Supplementation of Food with *Enterococcus faecium* (SF68) Stimulates Immune Functions in Young Dogs[1]

Jalil Benyacoub, Gail L. Czarnecki-Maulden,* Christoph Cavadini, Thérèse Sauthier, Rachel E. Anderson,* Eduardo J. Schiffrin and Thierry von der Weid[2]

*Nestec Limited, Centre de Recherche, Vers-chez-les-Blanc, 1000 Lausanne 26, Switzerland and *Nestlé Purina Petcare Research, St. Joseph, MO 64503*

ABSTRACT The gut microflora play a crucial role in several physiologic functions of the host, including maturation of the gut-associated lymphoid tissues during the first months of life. Oral administration of probiotic lactic acid bacteria (LAB) modulates the immune system of humans and some laboratory animals. This effect has never been examined in dogs; therefore, our aim was to study the capacity of a probiotic LAB to stimulate immune functions in young dogs. Puppies were allotted to two groups receiving either a control diet or a diet supplemented with 5×10^8 colony forming units (cfu)/d of probiotic *Enterococcus faecium* (SF68) from weaning to 1 y of age. Fecal and blood samples were collected from the dogs at different time points for the measurement of fecal immunoglobulin (Ig)A, circulating IgG and IgA, and the proportions of lymphoid cell subsets. Fecal IgA and canine distemper virus (CDV) vaccine–specific circulating IgG and IgA were higher in the group receiving the probiotic than in controls. There were no differences in the percentages of $CD4^+$ and $CD8^+$ T cells between the groups, but the proportion of mature B cells [$CD21^+$/major histocompatibility complex (MHC) class II^+] was greater in those fed the probiotic. These data show for the first time that a dietary probiotic LAB enhance specific immune functions in young dogs, thus offering new opportunities for the utilization of probiotics in canine nutrition. J. Nutr. 133: 1158–1162, 2003.

KEY WORDS: • *dogs* • *Enterococci* • *probiotic* • *immunity* • *vaccine*

The intestinal microflora play a crucial role in host defense as demonstrated by their ability to modulate both innate and acquired immunity at the local as well as systemic levels (1,2). Due to these immunological properties, specific strains of lactic acid bacteria (LAB),[3] defined as probiotics, have raised considerable interest in recent years. When ingested as a feed supplement in sufficient numbers, probiotics are live microorganisms that beneficially affect the gastrointestinal balance, going far beyond the conventional nutritional effect (3). The mechanisms underlying the immune modulating properties of probiotics are not fully understood. However, they may be due indirectly to the ability of probiotics to balance the intestinal microflora and/or be a consequence of a direct adjuvant effect on the production of immune factors, such as cytokines (2). In fact, several strains of LAB were shown to enhance nonspecific immunity in vitro as well as in vivo, including the release of tumor necrosis factor-α and interleukin 6 (4), increased phagocytosis in mice and humans (5,6) and stimulated natural killer cell activity (7,8). Their ability to specifically modulate the host's immune responses to pathogens was also demonstrated (9). An increase in rotavirus-specific antibodies was detected in children with acute rotavirus diarrhea who received *Lactobacillus rhamnosus* (10). Moreover, it was shown that administration of *L. johnsonii* to human volunteers boosted the specific immunoglobulin IgA response to the *Salmonella typhi* vaccine Ty21a (11).

Enterococci are normal inhabitants of the gut flora of humans (12) and animals (13), thereby giving a rationale for their use as a component of functional foods. *Enterococcus faecium* (strain NCIMB10415; SF68) is a LAB with inhibitory effects against important enteropathogens, including enterotoxigenic *Escherechia coli*, salmonellae, shigellae and clostridia (14). Therefore, it was suggested that this bacterium might be useful as an antidiarrheal agent. In fact, the efficacy of SF68 in the treatment of antibiotic-associated as well as acute diarrhea in humans was later demonstrated (15).

Stress and dietary changes are conditions that affect the intestinal microflora of dogs and for which probiotics might be beneficial. Thus, probiotics may improve health conditions in dogs exposed to stress and infections. Important changes in the microflora also occur at weaning, and events during this period of life may have a strong effect on the overall health of the dog throughout its life, in particular on the development of its immune system. Therefore, the rational for adding probiotics to certain types of pet foods, particularly for puppies, seems

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[2] To whom correspondence should be addressed.
E-mail: thierry.von-der-weid@rdls.nestle.com.

