Enterococcus faecium SF68 Enhances the Immune Response to Giardia intestinalis in Mice

J. Benyacoub,‡ P. F. Pérez,† F. Rochat,‡ K. Y. Saudan,‡ G. Reuteler,‡ N. Antille,‡ M. Humen,† G. L. De Antoni,† C. Cavadini,‡ S. Blum,‡ and E. J. Schiffrin*

* Nestlé Research Center, CH-1000, Lausanne 26, Switzerland; †Centro de Investigación y Desarrollo en Criotecnología de Alimentos, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, 553-1900 La Plata, Argentina; **Cátedra de Microbiología, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, 115-1900 La Plata, Argentina; ‡CONICET, Argentina; and ††CIC-PBA, Argentina

ABSTRACT We studied the ability of the probiotic organism Enterococcus faecium SF68 to antagonize Giardia intestinalis infection in mice. Oral feeding of E. faecium strain SF68 starting 7 d before inoculation with Giardia trophozoites significantly increased the production of specific anti-Giardia intestinal IgA and blood IgG. This humoral response was mirrored at the cellular level by an increased percentage of CD4+ T cells in the Peyer’s patches and in the spleens of SF68-fed mice. The improvement of specific immune responses in probiotic-fed mice was associated with a diminution in the number of active trophozoites in the small intestine as well as decreased shedding of fecal Giardia antigens (GSA65 protein). The ability of SF68 to stimulate the immune system at both mucosal and systemic levels highlights mechanisms by which this probiotic might antagonize pathogens in vivo. Taken together, the data demonstrate the strong potential of strain SF68 to prevent protozoa from causing intestinal infections. J. Nutr. 135: 1171–1176, 2005.

KEY WORDS: • Enterococcus faecium • probiotics • Giardia intestinalis • animal model • immune response

Giardia intestinalis is a flagellated protozoan that often infects companion animals, including dogs and cats (1,2). It is associated with diarrhea in young dogs and is also responsible for zoonotic transmission to humans (3). G. intestinalis has a biphasic developmental cycle with 2 morphologically different forms, i.e., cysts and trophozoites. After ingestion, cysts give rise to trophozoites in the upper intestinal tract. Trophozoites are the vegetative form of the parasite responsible for infection and pathological manifestations (4).

Nitroimidazoles and nitrofurans are widely used in the treatment of Giardia infections. However, the occurrence of resistant strains, therapeutic failures, and side effects of anti-Giardia drugs (5–7) encourage further research on new therapeutic strategies (8). Indeed, the use of some natural formulations including plant extracts and bee-derived products was reported (8).

Both secretory IgA and T cell responses were reported to play an important role in the control of Giardia infection (9, 10, 11). In addition, environmental factors such as the composition and/or metabolic activity of intestinal microbiota can play a role in the elimination of the protozoan. Indeed, colonization with a defined microbiota (altered Schaedler biota, 2 fusiform bacteria species) was shown to protect B and T cell-deficient recombinant-activating gene–knockout mice from Giardia infection. It follows therefore that probiotic bacteria may provide protection against such infection (12).

Probiotics are live microorganisms that beneficially affect the gastrointestinal balance if ingested in sufficient numbers, and provide benefits that go beyond normal nutritional effects (13). Specific strains of probiotic lactic acid bacteria (LAB)3 were shown to beneficially influence the composition and/or metabolic activity of the endogenous microbiota (14,15), and some of these were shown to inhibit the growth of a wide range of enteropathogens (16,17). Competition for essential nutrients, aggregation with pathogenic microorganisms (18), competition for receptor sites (19), and production of antimicrobial metabolites (16,17) were all postulated to play a role. It was also demonstrated that some probiotic strains are able to modulate both innate and acquired immunity at the local and systemic level (14,15).

