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Safety profile and effects on the peripheral immune response of fecal microbiota transplantation in clinically healthy dogs

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Abstract

Background: Fecal microbiota transplantation (FMT) is increasingly used for gastrointestinal and extra-gastrointestinal diseases in veterinary medicine. However, its effects on immune responses and possible adverse events have not been systematically investigated.

Hypothesis/Objectives: Determine the short-term safety profile and changes in the peripheral immune system after a single FMT administration in healthy dogs.

Animals: Ten client-owned, clinically healthy dogs as FMT recipients, and 2 client-owned clinically healthy dogs as FMT donors.

Methods: Prospective non-randomized clinical trial. A single rectal enema of 5 g/kg was given to clinically healthy canine recipients. During the 28 days after FMT administration, owners self-reported adverse events and fecal scores. On Days 0 (baseline), 1, 4, 10, and 28 after FMT, fecal and blood samples were collected. The canine fecal dysbiosis index (DI) was calculated using qPCR.

Results: No significant changes were found in the following variables: CBC, serum biochemistry, C-reactive protein, serum cytokines (interleukins [IL]-2, -6, -8, tumor necrosis factor [TNF]-α), peripheral leukocytes (B cells, T cells, cluster of differentiation [CD]4+ T cells, CD8+ T cells, T regulatory cells), and the canine DI. Mild vomiting (n = 3), diarrhea (n = 4), decreased activity (n = 2), and inappetence (n = 1) were reported, and resolved without intervention.

Abbreviations: AE, adverse event; ANOVA, analysis of variance; CD, cluster of differentiation; CDI, *Clostrioides difficile* infections; CRP, C-reactive protein; DI, dysbiosis index; FMT, fecal microbiota transplantation; FoxP3, forkhead Box P3; GI, gastrointestinal; IBD, inflammatory bowel disease; IL, interleukin; PBMC, peripheral blood mononuclear cells; SAE, serious adverse event; SIRS, systemic inflammatory response syndrome; TNF-α, tumor necrosis factor-α; VMTH, Veterinary Medical Teaching Hospital.

Conclusions and Clinical Importance: Fecal microbiota transplantation did not significantly alter the evaluated variables and recipients experienced minimal adverse events associated with FMT administration. Fecal microbiota transplantation was not associated with serious adverse events, changes in peripheral immunologic variables, or the canine DI in the short-term.

KEYWORDS
canine, C-reactive protein, cytokines, fecal microbiota transplantation, FMT, peripheral immune modulation

1 | INTRODUCTION

Similar to humans, tens of trillions of microbes are likely present in the gastrointestinal (GI) microbiome of dogs.[1-4] Gut microbiome may influence the local and systemic immune system by regulation of inflammation and peripheral T cell populations.[5-7] Although "dysbiosis" has not been universally defined, it is often referred to as decreased microbial diversity, lower abundance of beneficial or commensal organisms, an increased abundance of pathogenic organisms, or some combination of these.[1,7] Similarly, a consistent definition for "normobiosis" is lacking, but the general compositions of the gut microbiomes in healthy individuals have been described.[8,9] Dysbiosis has been characterized in veterinary medicine in conjunction with immune-modulated diseases, such as chronic enteropathies and atopic dermatitis.[10-13]

Fecal microbiota transplantation (FMT) is a procedure to restore normobiosis.[14] Two products for humans have been approved by the Federal Drug Administration for recurrent *Clostrioides difficile* infections (CDI).[14-17] Possible benefits may be seen in inflammatory bowel disease (IBD), irritable bowel syndrome, and hepatic encephalopathy.[18-21] In veterinary medicine, FMT has been utilized for IBD, acute diarrhea, chronic enteropathy, and parvoviral infections.[22-28]

Given the relationship between the gut microbiome and the host's immune system, FMT can lead to changes in their complex signaling and interactions. Human- and mouse-based FMT studies have shown changes affecting the peripheral immune system, such as a decrease in serum pro-inflammatory cytokines, and an increase in anti-inflammatory bacteria and T regulatory cells.[29,30] So far, FMT studies in veterinary medicine have not explored changes in the peripheral immune system in depth.

One of the challenges of understanding FMT safety is the difficulty of uncoupling the existing disease-related clinical signs and immune changes in diseased patients from those that are FMT-related. To date, studies in veterinary medicine on FMT in dogs have been performed in diseased dogs. Considering FMT as a treatment, it is reasonable to understand its effects on a healthy patient first. Traditionally, phase I clinical trials are conducted in healthy volunteers to isolate the therapeutic and adverse effects of the drug and assess safety.[31]

Investigations of FMT in humans have found it to be generally safe.[32,33] A review of human FMT recipients found adverse events to consist of mostly mild, self-limiting GI symptoms.[34] Serious adverse events (SAE) are rare; in total, 59 FMT-related SAEs (1.39%) including 5 deaths were reported.[35,36] A study involving 3 healthy human recipients of PO capsule FMT reported that all participants experienced diarrhea, flatulence, GI pain, and nausea, and 1 developed systemic inflammatory response syndrome.[37] Despite increasing FMT usage in veterinary medicine, adverse events have not been investigated systematically, and the majority reported have been mild and GI in nature with no records of SAEs.[27,38-40] We aimed to investigate the short-term safety and possible effects of FMTs on the peripheral immune system after a 1-time rectal FMT in clinically healthy canine recipients.

2 | MATERIALS AND METHODS

2.1 | Animals and sampling

All animals, including FMT recipients and fecal donors, were privately owned and lived in home environments in the Sacramento area, CA, USA. The study was conducted between November 2021 and May 2022 at the William R. Pritchard Veterinary Medical Teaching Hospital (VMTH), School of Veterinary Medicine, University of California, Davis (IACUC #22312).

