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# Effects of *Enterococcus faecium* (SF68) on immune function in mice

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ABSTRACT

Probiotics exert health benefits on human and animals when administered in adequate amounts. The objective of this study was to assess the effects of *Enterococcus faecium* (SF68) on intestinal colonisation and immune function of BALB/c mice. Six-week-old female BALB/c mice were orally administered with *E. faecium* (SF68). Results showed that the total anaerobe and lactobacilli in the faeces increased ($P < 0.05$), while the number of faecal enterobacteria decreased ($P < 0.05$) in *E. faecium*-fed mice. Furthermore, supplementation of *E. faecium* (SF68) increased the percentage of double positive (DP) cells in peripheral blood, the concentration of plasma IgG, and the levels of interleukin-4 (IL-4), interleukin-6 (IL-6) and interferon-γ (IFN-γ) in splenocytes of the mice ($P < 0.05$). This study demonstrated that *E. faecium* SF68 affects the intestinal microbial flora and modulates the immune responses, which indicates a viable probiotic characteristic of *E. faecium* SF68 in modification of immune function.



## 1. Introduction

Probiotics are low-pathogenic or non-pathogenic microorganisms that have been shown to exert health benefits when administered in adequate amounts (Reid, Jass, Sebulsky, & McCormick, 2003; Schrezenmeir & de Vrese, 2001). Complex interactions occur between the different constituents of the intestinal ecosystem (resident microflora, epithelial and immune cells) and probiotics. These interactions play a major role in the development and maintenance of immune function linked to the gut associated lymphoid tissue (GALT), including IgA secretion and $CD4^+$ and $CD8^+$ T cells activation (Perdigón, Fuller, & Raya, 2001; Rhee, Sethupathi, Driks, Lanning, & Knight, 2004). Numerous experiments have indicated that changes in the intestinal microbiota can cause immunomodulation, both at the local and systemic levels (de Vrese & Schrezenmeir, 2002; Noverr & Huffnagle, 2004).

Lactic acid bacteria (LAB) are normally distributed in human gastrointestinal tract with a long application history of food additives. Several LAB strains have been shown to modulate innate host and acquired immune responses *in vitro* and *in vivo* experiments (Benyacoub et al., 2003, 2005; Miettinen, Vuopio-Varkila, & Varkila, 1996). As normal inhabitants of the gut flora (Franz, Holzapfel, & Stiles, 1999), *Enterococcus faecium* is a LAB that has presented inhibitory effects against some important enteropathogens, such as enterotoxigenic *Escherichia coli* and *Salmonella* (Lewenstein, Frigerio, & Moroni, 1979). *E. faecium* (SF68) has been proven to stimulate both the mucosal and the systemic immune responses in dogs recently (Benyacoub et al., 2003). Oral administration of SF68 increased intestinal IgA production and improved the immune responses to the canine distemper virus vaccine (Benyacoub et al., 2003). In the subsequent research, Benyacoub et al. (2005) found that oral feeding of SF68 starting 7 days before mice were inoculated with Giardia trophozoites increased the production of specific anti-Giardia intestinal IgA and blood IgG, which indicated that SF68 potentially prevented protozoa from intestinal infections.

The objective of this study was to investigate the effects of supplementation with SF68 on the intestinal microflora and nonspecific immunity in mice. Therefore, the indices of faecal bacteria amount, lymphocyte subsets in peripheral blood, plasma antibody and splenocytes secreted cytokines were determined in this study.

## 2. Materials and methods

### 2.1. Identification and assessment of probiotic potential of E. faecium SF68

The *E. faecium* SF68 used in this study was isolated from silage and identified by Institute of Microbiology, Chinese Academy of Sciences. The bacteria were prepared in sterile de Man-Rogosa-Sharpe (MRS) broth (Land Bridge Technology, Beijing, China) by anaerobic culture at 37 °C for 24 h. Appropriate dilutions of SF68 were plated onto MRS Agar plates and incubated at 37 °C for 48 h for determination of colony forming units (CFU).

The probiotic potential of *E. faecium* SF68 was determined by bile and transit tolerance assays. The bile tolerance was conducted

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as described by Bujalance, Moreno, Jimenez-Valera, and Ruiz-Bravo (2007) with minor modifications. Briefly, 400 μl bovine bile solution (1 g of bovine bile (Sigma) was dissolved in 11.5 ml of distilled water) was mixed with 100 μl of the bacterial suspension in Eppendorf tubes. The tubes were incubated at 37 °C under anaerobic conditions, and 100 μl aliquots were removed after 0, 30 and 60 min, thereafter, bacterial survival was determined by CFU count. Additionally, 100 μl of bacterial suspension diluted in 400 μl of saline served as the control.

