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Evaluation of Fecal Microbiota Transfer as Treatment for Postweaning Diarrhea in Research-Colony Puppies

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Frequently just prior to or at weaning (approximate age, 6 to 8 wk), puppies in research settings often develop diarrheal disease, which may be due, in part, to an immature and unstable intestinal microbiota that is permissive to opportunistic pathogens. The overall objective of this study was to assess whether fecal microbiota transfer (FMT) increased the transmission of a stable maternal microbiota to pups and decreased the incidence of postweaning diarrhea. Puppies were designated by litter as treated (FMT) or sham-treated. The FMT group received fecal inoculum orally for 5 consecutive days during weaning (at 6 to 8 wk of age). Diarrhea was evaluated according to a published scoring system for 11 d during the weaning period. Fresh feces were collected from dams and puppies at 3 d before weaning and 3, 10, and 24 d after weaning for analysis of the fecal microbiota by using 16S rRNA amplicon sequencing. The composition of fecal inoculum refrigerated at 3 to 5 °C was stable for at least 5 d. No diarrhea was reported in either group during the study period, making comparison of treated and control groups problematic. However, 16S rRNA gene analysis revealed microbial variability across time in both groups. Therefore, although the fecal microbiota of neither group of puppies mirrored the dam at any of the designated time points, the data provided fundamental and novel information regarding the dynamic maturation process of the fecal microbiota of puppies after weaning.

Abbreviations: FMT, fecal microbiota transfer; GM, gastrointestinal microbiota

Across all veterinary settings, diarrhea is a common occurrence for puppies younger than 6 mo[7,12] and poses a major problem, especially in research settings, because diarrhea can reduce daily weight gain and increase the risk of mortality.[8] Although puppies in conventional settings are exposed to myriad bacterial, viral, and parasitic agents, those in group-housed settings are more likely to have diarrhea due to a viral or parasitic etiology.[7,9]

In the research colonies we studied, puppies often develop diarrhea shortly after weaning. Pooled fecal samples have variably yielded *Cystoisospora* spp. on fecal flotation, but no correlation between coccidial colonization and diarrhea has been observed. Moreover, affected puppies treated with 5% sulfadimethoxine demonstrate inconsistent resolution. Therefore these infections have not been considered as the sole cause of weaning diarrhea, and other contributing factors are likely. Generally speaking, dietary indiscretion during the transition from maternal milk to commercial dog food, as well as environmental and behavioral stresses,[4] are considered to be possible cofactors associated with diarrheal disease in postweaning puppies.

Aberrant shifts in the composition of the gastrointestinal microbiota (GM), referred to as dysbiosis, have been associated with diverse diarrheal diseases.[16-18,20] The GM of puppies, like most mammals, undergoes multiple stressors at the time of weaning, including a switch in the availability of dietary energy sources. In most mammals studied to date, when living under similar environmental conditions, offspring 'inherit' the maternal GM.[2,11] However, its composition does not normalize to that of the birth dam until later in life, often in adolescence.[2,11] We therefore speculated that postweaning diarrhea in puppies may be due, at least partially, to transient GM changes associated with transition from weaning to adulthood.

The goals of this study were to evaluate fecal microbiota transfer (FMT) as a pragmatic and effective means of accelerating the transition of the GM to its adult composition and to assess the potential of FMT as a treatment or preventative measure for postweaning diarrhea in research-setting puppies. Our hypotheses were that FMT would hasten the transmission of a stable maternal microbiota, thereby decreasing the frequency of postweaning diarrhea, compared with those of untreated pups.

Materials and Methods

Population. All experiments were approved by the University of Missouri IACUC. The study population comprised 23 (7 litters) purpose-bred dachshund puppies (age, 6 to 8 wk) and their dams. Litters of puppies and their dams were assigned to groups (intervention or sham-treated) by using a random-number generator, such that 11 puppies received the intervention (FMT), and 12 puppies served as sham-treated controls. The dam of each litter in the FMT-treated group was designated as the fecal donor for her puppies. One puppy in a sham-treated group was removed from its biologic mother immediately after birth due to rejection. This puppy was nursed and housed with his foster mother and litter throughout the entire study.