2.2 | FMT fecal donors

Two fecal donors were identified based on modified guidelines for the selection of human fecal donors and canine blood donors.[41-44] Enrollment criteria included no history of GI disease or systemic diseases, no history of medication administration other than ectoparasite preventatives within 6 months, a normal fecal score of 2 using the 7-point Nestlé Purina Fecal Scoring System, normal body condition score,[45] and no clinically relevant abnormal physical examination findings.[46] Donors had normal results or clinically irrelevant deviations from reference intervals for CBC, serum biochemistry, trypsin-like immunoreactivity, pancreatic lipase immunoreactivity, folate, and cobalamin. Feces were examined for GI parasites, common enteropathogens and their toxins (PCR testing for *Clostridium perfringens* [*C. perfringens*] enterotoxin gene, net F toxin gene-*C. perfringens*, *Clostridioides difficile*, *Campylobacter jejuni*, canine parvovirus, *Salmonella* spp. on enrichment broth, and immunofluorescence assay testing for Giardia and Cryptosporidium),

and normal gut microbiota using the canine dysbiosis index (DI).[47] Donors were fed Purina Pro Plan Adult Dry and Hill's Adult Healthy Weight Dry, respectively, before the study and throughout the study period.

2.3 | Healthy FMT recipients

Clinically healthy dogs aged 1 to 10 years old were recruited from the student and staff population at VMTH. Ten dogs were enrolled based on the same history and physical examination variables as the donors. Dogs were fed different commercial diets. Spontaneously passed fecal samples were collected in plastic bags, transported to the VMTH, stored at 4°C, and aliquoted to be frozen at −80°C until analysis within 12 hours of defecation. Samples were stored at −80°C for a maximum of 8 months before analysis.

2.4 | FMT processing

Spontaneously passed donor fecal samples were collected in plastic bags daily, transported on ice to the VMTH, stored at 4°C, and processed within 12 hours of defecation.

Donor feces were processed by mixing with non-bacteriostatic sterile saline solution (2.5 mL of 0.9% NaCl added per gram of feces) and kneaded for homogenization. Then, the solution was filtered twice through mesh sieves to remove large particulates. Sterile glycerol (30%) was added to the mixture to a final solution with 10% glycerol as previously described.[48] Transplants were aliquoted in 60 mL catheter tip syringes, frozen at −80°C, and used within 6 months of sample processing.

2.5 | FMT administration

Canine participants (n = 10) were given a single FMT via a rectal enema using a polyvinyl catheter without sedation or fasting. The first recipient received 2.5 g/kg of previously frozen prepared FMT solution thawed within 2 hours of use in a 30°C water bath. Because no adverse events were observed, the dosage subsequently was increased to 5 g/kg for the remainder of the recipients.[27,49] Four dogs received FMT from donor 1's feces and 6 dogs received FMT from donor 2's feces. Owners were given the option to hospitalize their pets overnight for monitoring. Spontaneously passed feces were collected from each recipient dog on Days 0, 1, 4, 10, and 28 post-FMT administration. Blood was collected on the same days within 6 hours of defecation.

2.6 | Evaluation of clinical signs

Owners recorded clinical variables on Days 0 to 10 and 28 post-FMT. The following variables were recorded and scored as absent, mild, moderate, or severe based on a previously published scale for the recording of adverse events in veterinary medicine (Veterinary Cooperative Oncology Group—Common Terminology Criteria for Adverse Events v2 following investigational therapy in dogs and cats): attitude, activity, vomiting, stool frequency, mucus or blood in feces, and fecal score.[50] A full physical examination was performed on each sample collection day (0, 1, 4, 10, 28).

2.7 | Evaluation of safety and peripheral immune system

Blood samples were available on all study days from 9 of 10 dogs. One dog was excluded from blood sampling because of development of hospital-associated anxiety upon repeated visits. Complete blood count, serum biochemistry, and C-reactive protein (CRP) concentrations were performed using blood samples collected from recipients at the Central Laboratory of the VMTH. For cytokines, serum interleukins (IL)-2, -6, -8, and tumor necrosis factor (TNF)-α were measured using a multiplex electrochemiluminescence immunoassay and Canine Proinflammatory Panel 3 Ultrasensitive Kit (Meso Scale Discovery, Meso Scale Diagnostics, Rockville, Maryland) on a MSD Quickplex SQ 120 (Meso Scale Diagnostics, Rockville, Maryland), according to manufacturer instructions and as previously described.[51-53]

2.8 | Isolation of canine peripheral blood mononuclear cells from whole blood

Whole blood was obtained using EDTA-containing vacutainer tubes and processed as previously described.[54] Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation using Histopaque 1077 (Sigma-Aldrich, Saint Louis, Missouri, USA). The PBMC pellets were treated with RBC lysis buffer (Biolegend, San Diego, California, USA) and cryopreserved in media containing 45% heat inactivated fetal bovine serum, 45% heat inactivated dog serum (Equitechbio, Kerrville, Texas, USA), and 10% dimethyl sulfoxide. Cryopreserved PBMC were stored in liquid nitrogen until batch analysis. Cryopreserved PBMC were thawed and rested in culture media overnight before staining the next day for flow cytometry analysis as described previously.[55]

2.9 | Flow cytometry

Staining protocols were used as previously described.[54-56] When possible, a minimum of 1 million cells were stained for PBMC. For all experiments, cells were stained for viability with a fixable viability dye (LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, or LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Cells were stained with cell surface antibodies in Dulbecco's phosphate buffered saline with 3% heat inactivated fetal bovine serum. Permeabilization, fixation, and intracellular staining of cells were performed using eBioscience Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific). Cell surface staining,

permeabilization, fixation, and intracellular staining were performed for 20 minutes at 4°C.

Antibodies staining for cell surface markers were directed against cluster of differentiation (CD) 45, CD4, CD8α, and CD25. Antibodies staining for intracellular markers were directed against CD3, forkhead box P3 (FoxP3), and CD79a. All antibodies are described for species, clone number, fluorochrome, and vendor in Table S1.