Simulated gastric and small intestinal transit tolerance were determined following Charteris, Kelly, Morelli, and Collins (1998) with minor modifications. A sample of washed cell suspension of SF68 (200 μl) was diluted in a 5.0 ml capacity tube with either 1.0 ml of simulated gastric (pH 2.0) or pancreatic (pH 8.0) juice to which 0.3 ml NaCl (0.5% w/v) was added. The tubes were mixed using a Vortex-5 Mixer (Qilinbeier Apparatus Co, China) for 10 s at a setting of 5 and then incubated at 37 °C in an anaerobic incubator (YQX-I anaerobic incubator). The gastric transit tolerance assay was performed on the resultant samples, and an aliquot of 0.1 ml was removed after 0, 20 and 60 min for determination of the total viable count. When assaying for small intestinal transit tolerance aliquots were removed after 0, 60 and 120 min for determination of the total viable count. Total viable counts of *Lactobacillus* species were determined with a pour plate method with MRS agar after serially diluting the sample. Plates were incubated in an anaerobic incubator at 37 °C for 48 h and then counted.

### 2.2. Animals, feeding procedure and sample collection

All animals used in this experiment were maintained according to the principles of Chinese Academy of Agricultural Sciences Animal Care and Use Committee. Forty-six-week-old female BALB/c mice weighing 17–20 g purchased from the Beijing Laboratory Animal Research Centre (Beijing, China) were randomly divided into two treatments. Each treatment had five replicates with four mice per replicate. All the mice were housed in plastic cages in a mechanically ventilated nursery room with set conditions: 12 h light: 12 h dark, constant temperature at 23–25 °C and relative humidity of 50–60%. All the mice were kept under pathogen-free conditions with free access to sterilised commercial chow (Beijing Laboratory Animal Research Centre) and water. The whole trial lasted for a 21-day experimental period. All mice received 200 μl of physical saline by intragastric gavage for 3 days just before the experimental period started.

For assessment of the number of viable bacteria, suitable dilutions of the culture were plated onto MRS Agar plates and colony forming units were counted after being incubated for 48 h at 37 °C. Mice in the experimental group received a daily dose of about $10^8$ CFU of viable bacteria in 200 μl of culture while the control mice received 200 μl of MRS broth. Bacterial concentrations were checked each week and dilutions of the culture were made in order to obtain a concentration of $10^8$ CFU of viable bacteria.

Faeces and blood samples were collected on day 0, 7, 14 and 21 of the trial. Immediately after the mice were killed on day 21, they were immersed into 75% ethanol for 5 min. Thereafter, the peritoneal cavity of each mouse was opened, and the spleen was removed followed by the recovery of intestinal fluid by truncating at the stomach/duodenum junction and the ileum/ascending colon junction and flushed with 1 ml of phosphate buffered saline (PBS) containing protease inhibitor.

### 2.3. Quantification of bacteria in faeces

Fresh faeces were weighed, homogenised in sterile saline solution, and suitable dilutions of the homogenates were plated onto Trypticase Soy Agar (TSA) plates and incubated aerobically at 37 °C for 48 h. Both MacConkey agar plates and MRS agar plates were incubated under anaerobic conditions for 48 h at 37 °C. The TSA was used for determination of aerobic bacteria, MacConkey agar for enterobacteria, and MRS for lactobacilli bacteria. Bacterial counts expressed as $\log_{10}$ (CFU/g faeces) were used for the determination of enterobacteria.

### 2.4. Spleen cell culture

The spleens were aseptically removed from all mice immediately after slaughter. The spleen tissue was minced by syringe and washed twice with RPMI 1640 containing 10% faecal bovine serum (HyClone Laboratories Inc., Logan, UT), 10 mM Hepes, 100 μg/ml penicillin and 100 μg/ml streptomycin. After erythrocyte lysis, splenocytes were removed and cultured as described by Tejada-Simon, Lee, Ustunol, and Pestka (1999). Briefly, splenocytes were incubated in 24-well plates at a density of $5 \times 10^5$ cells per well in the presence or absence of 2.5 μg/ml concanavalin A (Sigma, St. Louis, MO) for 48 h at 37 °C and 5% $CO_2$. The supernatant was harvested and stored at −70 °C for cytokine assay using an Enzyme-Linked Immunosorbent Assay Kit.