Study design. ***Housing.*** Each litter was housed individually on elevated pens with a plastic, slat flooring system (Tenderfoot, Tandem Products, Minneapolis, MN). The litters did not comingle at any point during the study but were housed in the same room. The ambient room temperature ranged from

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70 to 75 °F (21.1 to 23.9 °C) with 35% to 50% humidity and 12:12-h light:dark cycles. All puppies were weaned over a 5-d period and had unrestricted access to commercial diet (ProPlan Puppy Dry and Wet Formulas, Purina, St Louis, MO) and water throughout the study period.

Intestinal parasite screening. Each dam was evaluated for intestinal parasites once at 3 d prior to the collection of feces for inoculum, by using a routine sucrose-gradient centrifugation–flotation technique (specific gravity, 1.027) and microscopic examination. Other than coccidian parasites in a single dam, no intestinal parasites were found. Because previous data from this colony showed no correlation between coccidian status and diarrhea and given that the environment in which the animals were housed leads to repeated exposure, we chose to assess FMT independently of coccidian status. To this end, no dams were excluded from study, and puppies were not tested individually for coccidia.

FMT. Puppies designated for FMT received inoculum prepared from the feces of their cognate dam for 5 consecutive days beginning 3 d prior to weaning. Dam feces were collected and refrigerated (3 to 5 °C) each morning beginning 3 d prior to FMT. Fecal inoculum was prepared by blending 100 g of pooled dam feces with 200 mL of 2% reduced-fat bovine milk. The blended inoculum was filtered through cheesecloth to remove large pieces of organic material. The inoculum was stored between 3 and 5 °C in a conventional refrigerator between treatments. Each puppy received 10 mL of the inoculum by oral gavage with a syringe once daily, between 1100 and 1300, for 5 consecutive days. Puppies designated as sham-treated controls received 10 mL of 2% reduced-fat bovine milk by oral gavage according to the described schedule.

Just prior to oral gavage, 0.25 mL of the fecal inoculum to be used that day was placed directly into a sterile 2.0-mL round-bottom tube containing a stainless steel ball (diameter, 0.5 cm) and 800 µL lysis buffer, mechanically disrupted (TissueLyser, Qiagen, Venlo, Netherlands), and stored at –80 °C until DNA extraction.[22]

Fecal scoring. Beginning 3 d prior to weaning, stool samples were scored for 11 consecutive days by a single operator (EB) using the 7-point Nestlé Purina Fecal Scoring System (Figure 1) to identify and monitor diarrhea.[19] Any litter of puppies that received a fecal score of 6 or greater was considered to have diarrhea.

Sample collection and DNA extraction. For both FMT- and sham-treated groups, approximately 2 g of fresh feces for 16S rRNA amplicon sequencing was obtained by using a fecal loop directly from the rectum of each puppy and dam at 3 d prior to weaning and at 3, 10, and 24 d after weaning. Fecal samples were collected between 0600 and 0800 for each time point. The feces collected from each puppy was placed directly into a 2.0-mL sterile round-bottom tube, processed as described previously, and stored at –80 °C until DNA extraction.

DNA was extracted manually by using an adaptation of a published technique.[22] The purity of the DNA preparation was assessed by using spectrophotometry (Nanodrop, Thermo Fisher Scientific, Waltham, MA); yield was determined by using fluorometry (Qubit system and dsDNA BR assay kits, Life Technologies, Carlsbad, CA).