Stained PBMC were washed in buffers under conditions previously described,[55] suspended in 1% paraformaldehyde (Affymetrix, Thermo Fisher Scientific), and stored at 4°C for subsequent flow cytometer acquisition. A 4-laser Cytoflex S flow cytometer (Beckman Coulter, Miami, Florida, USA) was utilized for flow acquisitions. Single color antibody capture bead controls were prepared and recorded for each color to assist with multi-color fluorescence compensation. A separate aliquot of heat-treated cells was used to prepare the single-color control for the Fixable Live/Dead viability stain. At least 20 000 events were recorded for single color controls. To aid in gating of the compensated fully stained samples, selected fluorescence minus 1 controls were prepared for the FoxP3, CD25, and CD79a reagents and recorded at each time point. A minimum of 50 000 fluorescence minus 1 and 100 000 fully stained PBMCs were acquired per session. Flow cytometric data were compensated and analyzed using FlowJo v10.8.1 (BD Biosciences) software.

2.10 | Statistical analysis

Continuous variables were evaluated for normal distribution using the D'Agostino & Pearson test. Normally distributed data were analyzed using a 2-tailed student's *t*-test or analysis of variance (ANOVA). Non-normally distributed data were analyzed using the Wilcoxon signed-rank test or, if single data points were missing, a linear mixed effects model was performed. Unpaired 2-tailed *t*-tests were used to compare participant weight between dates 0 and 28 post-FMT. A repeated-measures 1-way ANOVA was used to compare results for all CBCs (Days 0, 1, 4, 10, 28). A repeated-measures linear mixed-effects model was used to compare serum biochemistry, CRP, cytokines, and canine DI values among Days 0, 1, 4, 10, and 28. Flow cytometry data were analyzed using a repeated-measures 1-way ANOVA. *P* values <.05 were considered significant. The Benjamini-Hochberg procedure was used to adjust for the false discovery rate of significant raw *P* values ≤.05. Both adjusted and unadjusted *P* values are reported in the results. All results are available in Table S2.

Statistical analyses were performed using GraphPad Prism version 9.4.0 for Mac (GraphPad Software, San Diego, California).

3 | RESULTS

3.1 | Animals

Ten FMT recipients and 2 FMT donors were recruited during the trial period. Recipient and donor characteristics are presented in Table S3. All dogs had a normal clinical examination. The weight of FMT recipients did not change significantly between Days 0 (21.3 ± 10.6 kg) and 28 (21.4 ± 10.7 kg; $P = .57$).

3.2 | Clinical survey and adverse events

Results of clinical survey are reported in Table S4. Owners of 2 patients reported slightly decreased attitude or activity. One patient's owner reported a slightly decreased attitude or activity on Days 1 and 28. The other patient had a minor limb injury on Day 7 post-FMT and its owner reported decreased activity between Days 7 and 8 post-FMT. One patient's owner reported slightly decreased appetite on Day 2 after FMT, and appetite was reported normal in all other patients throughout 28 days.

Owners of 3 patients reported vomiting, with 1 patient vomiting on Days 1 and 2 and another patient on Day 1 post-FMT. A third patient's owner reported vomiting on Day 28 after FMT, having been fed an edible chew toy the night before. All 3 patients were otherwise normal in attitude, appetite, fecal score, frequency of defecation, and general disposition on the day of the vomiting event.

Diarrhea was reported in 4 patients with fecal score above the reference range (2-3 on the 7-point Nestlé Purina Fecal Scoring System). On Day 1 after FMT, owners of 2 patients reported fecal scores at 4. One of the 2 patients' owners also reported an increased fecal score of 4 on Day 5. One patient's owner who reported an increased fecal score of 4 on Day 4 post-FMT also noted that the patient had incidentally ingested chicken feed, which may have been related to the increased fecal score. Another patient's owner reported a fecal score of 5 on Day 6 with slightly increased frequency of defecation and a fecal score of 4 on Day 9 with normal frequency of defecation. The same patient had a slightly increased frequency of defecation with a fecal score of 2 on Day 2 post-FMT. No patient's owner reported mucus or blood in feces or other illnesses.

3.3 | Complete blood count and serum biochemistry

Laboratory results were available for 9 of 10 dogs. Results of CBCs and serum biochemistry panels were not significantly different before and after FMT in any of the study participants (Tables S5 and S6).

3.4 | Markers of inflammation

Serum CRP concentration remained within the reference range (0-10 mg/L) for all recipients and did not differ between days ($P = .08$; Figure 1).

Serum cytokine measurements for IL-2 ($P = .16$), IL-6 ($P = .08$), IL-8 (chemokine; $P = .31$), and TNF-α ($P = .58$) did not change significantly during 28 days (Figure 2). A single recipient (patient #3) had relatively higher results of IL-2 on baseline (Day 0; 165.55 pg/mL) and

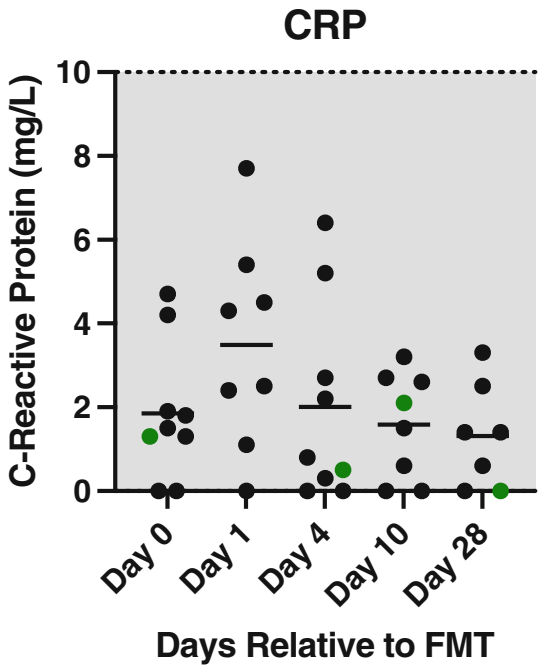

FIGURE 1 Longitudinal measurements of C-reactive protein (CRP) in 9 healthy dogs before (Day 0) and after (Days 1, 4, 10, 28) receiving a single, unsedated rectal fecal microbiota transplantation (FMT). Gray area indicates reference range in dogs (0-10 mg/L). All but 1 recipient received 5 g/kg of FMT. The first FMT recipient received a decreased dose of 2.5 g/kg FMT and is depicted as a green dot.

