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Pilot study to evaluate the effect of oral supplementation of *Enterococcus faecium* SF68 on cats with latent feline herpesvirus 1

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Feline herpesvirus 1 (FHV-1) infection is extremely common in cats and is frequently associated with morbidity because of recurrent ocular and respiratory clinical signs of disease. *Enterococcus faecium* strain SF68 is an immune-enhancing probiotic used as a dietary supplement. In this pilot study, 12 cats with chronic FHV-1 infection were administered either SF68 or a placebo, monitored for clinical signs of disease, monitored for FHV-1 shedding, and evaluated for FHV-1 specific humoral and cell-mediated immune responses and fecal microbiome stability. Fecal microbial diversity was maintained throughout the study in cats supplemented with SF68, but decreased in cats fed the placebo, indicating a more stable microbiome in cats fed SF68. While clinical results varied among individual cats, the overall findings suggest that administration of the probiotic lessened morbidity associated with chronic FHV-1 infection in some cats. Additional study is warranted to determine efficacy in a clinical setting.

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Feline herpesvirus 1 (FHV-1) infection is common in cats, is extremely contagious between cats, and frequently results in severe clinical disease in cats around the world.[1–6] For example, in one of our recent prevalence studies at a humane society in north central Colorado, FHV-1 DNA was amplified from throat swabs (42/45 samples; 93.3%) or nasal discharges (41/45 samples; 91.1%) from most cats tested.[1] In the acute phase of infection, fever, sneezing, nasal discharge, conjunctivitis, cough, dyspnea, and death can occur. FHV-1 persists as a latent infection after primary infection. While FHV-1 infected cats can be clinically normal for periods of time, the infection can be activated by crowding, other concurrent diseases, and other forms of stress. Recurrent conjunctivitis, keratitis, sneezing, and nasal discharge are common manifestations. In addition, during periods of activation, FHV-1 shedding rates are high, potentially resulting in the infection of other cats.

Administration of FHV-1 containing vaccines does not prevent infection[7] and there are currently no drugs that eliminate FHV-1 from the body. The drugs used orally are expensive, can be ineffective, and can have toxicity.[8] Daily administration of lysine is used to attempt to lessen severity of recurrent FHV-1 associated disease.[9,10] While there are multiple lysine products available, they are inconvenient for owners to administer and are often ineffective. In addition, cats are very difficult to medicate orally unless the substance is incorporated into their diet. Several topical medications have been used for the treatment of ocular FHV-1 infections, but none are labeled for use in the cat, the products are often irritating as well as being expensive and difficult for owners to administer.[11] Additional protocols that can be used in the management of the clinical manifestations of chronic, recurrent FHV-1 infection are needed.

Chronic feeding of the probiotic *Enterococcus faecium* SF68 has been shown to be safe and improved several specific and non-specific immune responses when fed to cats or dogs.[12,13] In the study described here, we hypothesized that feeding of SF68 would decrease clinical disease, episodes of FHV-1 shedding,

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and numbers of FHV-1 DNA copies shed over time in cats with chronic FHV-1 infection.

Materials and methods

Feline study population

One-year-old, mixed sex cats ($n = 12$) with chronic FHV-1 infection were available for study.[11] Each of these cats developed conjunctivitis and FHV-1 infection in the previous study and most had intermittent clinical signs of conjunctivitis in the intervening time period prior to use in this study. The cats were seronegative for feline leukemia virus antigen and feline immunodeficiency virus antibodies (Snap FeLV/FIV Combo, IDEXX Laboratories, Portland, Maine) and had been housed in a specific pathogen-free facility. Protocols followed in this study were approved by a Colorado State University (CSU) campus-wide Animal Care and Use Committee in accordance with federal guidelines.

Experimental design

Over a 2-week period (equilibration period), the cats were group housed together, ad libitum-fed the nutritionally complete dry cat food to be used throughout the study, and evaluated daily for the presence of conjunctivitis, sneezing, and nasal discharge. At the end of the 2-week period, the cats were randomly distributed, regardless of whether they had been administered topical cidofovir in the previous study,[11] into two groups of six cats and moved into different rooms of the specific pathogen-free facility to begin the supplementation period of the study. At this time and for the duration of the study, one group of cats was administered *E faecium* SF68 NCIMB10415 (5×10^8 CFU/day) in palatability enhancer (chicken digest; 15 mg per cat) and one group (placebo) was administered the palatability enhancer alone (15 mg per cat). To evaluate the impact of relocation on health status and probiotic response, the cats were group housed for 28 days, individually housed for 28 days, and then group housed for an additional 84 days. On day 56 of the last group housing period, ovariohysterectomy or neutering was performed using standard CSU protocols for the procedures, including postoperative pain management. The cats were adopted to private homes at the end of the study.