16s rRNA library preparation, sequencing, and informatics analysis. Extracted fecal DNA was processed at the University of Missouri DNA Core Facility. Bacterial 16S rRNA amplicons were generated through amplification of the V4 hypervariable region of the 16S rRNA gene by using single-indexed universal primers (U515F and 806R, Illumina, San Diego, CA) flanked by standard adapter sequences (Illumina) and the following parameters: initial denaturation, 98 °C for 3 min; 25 cycles of 98 °C for 15 s, 50 °C for 30 s, and 72 °C for 30 s; and final elongation, 72 °C for 7 min. Amplicons were then pooled for sequencing by using the MiSeq platform (Illumina) and V2 chemistry with two 250-bp paired-end reads, as previously described.[6]

Briefly, contiguous DNA sequences were assembled by using FLASH software[13] and were culled when too short after trimming for a base quality of less than 31. Qiime version 1.8[10] software was used to perform de novo and reference-based chimera detection and removal, and remaining contiguous sequences were assigned to operational taxonomic units (OTU) through de novo OTU clustering and a criterion of 97% nucleotide identity. Taxonomy was assigned to selected OTU by using BLAST[1] searches of the Greengenes database[5] of 16S rRNA sequences and taxonomy.

Data analysis. Fecal score data were analyzed by using 2-way repeated-measures ANOVA, with litter as the subject and day and treatment as factors. Posthoc pairwise analysis was performed by using the Student–Newman–Keuls method. For microbiota analysis, OTU with reads of less than 10,000 were excluded from the data set. Bar graphs and principal component analyses of data were generated by using Excel (Microsoft, Redmond, WA) and visually inspected for descriptive analysis of consistency between animals (bar graphs) or clustering of animals within groups (principal component analysis). We computed α diversity on the basis of Chao1 indices. A 2-tailed *t* test was performed by using Prism version 6.00c (GraphPad Software, San Diego, CA) for analysis of the α diversity index.

Results

Variability of microbiota in dams. To evaluate microbiota variability, fecal samples were collected at 4 time points and processed for characterization of the fecal microbiota. Representative data from 2 dams yielding complete datasets demonstrate that fecal microbiota varied throughout the study (Figure 2). Complete datasets were not obtained from the remaining 4 dams, but similar instability was apparent among the time points evaluated (data not shown). The 4 most commonly detected OTU from the dams were *Fusobacterium* sp. (mean ± SE, 20.2% ± 12.7%), *Bacteroides* sp. (10.6% ± 9.6%), *Faecalibacterium prausnitzii* (10.4% ± 12.1%), and *Prevotella copri* (9.6% ± 9.4%). The variability in the fecal samples from the dams over time was unexpected and prompted us to assess the fecal inoculum to evaluate stability over the course of the FMT.

Inoculum stability. To evaluate the bacterial composition and consistency of the FMT inoculum over the inoculation period (5 d), a portion of the refrigerated fecal inoculum from a single dam was processed and characterized daily prior to gavage. Only data from days 1, 3, and 5 are shown, because we obtained insufficient sequencing data from the other days' samples. Overall, the bacterial composition of the inoculum remained consistent over the 5 d inoculation period when stored at 3 to 5 °C (Figure 3). The 4 OTU most commonly isolated from the fecal inoculum were *Fusobacterium* sp. (mean ± SE, 32.2% ± 2.0%), *P. copri* (26.0% ± 3.8%), *Bacteroides* sp. (12.8% ± 2.8%), and *Prevotella* sp. (6.2% ± 1.2%). Although culture-independent techniques were used to assess diversity and stability, the data were limited regarding evaluation of bacterial viability to determine whether the bacteria within the fecal inoculum would survive and proliferate once reaching the intestine after transplantation. However, the data do demonstrate that FMT-treated puppies received a similar inoculum each day.

Score	
1	Very hard and dry; requires much effort to expel from body; no residue left on the ground when picked up. Often expelled as individual pellets.
2	Firm, but not hard; should be pliable; segmented appearance; little or no residue left on ground when picked up.
3	Log-like; little or no segmentation visible; moist surface; leaves residue but holds form when picked up.
4	Very moist (soggy); distinct log shape visible; leaves residue and loses form when picked up.
5	Very moist, but has distinct shape; present in piles rather than as distinct logs; leaves residue and loses form when picked up.
6	Has texture, but no defined shape; occurs as piles or spots; leaves residue when picked up.
7	Watery, no texture, flat; occurs as puddles.

