Effect of Supplementation with *Enterococcus faecium* (SF68) on Immune Functions in Cats*

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**CLINICAL RELEVANCE**

To evaluate the effect of supplementation with *Enterococcus faecium* strain SF68 (NCIMB10415) on immune function, responses to a multivalent vaccine were investigated in kittens given palatability enhancer with or without *E. faecium* SF68 daily. *E. faecium* SF68 was detected in the feces of seven of nine treated cats. Supplementation of kittens with *E. faecium* SF68 did not affect developmental parameters. The percentage of CD4+ lymphocytes was significantly higher in the treatment group. There were no statistical differences in measurements of any other nonspecific or specific immune parameters between groups.

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**INTRODUCTION**

Probiotics have been defined as live microorganisms that, when administered in adequate amounts, confer a health effect on the host. It is theorized that probiotics may impart their beneficial health effects either by increasing the resistance to colonization of mucosal surfaces by pathogenic bacteria (colonization resistance) or by exerting an effect on gut-associated lymphoid tissue that results in the production of immunomodulating substances.

Probiotics have commonly been used to modulate the course of a variety of infectious diseases in human medicine. Moreover, the immunomodulating properties of some specific strains of probiotics have been well documented in humans and rodents. In contrast, the few studies that
have been performed in veterinary medicine have focused primarily on large animals, in which probiotics have been used in an attempt to alter the shedding of fecal pathogens or to improve such production parameters as weight gain and feed conversion rate and reduce mortality. In one animal study, *Enterococcus faecium* strain SF68 (NCIMB10415) was fed to a group of puppies vaccinated against canine distemper virus (CDV) while the control group received vaccinations only. Puppies supplemented with *E. faecium* SF68 had increased serum and fecal total IgA concentrations, increased CDV-specific IgG and IgA serum concentrations, and an increased percentage of circulating B lymphocytes versus control puppies, findings that support an immune-enhancing effect induced by this probiotic.

Infection with feline panleukopenia virus (FPV) results in viremia followed by severe gastrointestinal disease. Appropriately vaccinated kittens have sterilizing immunity, but viral upper respiratory tract infections continue to be a major problem in feline medicine. At Colorado State University, approximately 10% of the feline admissions are for the evaluation of respiratory disease. Feline herpesvirus-1 (FHV-1) and feline calicivirus (FCV) are the two viral pathogens implicated in the syndrome. Whereas FCV vaccines are more than 95% effective in preventing disease in vaccinated versus unvaccinated controls after the animals are inoculated with a pathogenic challenge strain, FHV-1 vaccines offer only approximately 60% relative efficacy. Thus, despite widespread vaccination, FHV-1 continues to be a major problem in humane shelters and client-owned cats. Previous attempts to improve efficacy of vaccination have included intranasal administration, which leads to greater side effects, and genetic manipulation of virulent strains, which decreases disease severity but not the prevalence of the carrier state. The carrier state can lead to recrudescence or reinfection of the host as well as transmission to housemates; furthermore, because no therapy has been able to clear chronic viral infection, recurrences of viral shedding and clinical illness are common. Both cell-mediated and IgA mucosal immune responses are considered important in the prevention and control of α-herpesvirus infections. Improved FHV-1 vaccines or responses to vaccinations are needed to lessen morbidity induced by this pathogen.

In this study, we hypothesized that feeding *E. faecium* SF68 to kittens would enhance nonspecific immune responses; FHV-1–, FCV–, and FPV–specific humoral immune responses; and FHV-1–specific cell-mediated immune responses of kittens.

**Materials and Methods**

**Feline Study Population**

Six-week-old specific pathogen–free kittens (*N = 20*) were purchased from a commercial vendor (Liberty Laboratories, Liberty, NY). The kittens were shown to be seronegative for feline leukemia virus antigen and feline immunodeficiency virus antibodies by ELISA (Snap Combo, IDEXX Laboratories, Portland, ME).