continued to have higher results of IL-2 (range, 141.08-165.55 pg/mL) throughout the data collection period. The same patient (#3) had a relatively higher result of IL-8 at baseline (Day 0; 2014.5 pg/mL) that remained increased throughout (range, 1421.30-2021.84 pg/mL). Another recipient (patient #6) had relatively higher results of IL-8 on baseline (Day 0; 1716.15 pg/mL) that remained increased (range, 1259.28-2296.84 pg/mL).

3.5 | Flow cytometry

Results of flow cytometry measurements of peripheral leukocytes, B cells, T cells, CD4 T cells, CD8 T cells, and T reg cells did not change after FMT administration (Table 1; Figure 3).

3.6 | Dysbiosis index

No significant changes were noted during the 28-day trial period in canine DI results of all recipients ($P = .18$; Figure 4). Two recipients had a DI above 2 on Day 28 after FMT administration. Upon pre-FMT screening examination, the first recipient had a DI of 1.3 that increased to 3.8 on the day of FMT (Day 0), and ranged between DI scores of 1.1 and 2.3 in the next 28 days. This patient experienced slightly decreased appetite on Day 2 and did not show other signs of GI disease. The second recipient had a DI of 0.6 on Day 0, and DI ranged between 1.3 and 2.9 in the next 28 days. This patient had a mildly increased fecal score of 4 on Days 1 and 5 and no other adverse events were reported. Follow-up 6 months after enrollment (Day 0) showed no development of clinical signs of GI disease in these 2 patients.

4 | DISCUSSION

Fecal microbiota transplantation usage has been rapidly increasing in the last 2 decades. In human medicine, FMT is a promising adjunctive treatment modality for recurrent CDI and is used as exploratory treatment in multiple diseases, including IBD and metabolic syndrome.[15,35] In veterinary medicine, FMT also is being explored in many diseases, such as parvoviral enteritis, chronic enteropathies, acute hemorrhagic diarrhea syndrome, and atopic dermatitis.[23,24,27,40,57,58] With the increase in veterinary use, concerns over the lack of data on the short- and long-term safety of FMT have been raised. In companion animal veterinary medicine, few published studies on FMT are available, and safety has not been thoroughly investigated.[38]

We performed 5 g/kg rectal enema FMT in 9 clinically healthy recipients, as well as a 2.5 g/kg rectal enema FMT in 1 clinically healthy recipient, and found no SAEs during the 28 days after FMT.[27,49] Safety concerns in FMT can be divided into 2 major categories: microbiota-related adverse events (result of microbiota transplantation interactions with the host) and delivery-related adverse events.[34] No SAEs such as infection, systemic inflammatory response syndrome, septicemia, hospitalization, or deaths were encountered by any of the 10 FMT recipients in our study. Nine patients experienced mild adverse effects after FMT according to the owner surveys, including self-resolving diarrhea in 4 patients, transient vomiting in 3 patients, slightly decreased attitude or activity in 2 patients, and slightly decreased appetite in 1 patient. Only a single patient had >1 adverse effect (decreased attitude and diarrhea). In addition, events unrelated to the FMT administration may have contributed to the events recorded in the 28 days after FMT administration including 1 owner report of dietary indiscretion. Given that all clinical signs were mild and self-resolving without further medical intervention, no SAEs were reported. This observation is consistent with studies in humans, where FMT is considered a safe procedure with <1.4% SAEs reported.[34] Hence, we conclude that FMT likely carries low risk of SAEs in healthy dogs in the short term. However, as in all therapeutic drug trials, SAEs may be observed in larger scale studies. With regard to why we found a higher percentage of dogs with adverse events compared to previous studies, we postulate that it may be a consequence of using a systematic scale to specifically track adverse events, which had not been done in previous studies to our knowledge.

The delivery method (rectal enema) and preservation method (glycerol) also could have contributed to the transient self-resolving diarrhea after FMT. In a study of tylosin-induced dysbiosis, all dogs that received rectal enema FMT cryopreserved with 10% glycerol had an episode of diarrhea within 24 hours that resolved on its own.[59] Although a previous study in humans did not find differences in

