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Effects of *Giardia lamblia* Colonization and Fenbendazole Treatment on Canine Fecal Microbiota

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The gut microbiota (GM) is the sum of hundreds of distinct microbial species that can equal or outnumber their host's somatic cells. The GM influences a multitude of physiologic and immunologic processes in the host, and changes in the GM have been shown to alter the phenotypes of animal models. Previous studies using rodents have also shown that the composition of the GM is affected by many factors, including diet, husbandry, housing, and the genetic background of the animals. However, limited information exists about factors that may modulate GM in other laboratory species, such as dogs. We sought to eliminate sporadic *Giardia* colonization of dogs using fenbendazole (FBZ), an antiprotozoal widely used in biomedical research dog colonies. Concerns that FBZ could have inadvertent effects on the canine GM led us to assess GM over the course of treatment. FBZ (50 mg/kg) was given orally to all dogs in 3 different facilities (n = 19 to 25) for 10 consecutive days. Fecal samples were obtained 2 d before the initiation of treatment, on the last day of treatment, and 2 wk after the completion of treatment. Targeted 16S rRNA gene sequencing was used to analyze fecal microbiota. All dogs were clinically normal throughout the sample collection period. Statistical analyses of data showed significant differences between dogs housed in the 3 different facilities, further emphasizing the effect of housing and husbandry factors on the GM. However, negligible differences were seen between time points, indicating that FBZ did not significantly alter the canine GM. Comparison of the GM of *Giardia lamblia* positive and negative dogs revealed no significant difference between the 2 groups. These findings suggest that FBZ can be used therapeutically in dogs with minimal impact on the GM. Furthermore, the presence of *G. lamblia* in clinically normal animals may not be sufficient to influence the normal canine microbiota.

Abbreviations: GM, gut microbiota; FBZ, fenbendazole; PCR, polymerase chain reaction; IFA, immunofluorescent assay; OTU, operational taxonomic unit; gdh, glutamate dehydrogenase; PCoA, Principal coordinate analysis; PERMANOVA, permutational multivariate analysis of variance; MU, University of Missouri

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Giardia lamblia is a flagellated parasite that is also known as *Giardia duodenalis*. It has a wide range of host species and infects humans, wildlife, livestock, and companion animals, such as dogs and cats. It is one of the most common intestinal protozoan infections reported globally. *Giardia lamblia* has 2 life stages, the trophozoite, and the cyst. Trophozoites can be found in diarrhea of infected dogs and cats, but do not survive for a prolonged period outside the host.

In contrast, cysts are resistant in the environment, surviving several months outside the host in wet and cold conditions. Domesticated dogs may either harbor the parasite subclinically or suffer a similar array of clinical signs similar to those observed in humans. The latter include soft to watery feces, often with a strong odor and laden with mucus. Because giardiasis is a recognized veterinary problem, testing for this agent is often performed when canine patients are presented to veterinarians.

Even though *Giardia lamblia* has a significant impact on both public and veterinary health, little is known about interactions between this protozoan parasite and the canine gut microbiota (GM). The commensal GM consists of hundreds of microbial species that in recent years, have been implicated as a vital factor that influences hosts physiologic and immune responses.[20] *Giardia*-infected mice have been found to have altered gut microbiota (GM) and compromised epithelial barrier function in the presence of this protozoan.[3,9] Based on previous studies in murine models, giardiasis has been suggested as a factor that may alter[3,5,9,20,28] resident microbiota, and that this alteration may lead to phenotypic changes in canine models of disease.

Along with parasitic gastrointestinal infections, husbandry and medical treatments are factors that have been found to alter host microbiota.[13] This has been best described in murine models, with factors such as diet (formulation, shelf life, manufacturer, and sterilization process), husbandry (cage type, bedding, water source and additives, and housing density),[7,12] source of animals (commercial vendor, repository, and collaborators),[7,11,24] therapeutics,[13] and experimental[16] procedures which all inducing alterations in host microbiota. One of the most commonly used drugs in treating *Giardia* and other protozoa infections is metronidazole. This drug also has pronounced effects on anaerobic bacteria, thus impacting the gut microbiota.[10,19] Fenbendazole (FBZ) is a commonly used drug that treats a wide range of intestinal parasites. Although no drugs have been approved by the FDA for the treatment of giardiasis in dogs in the United States, FBZ is often used off-label for this purpose. FBZ prescribed at anthelmintic dosage was effective for treatment of

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G. lamblia infection in several studies.[4,22,23,26,27,31] However, the potential impact of FBZ on canine GM is unknown.