**Experimental Design**

All procedures were approved by the Colorado State University Animal Care and Use Committee. After a 10-day equilibration period, the kittens were randomized into two groups of 10 kittens each; the treatment study started when kittens were 7 weeks old. Aliquots of 0.25 to 0.28 g of dry probiotic powder *E. faecium* SF68 (NCIMB10415, LBC ME5 PET, Cerbios-Pharma SA, Switzerland) (approximately 5 × 10⁹ CFU based on dilution count assays) were stored at 4°C for the duration of the study. Similar preparations were used for aliquots of the palatability enhancer (typical pet food coating [main component, liver digest]; 150 mg/tube). Aliquots were
monitored for water absorption and were to be discarded if there appeared to be any clumping of the probiotic or palatability enhancer. Just before administration, one aliquot of palatability enhancer was transferred to one of the stored *E. faecium* SF68 tubes (treatment group) or an empty tube (placebo group) and diluted using room temperature tap water to a total volume of 10 ml. Contents were vortexed for at least 3 minutes and aspirated into a 12-ml syringe. Immediately after vortexing the suspension, 1 ml of either the *E. faecium* SF68 (total daily dose, $5 \times 10^8$ CFU/day) or the palatability enhancer alone (placebo kittens) was given orally to kittens daily until they were 27 weeks old (i.e., for 20 weeks). Both groups were fed dry kitten food ad libitum (typical kitten growth formula meeting all requirements of the Association of American Feed Control Officials; chicken and rice were the main ingredients) and group housed in two separate rooms to avoid cross-contamination with the probiotic. At 9 and 12 weeks of age, all kittens were vaccinated subcutaneously with a modified-live combination vaccine (Felocell 3, Pfizer Animal Health) for FHV-1, calicivirus, and FPV virus as recommended by the American Association of Feline Practitioners.

Sample Collection and Clinical Monitoring
The attitudes and behavior of the kittens were monitored daily throughout the study; body weight was measured weekly. Blood, saliva, and feces were collected from all cats at 7 weeks of age (before initiation of probiotic or palatability enhancer supplementation) and at 9, 15, 21, and 27 weeks of age. In addition, feces were collected from kittens in the treatment group at 28 weeks of age. For each group of kittens, five fecal samples/day were randomly selected from the shared litterbox and scored using a standardized graphic scoring card (Nestlé Purina PetCare), and the daily group means were determined. Fecal extracts from samples taken at 9 and 27 weeks of age were analyzed for total IgA and total IgG measurement according to a previously published protocol. All samples were stored at –80°C until assayed in batches.

**Fecal Assays**
On each sample date, feces from each kitten were plated in eight serial 10-fold dilutions onto KF *Streptococcus* agar (catalog 249610, BD, Franklin Lakes, NJ) and incubated aerobically for 48 hours at 37°C. Ten colonies of each morphology type were picked off using sterile loops and placed in 1.2 ml of brain heart infusion (BD) and stored at –80°C pending analysis. Randomly amplified polymorphic DNA polymerase chain reaction (PCR) was conducted on bacterial isolates from each sample to determine whether viable *E. faecium* SF68 was in the stools of treated cats and to assess whether the probiotic was accidentally transmitted from the treated kittens to the control kittens. The thermocycler parameters were as follows: 30 cycles of 1 minute of denaturation at 95°C, 1 minute of annealing at 40°C, and 4 minutes of extension at 72°C. The 25.5-µl reaction mixture included 2.45 µl of 10× magnesium-free buffer (100 mM Tris-hydrogen chloride [pH 8.3] and 500 mM potassium chloride), 3.22 mM magnesium chloride, 0.4 µl (1 Unit) JumpStart Taq DNA

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**Probiotics have commonly been used to modulate the course of a variety of infectious diseases in human medicine.**
polymerase (Sigma D-4184, Sigma-Aldrich, St. Louis, MO), 1.9 µl deoxyribonucleotide triphosphate mix (2.5 mM), 1 µl primer (100 µM), 15.47 µl PCR water, and 1 µl bacterial culture. The sequence of the primer used was:

GGT TGG GTG AGA ATT GCA CG

Five to 10 µl of the PCR product was run on a 2% agarose gel, and patterns of banding were compared with a positive E. faecium SF68 control. Commercially available ELISAs (Clostridium perfringens [ELISA, Kit No. 92-000-22] and Clostridium difficile [ELISA, Kit No. 94-0150-KT], Techlabs, Blacksburg, VA) were used to determine whether C. perfringens enterotoxins or C. difficile toxin A or B was present in the feces of all kittens. Routine aerobic fecal cultures for Salmonella spp and Campylobacter spp were conducted at the Colorado State University Diagnostic Laboratory.

