Influence of Enterococcus faecium SF68 Probiotic on Giardiasis in Dogs

K.W. Simpson, M. Rishniw, M. Bellosa, J. Liotta, A. Lucio, M. Baumgart, G. Czarnecki-Maulden, J. Benyacoub, and D. Bowman

Background: Giardiasis is a common, potentially zoonotic disease, and dogs often harbor and shed cysts without showing clinical signs. Treatment with the probiotic Enterococcus faecium SF68 has been shown to stimulate mucosal and systemic immunity in a variety of animal models and in young dogs, and to reduce giardial cyst and antigen shedding in rodents.

Hypothesis: Adult dogs with chronic naturally acquired giardiasis will have decreased giardial fecal cyst and antigen shedding and increased innate and adaptive immunity after 6 weeks probiotic treatment with E. faecium SF68.

Animals: Twenty adult dogs.

Methods: After a 6-week dietary equilibration period, dogs were randomized to receive E. faecium SF68 or placebo for 6 weeks, and then crossed over to the alternate treatment. We measured cyst shedding, fecal giardial antigen, fecal immunoglobulin A (IgA) concentration, and circulating leukocyte phagocytic activity at multiple timepoints to determine the effect of E. faecium SF68 on giardiasis and immune responses in these dogs.

Results: No differences were observed between placebo or E. faecium SF68 treatment for giardial cyst shedding, fecal antigen shedding, fecal IgA concentration, or leukocyte phagocytic activity.

Conclusions: Short-term treatment with E. faecium SF68 of dogs with chronic naturally acquired subclinical giardiasis fails to affect giardial cyst shedding or antigen content and does not alter innate or adaptive immune responses.

Key words: Fecal IgA; Immunity; Leukocyte phagocytosis; Microflora; Protozoa.

Giardia is a ubiquitous environmental protozoal pathogen that often results in clinical infection in domestic animals and humans, producing acute or chronic gastrointestinal signs. Prevalence of Giardia in dogs ranges from 5 to 35%, depending on age, whereas prevalences approaching 100% are possible in breeding colonies.1,2 A subclinical carrier state is also observed in dogs, which can serve as a reservoir for infection of susceptible individuals.3–5

Whether dogs (or cats) can serve as reservoirs for human giardiasis is debated. Epidemiological correlations between human and canine or feline populations and the risk of human giardiasis have variably documented potentially zoonotic associations. Although some studies showed associations between pet ownership and risk of human giardiasis,6,7 others failed to show any correlation.8 Several assemblages of Giardia exist (A-F), and most are considered potentially zoonotic, although humans appear to be almost exclusively susceptible to infection with assemblages A and B and no epidemiological evidence of substantial infection in humans with other assemblages exists.9,9 Detection of genetically identical assemblages of Giardia in humans, dogs, and cats supports the hypothesis that dogs and cats can serve as vectors for human giardiasis in some instances.7,10–12 However, other studies have revealed almost complete separation of assemblages in dogs and people living in the same area.13 Additionally, the finding of low prevalence of human assemblage types (A or B) in Giardia-infected dogs in areas where the prevalence of human giardiasis is low suggests that the infection of dogs with human assemblage types may be an anthropoposis—an animal infection with a human pathogen (or a “reverse zoonosis”), and questions the interpretation of previous epidemiological studies that implicated a traditional zoonotic transmission.14

Control of giardiasis can be problematic. Treatment with metronidazole or fenbendazole usually clears infection, but environmental decontamination can be difficult to implement, resulting in chronic infections because of repeated reinfection in kennel situations.4,15–18 Vaccination against Giardia is available, but efficacy has been questioned. One group indicated a protective effect of vaccination, but after subsequent studies by others failed to document any protective or therapeutic benefit of Giardia vaccination in dogs.19–22 Dietary methods aimed at decreasing or eliminating Giardia from infected dogs may provide a valuable means of dealing with endemic giardiasis. Probiotics offer a nonpharmacological approach to management of various gastrointestinal pathogens, including Giardia. Enterococcus faecium SF68 and Lactobacillus casei have been shown to reduce Giardia load and shedding in mice.23,24 The beneficial effect of probiotics in mice may reflect a direct effect on Giardia, as has been reported for probiotic Lactobacillus strains,25 as well as enhanced adaptive and innate immune responses, such as the local production of immunoglobulin A (IgA) and enhanced phagocytosis.26–29 Similarly, E. faecium SF68 has been suggested to possess immunomodulatory properties in mice, cats, dogs, and pigs.23,30–32 However, evidence of efficacy against Giardia in nonrodent animals is lacking.