FBZ has been widely and safely used in many species to address giardiasis for more than 2 decades.[6,15,31] Recently, FBZ treatment of mice for pinworm prophylaxis was shown to cause minimal GM changes.[21] Although FBZ is a commonly used drug, to the authors' knowledge, little is known about the potential effects of FBZ in species other than mice in respect to FBZ's influence on the canine GM. The current study aimed to address sporadic *Giardia* colonization identified in canine colonies at the University of Missouri, and to determine whether treatment with FBZ has any impact on host GM. Analysis of initial samples also aided in the assessment of the potential influence of *Giardia* on the GM in subclinically infected animals. We hypothesized that FBZ would have minimal impact on the GM of clinically normal dogs, regardless of their *Giardia* colonization status. For this study, all dogs were treated with FBZ according to a previously published protocol.[27] Fresh fecal samples were collected noninvasively from dogs housed in 3 different facilities with confirmed cases of *Giardia.* Samples were processed for DNA extraction and targeted 16S rRNA amplicon sequencing, which allowed statistical comparisons of both pre- and posttreatment GM, and of the GM of *Giardia* infected and noninfected samples.

Materials and Methods

Animals. Our study included dogs of various ages and sizes housed in 3 different facilities. All the dogs were routinely socialized and exercised indoors under the supervision of veterinary, husbandry, and research staff. Dogs were group-housed unless there were veterinary or scientific reasons that required them to be singly housed. All dogs were cared for according to federal and institutional regulations in AAALAC-accredited facilities and all procedures were approved by the University of Missouri IACUC.

Facilities. Facility 1 (n = 24) housed adult (aged 1 approximately 4 y) female hound dogs purchased from a commercial vendor (Marshall BioResources, NY). Upon arrival, all dogs from the vendor received physical and fecal examinations. None of the dogs had *Giardia* cysts or other parasites when screened by fecal floats and microscopic examinations of fecal smears. Dogs in this facility were fed with Purina ProPlan Puppy food (Purina, St Louis, MO). Facility 2 (n = 16) housed dogs of various ages (aged 8 wk to approximately 5 y) and breeds. Juvenile and adult Dachshunds bred inhouse were fed Purina ProPlan Puppy food (Purina, St Louis, MO). Adult Beagles (Marshall BioResources, NY) were fed Lab Diet 5006, and adult Creagles (Beagle and Chinese Crested dog crosses bred inhouse) were fed custom diets as they were already enrolled on a diet study. Facility 3 (n = 20) housed puppies and adult (aged 8wk approximately 6 y) dogs of Golden retriever, Labrador retriever, and Beagle backgrounds, bred inhouse and fed Purina ProPlan Puppy food (Purina, St Louis, MO).

Fenbendazole (FBZ) treatment. A small number of dogs in each facility developed diarrhea and tested positive for *Giardia* using a SNAP test kit (IDEXX Laboratories, Columbia, MO). FBZ (50 mg/kg) was used to treat all the dogs in the 3 facilities, regardless of infection status, for 10 consecutive days according to a published protocol.[27] Dogs were bathed with Dawn Ultra liquid soap, and vacated rooms were sanitized with accelerated hydrogen peroxide solution (Rescue, Virox Animal Health, ON, Canada) on the 5th and 10th day of treatment for environmental control of *Giardia lamblia* cysts.

Sample collection and DNA extraction. Fresh fecal samples were collected 2 d before the initiation of FBZ treatment, on the last day of treatment, and 2 wk after cessation of treatment. Fecal samples were placed in 2 mL round bottom microfuge tubes with a 0.5 cm-diameter stainless steel ball bead. Eight hundred microliters of lysis buffer were added into each tube, and they were mechanically agitated for 3 min using a TissueLyser II (Qiagen, Venlo, Netherlands). The samples were incubated at 70 °C for 20 min with brief vortexing every 5 min. The tubes were spun at 5000 × g for 5 min, and supernatants were transferred to 1.5 mL microfuge tubes. Two hundred microliters of 10 mM ammonium acetate were added to supernatant and mixed well. These samples were then incubated on ice for 5 min. After incubation, the tubes were spun at 5000 × g for 5 min. Seventy-five microliters of supernatant were moved to 1.5 mL microfuge tubes, 750 µL of chilled isopropanol were added, and the suspension was vortexed. The tubes were spun at 16,000 × g at 4 °C. Supernatant was carefully aspirated and discarded, leaving DNA pellets at the bottom. The pellets were resuspended in 150 µL of Tris-EDTA in warm water bath at 37 °C for 10 to 30 min with intermittent vortexing to loosen the pellets. Fifteen microliters of proteinase-K and 200 µL of Buffer AL from DNeasy Blood and Tissue kit (Qiagen, Venlo, Netherlands) were added, and the samples were incubated at 70 °C for 10 min. 200 µL of 100% ethanol was added and mixed well. Samples were then transferred to DNeasy columns and spun at 16,000 × g for 1 min. Flow-through was discarded, and the columns were washed with 500 µL of Buffer AW1 at 16,000 × g for 1 min, and this process was repeated with 500 µL of Buffer AW2 at 16,000 × g for 3 min. DNA was eluted in 200 µL of EB buffer into clean 1.5 mL microfuge tubes. DNA yields were determined using fluorometry (Qubit, Life Technologies, Carlsbad, CA) using quant-iT BR ds DNA reagent kit (Invitrogen, Carlsbad, CA).