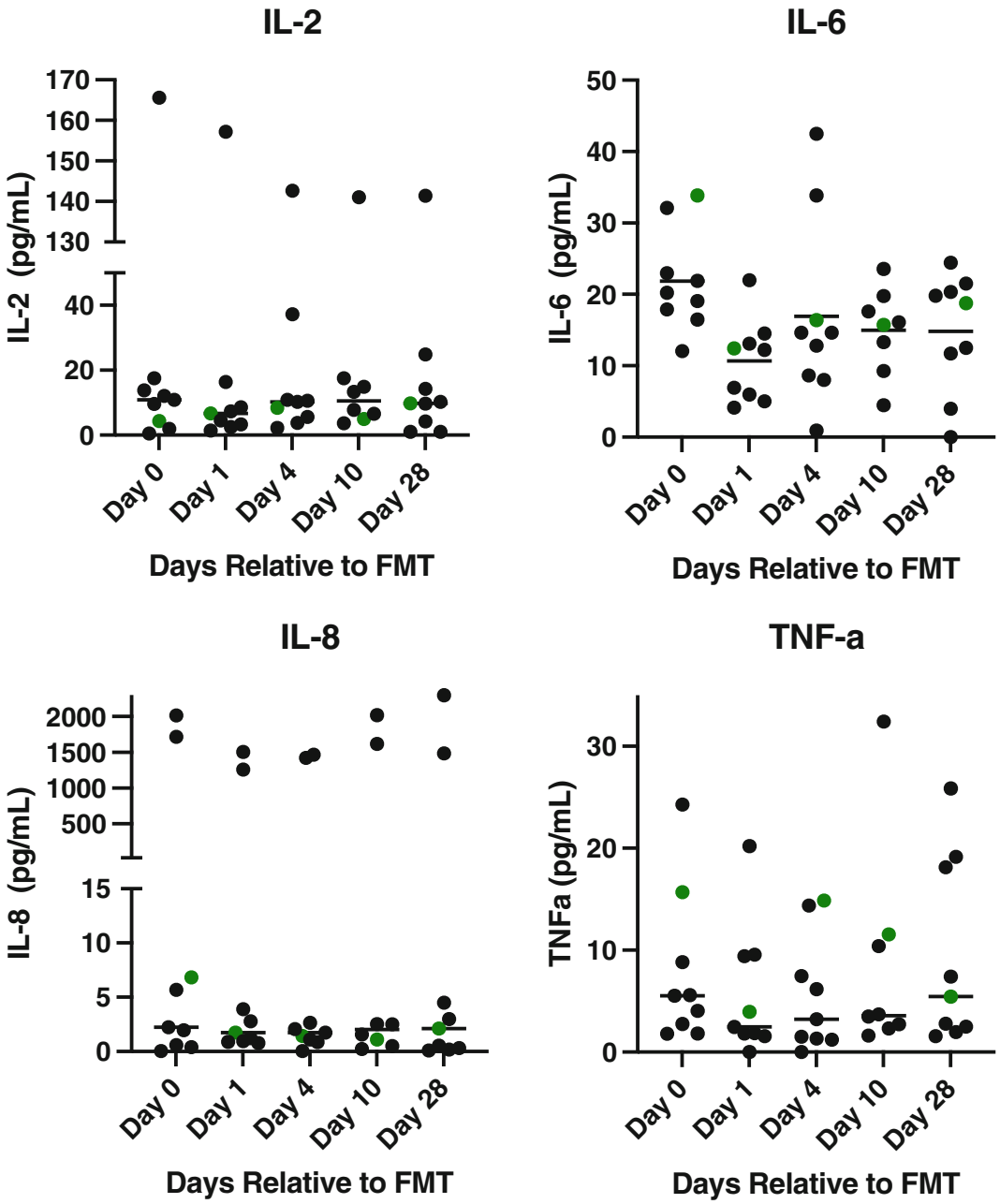

FIGURE 2 Longitudinal measurements of serum concentrations of inflammatory cytokines Interleukins (IL)-2, -6, -8, and tumor necrosis factor (TNF)-α in 9 healthy dogs before (Day 0) and after (Days 1, 4, 10, 28) receiving a single, unsedated rectal fecal microbiota transplantation (FMT). All but 1 recipient received 5 g/kg of FMT. The first FMT recipient received a decreased dose of 2.5 g/kg FMT and is depicted as a green dot.

adverse events of diarrhea within the first 7 days after FMT between capsule FMT and rectal enema FMT, the subjects had pre-existing CDI and disease-related diarrhea, and the rectal enema was retained for 1 hour after FMT.[60] We are not aware of a placebo-controlled, randomized study comparing the effects of the addition of glycerol in FMT in human or veterinary medicine. However, PO glycerol has been reported to cause diarrhea and vomiting in humans, as well as vomiting in dogs.[61,62]

A systematic, 20-year review of adverse events (AEs) related to FMT in humans reported that, of 4241 patients, the most common Aes were diarrhea (10%), abdominal discomfort, pain or cramping (7%), nausea and vomiting (3%), excessive flatulence (3%), constipation (2%), fever (2%), and fatigue or malaise (1%).[34] Serious adverse events (infections, hospitalizations, and deaths) also have been reported in 1.4% of patients, with 5 (0.12%) deaths related to FMT.[34] Out of the 5 deaths, 4 were deaths related to FMT delivery (upper GI tract) and 1 patient developed drug-resistant *Escherichia coli* bacteremia and sepsis, presumably transmitted by FMT.[36,63-66] All FMT-related deaths were observed in patients with mucosal barrier injury, severe comorbidities such as myelodysplastic

TABLE 1 Longitudinal flow cytometry data for peripheral leukocytes, B cells, T cells, cluster of differentiation (CD)4+ T cells, CD8+ T cells, T reg cells in 9 healthy dogs before (Day 0) and after (Days 1, 4, 10, 28) receiving a single, rectal fecal microbiota transplantation.

	Day 0	Day 1	Day 4	Day 10	Day 28	ANOVA
	% Median (range)	% Median (range)	% Median (range)	% Median (range)	% Median (range)	*P* value
Leukocytes	92.5 (87.3-98.1)	96.8 (81.0-98.0)	92.4 (87.9-98.0)	90.4 (73.4-99.0)	96.9 (84.8-99.1)	.20
B cells	8.6 (4.4-20.7)	8.2 (4.7-16.7)	10.0 (3.1-18.2)	6.3 (3.7-12.1)	8.6 (4.5-14.7)	.41
T cells	70.6 (63.6-90.0)	76.1 (56.1-93.2)	75.1 (64.0-89.5)	73.2 (53.9-94.1)	82.3 (69.8-91.6)	.20
CD4+ T cells	40.0 (30.4-53.6)	38.5 (25.4-49.9)	40.2 (27.2-51.9)	43.8 (29.6-49.5)	43.2 (35.8-51.2)	.14
CD8+ T cells	28.5 (18.0-45.8)	31.5 (22.3-44.2)	32.6 (20.0-42.2)	32.7 (17.3-36.0)	32.4 (20.3-44.8)	.80
Treg cells	5.0 (1.79-7.7)	4.5 (1.4-7.5)	5.6 (2.1-10.0)	4.7 (1.8-7.9)	3.6 (1.9-7.0)	.24

Note: Leukocytes were defined as CD45+ cells, B cells were defined as CD45+ CD3− CD79a+ cells, CD4+ T cells were defined as CD45+ CD3+ CD8− CD4+ cells, CD8+ cells were defined as CD45+ CD3+ CD8+ CD4− cells, and regulatory T cells (Treg cells) were defined as CD45+ CD3+ CD8− CD4+ forkhead box P3+ CD25+ cells.

