td></td>
<td>R-ATTACCGGCGGTGCTG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Originally described by Walter et al. (2001).
†Originally described by Heilig et al. (2002).

Quantitative real-time PCR

Quantitative changes in the fecal abundance of probiotic groups were assessed by qPCR before, during (days 1, 8, and 17 in cats and days 3, 5, and 17 in dogs) and after (days 26, 29, and 38 in cats and days 23, 26, and 38 in dogs) synbiotic administration. The decision of using different time points between dogs and cats depended primarily upon availability of remaining fecal DNA to conduct all qPCR assays on the same days for each individual subject within an animal species. Bacterial DNA was amplified using bacterial universal and 16S rRNA primers (Table 3) respectively, as described previously (Ritchie et al., 2010; Handl et al., 2011).

Massive parallel 16S rRNA gene pyrosequencing

Fecal bacterial communities were evaluated using pyrosequencing at baseline (day 0), after 5 days of feeding the synbiotic (day 5) and after 2 days of discontinuation of synbiotic administration (day 23) using a bacterial tag-encoded FLX-titanium 16S rRNA gene amplicon pyrosequencing (bTEFAP) as described previously for canine and feline fecal samples (Handl et al., 2011). Sequences with identity scores > 97% identity (< 3% divergence) were resolved at the species level, between 95% and 97% at the genus level, between 90% and 95% at the family level, and between 80% and 90% at the order level. Collection and sequence information is available through GenBank within a short read archive under accession SRA012231.

Parameters of gastrointestinal and immune function

Parameters in serum

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

Parameters in feces

Fecal IgA concentrations were measured in canine feces before (day 0), during (day 21, last day of synbiotic administration), and after (day 42) synbiotic administration using an ELISA that has been validated for measurement of IgA concentrations in canine feces (Tress et al., 2006). The analysis of fecal IgA in cats was not performed because to date there is no validated assay available. Canine and feline fecal α1-proteinase inhibitor (α1-PI) concentrations were measured at day 0, day 21, and day 42 using species specific in-house immunoassays (Melgarejo et al., 1998; Fetz et al., 2004), respectively.

Statistical analysis

To assess the diversity of the fecal microbiota, the Shannon–Weaver diversity index was calculated from the number of operation taxonomic units with < 1% dissimilarity. High values for this diversity index indicate higher bacterial diversity. Principal coordinate analysis (PCoA), based on the phylogeny-based Unifrac method (Lozupone & Knight, 2005), was used to investigate potential clustering of the microbial communities before, during, and after administering the synbiotic. A repeated measures ANOVA or its nonparametric counterpart, Friedman’s test, was used to compare the following dependent variables: the results of gastrointestinal and immune function tests, serum chemistry, complete blood counts, changes in microbial composition based on 16S rRNA gene pyrosequencing, bacterial abundance based on qPCRs, and Shannon–Weaver diversity indices, across different levels of the independent variable time (before, during, and after synbiotic administration) using Prism5 (GraphPad Software Inc., San Diego, CA). The assumption of normality was tested using the D’Agostino and Pearson normality test (GraphPad Software Inc.). For all tests, a value of P < 0.05 was considered statistically significant to reject the null hypotheses (time points are all equal). Multiple comparisons were adjusted by the Tukey–Kramer method (ANOVA) and Dunns’ test (Friedman’s test). To adjust for falsely rejected null hypotheses, the Benjamin–Hochberg critical values were calculated (Benjamini & Hochberg, 1995) and compared with the P values from the comparison of proportions of pyrosequencing tags at each phylogenetic level separately.