Figure 1. Nestlé Purina Fecal Scoring System. Any litter of puppies that received an average fecal score of 6 or greater was considered to have diarrhea.

Variability of microbiota in pups. To evaluate microbiota variability in puppies, fecal samples were collected over 4 time points and processed for characterization of the microbiota. Data from 7 FMT-treated and 3 sham-treated puppies from which complete data sets were obtained are shown in Figure 4; similar trends were seen in the remaining pups (data not shown). Both FMT- and sham-treated puppies displayed variability in fecal microbiota composition at each time point throughout the study (Figure 4 A and B). Despite the high degree of variability, α diversity as determined by the Chao 1 index was not detected (Figure 4 C). To avoid any bias in estimates of diversity due to differences in coverage, Chao1 indices were determined at a uniform coverage attained for all samples. Therefore, at a coverage of 26,281 reads per sample, the Chao1 indices for FMT- and sham-treated groups were 192.6 and 190.6, respectively. The 4 most common OTU found in the FMT litters were *Fusobacterium* sp. (mean ± SE, 27.2% ± 15.1%, *Bacteroides* sp. (12.8% ± 8.5%), *Anaerobiospirillum* sp. (7.9% ± 8.4%), and *P. copri* (7.7% ± 9.2%). In the sham-treated litters, *Fusobacterium* sp. (30.8% ± 11.5%), *Bacteroides* sp. (12.5% ± 7.3%), *Anaerobiospirillum* sp. (10.7% ± 9.9%), and *Sutterella* sp. (7.6% ± 7.9%) were the most common OTU.

Principal component analysis of puppy GM from each litter confirmed marked variability among subjects, with some clustering of litters as the study period advanced. No clustering according to treatment (FMT compared with sham) emerged, but this finding was expected, given that each litter had a different dam and thus source of fecal inoculum (Figure 5). Although litters clustered as time progressed, the fecal microbiota of both FMT- and sham-treated puppies varied from that of their dam and did not trend toward her fecal microbiota at any time point over the course of this study. This phenomenon is shown in Figure 6, in which the dam and her puppy had dissimilar microbiota profiles at the first time point (–3 d). Over time, this puppy did not trend toward her fecal microbiota, as initially expected. Interestingly, the bacterium *P. copri* (Figure 6, brown bar) was 1 of the 4 most common isolates identified in FMT-treated puppies and all dams but not sham-treated puppies. This finding may reflect some degree of successful fecal inoculum transfer from dam to puppy.

Efficacy of FMT for the amelioration of diarrhea. Using the pooled daily fecal scores, we calculated an overall average for each litter during the study period (Figure 7). Evaluation of fecal scores by using repeated-measures ANOVA revealed a difference ($P = 0.023$) in scores between days 2 and 4 of weaning, which was interpreted to be an incidental finding. Importantly, FMT- and sham-treated litters did not differ at any point. The overall average fecal score over 11 d was 4.31 for the 4 FMT-treated groups and 4.44 for the 3 sham-treated groups.

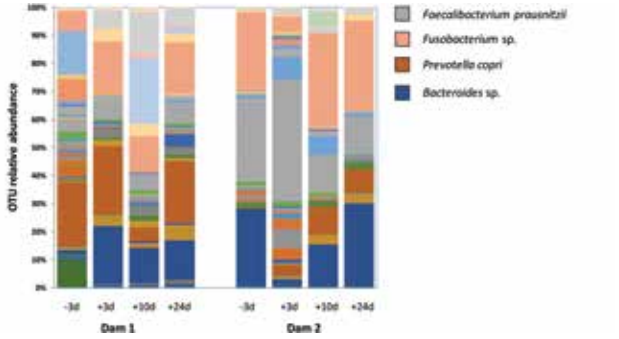

Figure 2. Composition and variability of fecal bacterial species in 2 dams over time. Each bar represents a different fecal sample, including 3 d prior to weaning (–3d) and 3, 10, and 24 d after weaning (+3d, +10d, and +24d, respectively). Each color within a bar represents the relative abundance of an individual operational taxonomic unit (OTU).