It was reported previously (20) that some strains of lacto-

3 Abbreviations used: FITC, fluorescein isothiocyanate; LAB, lactic acid bacteria; mAbs, monoclonal antibodies; MHC, major histocompatibility complex; MLN, mesenteric lymph node; PP, Peyer’s patches.
bacilli of human and animal origin have a strong inhibitory effect on G. intestinalis in vitro. Enterococcus faecium SF68 (NCIMB10415) is a LAB, originally isolated from a healthy Swedish baby, with inhibitory effects against important enteropathogens, including enterotoxigenic Escherichia coli, Salmonella, Shigella, and clostridia (21). Moreover, the efficacy of SF68 in the treatment of antibiotic-associated diarrhea as well as acute diarrhea was demonstrated in humans (22). The presence of enterococci as normal inhabitants of the gut flora of humans (23) and animals (24) justifies their use as a component of functional foods.

It was shown recently that E. faecium SF68 stimulates both the mucosal and systemic immune system in dogs. More specifically, oral administration of SF68 increases intestinal IgA production and improves the immune response to canine vaccine (25). The objective of the present study was to evaluate the physiologic importance of changes in immune variables, particularly increased intestinal IgA production, on the evolution of intestinal infections in mice. To this end, a mouse model of Giardia infection was used to investigate the protective effect of SF68 on such infection and to further delineate the immune responses that result from interactions among the host, the probiotic, and the pathogen.

MATERIALS AND METHODS

Bacterial strain, bacterial culture and animal feeding. E. faecium SF68 (NCIMB10415) was provided by Cerbios Pharma. Bacteria were grown in MRS (Difco) broth at 37°C for 16 h. The number of bacteria was determined by colony plating. After washing and resuspension in PBS containing 10% sucrose, the bacterial suspension was divided into aliquots and frozen at −80°C. The concentration of viable bacteria after thawing was assessed by plate counts. Each day, a vial was thawed and 20 μL (containing 5 × 10⁹ to 1 × 10¹⁰ cfu) or PBS containing 10% sucrose (placebo) was administered orally to each mouse (4 consecutive drops of 5 μL each placed into the mouth). Probiotic or placebo administration began on d 0 of the trial and continued throughout the study. The placebo and probiotic groups comprised 19 and 18 mice, respectively. Conventional 6-wk-old female C57BL/6 mice were used in this study (Iffa-Credo). Mice were housed under specific pathogen–free conditions at the Nestlé Research Center animal facility and were fed a standard rodent diet (Kliba 3434, Provimi Kliba SA). The experimental procedure was approved by the Nestlé ethical committee and the cantonal committee for permission of animal experimentation (Swiss Animal Experiment Grand Number 1570).

Culture of trophozoites. G. intestinalis H7 (ATCC 50581) was purchased from the American Type Culture Collection. Trophozoites were grown in Keister’s modified TYI-S-33 medium (26) supplemented with 15 mL of an antibiotic solution (1000 kIU/L of penicillin and 1 g/L of streptomycin, Gibco BRL, Life Technologies). The pH was adjusted to 6.9 before sterilization with a 0.22-μm filter. Parasites were cultured in 25-cm² polystyrene tissue culture flasks (LUX, Miles Laboratories) and harvested as previously described (20).

Infection protocol. On d 7 of the trial, i.e., 1 wk after starting probiotic feeding, a single dose of parasites in TYI-S-33 medium was administered by gavage (0.1 mL/mouse: 5 × 10⁶ trophozoites, as determined by microscopic count of motile trophozoites) using a blunt-ended needle. Cages were covered with micro-isoler lids to ensure disease containment.

Analysis of trophozoites in the small intestine. After being anesthetized by isoflurane inhalation, groups of mice were killed by cervical dislocation on d 14 (n = 10 for each group) and d 28 (n = 9 for placebo group and n = 8 for probiotic group) of the trial, i.e., 1 and 3 wk after infection, respectively. To evaluate the concentration of trophozoites in the intestine, 5-cm long segments of the small intestine collected 11 cm from the pylorus, were removed and placed in 2 mL of ice-cold TYI-S-33 medium for at least 15 min. After washing, opening of tissues longitudinally, and mixing on a vortex, the number of trophozoites was counted in a hemocytometer. Data were expressed as log10 trophozoites/cm intestine.