16S rRNA library preparation and sequencing. The extracted fecal DNA was submitted to the University of Missouri DNA Core Facility for amplification and sequencing as previously described.[18] Briefly, an amplicon library of the V4 region of the 16S rRNA gene was generated using normalized DNA as a template. Single-indexed universal primers (U515F/806R) flanked by Illumina standard adapter sequences and PCR parameters of 98 °C (3 m) + [98 °C (15 s) + 50 °C (30 s) + 72 °C (30 s)] × 25 cycles + 72 °C (7 m) were used. Amplicons were then pooled for sequencing using Illumina MiSeq and V2 chemistry with 2 × 250 bp paired-end reads.

Informatics. All assembly, filtering, binning, and annotation of contiguous sequences was performed at the University of Missouri Informatics Research Core Facility, as previously described,[18] with the exception that selected operational taxonomic units (OTUs) were annotated using BLAST[1] against the SILVA database[25] of 16S rRNA sequences and taxonomy.

Statistical analysis. Based on rarefaction curves, samples with less than 10,000 sequence reads were excluded from analysis. Differences in β-diversity were determined using permutational multivariate analysis of variance (PERMANOVA) of Bray-Curtis (shared abundances of OTUs) and Jaccard (unique presence or absence of OTUs) similarities using the open-access Past 3.16 software. Principal coordinate analysis (PCoA) was performed using Past 3.16 software, and the relative abundance data was fourth-root transformed to normalize the data. OTU richness and diversity indices were tested for normality using the Shapiro-Wilk method. Differences in richness and diversity were then statistically analyzed by ANOVA with Tukey posthoc test on SigmaPlot 12.3 (Systat software, San Jose, CA). The threshold for significance in all cases was $P \leq 0.01$.

Conventional *Giardia lamblia* PCR. Primers were designed to amplify fragments from *gdh* gene of *G. lamblia* using Primer3

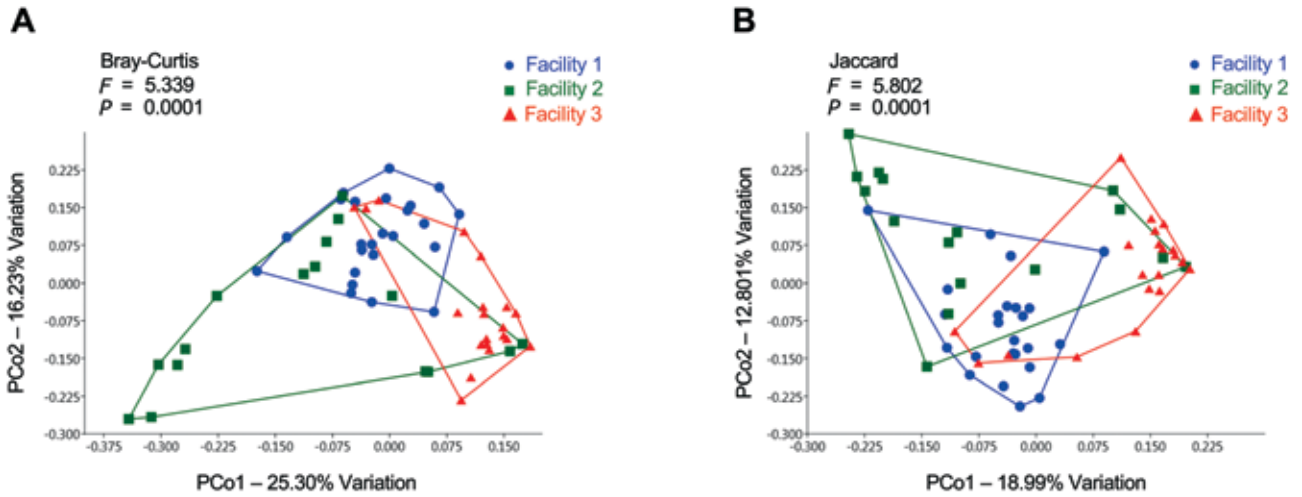

Figure 1. Principal Coordinate Analysis (PCoA) of fecal GM from dogs in 3 different housing facilities. PCoA plots of ranked Bray-Curtis (A) and Jaccard (B) similarity indices from all 3 canine housing facilities at the pretreatment time point. Dogs in the 3 facilities showed significant differences in canine fecal microbiota, indicated by distinctive grouping. PERMANOVA with $P \leq 0.01$ considered significant.