### 2.5. Lymphocyte subset analysis by flow cytometry

Peripheral blood lymphocyte $CD3^+$, $CD4^+$ and $CD8^+$ subset phenotypes were analysed by flow cytometry as described by Lai, Yin, Li, Zhao, and Chen (2005) with some modifications. The primary antibodies used were Fluorescein isothiocyanate (FITC) Hamster anti-mouse CD3 (epsilon subunit; CD3e), FITC anti-mouse CD4 (L3T4) and FITC anti-mouse CD8 (beta subunit; CD8b, Ly-3) (Southern Biotechnology Associates Inc., Birmingham, AL). The negative monoclonal antibodies used were mouse IgG1-FITC and mouse IgG1-RPE (Serotec).

### 2.6. Measurement of plasma and intestinal antibodies by ELISA

The intestinal fluid sample was centrifuged at 10,000*g* at 4 °C for 10 min, and the supernatant was obtained for IgA determination. The measurement of plasma IgA, IgG, IgM and intestinal IgA levels was performed by enzyme-linked immunosorbent assay (ELISA) using the mouse IgA, IgG and IgM ELISA kit produced by Bethyl Laboratories (Montgomery, TX, USA). A 96 well microtiter plate (BioFil, Canada) was coated overnight at 4 °C with capture antibody in carbonate buffer (pH 7) and then free binding sites were blocked with PBS containing 1% bovine serum albumin (BSA) and 0.1% Tween (ELISA buffer) for 1 h at 37 °C. Duplicate plasma and intestinal fluid samples were diluted in ELISA buffer and incubated for 2 h at 37 °C. The plates were then incubated with ELISA buffer containing IgA, IgG or IgM antibodies conjugated with horseradish peroxidase (HRP; Serotec) for 1 h at 37 °C. Samples were washed five times with PBS containing 0.1% Tween between each incubation step. Then the plates were developed with tetramethylbenzidine (TMB) to measure the absorbance at 450 nm. The data were expressed as optical density (OD) units.

### 2.7. Determination of cytokines in the cultured splenocyte supernatant

Concentrations of IL-2, IL-4, IL-6 and IFN-γ in the cultured splenocyte supernatant were determined using the mouse Enzyme-Linked Immunosorbent Assay Kit (ELISA Kit; Jingmei Biotech, Shanghai, China) according to the manufacturer's instructions.

### 2.8. Statistical analysis

*In vitro* probiotic potential and intestinal impact were analysed using unpaired Student's *t* test. All other data were analysed using

the MIXED Model of SAS system (version 9.0, SAS Institute Inc., Cary, NC, USA). *P* values less than 0.05 were considered statistically significant.

## 3. Results

### 3.1. Probiotic potential of E. faecium SF68

*In vitro* assessment of probiotic potential was examined by the tolerance of bovine bile and simulated gastric and small intestine (Table 1). SF68 retained viability and increased in number after 30 min and 60 min of exposure to bovine bile, exhibiting an intrinsic tolerance towards bovine bile. When exposed to simulated gastric juices for 20 min and 60 min, the SF68 exhibited 62.73% and 56.03% survival of the initial count, respectively. The SF68 exhibited a survival rate that would allow it to pass through the stomach. The SF68 retained 96.14% and 92.16% viability of the initial count when exposed to simulated small intestinal juice for 60 min and 120 min, respectively. The results indicated that SF68 can be classified as tolerant to the small intestinal digestive secretions.

### 3.2. Fecal flora composition

The bacteria numbers in the faeces are presented in Table 2. The number of enterobacteria was found to be significantly lower ($P < 0.05$), but the fecal lactobacilli count was dramatically higher ($P < 0.05$) in the probiotic supplemented group than in the control on day 7, 14 and 21. The aerobic bacteria count was also increased in the SF68 supplemented group on day 7 and 14 ($P < 0.05$), although no difference was observed between the two treatments on day 21.