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We sought to determine if adult dogs, chronically infected with Giardia, would exhibit an increase in their innate and adaptive immune responses and a decrease in Giardia cyst shedding, when fed a diet containing *E. faecium* SF68.

Materials and Methods

**Animals and Study Design**

Twenty adult dogs (Marshall Farms) with chronic, naturally acquired subclinical giardiasis were included in the study. The study protocol was approved by the Cornell University Institutional Animal Care and Use Committee. All dogs were housed in AALAC-accredited facilities at Cornell University.

Dogs were stratified by sex and Giardia cyst shedding, and randomly assigned into 1 of 2 groups by means of coin toss, such that each group had 10 dogs. Each group of dogs were housed in individual pens in separate facilities to prevent cross-contamination during the study. All dogs initially were screened for the presence of Giardia, and the absence of other fecal parasites and pathogenic bacteria (*Salmonella, Campylobacter*, and *Yersinia*) by ZnSO4 flotation and culture, respectively. Giardia infection was confirmed by direct immunofluorescence coproscopy, which is regarded as the gold standard for identifying fecal Giardia cysts.

Figure 1 illustrates the trial design. The dogs were fed a balanced and complete control diet\(^*\) twice daily for 6 weeks to equilibrate the counted number by 10,000 (dilution factor). The number of cysts per gram of wet feces was determined by multiplying the direct immunofluorescence coproscopy, which is regarded as the gold standard for identifying fecal Giardia cysts.

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
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<tbody>
<tr>
<td>1</td>
<td>Very hard and dry; requires much effort to expel from body; no residue left on ground when collected. Often expelled as individual pellets</td>
</tr>
<tr>
<td>2</td>
<td>Firm, but not hard; segmented appearance; little or no residue left on ground when collected. Considered normal</td>
</tr>
<tr>
<td>3</td>
<td>Log-like; little or no segmentation visible; moist surface; leaves residue, but holds form when picked up. Considered normal</td>
</tr>
<tr>
<td>4</td>
<td>Very moist (soggy); distinct log shape visible; leaves residue and loses form when picked up</td>
</tr>
<tr>
<td>5</td>
<td>Very moist but has distinct shape; present in piles rather than as distinct logs; leaves residue and loses form when picked up</td>
</tr>
<tr>
<td>6</td>
<td>Has texture, but no defined shape; occurs as piles or as spots; leaves residue when picked up. Diarrhea consistency</td>
</tr>
<tr>
<td>7</td>
<td>Watery, no texture; flat; occurs as puddles</td>
</tr>
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**Quantitation of Giardia Cyst Shedding and Antigen Load**

Fecal samples were collected from dogs on 3 consecutive days each week and feces were scored by a standardized scoring chart that assigned a score that ranged from 1 (hard feces) to 7 (liquid feces) (Table 1). We quantified the fecal cyst load in each dog on the 2nd day.

Figure 1 illustrates the trial design. The dogs were fed a balanced and complete control diet\(^*\) twice daily for 6 weeks to equilibrate the 2 groups. From the 7th to the 13th week, dogs in group A were maintained on the control diet and a placebo powder, whereas dogs in group B were fed control diet and probiotic powder (three-fourth teaspoon, approximating 5 \(\times\) \(10^{8}\) CFU *E. faecium* SF68 [NCIMBI0415])d, which was mixed into the food at every meal. From the 13th to the 18th week, dogs in group B (probiotic) were crossed over to the control diet with placebo whereas dogs in group A (control) were crossed over to the control diet with probiotic.