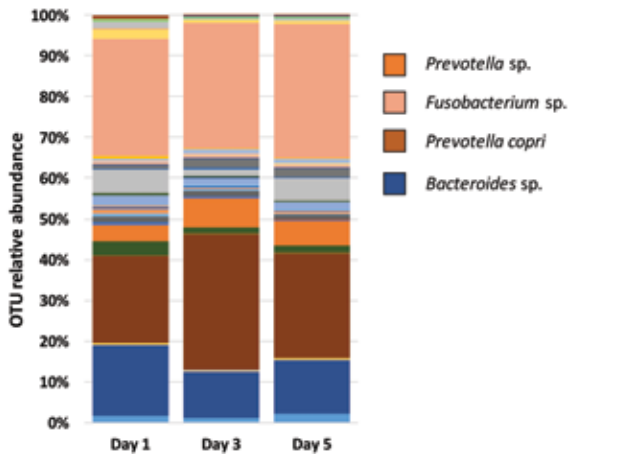

Figure 3. Stability and bacterial composition of the fecal inoculum during refrigerated storage. Individual bars represent bacterial species composition of the fecal inoculum given to a single litter of puppies on days 1, 3, and 5 of inoculation. Each color within a bar represents the relative abundance of an individual operational taxonomic unit (OTU).

One litter received FMT from a dam that tested positive for coccidia on fecal flotation. This group was included in the analysis because these puppies did not develop diarrhea and because coccidia are considered ubiquitous within the housing environment. In addition, neither FMT- nor sham-treated groups received a fecal score of 6 or greater.