**Table 1**
Effect of bovine bile, simulated gastric and small intestinal transit on viability of *Enterococcus faecium* SF68.

| Item | Viable count ($\log_{10}$ CFU/ml) | | |
|---|---|---|---|
| Bovine bile tolerance | 0 min | 30 min | 60 min |
| | 8.11 ± 0.03 | 8.26 ± 0.07 | 8.31 ± 0.04* |
| Simulated gastric tolerance | 0 min | 20 min | 60 min |
| | 8.21 ± 0.02** | 5.15 ± 0.02* | 4.60 ± 0.01 |
| Simulated small intestinal tolerance | 0 min | 60 min | 120 min |
| | 8.29 ± 0.06** | 7.97 ± 0.02* | 7.64 ± 0.04 |

Results are shown as means ± SEM ($n = 3$).
* Student's *t* test with $P < 0.05$.
** Student's *t* test with $P < 0.01$.

**Table 2**
Effect of feeding *E. faecium* SF68 on fecal flora composition of control and probiotic-fed mice.[a]

| Item | Day 0 | Day 7 | Day 14 | Day 21 |
|---|---|---|---|---|
| *Enterobacteria*[b] | | | | |
| Control | 4.18 ± 0.18 | 5.08 ± 0.06** | 4.56 ± 0.21** | 5.35 ± 0.07** |
| Probiotic-fed | 4.21 ± 0.15 | 3.82 ± 0.08 | 2.84 ± 0.28 | 2.69 ± 0.09 |
| *Aerobic bacteria*[b] | | | | |
| Control | 5.17 ± 0.14 | 5.51 ± 0.06 | 4.26 ± 0.24 | 5.39 ± 0.15 |
| Probiotic-fed | 5.14 ± 0.15 | 7.71 ± 0.16** | 6.15 ± 0.08** | 5.67 ± 0.24 |
| *Lactobacillus*[b] | | | | |
| Control | 8.75 ± 0.08 | 8.30 ± 0.06 | 7.72 ± 0.09 | 8.17 ± 0.03 |
| Probiotic-fed | 8.80 ± 0.11 | 9.10 ± 0.15* | 8.60 ± 0.66** | 9.22 ± 0.07** |

* Statistically significant difference with respect to control, $P < 0.05$.
** Statistically significant difference with respect to control, $P < 0.01$.
[a] Values are presented as means ± SEM ($n = 5$).
[b] Bacterial count ($\log_{10}$ CFU/g faeces).

**Table 3**
Effect of feeding *E. faecium* SF68 on lymphocyte subsets in peripheral blood of control and probiotic-fed mice.[a]

| Item | Treatment | | SEM[b] | *P* value | |
|---|---|---|---|---|---|
| | Control | Probiotic-fed | | Treatment | Treatment × period |
| γδT cell (%) | 0.67 | 0.71 | 0.07 | 0.67 | 0.99 |
| Th cell (%) | 55.81 | 53.96 | 1.16 | 0.27 | 0.14 |
| $T_c$ cell (%) | 10.36 | 9.56 | 0.28 | 0.05 | 0.05 |
| NK cell (%) | 0.20 | 0.25 | 0.05 | 0.57 | 0.23 |
| DP cell (%) | 0.88b | 1.10a | 0.07 | 0.03 | 0.01 |
| T cell (%) | 67.70 | 65.23 | 1.03 | 0.10 | 0.36 |

Mean values with different superscripts are different at $P < 0.05$.
[a] Each value is the mean of data from five replicates per treatment. The γδT cell, Th cell, $T_c$ cell, NK cell, DP cell and T cell in peripheral blood were expressed by $CD3^+CD4^-CD8^-$, $CD3^+CD4^+CD8^-$, $CD3^+CD4^-CD8^+$, $CD3^-CD4^-CD8^+$, $CD3^+CD4^+CD8^+$ and $CD3^+$, respectively.
[b] Standard error of the mean.

### 3.3. Lymphocyte subset phenotype

Since different immune cells are expressed by different lymphocyte subset phenotypes, the results of lymphocyte subset phenotype were presented by the percentage of γδT cell, Th cell, $T_c$ cell, NK cell, DP cell and T cell in peripheral blood, which were expressed by $CD3^+CD4^-CD8^-$, $CD3^+CD4^+CD8^-$, $CD3^+CD4^-CD8^+$, $CD3^-CD4^-CD8^+$, $CD3^+CD4^+CD8^+$ and $CD3^+$, respectively. As shown in Table 3, no difference was observed in the percentage of γδT cell, Th cell, NK cell and T cell in peripheral blood of control and probiotic supplemented mice. However, the proportion of $T_c$ cell tended to decrease while the percentage of DP cells was higher ($P < 0.05$) in the blood of mice fed SF68.