<table>
<thead>
<tr>
<th>wk 1 - wk 3</th>
<th>wk 7 - wk 12</th>
<th>wk 13 - wk 18</th>
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</thead>
<tbody>
<tr>
<td>wk A</td>
<td>control</td>
<td>placebo</td>
</tr>
<tr>
<td>wk B</td>
<td>control</td>
<td>placebo</td>
</tr>
<tr>
<td></td>
<td>probiotic</td>
<td>probiotic</td>
</tr>
</tbody>
</table>

\* Fecal analysis for Giardia cysts and antigen
\* Fecal IgA analysis & leukocyte phagocytosis analysis

**Fecal Dry Weight**

Fecal dry weight (and percentage dry weight) was determined by drying a measured quantity of feces (10–20 g) overnight at 108°C, and measuring the weight on the 2nd day.

**Qualitative ELISA**

Fecal samples were analyzed by a commercial qualitative ELISA microplate assay\(^*\) according to the manufacturer’s directions with the following modifications for preparing and analyzing uniform fecal wet weight samples. Fecal samples (80 mg) were collected and stored at −80°C until analysis. At time of analysis, the stored fecal samples and reagent diluent were brought to room temperature. Each fecal sample was combined with 400 μL diluent and stored at 4°C overnight to soften the feces and allow for even fecal distribution throughout the diluent. The next day the sample was briefly agitated to homogenize the particulate matter, and 50 μL of the prepared fecal sample were added the ELISA microwell as a test sample by means of a wide orifice micropipet. Negative and positive controls provided by the company were run with each assay. Samples were considered positive if the absorbance at 450 nm was >0.150.

**Assessment of Adaptive and Innate Immune Response**

To assess the adaptive and innate immune responses as potential mechanisms by which the probiotic could be exerting an effect, total fecal secretory IgA and phagocytic ability of dog leukocytes were measured on weeks 3, 9, and 15 by methods described previously.

**Fecal IgA Analysis**

Total fecal IgA was measured by ELISA. Feces from each dog (0.5 g) were collected and processed as follows: fresh feces were vortexed with 1.5 mL of extraction buffer (50 mMol/L EDTA, 100 mg/L soybean trypsin inhibitor in PBS/1% bovine serum albumin).\(^d\) Phenylmethanesulfonyl fluoride (50 μL; 350 mg/L) was added to each tube and the samples centrifuged at 10,000 \(\times\) g for 20 min. The supernatants then were collected and frozen at −80°C until tested for IgA by ELISA as follows: for total IgA analysis, 96-well microtiter plates\(^e\) were coated overnight at 4°C with 250 ng/well of mouse anti-canine IgA antibody\(^f\) in borate buffer (pH 7). Free binding sites were blocked with PBS containing 5% fetal calf serum and 0.1% Tween (ELISA buffer) for 1 hour at 37°C. Duplicate fecal extracts were diluted in ELISA buffer and incubated for 2 hour at 37°C. ELISA plates then were incubated with ELISA buffer containing...
0.1 mg/L of polyclonal goat anti-canine IgA conjugated with horseradish peroxidase for 1 hour at 37 °C. Several washes with PBS 0.1% Tween were performed between each incubation step. Finally, the plates were developed with the TMB microwell peroxidase substrate system according to the manufacturer’s instructions. Because a monoclonal canine IgA standard was not available, values were expressed as optical densities (OD 450 nm). Total IgA was normalized for total fecal protein content. Total fecal protein content was determined by means of a bichinchoninic acid protein assay according to the manufacturer’s instructions.