Immunologic Assays

Complete blood counts, serum biochemical panels, and urinalyses were conducted at the Colorado State University Clinical Pathology Laboratory. Antigen-specific humoral immune responses were estimated by measuring FHV-1–specific IgG, FCV–specific IgG, and FPV–specific IgG in sera; in addition, FHV-1–specific IgG and IgA levels were measured in saliva using adaptations of previously published ELISA assays. For FHV-1–specific IgG and IgA, results were calculated by both the mean absorbance for the triplicate test wells for each sample and by calculation of percentage of ELISA units (see equation, above). For FCV and FPV, mean absorbances were used. Total IgG and IgA concentrations in sera, fecal extracts, and saliva were estimated via commercially available ELISA assays or radial immunodiffusion assay (Bethyl Laboratories, Montgomery, TX).

Cellular immune responses were assessed via flow cytometry and whole blood proliferation assays. Flow cytometry was performed within 12 hours of blood collection using 500 µl of anticoagulated (EDTA) blood incubated at room temperature in erythrocyte lysis buffer (ammonium chloride [buffered], 0.155 M ammonium chloride, 0.010 M potassium bicarbonate, and phenol red [0.0005%]). Cells were washed twice with phosphate-buffered saline, and the resultant cell pellets were resuspended in flow cytometry buffer containing phosphate-buffered saline, 0.1% sodium azide, and 2% fetal bovine serum to attain a concentration of 1 × 10^6 cells/100 µl if possible. Samples with insufficient volume for at least 500 µl of the above suspension were counted, and the cell concentration was recorded. One hundred microliters of each cell suspension was added to individual wells in a round-bottom 96-well plate for immunostaining. Nonspecific binding was blocked by addition of 10% normal cat serum (Jackson ImmunoResearch Laboratories, West Grove, PA). Immunostaining was done at 4°C in the dark in flow cytometry buffer. Lymphocytes were stained for expression of CD4 and CD8 (vpg34; CD4-fitc, vpg9; CD8-rpe; Serotec, Raleigh, NC [Oxford, UK]) and expression of CD44 (IM7; CD44-pe/cy5; Pharmingen, Franklin Lakes, NJ). For analysis of B cells, lysed whole blood was immunostained with cross-reactive antibodies to B220 (ra3-b62; B220-biotinylated eBioscience, San Francisco, CA).
Diego, CA) and CD21 (b-ly4; CD21-apc; BD-Biosciences, Franklin Lakes, NJ) and major histocompatibility class II (MHC class II-fitc; clone CAG5-3D1, Serotec). Cells for analysis were gated on live lymphocyte populations based on forward and side-scatter characteristics. Data were collected on a Cyan MLE cytometer (ra3-b62; B220-biotinylated eBio-science) and analyzed using Summit software (Dako-Cytomation, Fort Collins, CO).

Previously optimized proliferation assays were performed in triplicate using 10 µl of whole heparinized blood preconditioned by incubating in 100 µl of complete tumor medium (modified Eagle’s medium supplemented with essential and nonessential amino acids + 10% fetal bovine serum) at 37˚C with 5% carbon dioxide for 30 minutes before addition of the mitogen or antigen. Cells were stimulated with no antigen (unstimulated), concanavalin A (Sigma-Aldrich; 10 µg/ml), or a crude FHV-1 antigen preparation (1 µl/well, prepared before the start of the study and stored aliquoted at −80˚C) for 96 hours at 37˚C with 5% carbon dioxide. Cells were pulsed with 1 µCi tritiated thymidine/well and harvested 18 hours later onto fiberglass filter mats (Wallac–MicroBeta Perkin–Elmer, Boston, MA). Mats were read using a MicroBeta liquid scintillation counter (Wallac–MicroBeta). The mean stimulation index was calculated for all samples as follows:

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\text{Statistical Evaluation}
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On each sample date, group mean values for all measured parameters were calculated. Differences between the probiotic-treated group and placebo group were analyzed using a mixed analysis of variance model appropriate for a repeated measures experiment. Time was included in the model as a continuous variable. Percentages of cat samples positive for C. perfringens enterotoxin or C. difficile toxins A or B and percentages of gated cells positive for cell surface markers were calculated for each group of cats over the duration of the study and compared by a two-tailed t-test (GRAPHPAD Prism, GRAPHPAD Software, San Diego, CA). Statistical significance was considered to be \( P < .05 \).