Sample collection and clinical monitoring

The presence or absence of sneezing, nasal discharge, or conjunctivitis was recorded daily by two trained observers that were masked to the supplementation groups. Body weight was measured weekly. Presence of abnormal stool character was to be noted daily if it occurred. Venous blood was collected from all cats twice prior to supplementing with SF68 or placebo and then approximately every 14 days for the duration of the study. Throat swabs were obtained weekly by gently rolling standard cotton tipped wooden applicators on the roof of the mouth of the awake, non-sedated cats. The swabs were placed in 1.0 ml sterile 0.01 M phosphate buffered saline and allowed to sit at room temperature for 2–3 h before being stored at −70°C until analyzed. Feces were collected from each cat twice prior to supplementing with SF68 or placebo and then throughout the supplementation phase.

Assays

Temporal temperature gradient electrophoresis (TTGE), a molecular microbial ecology technique using polymerase chain reaction (PCR) amplified ribosomal DNA fragments, was used to examine qualitative differences in bacterial populations.[14] Feces were extracted by the phenol/chloroform method in order to retrieve the DNA. The DNA was then subjected to PCR, which uses specific, fluorescently tagged, primers that bind to the conserved region of the bacterial 16S rDNA. Fragments of DNA with different sequences were separated based on electrophoretic mobility on polyacrylamide gels containing a linear temperature gradient. Melting temperatures of different bacteria are different, causing the various sequences to stop at different positions on the gel. The resulting pattern of bands corresponds to the predominant members in the microbial community (the microbiome). Gel banding patterns were evaluated for microbial population diversity using Simpson's index and Shannon–Wiener index.[15,16]

Serum FHV-1 IgG antibody enyzme-linked immunosorbent assay (ELISA) was performed as previously described on serum separated at each blood collection (Diagnostic Laboratory, Heska Corporation, Loveland, Colorado).[17] Concanavalin A stimulation of lymphocytes (10 μg/ml) and FHV-1 antigen stimulation of lymphocytes were performed at the beginning of the study and then monthly.[12] The DNA extracted from throat swabs was amplified in conventional and fluorogenic PCR assays weekly.[11]

Statistical evaluation

The number of days each cat had evidence of conjunctivitis within each period, lymphocyte responses to concanavalin A or FHV-1 antigens, and FHV-1 IgG levels between the supplementation groups were analyzed using a mixed analysis of variance (ANOVA) model appropriate for a repeated measures experiment using the values collected during the equilibration period as a covariate. The percentage of positive results for sneezing, nasal discharge, conjunctivitis, and end-point FHV-1 PCR assay was compared between the equilibration period and the supplementation period for each cat and between the two groups during the equilibration period and the supplementation period by Fisher's exact test. Differences in microbial diversity were compared by Student's *t*-test. Statistical significance was defined as $P < 0.05$.

Results

The palatability enhancer with or without SF68 was accepted by all cats, each of which maintained body weight and good fecal quality. Cats fed the placebo had decreased ($P < 0.05$) fecal microbiota diversity, as calculated by either the Simpson or Shannon–Wiener index, during the supplementation phase (Table 1). Microbiota diversity was maintained throughout SF68 supplementation.

Regardless of the study period, sneezing or nasal discharges were uncommon in all 12 cats and statistical differences for individual cats and between groups of cats were not evaluated. The repeated measures analysis showed a trend towards significance between groups after sterilization within the last group housing period (days 56–84), with SF68-supplemented cats (16.8%) having less days with conjunctivitis than placebo cats (30.9%). Results varied among individual cats. In the SF68-supplemented group, two cats had a decrease in percentage conjunctivitis (one statistically significant) and one cat had a significant increase in percentage conjunctivitis in the supplementation period when compared to the equilibration period (Table 2). The SF68-supplemented cat with the significant increase in percentage conjunctivitis developed spastic entropion early in the supplementation period which ultimately required two surgical eyelid tacking procedures. Thus, on many days of the supplementation period, it could not be determined whether conjunctivitis was related to physical inflammation from the entropion or activated FHV-1 infection. In the placebo group, two cats had an increase in percentage conjunctivitis (one was statistically significant) during the supplementation period when compared to the equilibration period (Table 2). In the equilibration period, conjunctivitis was detected on 11/84 (13.1%) observation points from SF68-supplemented cats and on 18/84 (21.4%) observation points from placebo cats; this difference was not significantly different ($P = 0.221$). In the supplementation period, conjunctivitis was detected on 136/835 (16.3%) observation points from SF68-supplemented cats and on 246/839 (29.3%) observation points from placebo cats; this difference was significantly different ($P < 0.001$).