[3] Abbreviations used: BSA, bovine serum albumin; CDV, canine distemper virus; cfu, colony forming units; FACS, fluorescence activating cell sorter; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; Ig, immunoglobulin; LAB, lactic acid bacteria; mAb, monoclonal antibody; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cell; RPE, R-phycoerythrin.

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well justified. Our objective was to address the capacity of *Enterococcus faecium* (SF68) to stimulate immune functions in puppies when added to dry dog food during the growth period (from weaning to 1 y of age).

MATERIALS AND METHODS

Animals and diets. Fourteen puppies were used in the trial. All puppies were housed at Nestlé Purina Product Technology Center, St. Joseph, MO. The puppies were born at the facility and had not been exposed to probiotics before the beginning of the trial. In addition, the mothers had not been fed probiotic-containing diets during gestation or lactation. Both males and females from several breeds, including Labrador Retriever, Manchester Terrier, Beagle and Fox Terrier were used. Puppies were allotted to treatment according to litter, with one puppy from the litter assigned to the control and a littermate assigned to the test diet. The sex distribution of puppies was equal in the control and test groups. Puppies were housed in pairs in indoor/outdoor kennel runs. The pairs consisted of puppies in the same treatment group. Housing in pairs provided the puppies with social interaction and exercise. Pairs were separated during feeding to allow measurement of individual food intake and during fecal sample collection. Puppies consumed fresh water ad libitum and food was offered for 20 min twice daily. The trial protocol was approved by the Nestlé Purina Pet Care Advisory Committee.

After weaning (8 wk of age), 7 puppies were assigned to each group and were fed one of two diets until 52 wk of age. The control group received a commercial, nutritionally complete, extruded dry dog food (FRISKIES ALPO Complete dry dog food; Nestlé Purina Petcare, Glendale, CA; 22% protein, 10% fat, 8% ash, 13.8 kJ metabolizable energy/g). The test group received the same dry dog food supplemented with *Enterococcus faecium* (strain NCIMB10415; SF68). A highly stable encapsulated form of SF68 (Cerbios-Pharma, Barbengo, Switzerland) was added to the diet at a dose of 5×10^8 colony forming units (cfu)/d. To ensure that administration of the probiotic did not adversely affect food palatability and promoted healthy growth of the puppies, food intake, body weight, complete blood counts and serum chemistry were measured throughout the trial. Food intake was recorded daily and body weight was measured weekly. Blood was collected by jugular venipuncture into heparinized tubes at wk 0, 10, 18, 31 and 44 of trial and analyzed for complete blood count or immune markers.

All dogs included in this study were vaccinated subcutaneously with a live attenuated canine distemper virus (CDV) vaccine (Progard 5, Intervet, Millsboro, DE) at 9 wk of age (wk 1 of trial) and boosted at 12 wk of age (wk 4 of trial).

Measurement of antibodies in fecal contents. Because SF68 was administered orally and was expected to act primarily at the mucosal level, secretory IgA was analyzed in the feces. Total IgA in fecal contents was measured by ELISA. Feces from each puppy (0.5 g) were collected and processed. 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 (BSA), all from Sigma, St. Louis, MO]. Phenylmethanesulfonyl fluoride (50 μL; 350 mg/L from Sigma) was added to each tube and the samples were centrifuged at 10,000 $\times$ *g* for 20 min. The supernatants were then collected and frozen at −80°C until tested for IgA by ELISA as follows. For total IgA analysis, 96-well microtiter plates (Life Technologies AG, Basel, Switzerland) were coated overnight at 4°C with 250 ng/well of mouse anti-canine IgA (Serotec, Oxford, UK) 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 h at 37°C. Duplicate fecal extracts were diluted in ELISA buffer and incubated for 2 h at 37°C. ELISA plates were then incubated with ELISA buffer containing 0.1 mg/L of polyclonal goat anti-canine IgA conjugated with horseradish peroxidase (HRP; Serotec) for 1 h 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 (KPL, Gaithersburg, MD). Because a monoclonal canine IgA standard was not available, values were expressed as optical densities (O.D. 450 nm).