\section*{RESULTS}

**Clinical Monitoring**

The stools of all kittens were normal at the beginning of the supplementation period (7 weeks of age). One kitten in each group was removed from the study for reasons unrelated to the study; data from these kittens were therefore removed from the final analysis. Body weight and fecal scores were not statistically different between the two groups over time or at any individual time point (Figure 1).

**Fecal Assays**

Feces from seven of nine treatment cats were positive for E. faecium SF68 at least once point during the study, whereas feces from all control cats were negative for E. faecium SF68 at all time points.
control cats were negative for *E. faecium* SF68 at all time points. *E. faecium* SF68 DNA was not amplified from feces of any treated cat 1 week after supplementation was stopped (week 28). Neither *Salmonella* spp nor *Campylobacter* spp were grown from feces. All samples from placebo cats were negative for *E. faecium* SF68 by randomly amplified polymorphic DNA PCR. Numbers of positive samples for *C. difficile* toxin A or B or *C. perfringens* enterotoxin (Table 1) were not significantly different between the groups over the course of the study.

### Table 1. Percent of Cats Positive for *Clostridium difficile* Toxin A or B and *Clostridium perfringens* Enterotoxin during and after 20 Weeks of Daily Feeding of a Diet Containing *E. faecium* SF68 (5 × 10⁶ CFU/kitten/day)*

<table>
<thead>
<tr>
<th>Age (wk)</th>
<th>Weeks on Trial</th>
<th>C. difficile</th>
<th>C. perfringens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo (n = 9)</td>
<td>Treatment (n = 9)</td>
<td>Placebo (n = 9)</td>
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<tr>
<td>7</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
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<tr>
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</tr>
<tr>
<td>27</td>
<td>20</td>
<td>11.1</td>
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</tr>
</tbody>
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Mean % positive 8.8 11.1 22.2 15.6

*P* value .69 .75 .75

*See Figure 1 for additional details.*

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**Figure 1.** Body weights and fecal scores over time of kittens supplemented daily with either 150 mg chicken digest PO (placebo; n = 9) or 150 mg chicken digest mixed with 5 × 10⁶ CFU/day *E. faecium* SF68 (treatment; n = 9) starting at 7 weeks of age and continuing until 27 weeks of age. Kittens were vaccinated subcutaneously with a commercially available modified-live vaccine at 9 and 12 weeks of age. Box and whiskers represent the minimum, maximum, median, and 25th and 75th percentiles. *P* > .05 at all time points.
Immunologic Assays

Complete blood counts and biochemical profiles were within normal limits for the age group for all cats at all time points. There was no statistical difference between the groups over time or at any individual time point among the assays analyzed. At 21 and 27 weeks of age, the mean levels of FHV-1–specific IgA in serum and saliva were numerically greater in the treatment group than in the placebo group (Figure 2). Moreover, the mean FHV-1–specific serum IgG levels were numerically greater in the treatment group than in the placebo group at 15, 21, and 27 weeks of age (Figure 3). However, these differences in FHV-1 antibody levels did not reach statistical significance. No FHV-1–specific IgG was detected in saliva. FCV-specific IgG levels in serum were similar between groups (Figure 4). At 15 weeks of age, the treatment group serum mean FPV-specific IgG levels were numerically greater than those of the placebo group, but the differences were not statistically significant (Figure 5).

Concentrations of total IgG and IgA in serum were similar between groups (data not shown). Total IgG was not detected in saliva, and total IgA concentrations in saliva were similar between groups (data not shown). Total IgA and IgG concentrations in fecal extracts

Figure 2. FHV-1–specific IgA results in serum and saliva from kittens with (treatment) or without (placebo) E. faecium SF68 supplementation. See Figure 1 for the complete legend. P > .05 for all time points.