Analysis of Giardia antigens in the feces. Fecal samples were collected on d 7 (before infection), 12, 14, 21, and 28 of the trial. For extraction of proteins from feces, samples were homogenized with extraction buffer (50 mmol/L EDTA, 100 mg/L soybean trypsin inhibitor in PBS, all purchased from Sigma). After being mixed on a vortex for 120 s, samples were centrifuged at 10,000 × g for 5 min and the pellets rapidly lysed with 1 mL of sterile distilled water before centrifugation at 250 × g for 5 min. The cells pellets were then suspended in complete RPMI-1640 and the concentration of viable cells determined by Trypan blue exclusion.

Cell subsets were quantified by 2-color flow cytometry using the following monoclonal antibodies (mAbs): biotinylated rat IgG2a anti-mouse CD4 (H129.19), rat IgG2a anti-mouse CD8 (53-6.7) conjugated with fluorescein isothiocyanate (FITC), biotinylated rat IgG2a anti-mouse B220 (RA3-6B2), and unconjugated rat IgG2a anti-mouse major histocompatibility complex (MHC) class II (M5) (all purchased from Pharmingen, Becton Dickinson). Briefly, 2 sets of tubes for each sample were analyzed as follows: 3 × 10⁵ cells were washed with FACS buffer (PBS, 1% bovine serum albumin) and incubated for 30 min on ice with either anti-CD4 and anti-CD8 or anti-B220 and anti-MHC class II mAbs or their respective isotype controls. The cells were then washed twice (250 × g, 5 min, 4°C) Cells incubated with anti-CD4*, anti-B220*, antibodies or their isotypes controls were further incubated with streptavidin-phycocyanin-erythrin. Because the anti-CD8 antibody was already conjugated with FITC, the cell labeling did not require the use of further enzymatic coupling or secondary antibody. Cells incubated with anti-MHCCI unlabeled antibody or its isotype control were further incubated with an FITC-conjugated goat anti-rat IgG (SeroTec). After 30 min on ice, the second antibodies were washed twice and cells were suspended in 500 μL of FACS buffer. The differential cell count was determined by flow cytometry (FACSCalibur, Becton Dickinson) after gating the lymphocyte population for forward and side scatter analysis. Unlabeled cells were used as the negative control and cells labeled with a single antibody, anti-CD4, anti-CD8, anti-B220, or anti-MHCCI were used as the positive controls. Acquisition was done on 10,000 cells and the data expressed as the percentage of positive cells.

Analysis of antibody responses. Blood samples were collected from the tail vein on d 0, 7, 14, and 28 of the trial. After coagulation samples were centrifuged at 10,000 × g for 15 min, sera were collected and frozen at −20°C until analysis for total and specific IgG. Proteins were extracted from feces as described above, and total as well as specific IgA measured in samples collected on d 0, 7, 14, and 28 of the trial.

Total antibody levels were measured by ELISA using antibodies and conjugates purchased from Southern Biotech. Briefly, 96-well microtiter plates (Life Technologies) were coated overnight at 4°C with 0.1 μg/well of rabbit anti-mouse IgA in borate buffer (pH 7). Free binding sites were blocked with PBS containing 5% fetal calf serum and 0.1% Tween 20 (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 0.05 mg/L of purified biotinylated goat anti-mouse IgA for 1 h at 37°C. The ELISA plates were further incubated for 30 min at room temperature with peroxidase-labeled streptavidin (KPL). Several washes with PBS 0.1% Tween 20 were performed between each incubation step. Finally, the plates were developed with the TMB.
**RESULTS**