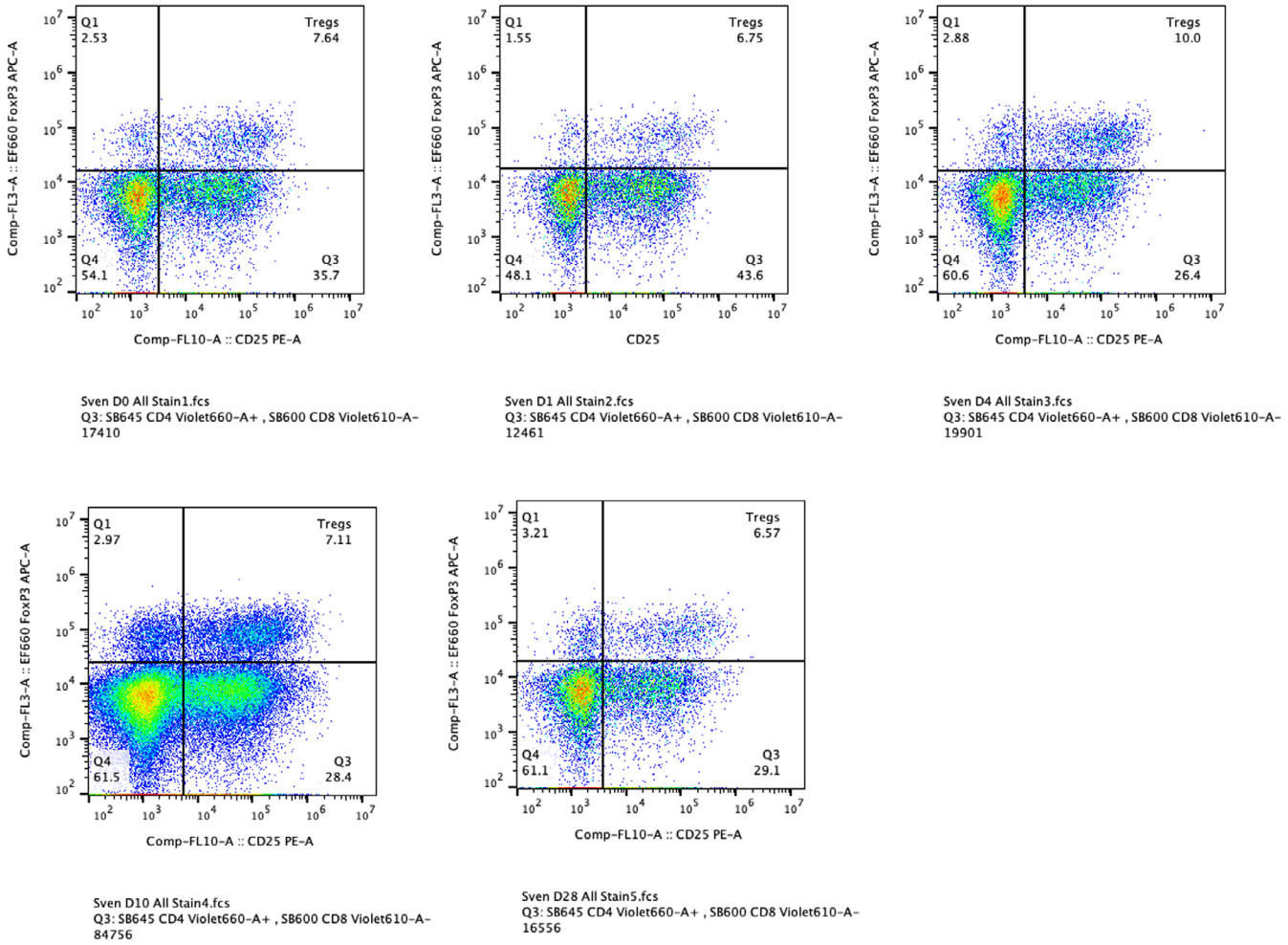

FIGURE 3 Longitudinal flow cytometry data for cluster of differentiation (CD)45+ CD3+ CD8− CD4+ forkhead box P3+ CD25+ cells (regulatory T cells) in 1 recipient before (Day 0, A) and after (Days 1, 4, 10, 28, B-E) receiving a single, rectal fecal microbiota transplantation.

syndrome, or both. The authors suggested that most FMT-related SAEs can be avoided by minimizing delivery-related risks. In a separate systematic review of FMT-related AEs, the rate of Aes was higher in upper-GI routes (43%) compared to lower-GI routes (18%).[67]

Few studies on FMT have been published in the veterinary medical literature with 5 studies reporting AEs related to FMT, including the aforementioned study of tylosin-induced dysbiosis. One study reported a dog with IBD and diarrhea that had gotten transiently worse after FMT, and a survey-based observational study reported