All of the cats had previously been shown to be FHV-1 infected by fluorogenic PCR assay performed on DNA collected from conjunctiva cells[11] and so were known to be FHV-1 carriers. Over the course of the study described here, FHV-1 DNA was detected in cells collected by throat swab in 8/12 cats by conventional PCR assay and detection was intermittent (overall − 10 positive samples); no statistical differences were noted in individual cats between periods or between groups in the supplementation period.

Table 1. Microbiota stability before and during supplementation with SF68 or a placebo

Group	Equilibration	Supplementation	*P* value (vs equilibration)
Number of bands			
SF68 supplemented	22.40	22.09	0.880
Placebo supplemented	24.40	20.53	0.092
P value	0.449	0.593	
Simpson's index of microbiota diversity			
SF68 supplemented	0.863	0.869	0.851
Placebo supplemented	0.899	0.839	0.050
P value	0.114	0.513	
Shannon–Wiener index of microbiota diversity			
SF68 supplemented	2.457	2.538	0.624
Placebo supplemented	2.689	2.385	0.046
P value	0.079	0.492	

The equilibration period was 14 days and the supplementation period was 140 days. Results of supplementation samples were pooled within cat.

Table 2. Percentage of days that conjunctivitis was detected in FHV-1 infected cats before and during supplementation with SF68 or a placebo

Group/cat	Equilibration	Supplementation	*P* value
SF68 supplemented			
1	0	0	1
2†	7.7	0	0.085
3†	0	0	1
4	42.9	7.1	0.006
5†	0	2.9	1
6*,†	28.6	82.9	0.00004
Placebo supplemented			
1†	0	0	1
2†	64.3	85.7	0.053
3	7.1	0.7	0.174
4†	0	0	1
5†	50	88.6	0.001
6	7.1	0.7	0.174

The equilibration period was 14 days and the supplementation period was 140 days. Comparisons were made by Fisher's exact test with significance defined as $P < 0.05$.

*This cat developed spastic entropion which required two surgical procedures during the supplementation period.

†Cats that were FHV-1 PCR positive during the course of the study.

Of the eight FHV-1 PCR positive cats, four had previously been administered cidofovir. None of the cats were positive for FHV-1 DNA by fluorogenic PCR assay and so the effect of SF68 supplementation on the level of FHV-1 shedding could not be determined.

All of the cats had high FHV-1 antibody ELISA unit values at the beginning of the study and there were no statistical differences detected between groups over time. Lymphocytes from all cats responded to concanavalin A (Fig 1) and FHV-1 antigens (Fig 2) but statistical differences between groups were not detected.

Discussion

As previously noted, SF68 supplementation is safe for use in cats.[12] During the supplementation phase cats were exposed to minor stress from neutering and housing relocation. Consequently, microbiota diversity decreased in the placebo cats, but not the SF68-supplemented cats, during this phase of the study. This was, most likely, a response to the minor stress experienced during this phase. Decreased microbiota diversity has previously been observed in feces of infants with atopic eczema[18] and in plaque of children with gingivitis. While diarrhea was not observed in the present study, fecal quality was not specifically measured. SF68 ingestion has previously been shown to improve fecal quality in both puppies and kittens.[13,19] The improved microbiota diversity in cats fed SF68 in this study may provide the mechanism for improved fecal quality observed in these previous studies.