Measurement of antibodies in plasma. To assess the effect of the probiotic on systemic humoral responses, circulating total and vaccine-specific IgG and IgA levels were measured in the plasma. Plasma was recovered from whole blood after fractionation through vacutainer columns (Becton Dickinson, Franklin Lakes, NJ) and the same ELISA described above was used to analyze the total level of IgA in the plasma. Because a monoclonal canine IgA standard was not available, values were expressed as O.D. 450 nm.

The total amount of IgG in the plasma was determined using ELISA plates coated with 100 ng/well of rabbit anti-canine IgG (Jackson Immunoresearch, West Grove, PA). A monoclonal canine IgG (Europa Bioproducts, Cambridge UK) was used as a standard; values were therefore expressed as g/L of IgG. ELISA plates were revealed with a sheep anti-canine IgG conjugated with HRP (Serotec) as the secondary antibody.

Specific antibodies to CDV were measured using the same ELISA, except that the microtiter plates were coated with 100 ng/well of CDV antigen preparation purchased from VMRD (Pullman, WA). Each isotype of antibody (IgG or IgA) was determined using the respective secondary antibody.

Flow cytometric analysis. Blood was collected from the puppies and fractionated using vacutainer columns (Becton Dickinson). Peripheral blood mononuclear cells (PBMC) were recovered according to the manufacturer's recommendations. Canine lymphocyte subsets were quantified by two-color flow cytometry using anti-canine monoclonal antibodies (mAB) purchased from Serotec, Oxford, UK. These mAb were: rat anti-CD4 conjugated with fluorescein isothiocyanate (FITC), rat anti-CD8:R-phycoerythrin (RPE), rat anti-major histocompatibility complex (MHC) class II:FITC and unconjugated mouse anti-CD21. Briefly, 5×10^5 PBMC were washed with PBS/1% BSA buffer. After a saturation step with PBS containing 10% canine serum, the cells were incubated for 30 min on ice with the respective mAb. Cells were then washed twice and resuspended in 0.5 mL of PBS/1% BSA. $CD21^+$ cells were further labeled with RPE-conjugated goat anti-mouse IgG (Southern Biotech., Birmingham, AL). The differential cell count was determined using a flow cytometer (fluorescence activating cell sorter; FACScalibur, Becton Dickinson) after gating the lymphocyte population by forward and side scatter analysis (16). This gating was made because the canine CD21 cell surface molecule is not restricted to B cells but is also expressed by some monocytes and granulocytes (17). Furthermore, the canine CD4 molecule is not restricted to T cells but is also expressed diffusely on granulocytes (17–19). A double-labeling of the B cells with anti-MHC class II mAb was performed for targeting more precisely the mature B cell population ($CD21^+$/$MHCII^+$) (18). Unlabeled cells were used as negative controls. Furthermore, isotype control antibodies were used to control for nonspecific labeling. Data were expressed as the percentage of positive-staining cells. Dead cells were identified using propidium iodide and were excluded from the gated cells analyzed.

Statistical analysis. For food intake and weight gain, repeated-measures ANOVA was used to test for overall differences between treatments. The FACS analyses were performed separately at each time point, and the effect of time could not be investigated. Therefore, differences among means for each variable within a sampling period were evaluated by the two-tailed Student's *t* test using equal variance. For all other immune markers, repeated two-way ANOVA was used. For all tests, the level of significant difference was set at $P < 0.05$.

RESULTS

General physiological status. Food intake and body weight (**Table 1**) did not differ between the two groups during the trial. The blood cell count and serum chemistry were also not influenced by SF68 feeding (data not shown).

Antibodies in fecal contents. The total IgA in the fecal contents tended to be greater ($P = 0.056$) in the SF68-treated group than in controls at wk 44 (**Fig. 1**).

Antibodies in plasma. The concentration of total plasma IgG did not differ between the two groups throughout the trial

TABLE 1

Food intake and weight gain of growing puppies fed Enterococcus faecium[1]

		Week			
	Treatment	10	18	32	44
Intake,* *kg/wk*	Control	2.03 ± 0.04	2.15 ± 0.04	2.15 ± 0.04	2.08 ± 0.05
	E. faecium	2.01 ± 0.03	2.10 ± 0.05	2.20 ± 0.02	2.19 ± 0.03
Weight gain,* *kg*	Control	4.00 ± 0.86	7.02 ± 2.12	9.30 ± 3.86	10.12 ± 4.00
	E. faecium	4.02 ± 0.77	6.96 ± 2.00	8.71 ± 3.54	9.53 ± 3.46

[1] Data are means ± SEM, $n = 7$.
* Significant effect of time, $P < 0.0001$.