(http://bioinfo.ut.ee/primer3/). The primers used for the detection of *G. lamblia* were forward 5′-TCTCTGACTCCAACG-GAACC-3′ and reverse 5′-CCAGGGCTTCTTGTTTTCGT-3′, which resulted in 155 bp amplicons. PCR was conducted using FastStart taq (Roche). The amplification reactions (20 µL total) contained 1 to 2 µL of DNA, 10× PCR buffer, each deoxynucleotide triphosphate at a concentration of 1.25 mM, each primer at a concentration of 25 µM, and 5 U/ µL of FastStart Taq DNA polymerase (Roche). Cycling parameters were 15 min at 95 (initial heat activation step), followed by 45 cycles of 20 s at 94, 45 s at 58.8, and 34 s at 72, with a final extension of 10 min at 74. Positive controls were obtained from one of the dogs that tested positive with *Giardia* SNAP test (IDEXX Laboratories, Westbrook, ME). DNase-free water (Thermo Fisher Scientific, Waltham, MA) was used for the negative controls. PCR products were detected using a QIAxcel (Qiagen, Venlo, Netherlands), according to the manufacturer's instructions for DNA fragments analysis.

Results

GM differences by facilities. GM profiles from dogs housed in 3 different facilities were compared prior to FBZ treatment to evaluate any colony-dependent differences. When samples from each facility were visualized via PCoA, distinctive clustering patterns were found. These differences in GM profile were significantly different, based on one-way PERMANOVA (Figure 1) using both Bray-Curtis and Jaccard similarity indices. Facility 2 housed 3 different breeds of dogs that were on various diets, and greater variability in microbiota composition was observed in this facility.

GM richness and diversity differences by facilities. Canine GM richness and diversity were also compared among the 3 facilities. To measure richness of each group, the number of observed OTUs was counted for each sample. We found no significant difference in GM richness among the 3 facilities (Figure 2 A). Diversity of the samples was calculated using the Shannon diversity index, which combines richness and evenness of the OTUs. A significant difference ($P \leq 0.01$) in diversity was demonstrated between Facility 2 and 3 (Figure 2 B).

FBZ treatment impact on canine GM compositions by facilities. To determine the effect of FBZ treatment on the GM, fecal samples were collected one day after the completion of the 10-d treatment regimen (Figures 3 A through F). Comparison of pre- and post-FBZ treatment samples revealed no significant difference in GM compositions in Facility 1 ($P = 0.9982$) or Facility 2 ($P = 0.062$) using the Bray-Curtis index (Figures 3 A and 3 B). However, when Facility 1 and 2 were evaluated using the Jaccard index (Figures 3 D and 3 E), significant differences were detected between time points (Facility 1 $P = 0.0001$; Facility 2 $P = 0.0076$), despite substantial overlap on ordination. These findings likely reflect differences in the presence or absence of rare taxa, which the Jaccard index highlights; in contrast, the Bray-Curtis index puts greater emphasis on differences in the relative abundance of taxa. Although a significant difference in GM compositions was observed after FBZ treatment in Facility 3 using the Bray-Curtis index ($P = 0.0039$), PCoA analysis showed marked overlap in the 2 groups (Figure 3 C). No significant differences between the time points were detected when the GM of Facility 3 was evaluated using the Jaccard index (Figure 3 F). These disparate results may be due to the many environmental variables present in Facility 3. This facility was highly active, with research and veterinary care activities that involved high traffic of laboratory and veterinary personnel, and periodic movement of animals both within the facility and to the MU Veterinary Health Center. Furthermore, these dogs often had complicated clinical cases and presented with GI and respiratory signs due to their model phenotype. Diet changes made to address medical issues associated with the experimental models were also common for dogs housed in Facility 3. Collectively, these findings suggest that FBZ has only modest effects on the composition of canine GM. Serial *t* tests were performed for each operational taxonomic unit (OTU), to identify any taxa that were present at a significantly different relative abundance at either the pre- or posttreatment time-points. Although 8 OTUs yielded *P* values below 0.05, none withstood correction for multiple testing using the method of Benjamini and Hochberg and an allowed false discovery rate of 10%.

FBZ treatment impact on richness and diversity of canine GM by facilities. Figures 4 A through F show comparisons of GM richness and diversity in each facility pre- and post-FBZ treatment. None of the facilities had significant differences in either of these factors, indicating that FBZ did not influence GM richness or diversity.