**Trophozoites in the small intestine.** The number of trophozoites recovered from the intestine of SF68-fed mice on d 14 of the trial, corresponding to d 7 postinfection, was lower (p < 0.01) than that from control mice; 10^4 trophozoites/cm

**Giardia fecal antigens.** The kinetics of shedding of Giardia antigens in the feces were associated with that of the trophozoite load in the intestine. Indeed, both groups were negative for the presence of Giardia antigens in the feces on d 7 and 12 (below the cutoff value 0.05 U/mg protein). Titers of GSA65 protein peaked on d 14 of the trial (d 7 postinfection) and then decreased dramatically on d 21 and 28, corresponding to d 14 and 21 postinfection. Interestingly, SF68-fed mice had lower titers (p < 0.001) of Giardia antigens in their feces than control mice on d 14 and 21 (Fig. 1).

**Flow cytometry.** Differences in the clearance of Giardia trophozoites and Giardia antigens between the 2 treatment groups prompted us to investigate the immune response to Giardia infection. A significantly higher percentage of CD4+ T-cells in the PP was found in SF68-fed mice compared with controls (Table 1). Furthermore, there was a trend for more CD4+ cells in the spleen (P = 0.07), and more CD8+ cells in the PP and spleen of SF68-fed mice (P = 0.08 for each). In contrast, the percentages of B220+ and MHCII+ cells did not differ between the groups (Table 1). The groups also did not differ in the subsets of cells present in the MLN (data not shown).

**Antibody responses.** Total IgG levels were not influenced by feeding SF68 (data not shown). However, the total IgA levels in the intestine were already higher in SF68-fed mice on the day of Giardia infection, i.e., d 7 of the trial (d 7 after feeding of SF68 had begun) compared with control mice (Fig. 2). The amount of intestinal IgA remained significantly higher in SF68-fed mice compared with controls on d 14 of the trial. By d 28 of the trial, intestinal total IgA concentration did not differ between the groups.

As expected, specific anti-Giardia IgA in the intestine (Fig. 3A) and IgG in the systemic circulation (Fig. 3B) increased progressively after Giardia infection in both groups. Interestingly, the levels of specific anti-Giardia intestinal IgA were higher in SF68-fed mice than in controls on d 7, 14, and 28 of the trial (P < 0.05). Specific anti-Giardia serum IgG were also slightly higher in SF68-fed mice than in controls on d 28 of the trial.

**Microbiota.** Some representative components of the endogenous microbiota such as enterobacteria, lactobacilli, bifidobacteria, and enterococci, were enumerated on semisensitive agar media. As expected, the number of enterococci was

<table>
<thead>
<tr>
<th>Lymphocyte subsets</th>
<th>Peyer’s patches</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>8.9 ± 0.4</td>
<td>21.2 ± 1.2</td>
</tr>
<tr>
<td>CD8</td>
<td>2.9 ± 0.5</td>
<td>12.1 ± 1.4</td>
</tr>
<tr>
<td>B220</td>
<td>68.8 ± 2.5</td>
<td>49.3 ± 2.7</td>
</tr>
<tr>
<td>MHCII</td>
<td>69.6 ± 1.9</td>
<td>44.8 ± 1.6</td>
</tr>
</tbody>
</table>

E. faecium ANTAGONIZES Giardia INFECTION

**FIGURE 1** Fecal shedding of Giardia antigen GSA65 in control and probiotic-fed mice on d 7 (before infection), 12, 14, 21, and 28 of the trial. Values are means ± SEM, n = 18 or 19 (d 7, 12, 14); n = 8 or 9 (d 21, 28). *Different from control at that time, P < 0.05.
significantly greater in SF68-fed mice than in controls on d 7 and 21 of the trial (Table 2). SF68 was not detected in the feces from control or test groups on d 0 (0/19 and 0/18, respectively). However, the presence of SF68 was confirmed by PCR in 100% of SF68-fed mice on d 7 and d 21 (18/18 and 18/18, respectively), whereas SF68 was not detected in control mice at the same time points (0/19 and 0/9). SF68 did not affect Lactobacilli, Bifidobacteria, and Enterobacteraeaceae levels, which were maintained within the normal physiologic range in these mice. However, fecal Enterobacteria counts on d 21 were lower in the control group than in SF68-fed mice.