(**Fig. 2**, *upper panel*). In contrast, the amount of IgA was higher ($P < 0.05$) in the plasma from the test group than in controls (Fig. 2, *lower panel*) from wk 18 onward. Interestingly, the response to CDV vaccination was stronger in puppies supplemented with SF68 as evidenced by higher amounts of both CDV-specific IgG and IgA ($P < 0.05$) at wk 31 and 44, compared with the control group (**Fig. 3**).

Differential cell counts (FACS analysis). There were no differences in the percentages of $CD4^+$ and $CD8^+$ T cells or in the CD4/CD8 ratio between the two groups (**Table 2**). However, there was a greater proportion of mature B cells ($CD21^+/MHCII^+$) in puppies fed SF68 at wk 31 and 44 compared with controls ($P < 0.05$). The surface expression of the MHCII molecule in monocyte population (mean-fluorescent intensity) was higher in puppies fed SF68 at wk 44 (221.2 ± 27.8) than in controls (136.2 ± 17.3, $P < 0.05$).

DISCUSSION

This study show for the first time that feeding a dry dog food supplemented with a live probiotic LAB enhanced long-term immune functions in growing dogs. It did not have measurable side effects on the dogs because there were no differences in food intake, weight gain or blood composition between the two groups. A previous publication by Biourge et al. (20) reported the administration of a dry dog food containing a *Bacillus* strain, but no health benefits were associated with this bacterium. To our knowledge, only one previous report showed immune stimulation by *Enterococcus* in dogs. Kanasugi and colleagues (21) demonstrated that oral administration of a heat-killed *Enterococcus faecalis* (FK-23) stimulated nonspecific immune responses in healthy dogs, including lymphoid cells proliferation upon activation with mitogens and neutrophil phagocytosis.

Because probiotics were delivered orally in this study, we assumed that their primary target would be the gut-associated lymphoid tissue. Therefore, IgA was measured in the fecal contents. Total fecal IgA levels increased progressively in both groups, but was higher at wk 44 ($P = 0.056$) in the group receiving SF68 compared with the control group. This result argues in favor of a mucosal adjuvant effect of the orally administered SF68. The immunogenicity of SF68 was confirmed because the dogs developed a specific transient IgA response against SF68 (data not shown), showing that the bacteria directly triggered and stimulated the immune system underlying the intestinal mucosa. This is not surprising because it has been demonstrated that commensals are able to engender a self-limited humoral mucosal immune response in monoassociated germ-free mice (22). Furthermore, it was demonstrated recently that mucosal dendritic cells express tight junction proteins and penetrate the gut epithelial monolayer to sample bacteria directly in the intestinal lumen (23). Interestingly, although the fecal IgA isolated from all dogs were cross-reactive to canine commensals, including *E. faecium*,

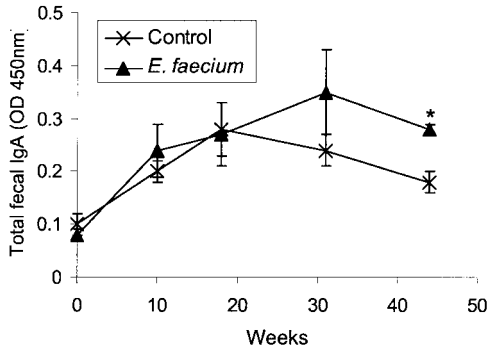

FIGURE 1 Total immunoglobulin IgA in the fecal contents collected at wk 0, 10, 18, 31 and 44 from puppies fed diets with or without SF68. Data are means ± SEM, $n = 7$. *Tended to be higher than control, $P = 0.056$.

