Evidence for Immune Modulation Induced by a Probiotic

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Introduction

Glossary of Abbreviations
CDV: Canine Distemper Virus
IFA: Immunofluorescent Antibody Testing

Probiotics are live microorganisms that when administered in adequate amounts confer a health effect on the host.[1] There have been many studies of the effects of probiotics on the health of humans, but few in small animals. In reviews of human studies involving probiotics. it was stated that well-established probiotic effects include:[1,2]

1. Prevention and/or reduction of duration and complaints of rotavirus-induced or antibiotic-associated diarrhea as well as alleviation of complaints due to lactose intolerance;
2. Reduction of the concentration of cancer-promoting enzymes and/or putrefactive (bacterial) metabolites in the gut;
3. Prevention and alleviation of unspecific and irregular complaints of the gastrointestinal tracts in healthy people;
4. Beneficial effects on microbial aberrancies, inflammation and other complaints in connection with inflammatory diseases of the gastrointestinal tract, *Helicobacter pylori* infection or bacterial overgrowth;
5. Normalization of passing stool and stool consistency in subjects suffering from obstipation or an irritable colon;
6. Prevention or alleviation of allergies and atopic diseases in infants; and
7. Prevention of respiratory tract infections (common cold, influenza) and other infectious diseases as well as treatment of urogenital infections.

Immune-mediated and infectious diseases are very common in small animals so the potential beneficial effects of probiotics that can immune modulate could significantly impact veterinary practice. All mechanisms of immune modulation have not been characterized, and it is clear that these effects vary by the probiotic. Some probiotics may beneficially influence innate and acquired immunity systemically by a variety of proposed mechanisms including inducing cytokine production, natural killer cell activity, and both specific and nonspecific immunoglobulin production.[2]

Several review articles in human medicine have recently suggested that the evidence indicating probiotics have provided a beneficial effect for human conditions such as *Clostridium difficile* diarrhea and hospital-acquired pneumonia is minimal and that larger, more rigorously controlled multicenter studies should be performed. These findings emphasize that biological effects of individual probiotics will vary and that each probiotic introduced should be rigorously evaluated in a controlled fashion to define the potential for clinical utility.[3-5]

In addition, the source of the probiotic should also be considered. For example, in recent veterinary studies, the majority of products claiming to contain probiotics generally did not meet the label claim when evaluated.[6,7] One exception is the *Purina Pro Plan Veterinary Diets* probiotic, *Enterococcus faecium* SF68 (*FortiFlora*). It was recently shown that this probiotic stored appropriately still met the label claim for bacterial numbers several years after production.

The potential benefit of immune-modulating probiotics to animal health could be considerable.[8] Some of the strongest data supporting immune-modulating properties of some probiotics in people and dogs are associated with the treatment of atopy and inflammatory bowel diseases.[9-13]

Enterococcus faecium strain SF68 (NCIMB10415) was originally isolated from the feces of a healthy baby and was initially shown to inhibit the growth of a number of enteropathogens.[14] The purpose of this lecture is to summarize key findings regarding past and ongoing studies of the potential immune-modulating effects of this probiotic.

Dog Immune Modulation Studies

Enterococcus faecium strain SF68 was fed to a group of puppies vaccinated for canine distemper virus (CDV) and compared over time to a control group that was similarly vaccinated, but not fed the probiotic.[15] A number of findings suggested an immune-modulating effect of the probiotic. The puppies supplemented with SF68 had increased serum and fecal total IgA concentrations, increased CDV-specific IgG and IgA serum concentrations, and increased percentage of circulating B lymphocytes when compared to control puppies. The effect on CDV-specific IgG and IgA antibodies in serum was seen only after the puppies had been supplemented for 31 and 44 weeks, and it was believed that SF68 prevented the decline in antibody titers observed in the controls by maintaining high levels of antibodies in the supplemented puppies.

In the first study, immunological parameters were not assessed until 10 weeks after starting supplementation with the probiotic. Clinical observations suggest that immune modulation induced by this probiotic may occur sooner than 10 weeks. Thus, a double-blind, placebo-controlled study

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in healthy young adult Beagles was recently performed at the Center for Companion Animal Studies. Due to prepublication restrictions, this proceedings will only present the general findings with more extensive review of the results presented at the Summit. Using flow cytometry for the measurement of cellular findings and a serum-based cytokine panel, evidence for immune modulation induced by the probiotic was shown as early as four weeks after supplementation.[16]

In another ongoing study at the Center for Companion Animal Studies, the effect of *E. faecium* SF68 on the clinical outcomes of American Pitbull Terriers with generalized demodecosis will be shared at the Summit.