**Phagocytic Ability of Circulating Leukocytes**

To assess nonspecific upregulation of the innate immune response by the probiotic, granulocyte, and monocyte phagocytic ability were assessed by means of a commercial phagocytosis assay according to the manufacturer’s instructions. Briefly, 1 mL fresh blood was collected into chilled heparinized tubes from each dog and allowed to chill for 10 minutes on ice. A 100-μL aliquot was transferred into a 5-mL tube. Precooled FITC-labeled *Escherichia coli* (20 μL) were added to the blood sample, vortexed, and incubated at 37 °C for 10 minutes, after which phagocytosis was stopped by rapidly chilling the samples on ice and adding 100 μL quenching solution to each sample. Washing solution (3 mL) was then added to each tube, mixed, and centrifuged (5 min, 250 × g, 4 °C). The solution was discarded and a second wash performed. Lysing solution (2 mL) was added to the whole blood, cells were incubated at room temperature for 20 minutes, centrifuged (5 min, 250 × g, 4 °C), and the supernatant was discarded. DNA staining solution (200 μL) was added to the pellet and incubated in the dark for 10 minutes on ice. The cell suspension then was analyzed by flow cytometry by means of a blue-green excitation light (488 nm wavelength), with 10–15 × 10^3 cells counted per sample. Both the percentage of leukocytes having phagocytosed *E. coli*, and the mean fluorescence intensity (which corresponds to the number of bacteria ingested) were analyzed.

**Statistical Analysis**

Data were visually examined and plotted. For cyst shedding and fecal antigen load, median cyst counts or median antigen load for each dog during each of the 3 periods (pretreatment, probiotic, or placebo) were determined, and the within-dog median cyst counts then were analyzed by a repeated-measures ANOVA and any significant differences were analyzed posthoc with a Tukey’s highest significant difference test. For the secretory IgA measurements, because no effect of time was evident, treatment effects were compared by Wilcoxon’s signed rank test because data were not normally distributed. A housing effect was observed with IgA concentrations—1 group of dogs had substantially lower IgA concentrations throughout the trial than did the other group. Innate immunity (phagocytic activity) was evaluated by a repeated-measures ANOVA, and any significant differences were analyzed posthoc with a Tukey’s highest significant difference test.

**Results**

All dogs remained subclinical throughout the study with 95% of fecal scores rating 2 and 5% rating 1 or 3 (3 dogs had a rating of 4 and 3 dogs had a rating of 5 at single time-points during the study out of 900 fecal samples). Fecal Giardia cyst shedding did not differ among periods (pretreatment, probiotic, or placebo) (P = .18) (Fig 2). Fecal Giardia antigen load did not differ between treatments. There was no evidence of upregulated adaptive immunity between groups—IgA concentrations did not differ between treatments (P = .55) (Fig 3). However, a group effect was observed, in that group B dogs had consistently higher IgA concentrations than group A dogs at all measured time points (weeks 3, 9, and 15) (Fig 3). Innate immunity, as assessed by phagocytosis, did not differ between groups. The percentage of cells phagocytosing bacteria was the same before treatment and during treatment (P = .47) (Fig 4A). However, the mean fluorescence index was higher during the acclimatization period before treatment than in either group after treatment with either placebo or probiotic (P < .0001, Fig 4B), suggesting an effect of change of environment, or the vehicle in both the placebo and SF68 powders.
Discussion

Consumption of an *E. faecium* SF68 probiotic for 6 weeks by adult dogs with naturally acquired subclinical chronic Giardia infections failed to affect Giardia cyst shedding or antigen load during, or immediately after the treatment period. After 3 weeks of supplementation, we found no changes in fecal IgA or leukocyte phagocytosis to support an effect of short-term ingestion of SF68 on innate or adaptive immunity in adult dogs. These data suggest that short-term probiotic supplementation with SF68 in adult dogs with chronic giardiasis is not a valid adjunct to therapy or management of giardiasis. These results differ from those of Benyacoub et al., who showed a decrease in Giardia load and shedding in mice fed SF68. Several reasons may account for these differences. The experimental design differed between the 2 studies. The mice in the previous study were treated with the probiotic for several weeks before experimental inoculation with Giardia, whereas the study population had a pre-existing naturally acquired infection before administration of the probiotic. Thus, although infection may be able to be limited with probiotics, possibly by competing for enteric adherence sites, nutrients, and creating an enteric environment less favorable for colonization by Giardia, they may be less effective in affecting a pre-existing Giardia infection. The lack of effect we observed is similar to studies in people that have examined administration of SF68 after exposure to pathogens such as *Vibrio cholerae* and enterotoxigenic *E. coli*, and acute diarrhea in children, although the latter study did find some probiotic combinations beneficial. These findings suggest that short-term posthoc intervention with SF68 is of no value in decreasing Giardia cyst shedding, but additional studies are necessary to determine whether longer-term treatment would effectively decrease cyst shedding or prevent or decrease reinfection, or if pretreatment of dogs with SF68 would prevent or decrease colonization by Giardia.