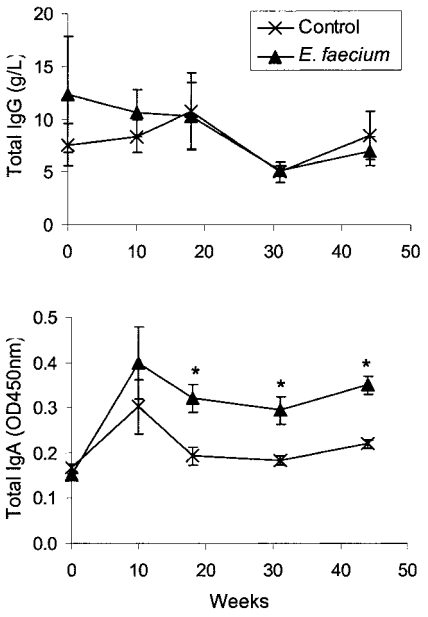

FIGURE 2 Total immunoglobulin IgG (*upper panel*) and IgA (*lower panel*) in the plasma collected at wk 0, 10, 18, 31 and 44 from puppies fed diets with or without SF68. Values are means ± SEM, $n = 7$. *Different from control, $P < 0.05$.

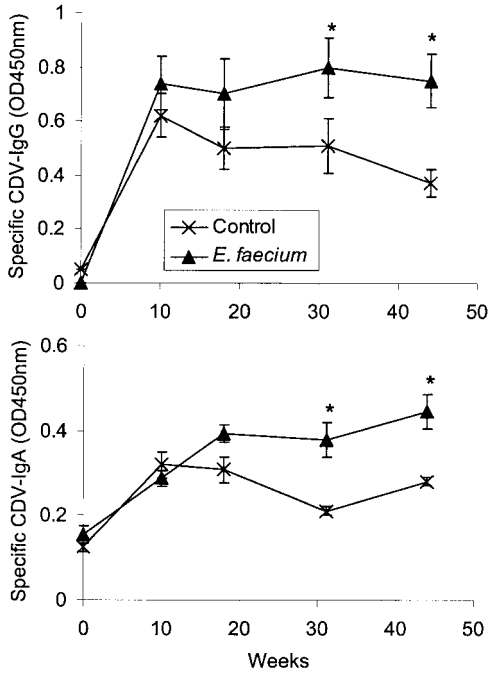

FIGURE 3 Specific anti-canine distemper virus immunoglobulin IgG (*upper panel*) and IgA (*lower panel*) in the plasma collected at wk 0, 10, 18, 31 and 44 from puppies fed diets with or without SF68. Values are means ± SEM, $n = 7$. *Different from control, $P < 0.05$.

Lactobacillus acidophilus, *E. coli* and *Clostridium perfringens* (data not shown), no differences between the two groups were observed, suggesting that this immune response was not deleterious to the endogenous bacterial flora.

Secretory IgA in the intestine is the most important protective humoral immune factor at this mucosal site (24). It inhibits microbial adherence, colonization and penetration, as well as food antigen uptake (25,26). In that respect, the increase in fecal polyclonal IgA observed in our study could play a role in nonspecific anti-enteropathogenic protection of the dogs (27).

Dogs included in this study were routinely vaccinated, including a CDV vaccine at wk 9 of life and a booster dose at wk 12. The fact that the probiotic was given to the puppies early in life, before starting the vaccination program, allowed investigation of effects on specific immune markers, such as response to CDV vaccination. No changes in the amounts of total IgG in the plasma were observed. However, the amounts of specific anti-CDV IgG were significantly higher in the puppies fed SF68 and were maintained at such high levels throughout the study (up to 1 y of age). No CDV-specific IgG were detected before vaccination (wk 0 of trial) in either group, excluding any possible contribution of maternal antibodies in the CDV-IgG levels observed upon treatment with SF68. These results suggest that SF68 administration increased the priming of naïve B cells in response to initial CDV vaccination. This prolonged improvement of antibody response may enhance the effectiveness of the vaccine in preventing CDV infection. Indeed, it was previously shown that specific antibodies were highly effective in neutralizing extracellular CDV as well as preventing the intercellular spread of the virus in vitro (28).

In contrast to the total amount of circulating IgG, total IgA levels were significantly higher in puppies receiving SF68. The specific anti-CDV IgA were also significantly increased. This could reflect a preferential IgA switch of mucosally primed B cells (29–31), and may be associated with the increase in mucosal IgA response that likely results from the specific homing of the IgA-producing B cells in the gut (32,33). The live attenuated virus vaccine, although avirulent, is still able to multiply in dogs (34). It is therefore likely that this virus migrates through the blood to lymphoid organs where it encounters immune cells activated at the mucosal level by the probiotic. This interaction, together with the highly activated monocytes (MHCII high) found in the blood of puppies fed SF68, may contribute to the improvement of the response to the vaccine. Indeed, it was shown that dendritic cell maturation can be induced by probiotics in vitro, as characterized by increased expression of MHC II (35).