### DISCUSSION

The present work shows that the ability of *E. faecium* SF68 (NCIMB10415) to improve the specific anti-*Giardia* immune response was associated with a significant decrease in *G. intestinale* infection in mice. There are several publications that provide experimental and clinical support for the use of probiotics in the maintenance of gut homeostasis, particularly in the prevention of intestinal infections and inflammatory conditions (31–34).

The ability of Enterococci spp., such as *E. faecalis* and *E. faecium* strains to modulate both innate and acquired immune responses was reported previously in different species (25,35,36). Furthermore, the safe use of *E. faecium* SF68 as a probiotic for humans and animals was investigated extensively and confirmed recently (37,38).

It was previously suggested that greater production of mucosal IgA when SF68 is fed may have an important protective effect against intestinal pathogens (25). To date, no studies have assessed the protective effect of SF68 on intestinal parasitosis. The present study therefore specifically addressed the effect of an SF68-mediated immune stimulation and in particular an enhanced intestinal IgA response in a murine model of *Giardia* infection.

*Giardia* strains WB, WB clone C6, and strain H7 were tested in different strains of immunocompetent mice, such as NIH (Swiss), B10.A, CF1, and C57BL/6. Results of these preliminary experiments revealed that *G. intestinale* strain H7, at an infective dose of $5 \times 10^6$ trophozoites, is able to transiently colonize the intestine of C57BL/6 mice. The infection peaked on d 7 and then the parasite was naturally cleared after d 21. Comparable results were not observed with other *Giardia* or mouse strains (data not shown). We also observed in the same preliminary study a progressive increase in the levels of specific anti-*Giardia* IgA in the intestine as well as IgG in the circulation, concomitant with a decline in live parasite as well as antigen load in the intestine. Furthermore, there was an increased proportion of T cells, mainly CD4+ in the PP and spleen suggesting that T-cell stimulation also...
contributed to the protective mechanisms (data not shown). This is in agreement with the findings of several previous studies showing that in addition to innate immune responses, adaptive humoral and cell-mediated immune responses participate in the control of *Giardia* infection (10,11,39,40).

Intestinal infection by *G. intestinalis* causes diarrhea and nutrient malabsorption. These effects are associated with the burden of parasites in the small intestine as well as with the intestinal mucosal damage triggered by the immune response of the host (4). These events, together with reduced enzymatic activity in the mucosa, contribute to the main pathological features of symptomatic infections (41). However, in our study, we did not observe intestinal damage or alteration in enzymatic activity (data not shown). This is probably associated with the incapacity of *G. intestinalis* to permanently colonize the mouse intestine and thereby establish a persistent more severe infection.

The protective effect of probiotics against intestinal pathogens has been ascribed to many factors. A role for bacterial exocellular factors (16,17,42), interference with pathogen-enterocyte interactions (43,44), and modulation of the immune response were suggested (45,46).

Feeding mice SF68 led to a more rapid clearance of *Giardia* infection (Figs. 1 and 2). This may be due, at least in part, to the production of some anti-*Giardia* substance(s) in situ as already shown in vitro with other probiotic microorganisms (20). However, our experiments showed that SF68-free spent culture supernatants are unable to inhibit the growth of *Giardia* trophozoites (data not shown). The ability of strain SF68 to stimulate the immune system (25) may be another important mechanism by which the anti-*Giardia* effect of this probiotic is mediated.