### 3.4. Antibody levels in the plasma and intestinal fluid

To investigate the effects of *E. faecium* SF68 on systemic or local immune response in mice, levels of circulating IgA, IgG and IgM antibodies in plasma, as well as IgA antibody in the intestinal fluid, were determined (Table 4). Plasma IgA, IgM or intestinal IgA concentrations between the control and probiotic supplemented groups were not statistically different. Nevertheless, plasma IgG was statistically higher ($P < 0.05$) in SF68 supplemented mice than in the control mice.

### 3.5. Cytokine concentrations in the cultured splenocyte supernatant

The IL-2 levels were not influenced in the two groups of mice throughout the whole experiment (Fig. 1A). Concentrations of IL-4, IL-6 and IFN-γ increased progressively in the supernatant of spleen cell culture of SF68 supplemented mice and peaked on day 14 compared to the control ($P < 0.05$) (Fig. 1B–D). On the contrary, the levels of IL-6 and IFN-γ decreased after day 14 and were lower than the control group on day 21 (Fig. 1C and D).

## 4. Discussion

The present study investigated the effects of oral supplementation of *E. faecium* SF68 on the fecal microbiota as well as the local and systemic responses in mice after assessment of probiotic potential. SF68 are facultative anaerobes and can survive in adverse environmental conditions, such as extreme pH, low and high temperatures, and inadaptive salinity (Foulquié Moreno, Sarantinopoulos, Tsakalidou, & De Vuyst, 2006). As one of the important strains of enterococcus, *E. faecium* SF68 has an inhibitory effect on the growth of some enterobacteria, such as *E. coli* and *Salmonella* spp. and has been extensively studied as a probiotic for diarrhoea treat-

**Table 4**
Effect of feeding *E. faecium* SF68 on plasma IgA, IgM, IgG levels and IgA concentrations in the intestinal fluid of control and probiotic-fed mice.[a]

| Item | Treatment | | SEM[b] | *P* value | |
|---|---|---|---|---|---|
| | Control | Probiotic-fed | | Treatment | Treatment × period |
| Plasma IgG (ng/ml) | 2481.34b | 2818.91a | 83.94 | 0.01 | 0.08 |
| Plasma IgA (ng/ml) | 3150.01 | 2952.94 | 123.71 | 0.27 | 0.29 |
| Plasma IgM (ng/ml) | 748.75 | 748.77 | 0.16 | 0.93 | 0.22 |
| Intestinal IgA (ng/ml) | 964.88 | 1005.38 | 118.08 | 0.81 | 0.76 |

Mean values with different superscripts are different at $P < 0.05$.
[a] Each value is the mean of data from five replicates per treatment.
[b] Standard error of the mean.