Results

Qualitative assessment of the fecal microbiota

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

Quantitative real-time PCR

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

Massive parallel pyrosequencing

454-Pyrosequencing in cats

A total of 187,396 pyrosequencing reads were generated: 75,350 at baseline, 60,355 on day 5 of synbiotic administration, and 51,691 on day 23 (2 days after end of synbiotic administration). The most abundant phylum was Firmicutes, followed by Actinobacteria (Fig. 2). Within these main phyla, Clostridiales, Lactobacillales, and Erysipelotrichales (Firmicutes) and Coriobacteriales (Actinobacteria), were the most abundant orders (Fig. 3). After adjustment for falsely rejected null hypotheses, there were no significant differences in relative proportions of pyrosequencing tags belonging to the phyla Firmicutes, Actinobacteria, Bacteroidetes, Proteobacteria, and Fusobacteria across the three time points evaluated (Supporting Information, Table S1). Also, there was no statistically significant difference in any class, order, family, or genus within these phyla. Based on unadjusted P values, the genus Collinsella (Actinobacteria) was found to be significantly higher at baseline when compared with both during and after synbiotic administration (P < 0.01). Also, Lactobacillus (Firmicutes) was found to be higher during synbiotic administration than before or after administration (P = 0.0390); however, multiple comparisons did not reach statistical significance. Differences in proportions of pyrosequencing reads among and within cats before, during, and after synbiotic administration were visualized by plotting a heatmap at the family level (Fig. 4), using a gplots library (Warnes, 2010) in the R software (R Development Core Team, 2004). These heatmaps showed a high variability among cats and a comparably much lower degree of variation within cats (i.e. proportions for time points within a subject usually clustered together).

454-Pyrosequencing in dogs

A total of 201,642 pyrosequencing reads were generated: 87,737 at baseline, 56,852 on day 5 of synbiotic administration, and 57,053 on day 23 (2 days after end of synbiotic administration). The most abundant phylum was Firmicutes, followed by Actinobacteria and Bacteroidetes (Fig. 2). Within these main phyla, Clostridiales, Erysipelotrichales, and Lactobacillales (Firmicutes), and Coriobacteriales (Actinobacteria) were the most abundant orders (Fig. 3). After adjustment for falsely rejected null hypotheses, there were no statistically significant differences in the relative proportions of pyrosequencing tags belonging to any phylogenetic level (Table S2). Based on unadjusted P values, the proportion of organisms belonging to the family Eubacteriaceae (Firmicutes) was found to be significantly increased during synbiotic administration when compared with baseline values (P < 0.05). Likewise, the proportion of organisms belonging to the genus Eubacterium was found to be significantly higher during synbiotic administration than before or after administration (P = 0.0390); however, multiple comparisons did not reach statistical significance. Differences in proportions of pyrosequencing reads among and within dogs before, during, and after synbiotic administration were visualized by plotting a heatmap at the family level (Fig. 4), using a gplots library (Warnes, 2010) in the R software (R Development Core Team, 2004). These heatmaps showed a high variability among dogs and a comparably much lower degree of variation within dogs (i.e. proportions for time points within a subject usually clustered together).

Fig. 1. Quantitative real-time PCR results for cats (a) and dogs (b) at baseline (BL), during synbiotic administration [days (D) 1 or 3, 5 or 8, and 17], and after synbiotic administration (days 23 or 26, 26 or 29, and 38) for Enterococcus, Lactobacillus, Bifidobacterium, and Streptococcus spp. Error bars represent mean normalized log_{10} 16S rRNA gene copies and standard deviation. †, significantly higher than baseline; ‡, significantly lower than day 17 (P < 0.05).
administration when compared to baseline results \((P < 0.05)\). Also, the proportion of organisms belonging to the genus *Roseburia* (*Firmicutes*) was found to be significantly lower 2 days after cessation of synbiotic administration than at both baseline and on day 5 of synbiotic administration \((P < 0.05)\). Also, based on unadjusted \(P\) values, relative proportions of sequencing tags belonging to the phylum *Fusobacteria* and all phylogenetic levels down to the genus *Fusobacterium* were found to be significantly higher during synbiotic administration when compared to baseline values \((P < 0.05)\) (Table S2). Differences in proportions of pyrosequencing reads among and within dogs before, during, and after synbiotic administration were also visualized by plotting a heatmap at the family level (Fig. 4). Similarly to results observed in the cats, these heatmaps showed a high variability among individual dogs and a comparably lower variation within dogs (i.e. proportions for time points within a subject were usually clustered together).