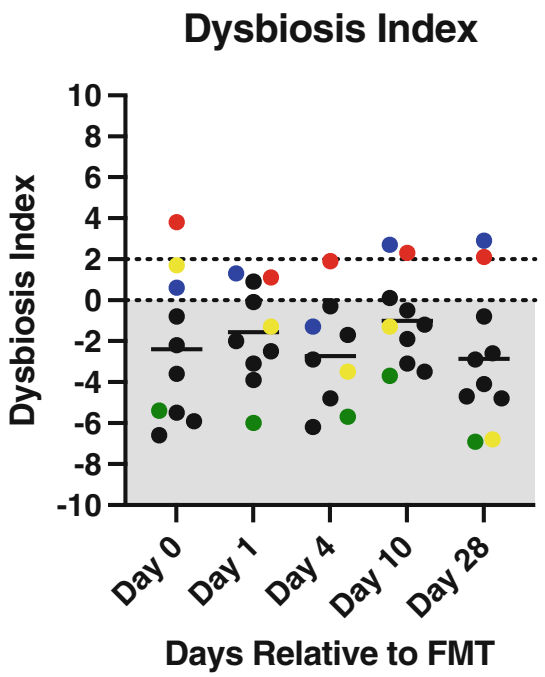

FIGURE 4 Longitudinal measurements of canine dysbiosis Index (DI) in 10 healthy dogs before (Day 0) and after (Days 1, 4, 10, 28) receiving a single, rectal fecal microbiota transplantation (FMT). Canine DI score < 0 indicates normobiosis. Blue, red, and yellow colored dots represent 3 individual patients whose canine DI score was above reference range on baseline (Day 0). All but 1 recipient received 5 g/kg of FMT. The first FMT recipient received a decreased dose of 2.5 g/kg FMT and is depicted as a green dot. The gray shaded area represents values considered normal canine DI. The area between the 2 dotted lines represents results reported as ambiguous for dysbiosis. Values >2 are considered as an abnormal canine DI.

4 survey responders (12%) who reported worsening diarrhea in 3 dogs that had received FMT and 1 with increased flatulence.[38,39] A recent study on dogs with chronic enteropathy that received 5 to 7 g/kg FMT by rectal enema reported that 10/41 dogs (24.4%) experienced AEs after FMT with diarrhea or worsening diarrhea (n = 7), flare up of diarrhea and occasional vomiting for a week (n = 1), flatulence, malodorous feces, and mild vomiting (n = 1), and tenesmus and dyschezia (n = 1).[27] In another study on dogs with atopic dermatitis receiving PO FMT, 4/12 (33.3%) dogs experienced mildly softer feces after PO FMT, but no SAEs.[40] Because of the variability of indications for FMT, preexisting diseases, FMT dosage, and administration routes, further investigation is needed to better characterize the role of each factor on the development of AEs.

Besides clinical safety, we also investigated the effects of FMT on laboratory data, inflammatory markers, and the peripheral immune system. We found no significant changes in the CBC and serum biochemistry results from healthy canine FMT recipients, further suggesting FMT safety. In the only study of humans published to date with healthy recipients of FMT, 2 participants had normal CBC and biochemistry results except for increased blood neutrophil count (n = 1) and decreased lymphocyte count (n = 2) on Day 2. In the single participant that developed systemic inflammatory response syndrome after the first FMT, leukocytosis, neutrophilia with left shift and toxic granulation, lymphopenia, and small increases in gamma-glutamyl transpeptidase and alanine transaminase activity were noted on Day 2.[37]

C-reactive protein is an acute phase protein and a reliable and sensitive marker of inflammation. It is secreted by the liver in response to inflammatory cytokines, increases rapidly with trauma, inflammation, and infection and then decreases rapidly when the stimulus is gone.[68] Serum CRP concentration changes after FMT in studies of humans have shown mixed results.[29,69,70] These differences may be related to the disease state of the patient before receiving FMT, the timepoint of measuring CRP concentrations, and clinical response. In a study of human patients with CDI, which is characterized by increased CRP concentrations, CRP was significantly decreased 3 weeks after FMT compared to baseline.[29] A study of humans with ulcerative colitis who received FMT (n = 19) found no differences between Days 0 and 3 but a significant decrease by month 3 in patients with clinical improvement (n = 11) whereas a different group of ulcerative colitis patients (n = 5) all had transient increases in CRP during the first few days after FMT but only 1 showed clinical improvement.[69,70]

In veterinary medicine, serum CRP concentrations in placebo and FMT treated dogs with IBD were not significantly changed from baseline to 7, 30, and 90 days after FMT or between placebo and FMT groups with remission in 8/12 patients that received FMT.[71] This observation is in agreement with our findings, despite the difference that FMT recipients from our study were clinically healthy and did not have baseline increases in CRP. No significant changes in systemic inflammation occurred based on CRP concentrations.

We did not find significant changes in the cytokines indicating inflammatory responses, including IL-2, IL-6, IL-8, and TNF-α between baseline and the days after FMT. The current understanding of cytokines in dogs does not include an established healthy dog reference range because of substantial variability of cytokine concentrations based on several factors, including age, breed, and body condition score.[72-74] For example, using the same electrochemiluminescence multiplex technology (QuickPlex Canine ProInflammatory Panel 3), a study in healthy control Labrador retrievers (n = 30) found a range of IL-6 in their samples of between 3.29 and 7.61 pg/mL, but a different study in healthy control dogs of various breeds (n = 25) found IL-6 concentrations from below detection to 65.0 pg/mL.[53,73]

Similarly, none of our dogs had significant changes in any of the peripheral immune cells involved in the initiation and mediation of generalized peripheral immune responses including $CD4^+$ and $CD8^+$ T cells. We also found no evidence of activation of $CD4^+/CD25^+$ regulatory T-cells. A previous study in healthy human volunteers found a transient decrease in lymphocytes, decreased CD8+ T and natural killer cells, and increased CD4/CD8 ratio, which was not found in our study population.[37]