The lack of alterations in immune response we observed in the present study contrasts with the changes in immune function (increased fecal IgA and increased circulating anti-Distemper–virus vaccine IgG and IgA, and increased proportion of mature B cells) observed in young dogs fed SF68 from weaning to 1 year of age. This is perhaps not surprising, considering the short duration of treatment in the current study. The differences in fecal IgA in the previous study between dogs fed placebo or SF68 only were apparent after 32–44 weeks, reflecting early immune stimulation that contributed to maintenance of fecal IgA concentrations in pups fed SF68, whereas placebo fed pups exhibited a decline starting from 20 weeks. A study in kittens showed increased percentage of CD4+ lymphocytes after the kittens had been fed SF68 for 20 weeks, but no effects of SF68 on other indicators of immune function. Similarly, studies in pigs report the absence of positive changes in immunity in sows or piglets given SF68. However,
Czarnecki-Maulden and colleagues observed increased serum IgA concentration and decreased incidence of naturally occurring diarrhea in kittens fed SF68 when compared with kittens fed a placebo.37 Time to resolution of diarrhea was significantly shorter in SF68 kittens than in controls. The diarrhea outbreak occurred after the kittens had been fed the test diets for 3 months. One can hypothesize that early life intervention might be crucial to impact immune defense mechanisms enabling better protection against pathogen colonization. Nevertheless, it remains to be determined if immune responsiveness to SF68 in dogs is age- or duration of supplementation dependent.

We assessed the effect of short-term ingestion of SF68 on innate immunity by examining nonspecific upregulation of phagocytosis by leukocytes and effect on adaptive immunity by measuring fecal IgA content. We could find no effect of SF68 on phagocytosis, but observed that mean fluorescence index (which corresponds to the number of bacteria phagocyted by leukocytes) decreased after the pretreatment (acclimatization) period in both groups and remained lower throughout the study. This observation would argue for either an environmental effect on phagocytosis, or an effect of the vehicle used in the placebo and SF68 powder formulations. Additionally, although fecal IgA content was not affected by treatment, we observed a group effect in the study, with dogs in group B having consistently higher fecal IgA content than dogs in group A. We cannot explain this observation. Finally, we observed a temporal effect on Giardia cyst shedding, with both groups showing a decrease in cysts during the final treatment phase of the study (weeks 13–17). This finding underscores the value of these cross-over design. Had we only examined one group, we may have concluded that there was an effect of the probiotic.

In summary, this study failed to demonstrate any effect of short-term ingestion of the probiotic SF68 on Giardia shedding or fecal antigen concentration, or any change in innate or adaptive immunity in adult dogs with naturally acquired subclinical chronic giardiasis. Further research is needed to determine whether longer-term supplementation would be effective or whether SF68 ingestion would prevent reinfection after treatment.

**Footnotes**

1. Purina Dog Chow, Nestlé-Purina, St. Louis, MO
2. Merilfluor Cryptosporidium/Giardia Test Kit, Meridian Bioscience Inc, Cincinnati, OH
3. PT5012 Giardia II test, Direct ELISA Techlaboratories Inc, Blacksburg, VA
4. Sigma Chemicals, St. Louis, MO
5. Life Technologies AG, Basel, Switzerland
6. Clone K117 IA8 Serotec, Oxford, UK
7. AA151P, Serotec
8. KPL, Gaithersburg, MD
9. BCA Protein Assay Kit, Pierce Biotechnology Rockford, IL
10. Phagotest, Orpegen, Heidelberg, Germany
11. BD FACSaria, BD BioSciences, San Jose, CA
12. BD FACSAria, BD BioSciences, San Jose, CA

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**References**