Figure 3. FHV-1–specific IgG results in serum from kittens with (treatment) or without (placebo) E. faecium SF68 supplementation. See Figure 1 for the complete legend. P > .05 for all time points.
were similar between groups (data not shown).

Proliferation assays using either 10 μg/ml concanavalin A or 1 μl FHV-1 antigen preparation did not produce significantly different mean maximum counts between groups at any time points. There were no statistical differences between the groups for any cell surface markers at the first four time points (Figure 6). At 27
weeks of age, the treatment group had a significantly higher percentage of gated lymphocytes positive for CD4 (mean, 13.87%) than did the placebo group (mean, 10.61%; \( P = .0220 \)). No other comparisons were significantly different.

**DISCUSSION**

Complete blood cell counts, biochemistry parameters, and body weights were similar between groups of cats over the course of the study. Fecal scores were similar between groups as well, suggesting that use of *E. faecium* SF68 at the dosage described here will induce no noticeable clinical abnormalities. *Salmonella* spp and *Campylobacter* spp shedding was not induced by *E. faecium* SF68 supplementation. Several fecal samples in both groups of kittens were positive for *C. difficile* or *C. perfringens* toxins; however, there was no significant difference in number of positive samples between groups, and positive results did not correlate to the presence of diarrhea. Although *E. faecium* SF68 was documented in the feces of the majority of treated cats, the organism could not be detected 1 week after stopping supplementation. This indicates that the organism persisted in the cats only transiently or was not in feces of the cats at the time tested. We conclude that administration of *E. faecium* SF68 using the dosage described here has no deleterious effects and is safe for administration in the time period studied.

After vaccinations, each of the kittens developed FHV-1−, FCV−, and FPV-specific serum antibody responses that are similar to those seen in other studies, suggesting that the kittens were immunocompetent and that the modified-live vaccine used was viable. There were no statistical differences in the levels of total serum or salivary IgG and IgA between groups.

In a previous study, *E. faecium* SF68 significantly enhanced CDV-specific IgG and IgA antibodies in serum only after the puppies had been supplemented for 31 and 44 weeks, respectively. It was suggested that in these puppies, *E. faecium* SF68 prevented the decline in antibody titers observed in the controls by maintaining high levels of antibodies throughout the year of the study. Based on these results, a longer collection period might be indicated in future feline studies of this type. In addition, one should consider that in the study of puppies, a monovalent vaccine was administered, whereas we used a polyvalent vaccine. The difference in findings between the previous study and the one described here may be species variability, or the administration of three modified-live viruses could have led to potentiated immune responses and masked any potential enhancing effects of the probiotics seen in the puppies. As with most IgA− or IgG−antigen specific antibody assays, there was a large degree of inter- and intra-individual variation for results of the assays. Because of this, challenge studies are more useful for true measure of protection.

The increase in CD4+ cell counts in the treatment group compared with the placebo group without a concurrent increase in CD8+ counts at 27 weeks of age demonstrates systemic immune-modulating effects by the probiotic. Because this study did not show a significant in-
crease in lymphocyte stimulation by FHV-1 or an increase in the expression of the memory cell marker CD44 on the CD4+ lymphocytes in the treatment group, the increase in CD4+ T lymphocytes may have been nonspecific because the cells appear to be unprimed. In humans, there is evidence that CD4+ T lymphocytes dominate the response to herpes simplex virus (HSV)–1 infection over CD8+ T lymphocytes. In mice, a subset of CD4+ T lymphocytes is believed to be responsible for decreased mucosal replication and latency when challenged with HSV-1. In addition, a subset of CD4+ antigen-specific T lymphocytes have been shown to be necessary for induction of protective immunity from HSV-2 infection in mice. Because the CD4+ T lymphocytes of kittens in this study were not additionally characterized via cytokine production profiles or additional cell surface marker characterization, it cannot be determined whether a Th1 or Th2 response predominated.

CONCLUSION

As administered, E. faecium SF68 did not alter developmental parameters in the study subjects but also did not alter most of the immune parameters measured. In future feline studies, challenge inoculation with virulent FHV-1 could be used to more fully evaluate protection from disease, the ultimate goal of immunomodulation.

ACKNOWLEDGMENT

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REFERENCES