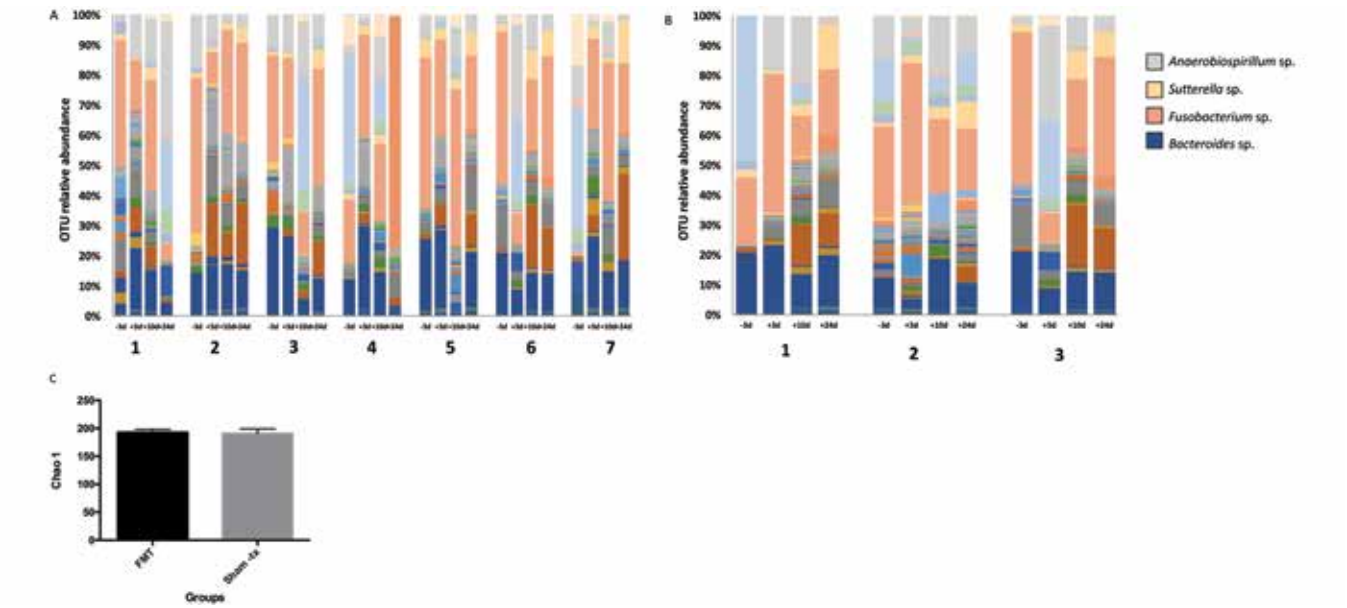

Figure 4. Fecal bacterial species composition and variability over time in (A) 7 FMT-treated puppies and 3 (B) sham-treated puppies. Each bar represents a different fecal collection including 3 d prior to weaning (–3d), and 3, 10, and 24 d after weaning (+3d, +10d, and +24d, respectively). Each color within a bar represents the relative abundance of an individual operational taxonomic unit (OTU). (C) The α diversity index, using Chao 1, shows that there is no difference in microbial diversity between FMT- and sham-treated puppies ($P = 0.0419$). The SE (bar) was 192.6 for FMT-treated pups and 190.6 for sham-treated puppies.