Intestinal IgA from both groups were tested by ELISA for their reactivity against SF68 antigen preparation. Specific fecal anti-SF68 IgA titers were significantly higher in SF68-fed mice compared with controls on d 7 and 14 of the trial and not on d 28 (data not shown). This transient intestinal IgA response against SF68 itself reflects the immunogenicity of this probiotic and suggests that these bacteria can trigger and stimulate a local mucosal immune response. This is not surprising because it was already demonstrated that commensal bacteria are able to initiate a self-limited humoral mucosal immune response in monoassociated germfree mice (47).

We also showed that the proportion of CD4+ T cells in PP and, to a lesser extent, in spleen was higher in SF68-fed mice than in controls on d 14 of the trial, corresponding to d 7 post-*Giardia* infection. At the same time, both local (fecal IgA) and systemic (serum IgG) anti-*Giardia* antibody concentrations were higher in SF68-fed mice compared with controls.

Higher levels of antibodies produced in the SF68-group during the trial are likely to have contributed to the significantly lower level of live trophozoites as well as *Giardia* antigen load found in the intestine of these mice compared with controls. In that sense, high levels of fecal IgA reacting against *Giardia* were already detected in the feces of SF68-fed mice compared with control mice on the day of infection (d 7 of the trial). It is unlikely that this was due to parasitic contamination in this group because these feces were collected before infection and were negative for the presence of *Giardia* antigens at this time point (d 7 of the trial, see Fig. 1). It is interesting to note that SF68-fed mice produced higher levels of total intestinal IgA at the same time point, i.e., d 7 of the trial (see Fig. 2) than controls, probably resulting from the priming of naive B cells by SF68 in these mice.

We hypothesize that part of this “total IgA” is cross-activating to intestinal pathogens such as *Giardia* and might therefore be responsible for the anti-*Giardia* response detected in SF68-fed mice on d 7 of the trial i.e., before experimental *Giardia* infection. This is in agreement with the previously reported important role that natural poly-reactive intestinal IgA can play in host defense against pathogens (48).

The ability of SF68 to persist during the feeding period in the intestine of mice was also assessed in the present study. Although the presence of SF68 in fecal bacterial isolates was confirmed by PCR in 100% of SF68-fed mice, the probiotic did not significantly affect either the level or the composition of endogenous bacteria. However, modification of other key components of the intestinal microbiota cannot be excluded.

The immune-stimulating ability of SF68 may be mediated in part by the priming of the gut-associated lymphoid tissue and the induction of secretory IgA. Indeed, it is documented that commensal bacteria are able to interact with dendritic cells by different routes and initiate a local immune response without any damaging immune activation (49,50).

It is possible that both immune and antiparasitic activities act synergistically to limit *Giardia* infection. Although we did not detect any significant changes in the composition of the intestinal microbiota in our model, it is likely that the in vivo antiparasitic activity of SF68 is multifactorial and involves changes in both the composition and/or the metabolic activity of the endogenous flora (12) and stimulation of immune functions.

The precise mechanism by which the probiotic antagonizes pathogens as well as the importance of colonization with live vs. dead bacteria warrants further investigation. For instance, it is interesting to note that the ability to interact in vitro with macrophage cell line (J774) and the induction of interleukin-1β secretion was much stronger with live cultures of SF68 than SF68-free spent culture supernatant, heat-killed, or UV-irradiated SF68 (Benyacoub et al. unpublished data). This suggests that SF68 does not secrete immune-active compounds and that its immunogenicity is decreased upon killing.

Taken together, our results show that *E. faecium* strain SF68 primed the immune system and in so doing, promoted a stronger specific anti-*Giardia* immune response. This increased immune response was concomitant with a rapid clearance of live trophozoites and parasite antigens from the intestine. These data support the idea that a probiotic such as SF68 might be useful in the treatment of *Giardia* infection.

ACKNOWLEDGMENTS

The authors thank S. Mafuala and Mary-Aude Rochat for technical assistance, and A. Donnet for critical reading of the manuscript.

LITERATURE CITED