Kitten Immune Modulation Study

In a follow-up study, a similar experimental design used to assess vaccine responses in puppies was applied to a study group of kittens.[17] In that study, it was 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. Twenty 6-week-old SPF kittens were purchased from a commercial vendor and divided into two groups. One group was fed SF68 daily, and the other group was fed the placebo starting at 7 weeks of age.

At 9 and 12 weeks of age, a commercially available FVRCP-modified live vaccine was administered SQ, and the kittens were followed until 27 weeks of age. 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 prior to starting probiotic or placebo supplementation at 7 weeks of age and at 9, 15, 21, and 27 weeks of age. In addition, feces were collected from kittens in the treatment group after the study was completed at 28 weeks of age.

For each group of kittens, five fecal samples per day were randomly selected from the shared litter box and scored using a standardized graphic scoring card. Fecal extracts from samples taken at 9 and 27 weeks of ages were analyzed for total IgA and total IgG. Other parameters monitored included randomly amplified polymorphic DNA (RAPD)-PCR on feces to determine if 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. Commercially available ELISAs were used to determine whether *Clostridium perfringens* enterotoxins or *C. difficile* toxins A/B were present in the feces of the kittens. Routine aerobic fecal cultures for *Salmonella* spp. and *Campylobacter* spp. were performed. Complete blood counts, serum biochemical panels, and urinalyses were performed to assess for adverse events induced by the probiotic. Antigen-specific humoral immune responses were estimated by measuring serum FHV-1-specific IgG, FHV-1-specific IgA, FCV-specific IgG, and feline panleukopenia-specific IgG in sera as well as FHV-1 specific IgG and IgA levels in saliva using adaptations of previously published ELISA assays. Total IgG and IgA concentrations in sera, fecal extracts, and saliva were estimated by use of commercially available ELISA assays or radial immunodiffusion assay. Cellular immune responses were assessed via flow cytometry and whole blood proliferation assays. Lymphocytes were stained for expression of CD4, CD8, CD44, MHC Class II, and B cells. In addition, lymphocyte proliferation in response to concanavalin A and FHV-1 antigens was assessed.

Body weight and fecal scores were not statistically different between the two groups over time or at any individual time points. Feces from seven of nine treatment cats were positive for SF68 on at least one time point during the study, whereas feces from all control cats were negative for SF68 at all time points. SF68 DNA was not detectible from feces of any treated cat one week after stopping supplementation (week 28). All samples from placebo cats were negative for SF68 by RAPD-PCR. Neither *Salmonella* spp. nor *Campylobacter* spp. were grown from feces. Numbers of positive samples for *C. difficile* toxins A/B or *C. perfringens* enterotoxin were not significantly different between the groups over the course of the study.

Complete blood counts and biochemical profiles were within normal limits for the age group for all cats at all time points. A number of the immune markers were numerically greater in the SF68 kittens versus the placebo group, but did not reach statistical significance. For example, at 21 and 27 weeks of age, the mean levels of FHV-1-specific IgA in serum and saliva were greater in the treatment group when compared to the placebo group. Moreover, the mean FHV-1-specific serum IgG levels were greater in the treatment group when compared to the placebo group at 15, 21, and 27 weeks of age. At 15 weeks of age, the treatment group serum mean FPV-specific IgG levels were greater than those of the placebo group. There were no statistical differences between the groups for any cell surface markers at the first four time points. However, at 27 weeks of age, the treatment group had a significantly higher percentage of gated lymphocytes positive for CD4 (mean 13.87%) than the placebo group (mean 10.61%, $p = 0.0220$).

In this study, we concluded that SF68 was safe to administer to cats and the increase in CD4+ cell counts in the treatment group compared to the placebo group without a concurrent increase in CD8+ counts at 27 weeks of age demonstrated a systemic immune modulating effect by the probiotic. Because we did not show a significant increase 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 as the cells appeared to be unprimed. As the CD4+ T lymphocytes of kittens in this study were not additionally characterized via cytokine production profiles or additional cell-surface marker characterization; it could not be determined whether

a Th1 or Th2 response predominated. We believed that sample size and/or the duration of the study may have precluded detection of statistical differences between the groups in regard to FPV, FCV, and FHV-1 antibody titers.