**Principal coordinate analysis**

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

**Diversity indices for pyrosequencing bacterial tags**

At 1% dissimilarity, no significant differences were found between the Shannon–Weaver diversity indices across the evaluated time points in the dogs. Cats had a significantly higher Shannon–Weaver diversity index before administration of the synbiotic (mean \(\pm\) SD: 4.9 \(\pm\) 0.3) when compared to the indices at 5 days of feeding the synbiotic \((4.3 \pm 0.6, P < 0.05)\) or at 2 days after the end of administration \((4.3 \pm 0.6, P < 0.01)\), respectively.

**Effect of the synbiotic on gastrointestinal and immune function**

With the exception of one cat and one dog that vomited once during synbiotic administration, all enrolled animals were reported by the owners to eat and behave normally during synbiotic administration. Similarly, fecal consistency was reported as normal by the owners with the exception of two cats and two dogs that were recorded to have pulpy feces for 2 days during the administration period. No flatulence was recorded in any cat during the 21 days of synbiotic administration. In contrast, owners reported that seven dogs had some flatulence (scores: \(0 = \) no flatulence, 5 dogs; \(1 = \) some flatulence, 7 dogs; \(2 = \) frequent flatulence, 0 dogs) for at least 1 day during synbiotic administration. Other clinical signs of gastrointestinal health (appetite, defecation frequency, volume of feces) in both cats and dogs were judged to be normal by the owners during treatment with the synbiotic (21 days). With the exception of lymphocytes in the cats and neutrophils in the dogs, which were decreased, although not significantly, during synbiotic administration, none of the evaluated serum (cobalamin, folate, IgA, TLI, and PLI) or fecal (IgA and \(\alpha_1\)-PI) markers of gastrointestinal and immune function changed significantly after 3 weeks of administration or 3 weeks after discontinuation of the preparation (Table S3).
Discussion

Despite the numerous applications of prebiotics, probiotics, and synbiotics in veterinary medicine, to date little is known about the in vivo effects of these agents on the composition of the intestinal microbiota of cats and dogs. Several studies have used traditional culture techniques to evaluate the effects of probiotics and prebiotics on fecal microbial composition of dogs (Swanson et al., 2002; Vahjen & Manner, 2003). Also, culture techniques have been used to assess the effect of fructooligosaccharides on duodenal bacterial populations of cats (Sparkes et al., 1998). However, it is well acknowledged today that traditional culture techniques have limitations in fully characterizing complex microbial communities (Ritz, 2007) like those found in the mammalian gastrointestinal tract.
Molecular tools are now widely available to identify intestinal microbial phylotypes (Furrie, 2006). Molecular methods, such as fluorescent in situ hybridization, have been used to evaluate the effect of the probiotic *L. acidophilus* DSM13241 on fecal bacterial populations in cats (Marshall-Jones et al., 2006) and dogs (Baillon et al., 2004). Also, one study has recently evaluated the effect of the probiotic *E. faecium* SF68 on fecal microbial diversity of cats with feline herpesvirus infection using DGGE (Lappin et al., 2009). However, the application of traditional molecular tools to fully characterize the composition of the gastrointestinal microbiota can also be technically and economically challenging. Recently developed high throughput technologies allow a more in depth phylogenetic coverage of the intestinal microbiota (Zoetendal et al., 2008; Suchodolksi et al., 2009) and thus could be useful in evaluating the overall effect of prebiotic, probiotic, or synbiotic formulations on intestinal microbial communities.

This study used various complementary molecular tools to evaluate the effect of a synbiotic formulation on fecal bacterial composition of healthy cats and dogs. Our results indicate that whereas the administration of the multi-species synbiotic preparation for 21 days induced several changes in the abundance of specific probiotic groups in both cats and dogs, the fecal microbiota was not altered on higher phylogenetic levels as evidenced by 454-pyrosequencing. We observed the appearance of DGGE bands in 11/12 cats and 5/11 dogs that were not present before or after synbiotic administration. Sequence analysis identified these bands as *E. faecium*, suggesting that these organisms were more abundant in feces during the ingestion of the synbiotic. Similarly to these results obtained with DGGE, the analysis of 16S rRNA gene clone libraries revealed that bacterial species contained in the administered synbiotic, such as *B. longum* and various *Lactobacillus* spp. matching those contained in the synbiotic formulation, were present in the feces of most cats (8/11) and dogs (9/11) during the administration of the synbiotic, but were undetectable at baseline or after administration was discontinued. This observation further suggests an increase in fecal abundance of the ingested probiotic organisms during the administration period. However, the phylogenetic coverage of 16S rRNA gene clone libraries is generally limited to the species level (Suchodolksi et al., 2008). Therefore, we cannot confirm if the appearance of the bacteria in feces were truly the ingested probiotic strains contained in the synbiotic, or if they belonged to the same bacterial species but were in fact different strains. Nonetheless, the fact that these particular species increased during the administration period, but were not detectable before and after the administration period in most of the animals, strongly suggests that the administration of the product led to an increase of these probiotic groups in the feces of the enrolled cats and dogs. Of further interest is that the detection of the probiotic species by the 16S rRNA gene clone libraries was not equal among individual cats and dogs. For instance, in some animals only one probiotic species was detected during the administration period, whereas in other animals several probiotic species could be identified during administration of the synbiotic. These observations suggest a highly individualized response of the host to administered probiotic species.