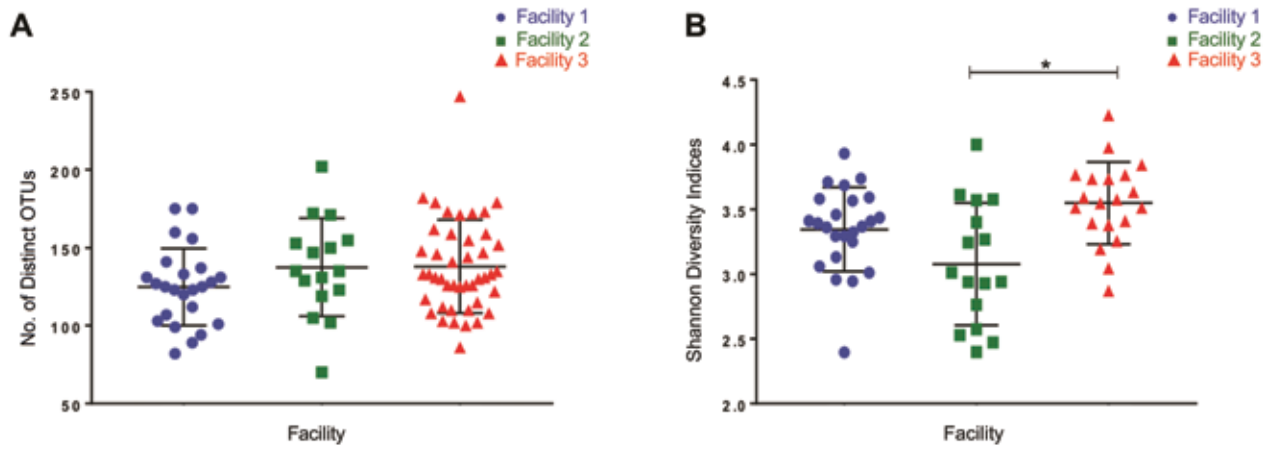

Figure 2. Richness and diversity of canine fecal microbiota by facility before FBZ treatment. No significant differences in the richness of fecal microbiota were found among the facilities prior to treatment with FBZ (A). Significant difference was noted between facilities 2 and 3 before treatment with FBZ (B). Facilities 2 and 3 housed a diverse group of breeds with variable diets. $P \leq 0.01$ considered significant. Bars within points represent mean ± SD.