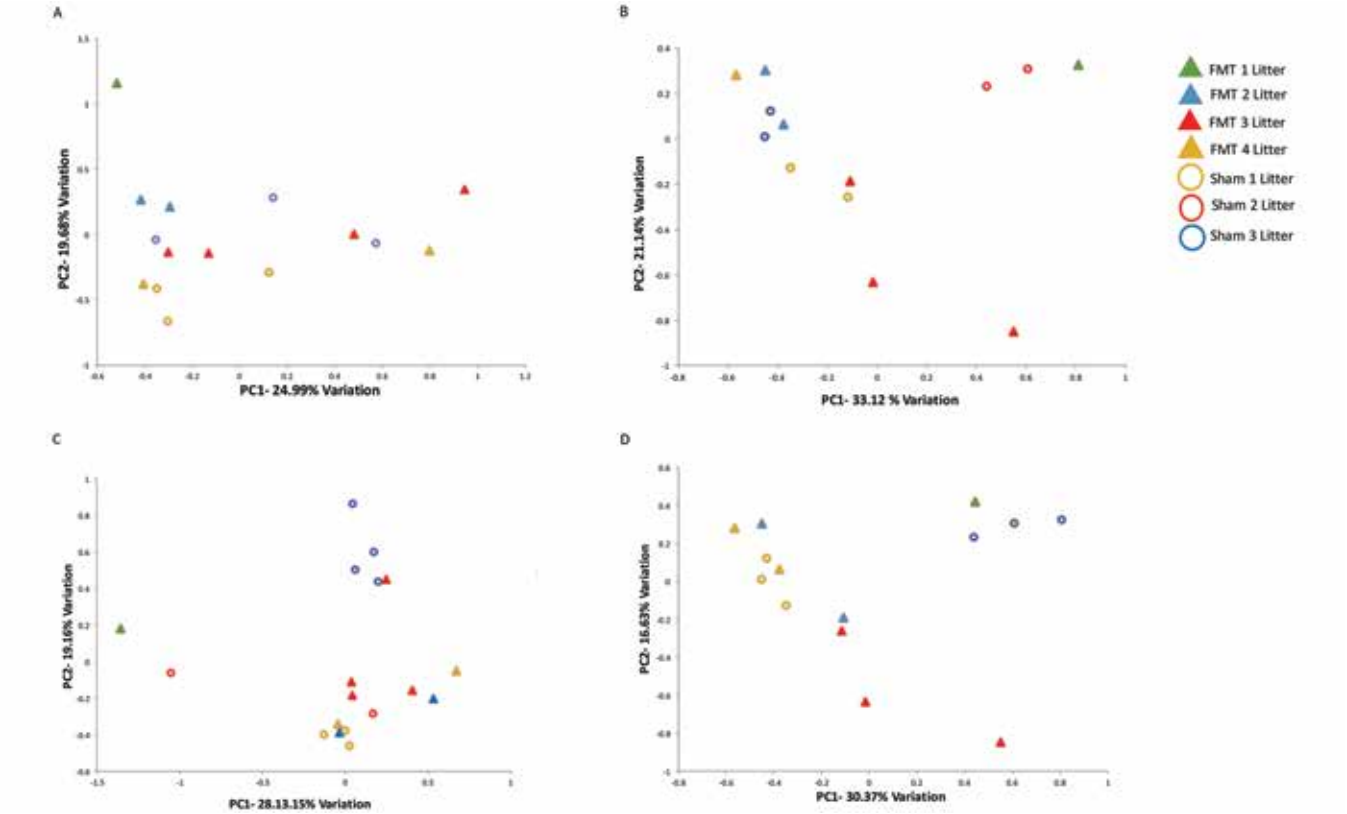

Figure 5. Principal component (PC) analysis of puppies (color-coded by litter) that received FMT (triangles) or sham treatment (circles), depicting highly variable microbial composition among subjects, with some clustering of litters over time. Variability of microbiota at (A) 3 d before weaning and (B) 3 d, (C) 10 d, and (D) 24 d after weaning.

Discussion

Although we had anticipated that the GM of puppies receiving FMT would closely resemble their dam's after treatment, the microbiota of both groups varied markedly over time. To our knowledge, no report in the current veterinary literature describes the degree of variability in the fecal microbiota of

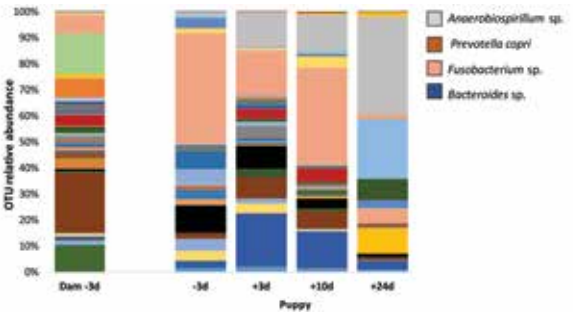

Figure 6. Composition and variability of fecal bacterial species in a FMT dam and cognate puppy. The single bar for the dam represents sample collection at 3 d prior (–3d) to weaning of the puppy. For the puppy, each bar represents samples collected 3 d before (–3d) and 3, 10, and 24 d after weaning (+3d, +10d, and +24d, respectively). Each color within a bar represents the relative abundance of an individual operational taxonomic unit (OTU).

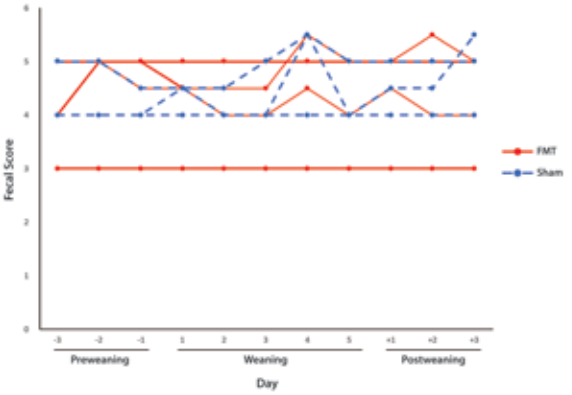

Figure 7. Line graph depicting fecal scores over time (days) beginning 3 d before weaning and concluding 3 d after weaning for FMT-treated litters (red) and sham-treated litters (blue). Average daily fecal score did not differ between FMT- and sham- treated litters at any time point. Any litter of puppies that received an average fecal score of 6 or greater was considered to have diarrhea.