The canine DI is a previously validated quantitative PCR-based assessment of fecal microbiome health in dogs that also has been shown to correlate with overall microbiota shifts based on shotgun sequencing.[47,75] Canine DI did not change significantly throughout our

study (ie, pre- vs post-FMT), and no dysbiosis was observed after FMT. Two patients had an abnormal (above 0) DI score at baseline. One of the patients transiently changed to normobiosis on Day 4 after FMT but DI increased again on Day 28. The other patient did not change to normobiosis after FMT administration. Because a DI score ≥2 is considered to be a clinically relevant increase, the first patient had been enrolled based on a baseline score of 1.3, which then increased to 3.8 on Day 0 of data collection, which was not a controllable factor. The cause of the variant composition of the second patient's fecal microbiome is unknown; although the dog was fed exclusively a vegan diet, other dogs fed a vegan diet have been found to have normal DI scores.[76] Although the healthy dog microbiome can vary based on several factors, including day-to-day fluctuation based on the individual, diet, and environment, it may be difficult to explain the magnitude of DI variation in this dog.[8,77,78] It is also possible that this dog had subclinical dysbiosis. A previous study reported a clinically healthy control dog with a DI score >6 and decreased *Clostridium hiranonis*, that developed chronic diarrhea 1 year after the study.[79] All other patients had DI scores ≤2 pre- and post-FMT administration. This finding further supports the relative short-term safety of FMT in healthy dogs as no major microbiome-related AE associated with induction of dysbiosis in relation to FMT administration was found.

Our study had several limitations. A limited number of dogs was included, and the study was underpowered. Another major limitation was the lack of a placebo group. Therefore, we cannot exclude that the mild AEs observed in this cohort could be unrelated to the FMT, and results should be interpreted cautiously. However, our study was intended to generate data on whether SAEs may be expected at an FMT dose approaching the upper end of previously described doses.[38,49] We only investigated FMT via rectal enema. We cannot exclude that AEs or immunologic changes could be more common in FMT administered through upper GI routes, such as PO capsules as described in people.[34,37] Fecal microbiota transplantation may elicit local immunological effects such as in the gut-associated lymphoid tissue (eg, the mucosa, Peyer's patches, or mesenteric lymph nodes) that were not detected in our study. Healthy dogs also may lack colonization of their GI tract in a manner necessary to provoke immune variable changes. Our data still can guide considerations of short-term safety for future studies and potential regulatory authorities considering the safety of FMT administered through lower GI routes in diseased animals. All participants were considered clinically healthy before FMT, and administering FMT in patients with pre-existing comorbidity may cause AEs associated with FMT not seen in clinically healthy patients. However, the assessment of patients with GI disease and whether the spectrum of possible AEs may be different between healthy dogs and dogs with GI disease was beyond the scope of our study. Nonetheless, our data may help differentiate true FMT-related AEs from multifactorial AEs after FMT administration in diseased dogs. Three recipients had DI considered abnormal on Day 0, before FMT, but had no clinical signs of GI disease with normal DI on screening. Upon follow-up, owners still reported those dogs to have no clinical signs of GI disease 11.5, 17, and 19 months after FMT, respectively. Our FMT recipients were young to middle-aged healthy dogs, and unobserved age-related changes in the parameters measured may have existed. We did not find significant changes in the CBC, serum biochemistry, markers of inflammation (pro-inflammatory cytokines, CRP) measured, or peripheral immune cell populations after rectal administration of 2.5-5 g/kg FMT. The clinical response observed post-FMT administration showed no major AEs and minor self-resolving clinical signs of vomiting, diarrhea, and lethargy. Lastly, a limited number of clinical and laboratory variables was assessed to determine the occurrence of AEs and effects on peripheral immune response. We cannot exclude that effects occurred that were outside of the assessed variables. Therefore, we conclude that FMT administration in a small number of clinically healthy dogs was not associated with SAEs, significant changes in the peripheral immunologic variables measured, or the canine DI in the short-term.

Our study used different FMT dosages. The first recipient received 2.5 g/kg FMT to observe potential AEs in healthy dogs that would warrant immediate discontinuation of the study, whereas the other 9 recipients received 5 g/kg FMT. Recommended dosages for FMT are variable in canine and human medicine and dosages between 0.15-5 g/kg have been described.[38,80] Therefore, our dosage was well within the range for FMT in dogs and humans. In addition, all results were non-significant. The FMT preparation used in our study followed previously published protocols, reflecting common use of FMT in clinical practice. Our FMTs were not processed under anaerobic conditions and the microbial composition stability was not validated during processing and storage. We cannot exclude that different processing techniques could have led to different results outcomes for FMT recipients. However, studies in humans using different processing techniques have not consistently been shown to affect results in a clinically meaningful way.[49,80] Lastly, our study only assessed peripheral immune responses in healthy dogs. The assessment of local immunologic changes and changes in diseased dogs were outside of the scope of our study. Further research is warranted to determine if FMT is safe in patients with GI diseases, as well as determine the appropriate application and indications of FMT in dogs.

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CONFLICT OF INTEREST DECLARATION

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Wanakumjorn are employed by the University of California Davis and declare no conflict of interest. Bridget Mclaughlin is employed by the University of California Davis Flow Cytometry Shared Resource Laboratory, which offers flow cytometry on a fee-for-service basis. Jan S. Suchodolski and Agostino Buono are employees of the Gastrointestinal Laboratory at Texas A&M University which offers microbiome assessment on a fee-for-service basis. Jan S. Suchodolski is the Purina PetCare Endowed Chair for Microbiome Research and receives support for microbiome research through the Purina PetCare Research Excellence Funds. Jan S. Suchodolski has also received consulting or speaking fees from Nestle Purina, IDEXX Laboratories, Royal Canin and Hill's Pet Nutrition, Inc. Sina Marsilio is a paid consultant for Dutch Pet, Inc., an online veterinary pet telehealth service and a paid speaker for IDEXX Laboratories.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

The study was approved by University of California, Davis IACUC (Protocol: IACUC #22312). Owners were informed about the purpose of the study and signed a written informed consent form.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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