In addition to these antibody analyses, plasma samples were sent to Imovet-Laupeneck laboratories (Bern, Switzerland) for the measurement of 20 food antigen–specific IgE levels, including chicken egg albumin, casein, α lactalbumin, soy proteins, rice, and BSA, as well as C-reactive protein, antinuclear autoantibodies and rheumatoid factors. There were no differences in the levels of any of these markers between the two groups of dogs (data not shown), strongly suggesting that SF68 was not overstimulating or dysregulating the immune system, leading to increased risks for allergy, autoimmunity or inflammation.

Whether the stimulation of humoral responses by SF68 could be mirrored at the cellular level was also investigated. PBMC proliferation in vitro was not different between the two groups, due to high variability in the responses (data not shown). Further research should be conducted with more animals and a more homogeneous study population to determine whether SF68 affects lymphocyte proliferation in vitro. However, FACS analysis showed a significant relative increase in mature B cells ($CD21^+$/MHC II^+) at later time points in puppies receiving SF68, suggesting an expansion of this lymphocyte population in probiotic-fed dogs. This is in line with the increase of fecal IgA and plasma IgG and IgA observed at later time points in the test group. In addition, monocytes were more activated in the test group. In contrast, the proportions and ratios of T cell subsets were not affected by the probiotic. Nevertheless, in future clinical trials, it will be interesting to study the activation state of these T cells by the analysis of activation and memory cell-surface markers that have recently become available for canine cells.

The ability of SF68 to colonize the intestine of the dogs was

TABLE 2

Percentage of different lymphoid cell subsets in growing puppies fed Enterococcus faecium[1]

Lymphocyte subsets	Treatment	Week 18	Week 31	Week 44
$CD4^+$ T cells	Control	32.3 ± 2.5	32.7 ± 3.7	37.0 ± 5.6
	E. faecium	33.0 ± 4.3	35.2 ± 3.0	39.0 ± 4.0
$CD8^+$ T cells	Control	18.3 ± 3.2	14.5 ± 2.5	18.3 ± 1.4
	E. faecium	18.0 ± 2.8	15.6 ± 0.6	16.8 ± 1.0
$CD4^+/CD8^+$ T cells	Control	1.7 ± 0.4	2.2 ± 0.8	2.0 ± 0.2
	E. faecium	1.8 ± 0.6	2.2 ± 0.4	2.3 ± 0.3
$CD21^+$/$MHCII^+$ B cells	Control	18.1 ± 4.2	10.2 ± 1.2	11.5 ± 1.5
	E. faecium	23.3 ± 4.7	17.6 ± 1.8*	16.0 ± 1.6*

[1] Data are means ± SEM, $n = 7$. MHC, major histocompatibility complex.

* Different from control, $P < 0.05$.

assessed in the present study. The presence of SF68 in the feces of the test group was confirmed in all dogs ($n = 7$) by pulsed field gel electrophoresis performed on bacterial isolates (36,37), whereas no SF68 bacteria were detected in the feces from the control group (data not shown). These data confirmed previous results obtained in a pilot study reporting that SF68, was able to persist in the canine intestine during feeding (14 d) with values ranging from 10^7 to 10^8 cfu/g of feces (personal communication, F. Rochat, Centre de Recherche Nestlé). This earlier pilot study also demonstrated that endogenous microflora were not significantly changed after intake of SF68. This suggests that the immune stimulating properties of SF68 were probably not elicited indirectly via modulation of the endogenous flora, but rather directly via an immunoadjuvant mechanism.

In conclusion, the results reported in this study support an adjuvant effect of *Enterococcus faecium* SF68 at both mucosal and systemic levels in puppies, an effect that could be relevant for improving protective immune responses against various infections during the critical weaning period as well as at later stages in life. This is particularly relevant if the long-term (up to 1 y of age) effect on antibody responses is considered. The precise mechanisms by which SF68 stimulates immune functions and boosts immune responses to a vaccine clearly deserve further investigation.

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