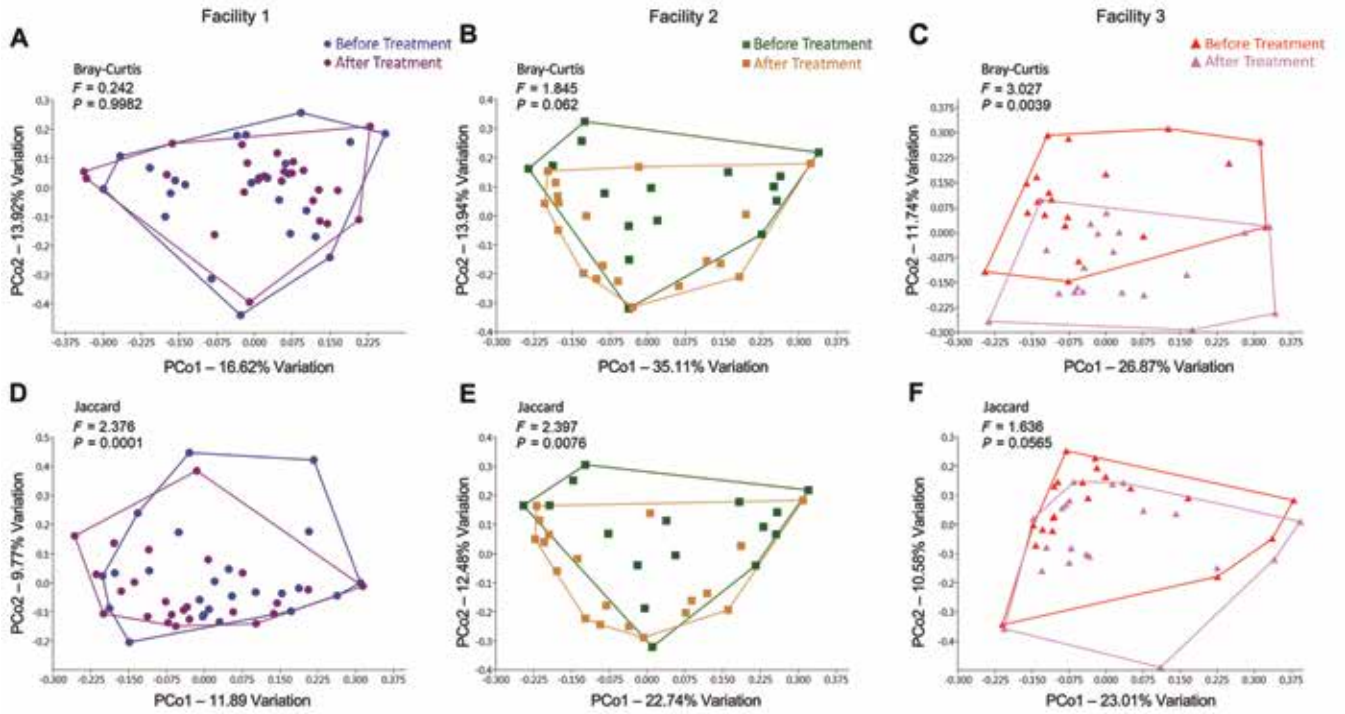

Figure 3. Principal Coordinate Analysis (PCoA) of GM from dogs before and after FBZ treatment. PCoA plots of ranked Bray-Curtis (A through C) and Jaccard (D through F) similarity indices results from before and after treatment fecal microbiota in the 3 different facilities. Dogs in facilities 1 (A) and 2 (B) did not show significant changes in the canine fecal microbiota after FBZ treatment using Bray-Curtis similarity indices. Fecal microbiota from dogs in facility 3 (C) showed a significant difference between before and after FBZ treatment. Analysis with Jaccard indices of Facility 1 (D) and 2 (E) showed significant differences. For Facility 3, analysis with the Jaccard index did not show significant differences (F). PERMANOVA with $P \leq 0.01$ considered significant.

***Giardia* status and GM.** The present study also allowed us to examine the relationship between patent *Giardia* colonization and GM. Figure 5 A shows a comparison of *Giardia* prevalence before and after the FBZ treatment in each facility. Prior to FBZ treatment, 10 samples out of 25 fecal samples (40%) from Facility 1 tested positive for *Giardia lamblia* using the described conventional PCR assay. Facility 2 had 7 samples out of 18 samples (38.9%) positive for *Giardia*, and Facility 3 had 15 animals positive for *Giardia* out of 20 samples total (75%). While FBZ treatment appeared to be largely effective, 2 animals remained positive after treatment. Using fecal samples obtained 2 wk after FBZ treatment, Facilities 1 and 3 each had one *Giardia*-positive animal. PCoA and PERMANOVA of *Giardia lamblia* positive and negative animals collected prior to FBZ treatment revealed no significant differences in GM using either Bray-Curtis (Figure 5 B) or Jaccard (Figure 5 C) indices. These results indicate that the presence of subclinical infection with *Giardia lamblia* in dogs does not significantly influence their GM.

Discussion

Giardia infections in laboratory dogs can potentially result in clinical symptoms due to altered gut barrier function. However,

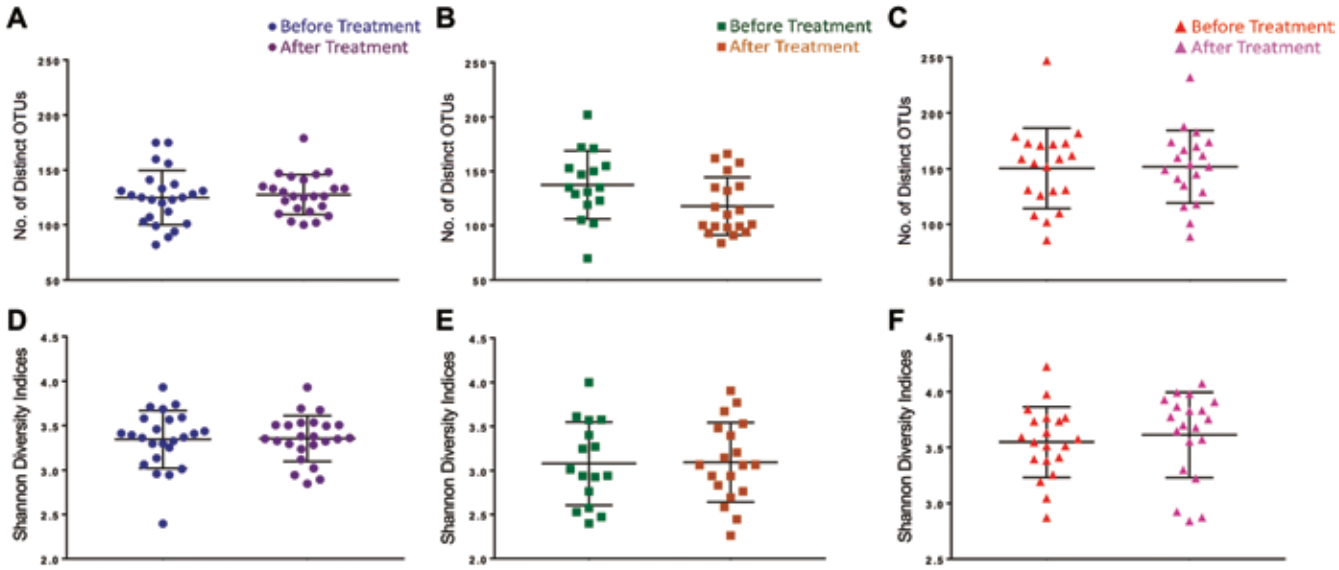

Figure 4. Richness and diversity of canine fecal microbiota by time point. There were no significant differences in canine GM before and after FBZ treatment in all 3 facilities (A through F). $P \leq 0.01$ considered significant. Bars within points represent mean ± SD.