postweaning puppies. In infants and children, similar variability is observed, and the intestinal microbiota does not stabilize until around 3 y of age.[2,11] These changes in children have been attributed to development and to increased exposure to diverse environmental stimuli.[2] Although similar studies have not been performed in dogs in a research or companion animal setting, we found that neither of our group's GM became similar to the dams' GM over the course of the study. However, some evidence suggested that the GM of sham-treated puppies became more similar to that of puppies in other sham-treated litters over time, a characteristic that was not noted in the FMT litters. Additional studies that include time points beyond 24 d after weaning are necessary before any conclusions regarding the utility of FMT and its effect on the GM can be drawn. Our data clearly show that, during the first month after weaning, intestinal microbial development and establishment is characterized by marked interindividual variability.

In human medicine, the link between fostering a healthy GM and a person's overall immune status has led to increased use of FMT as a therapy for reestablishment of the GM, especially in cases of nosocomial *Clostridium difficile* infections secondary to broad-spectrum antibiotic usage.[3,14] In these patients, the primary cure rate is approximately 79%, with a subsequent decrease in hospitalization time.[3,14] In contrast, our puppies' fecal microbiome had not been altered secondary to antibiotics. Furthermore, FMT is administered to human patients by using modalities that bypass the stomach, to decrease destruction of microbes from exposure to digestive enzymes.[15] Smaller studies have successfully explored the use of oral, frozen, and encapsulated FMT for the treatment of *C. difficile* in hope of providing a more accessible therapeutic modality than nasogastric tubing or colonic administration.[21] In addition, this pragmatic approach to FMT was the rationale behind using oral FMT in our group of puppies. Unlike humans, nasogastric tubing and colonic administration of companion animals often requires their heavy sedation or general anesthesia. Due to these constraints, performing consecutive FMT is infeasible, thus limiting the breadth of candidates qualifying for this treatment option.

Perhaps the puppies' resident microbiota outcompeted the introduced microbiota, resulting in minimal transfer of the dam's microbes. Further studies are needed to better characterize this potential phenomenon. Regardless, although FMT administration did not appear to be advantageous for the treatment of diarrhea in our target population, we made several other interesting observations during our study. The puppies were housed and weaned on raised pens. These pens allowed for excreta and debris to drop through the slats, subsequently improving hygiene and possibly decreasing exposure to coccidia. Previously, litters that were whelped during similar time frames were comingled at weaning. For our study, comingling of the litters did not occur until after 12 wk of age. We hypothesize that waiting until the puppies were more mature before comingling reduced overall stress, thus promoting gastrointestinal health.

In hindsight, we surmise that using a fecal scoring system adapted to postweaning puppies[8] may have been more appropriate than was the scoring system we used. That study scored postweaning puppies' stools using a 13-point scale that accounted for age (in weeks), weight, average daily gain, and large- compared with small-breed variables before making treatment decisions. The study reported that 6- to 8-wk-old puppies with a score of 7 or less often showed a reduction in the individual average daily weight gain compared with puppies that had a fecal score of 8 or greater. A fecal score of 7 on the weaned-puppy scale[8] roughly equates to a fecal score of 4 or 5 on the adult-dog scoring system we used here.[19] Although puppies in our facility historically have been scored according to the adult-dog system, implementation of the 13-point scale and monitoring daily weight gain may greatly reduce the number of puppies that are diagnosed and treated for diarrhea, given that the finding of coccidia on pooled fecal floatation may simply be incidental.

In conclusion, the fecal microbiota of puppies varies markedly immediately after weaning. Future studies that follow puppies beyond 1 mo after weaning may identify a defined time point at which the canine microbiome stabilizes. This characterization may provide a better understanding for diseases that result in canine intestinal dysbiosis and diarrhea. The use of oral FMT may be a pragmatic and convenient therapy for the treatment of postweaning diarrhea, but larger studies are needed for further characterization.

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