The fact that the probiotic groups were undetectable at baseline suggests that the bacterial species contained in the synbiotic were not present in the gastrointestinal tract of the enrolled cats and dogs. The probiotic strains in the synbiotic preparation have in fact been derived mostly from human and dairy sources. Although it has been suggested that canine-derived bacterial species exhibit host specificity (McCoy & Gilliland, 2007), to date there are no studies confirming this assumption. In fact, probiotics may not need to be native to colonize the intestinal tract of the recipient host, because the adherence of these agents to intestinal mucus has been shown not to be host-specific (Rinkinen et al., 2003). Furthermore, studies have shown that canine-derived probiotic strains can successfully transit the murine gastrointestinal tract (OMahony et al., 2009) and can adhere to both human and canine intestinal mucus in a similar fashion (Strompfova et al., 2004). To our knowledge there are only a few probiotic strains that have been derived from dogs (Strompfova et al., 2004; Biagi et al., 2007; McCoy & Gilliland, 2007; O’Mahony et al., 2009; Martin et al., 2010), and no canine-specific probiotic strains have been described in the literature to date.

Similarly to the findings using DGGE and 16S rRNA gene clone libraries, quantitative real-time PCR analyses showed increases in fecal abundance of probiotic bacterial groups within a few days after the subjects started consuming the synbiotic preparation (Fig. 1), with abundances returning to baseline levels after discontinuation of synbiotic administration. Several studies in humans have shown that qPCR can be useful in detecting quantitative increases of ingested probiotics in feces (Bartosch et al., 2005; Vitali et al., 2010), although an inter-individual host response to the dietary intervention is frequently noticed. This variation among subjects was also noticeable in our study, as evidenced by the wide variability in fecal abundances of the target groups within each time point (Fig. 1). Interestingly, in the current study the observed quantitative increases also varied among the four bacterial genera contained in the synbiotic preparation. The fecal abundance of *Lactobacillus* and *Bifidobacterium* spp. was not significantly increased during synbiotic administration,
whereas abundances of *Enterococcus* and *Streptococcus* spp. were significantly increased during at least one time point during synbiotic administration in both cats and dogs. This observation is likely due to the unequal proportions of each probiotic strain contained in the synbiotic preparation evaluated. It is common for commercially available multi-species probiotic formulations to contain unequal quantities of microorganisms. For instance, a multi-species probiotic for use in humans (VSL#3; VSL Pharmaceuticals) is known to contain different concentrations of at least one probiotic strain (Pagnini et al., 2010).