Chronic Feline Herpesvirus 1 Study

Since the normal kitten study documented potential effects of *E. faecium* SF68 on cell-mediated immunity in cats, we chose to study the potential effects on feline herpesvirus 1 (FHV-1).[18] This infectious agent is extremely common in cats and is frequently associated with morbidity because of recurrent ocular and respiratory clinical signs of disease. In addition, there is no known drug therapy that consistently eliminates the carrier state, and vaccination does not provide sterilizing immunity. In this study, it was hypothesized that feeding SF68 would decrease clinical disease, episodes of FHV-1 shedding, and numbers of FHV-1 DNA copies shed over time in cats with chronic FHV-1 infection.[11]

Overall, 12 cats with chronic FHV-1 infection were administered either SF68 or the palatability enhancer as 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 as well as for fecal microbiome stability. After an equilibration period, mild stress was induced over time by changing the housing of the cats from cages to group housing multiple times over a five-month period.

The SF68 was well-tolerated by all cats. 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. Upper respiratory signs of disease were not exacerbated in this model of stress. While results varied among cats, those administered SF68 had fewer episodes of conjunctivitis than the placebo group during the supplementation period suggesting that administration of the probiotic lessened morbidity associated with chronic FHV-1 infection exacerbated by stress.

Murine Acute *Giardia* Study

In previous work, mice administered SF68 and then infected with *Giardia intestinalis* shed fewer trophozoites and less *Giardia* antigen than the placebo group.[19] In addition, supplemented mice had increased CD4+ cells in Peyer's patches and the spleen as well as increased anti-*Giardia* intestinal IgA and serum IgG when compared to untreated mice.

Chronic Subclinical *Giardia* Study in Dogs

When SF68 was administered to 10 adult dogs with chronic, subclinical *Giardia* infection, no differences in cyst shedding or fecal antigen testing were found when compared to 10 placebo-treated dogs.[20] In addition, there were no differences between groups in fecal IgA concentrations. In contrast to the mouse study, the dogs were previously infected by *Giardia*, which may have affected the results. In addition, the study was only for six weeks; in the previously discussed puppy study, some of the significant immune-modulating effects were not seen until later in the supplementation period.[1]

Shelter Animals Acute Nonspecific Diarrhea Study

In a recent study, we hypothesized that cats and dogs housed in an animal shelter that were fed SF68 would have decreased episodes of diarrhea and improved fecal scores compared to untreated cats and dogs in the same environment.[21] The cats were housed in two different rooms, and the dogs were housed in two different rooms in a northern Colorado Animal Shelter. The cats and dogs were all fed a standardized diet by species. Animals in one room were supplemented daily with *E. faecium* SF68, and animals in the alternate room were supplemented daily with a placebo. Otherwise, management of the rooms was identical for the duration of the study. To reduce risk of a room influence on the results of the study, the room in which cats or dogs were being supplemented with *E. faecium* SF68 was switched after one month, with a one-week washout period to lessen the possibility that SF68 surviving in the environment could influence the results of the study.

During the study, routine shelter cleaning and disinfection protocols were being followed. Prior to cleaning the room each morning, feces in the cage of each animal was scored by one of the investigators using the Purina Fecal Scoring System for Dogs and Cats. This person was blinded to the treatment groups. After scoring, feces from dogs with a score from 4 to 7 (indicating mild to severe diarrhea) were collected and transported to Colorado State University for infectious disease testing, which included microscopic examination for parasites eggs, cysts, and oocysts after zinc sulfate centrifugation flotation and immunofluorescent antibody testing (IFA) for *Cryptosporidium* oocysts and *Giardia* cysts (Merifluor® *Cryptosporidium/Giardia*, Meridian Bioscience Inc., Cincinnati, OH). The percentages of dogs or cats with diarrhea of >2 days duration were calculated over the course of the study. A generalized linear mixed model using a bionomial distribution with treatment being a fixed effect and the room being a random effect was used to assess for statistical differences between treatment groups. Presence of parasites was included as a covariate. Significance was defined as $p < 0.05$.

Diarrhea prevalence rates were low for all dogs in the study, and statistical differences were not detected. However, the percentage of cats with diarrhea >2 days was 7.7% for the probiotic group and 20.7% for the placebo group. This result was significantly different ($p = 0.0297$). These results suggest that administration of SF68 to cats housed in shelters may lessen the numbers of days with diarrhea. As this was a short-term study, this effect may have been from probiotic influences on intestinal microbiota rather than systemic immune enhancing effects.

Summary and Conclusions

The evidence gathered to date suggests that *E. faecium* SF68 has immune-modulating effects in dogs and cats and may be effective as an aid in the management of select clinical disorders. Further data is needed to detail the range of immune modulation and to provide comparative data among probiotics.

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