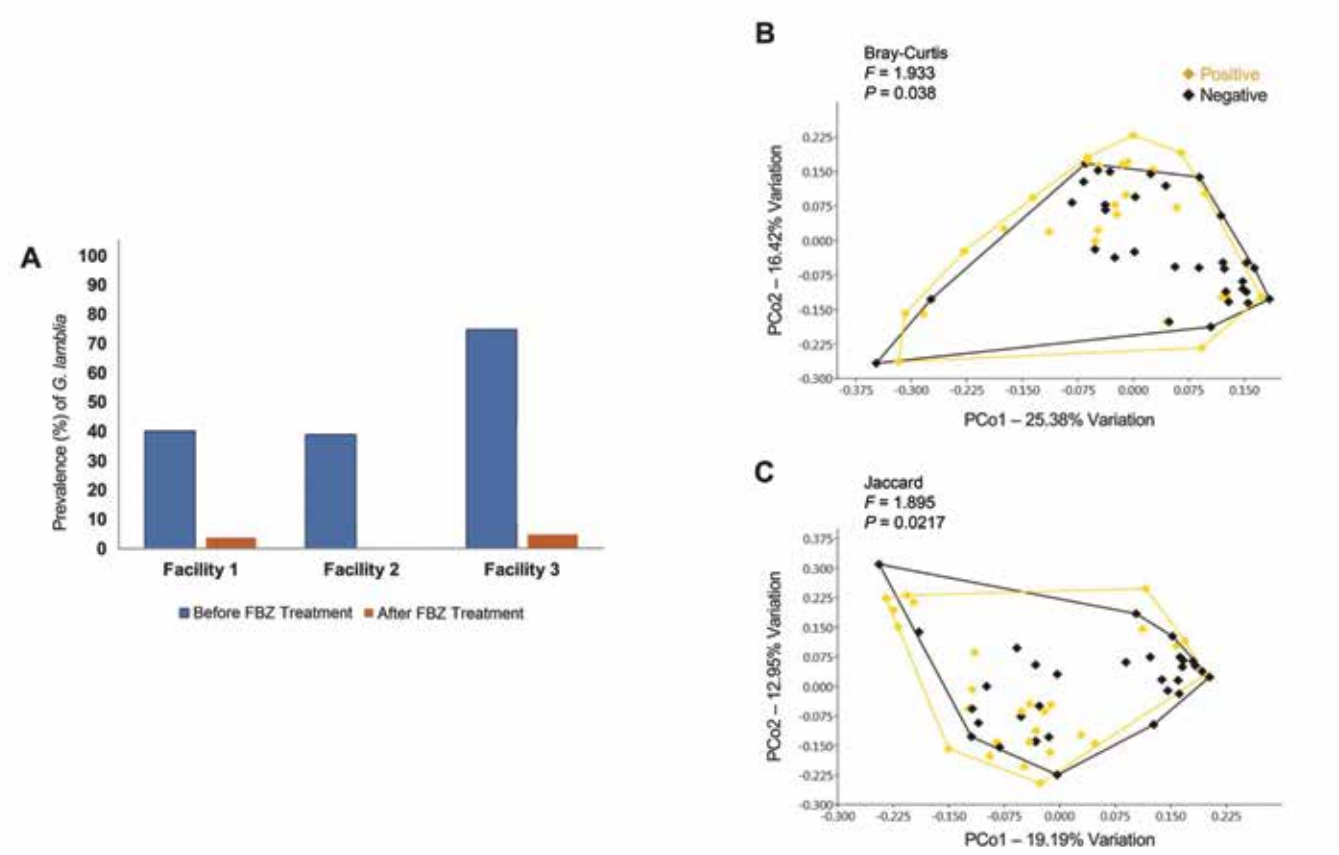

Figure 5. Efficacy of FBZ in treatment of *Giardia lamblia* in canine GM. All dogs had normal fecal consistency at the time of fecal collection. Prior to FBZ treatment, 50.8% of all dogs tested were positive for *Giardia;* 3.1% (2 dogs total) remained positive after FBZ treatment (A). PERMANOVA and PCoA of GM from dogs with different *Giardia* status showed no significant different between *Giardia* negative and positive dogs on Bray-Curtis (B) and Jaccard (C) indices. $P \leq 0.01$ considered significant.

most infections with this pathogen appear to be subclinical. Regardless, when detected, treatment is often initiated, and very little is known about the impact of either *Giardia* colonization or FBZ treatment on GM. While evidence indicates that *Giardia* alters gut barrier function in rodents and the immune responses of experimentally infected mice,[9,20] very little information is available on *Giardia* colonization and canine gut microbiota composition. Differences in the GM composition are associated

with protection or resistance against *Giardia* infection in mice,[28] indicating that *Giardia* infection with certain "normal" GM may not lead to clinical manifestations of *Giardia* infection. Based on these observations, *Giardia* infection may or may not have a significant impact on host physiology and GM composition, and the presence of *Giardia* alone does not always indicate altered GM or gut barrier function.