In spite of the observed increases of the ingested probiotic groups in feces using DGGE, 16S rRNA gene clone libraries, and qPCR, pyrosequencing of the 16S rRNA gene did not reveal major changes in the proportions of the most abundant fecal bacterial phyla such as *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Proteobacteria*, and * Fusobacteria* (Tables S1 and S2). This observation is in agreement with other studies showing that ingestion of probiotics leads to fecal colonization by the ingested groups but does not alter the major bacterial groups in the intestine (Venturi et al., 1999). This may be due in part to the low abundance of the targeted probiotic groups (i.e. *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, and *Streptococcus* spp.), as representatives of these groups accounted for <1% of all identified sequences in both cats and dogs at baseline (Tables S1 and S2). Also, the effect of probiotic/prebiotic formulations on the intestinal tract may be more evident at the functional level (i.e. production of lactic acid and/or short-chain fatty acids) rather than in addition to changes in the abundance of intestinal microbial groups. For example, it has been shown that as low as 0.2% or 0.4% of inulin or short-chain fructooligosaccharides can be effective at modifying fecal concentrations of short-chain fatty acids in dogs, while minimally altering the abundance of fecal bacterial populations (Barry et al., 2009). Also, in humans, the ingestion of two probiotic strains (i.e. *Lactobacillus helveticus* Bar13 and *B. longum* Bar33) led to significant increases of acetic and valeric acids but did not modify the overall structure of the fecal microbiota, as assessed by DGGE (Vitali et al., 2010). More studies are needed to investigate the effect of synbiotics on the functional (metabolic) activities of the gastrointestinal microbiota as a supplement to phylogenetic analysis.

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

In the present study we also showed that 21 days of oral administration of a synbiotic preparation did not lead to adverse gastrointestinal effects and may not interfere with markers of gastrointestinal (e.g. serum cobalamin and folate concentrations) or immune (e.g. fecal and serum IgA concentration) function. Similarly, one study showed that the administration of the probiotic *E. faecium* SF68 for 6 weeks did not change fecal IgA concentrations in adult dogs with chronic giardiasis (Simpson et al., 2009). In contrast, an increase in fecal IgA in young dogs has been described after oral administration of the same probiotic strain (i.e. *E. faecium* SF68), although this effect was only evident after 30 weeks of probiotic administration (Benyacoub et al., 2003). Possible explanations for the discrepancy between this study and our investigation may include a shorter period of probiotic administration (3 vs. 30 weeks) and also a more age-heterogeneous group of individuals, as age-related differences in immunological parameters have been observed in both cats (Campbell et al., 2004) and dogs (Blount et al., 2005).

Limitations of this study include the fact that each individual animal was fed a different diet, lived in a different environment, and/or received the formulation in a different manner, all factors that could have potentially influenced our ability to detect major alterations in the fecal microbiota due to the administration of probiotics. Moreover, all animals were fed the same dose of probiotic bacteria (5 × 10^8 CFU) with no regard to differences in body weight (e.g. the body weight among the dogs differed up to eightfold). Although there is growing evidence suggesting a dose-dependent clinical effect of probiotics (Pagnini et al., 2010), to our knowledge no information about the efficacy of various doses of synbiotic have been published for dogs and cats. Although preliminary data in our laboratory suggest that the body weight of dogs may not be a significant predictor to forecast an increased abundance of ingested probiotics in the feces (J.F. Garcia-Mazcorro et al., unpublished observations), clearly more studies are needed to evaluate if there is a dose-dependent effect of synbiotics on the microbiome of dogs and cats. Also, the separate contribution of each component in the synbiotic preparation (i.e. prebiotics and probiotic bacteria) to the changes observed in the fecal bacterial populations was not assessed independently. This is important because it
has been shown in both humans (Worthley et al., 2009) and dogs (Swanson et al., 2002) that the probiotic and the prebiotic component of a synbiotic formulation, when administered separately, may have different effects on fecal bacterial populations. Finally, molecular methods generally cannot confirm the viability of bacteria, and therefore it is possible that the isolated fecal bacterial DNA belonged to both viable and nonviable microorganisms (Palka-Santini et al., 2003).

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

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References


Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Median (interquartile range) pyrosequencing bacterial tags (percentages of all sequences) obtained before synbiotic administration at baseline (BL), after 5 days of administration of the synbiotic formulation (day 5), and 2 days after discontinuation of administration of the synbiotic formulation (day 23) in the enrolled cats.

Table S2. Median (interquartile range) pyrosequencing bacterial tags (percentages of all sequences) obtained before synbiotic administration at baseline (BL), after 5 days of administration of the synbiotic formulation (day 5), and 2 days after discontinuation of administration of the synbiotic formulation (day 23) in the enrolled dogs.

Table S3. Mean concentrations (± SD) of relevant blood, serum, and fecal markers obtained before (baseline), during (day 21), and after (day 42) synbiotic administration.

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