In this study, the minor stress the cats were exposed to was not associated with detectable recrudescence of FHV-1 shedding as determined by real time PCR assay. Glucocorticoid administration, which has been used successfully to activate FHV-1 in other studies, was not used in this group of cats because of humane reasons. Respiratory signs were infrequent before and

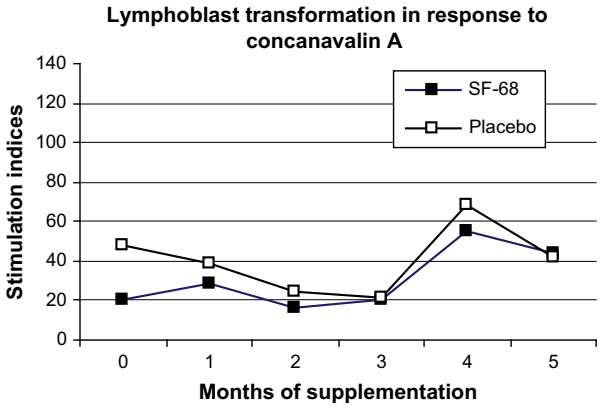

Fig 1. Lymphocyte responses to concanavalin A in cats with or without SF68 supplementation. There are six cats per group. No statistically significant differences were detected between groups.

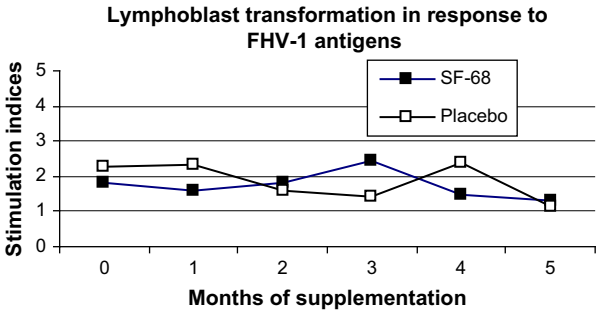

Fig 2. Lymphocyte responses to FHV-1 antigens in cats with or without SF68 supplementation. There are six cats per group. No statistically significant differences were detected between groups.

after the supplementation period in both groups of cats suggesting that minor stress failed to reactivate FHV-1 enough to cause repeated respiratory signs of disease. It is also possible that the application of cidofovir, a drug with activity against FHV-1, to the conjunctiva of some cats in the previous study[11] affected the results of the current study. However, we believe that hypothesis is unlikely as FHV-1 infection cannot be eliminated by any drug, the cats had not been treated for 12 weeks prior to entering this study, and of the eight FHV-1 positive cats in this study, four had been administered cidofovir.

Overall, 8/12 cats had active disease over the course of the study that was attributed to FHV-1 infection. Two of the six cats in the placebo group had numerically or statistically increased percentage conjunctivitis during the supplementation period when compared to the equilibration period suggesting reactivation of FHV-1 associated conjunctivitis in these cats. Detection of numerically or statistically decreased percentage conjunctivitis in two SF68-supplemented cats during the supplementation period when compared to the equilibration period and the detection of significantly less percentage conjunctivitis in the SF68-supplemented cats compared to placebo supplemented cats within the supplementation period but not within the equilibration period suggest that SF68 exerted a treatment effect. Results of this pilot study should be interpreted carefully, but suggest that further studies in naturally infected cats under field conditions should be performed.

The results of this study cannot be used to determine the mechanism by which SF68 exerted the apparent beneficial effect. There was no detectable -enhancement of FHV-1 serum IgG antibody responses or lymphocyte responses to concanavalin A or FHV-1 antigens in SF68-supplemented cats. However, in previous work, we showed a statistically significant increase in CD4+ lymphocyte numbers in SF68-supplemented cats over time which may have played a role in the current study.[12] In addition, in the previous work, we documented numerically greater FHV-1 specific IgA levels in serum and saliva of supplemented

cats.[12] In future studies of this type, FHV-1 specific IgG and IgA antibody responses should be determined on serum and tears.

In previous work, we showed FHV-1 DNA to be readily amplified from throat swabs from cats with respiratory disease.[1] Thus, in the design of the study described here, we chose to evaluate the FHV-1 PCR assays on throat swabs rather than conjunctival swabs because of potential irritation and FHV-1 activation from collecting multiple conjunctival samples over time. We hypothesize that our failure to amplify FHV-1 DNA from more cats related to the relative lack of clinical signs of respiratory disease and that higher numbers of positive samples would have been obtained from conjunctival cells. Results of the fluorogenic PCR assay on DNA extracted from conjunctival cells were used to show a statistical difference in FHV-1 shedding from cats with and without treatment with cidofovir.[11] In future studies of SF68 supplementation on cats with conjunctivitis, samples from the conjunctiva should be used to determine effects on viral shedding.

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