Reports on the prevalence of *G. lamblia* colonization vary in domestic dogs and cats, depending on populations, regions, and diagnostic methodologies. Young animals are more likely to be infected with *Giardia* than older animals. Literature reviews[2,8,22] of reports from various countries describe prevalence ranges as low as 0.1%, to as high as 69.9% in younger dogs in breeding kennels. Some report detection of *G. lamblia* in dogs displaying no clinical disease. 7 different genome assemblages (A through G) have been identified based on genetic analyses.[2] Host specificity of different assemblages has been determined, but cross-infections have also been observed. Genotyping of *Giardia lamblia* is not a test that is routinely performed when diagnosing giardiasis, and potential for cross-infection of assemblages makes *Giardia lamblia* a potential zoonotic pathogen.

Overall, evidence of the effects of *Giardia* colonization on the canine GM is generally lacking. Most studies have been performed in privately-owned animals or animals in shelters or kennels that are more prone to exposures to *Giardia lamblia*. No definitive rationale explains whether health or disease will occur after *Giardia* colonization. Some animals can carry *Giardia* without showing any clinical signs. One study investigated clinically normal village dogs of various regions of Australia;[29] the results from that study agree with our current findings of no significant differences in GM composition (that is, β-diversity), based on the *Giardia* status. Moreover, the village dogs enrolled in the earlier study encompassed much more variability in genetics, dietary, and husbandry factors than did our population of laboratory dogs housed in an AAALAC-accredited facility. Because none of the dogs in our study dogs developed clinical signs, comparison between clinical and nonclinical animals infected with *Giardia lamblia* could not be performed. We speculate that dogs showing signs of *Giardia* infection would more likely have GM dysbiosis as compared with subclinically infected animals, although this would require confirmation through further investigation. Investigations of dogs with acute diarrhea and inflammatory bowel disease have revealed a significant difference in GM compositions of diarrheic and healthy dogs.[14,30] Diarrhea is likely associated with changes in microbiota and gut barrier function; however, our results indicate that the presence of *Giardia* does not preclude a normal GM.

Our study found no consistent difference in β-diversity, α-diversity, and richness of GM based on *Giardia* status, but we did find significant differences in the GM between our 3 facilities. Significant differences from both Bray-Curtis and Jaccard indices indicate differences in global composition between facilities, regardless of the similarity metric used. This finding further highlights the importance of genetic, environmental, and husbandry as factors that potentially influence the gut microbiota. Although numerous reports indicate that of facility and husbandry factors contribute to variations of GM in rodents,[7,11-13] the effects of these GM-modulating factors have not previously been evaluated in a comprehensive controlled study in dogs. Dogs provide important models for numerous human and canine diseases. Differences in GM composition can be a confounding factor in research, and may negatively affect the reproducibility of studies. Recent concerns within the scientific community regarding the reproducibility of biomedical studies have been partially attributed to differences in housing and husbandry factors. Specifically, mice have significant differences in GM compositions depending on their sources[11] and husbandry conditions.[12] Effects of these GM differences can be profound enough to change disease phenotypes.[17] Researchers should be mindful of potential inconsistencies and reduced research reproducibility due to husbandry conditions that may influence the GM, and differences among populations housed in separate facilities.

In summary, we evaluated FBZ as a potential GM-modulating factor in *Giardia* outbreaks. FBZ is widely used to treat gastrointestinal parasites, including *Giardia spp.*, roundworms, hookworms, whipworms, and tapeworms of the genus *Taenia*. It is a safe drug with a wide therapeutic index that has been used in multiple different species and is commonly used by veterinarians caring for laboratory animals. Veterinarians at the University of Missouri used FBZ to address sporadic *Giardia* colonization. To the authors' knowledge, the effects of FBZ on the canine GM had not been studied, while metronidazole,[10] which also has been used to treat giardiasis, has been shown to alter the canine GM after 14 d of treatment.[19] The wide use of FBZ in both companion and research canines generates the need to understand the potential effects FBZ might have on the canine GM. Our results show that FBZ has a minimal effect on the canine GM composition, and that FBZ may be safely used in dogs without altering the composition, richness, or diversity of their GM.

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