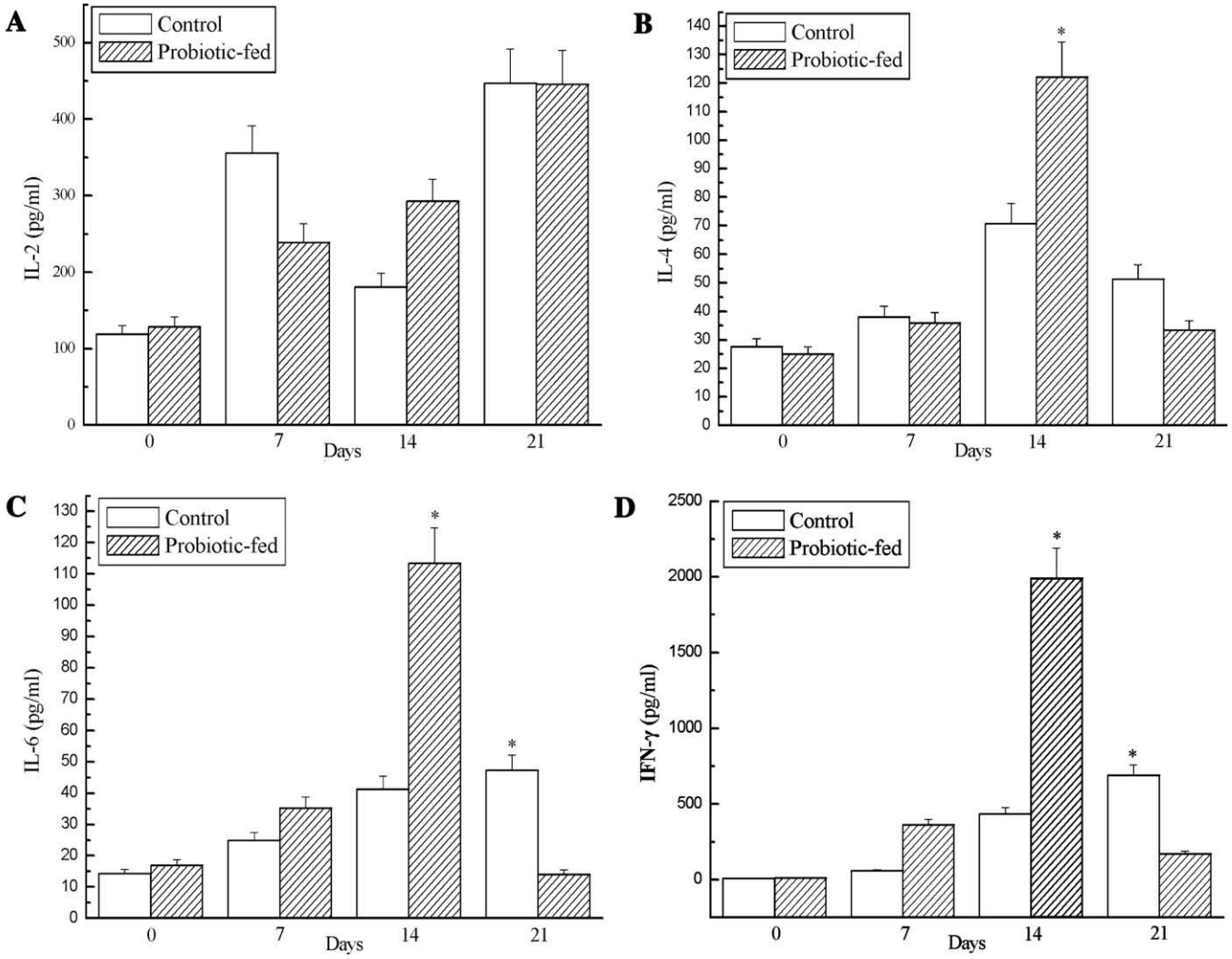


**Fig. 1.** The IL-2 (A), IL-4 (B), IL-6 (C) and IFN-γ (D) production in Con A-stimulated splenocytes at day 0, 7, 14, and 21 from probiotic-fed mice or control mice. Each data point is presented as the groups mean ± SEM of five replicates (with four mice per replicate) per group. $^{*}P < 0.05$ vs. control.

ment for improving the gastrointestinal balance of colonic microbiota (Foulquié Moreno et al., 2006; Franz et al., 1999). It is generally accepted that the successful probiotic bacteria need to be able to survive passage through the gastrointestinal tract and reach the distal part of the intestine in sufficient numbers (approximately $10^6$–$10^7$ cells/g of food) in order to exert a beneficial effect. The acidic environment of the stomach and the bile salts secreted in the duodenum are major impediments to the survival of ingested bacteria (Collado & Sanz, 2006). In this study the SF68 was shown to possess both gastrointestinal tract transit tolerance and bovine bile tolerance properties.

When the lymphocyte subsets in peripheral blood were assessed, the proportion of γδT cell, Th cell, NK cell, and T cell were not affected by SF68 supplementation. In a previous publication, Benyacoub et al. (2003) found no differences in the percentages of $CD4^+$ and $CD8^+$ T cells in peripheral blood of puppies fed SF68. Research conducted by Scharek et al. (2005) also showed that no difference was observed in the lymphocyte populations isolated

from the Peyers patches of piglets administrated *E. faecium* SF68. In fact, one of the important findings of the present study was that supplementation of *E. faecium* SF68 increased the proportion of DP cells in the probiotic-fed mice compared with the control. DP cells are an important indicator of the activation of the immune system, reflecting the active status of T cells which coexpress CD4 and CD8 (Kenny, Mason, Pombo, & Ramírez, 2000). The increase of DP cells in the peripheral blood of probiotic-fed mice suggested that the immune system was activated by *E. faecium* SF68. Opposite to DP cells, the percentage of $T_c$ cells tended to decrease although no significant difference was observed. It has been reported that $T_c$ cells and suppressor T cells ($CD8^+$ T-cell subsets) are lineages of $CD8^+$ T cells (Poggí & Zocchi, 2008). Therefore, the proportion of $CD3^+CD4^-CD8^+$ might contribute more to the expression of $CD8^+$ T-cell subsets including $CD8^+CD25^+$ and $CD8^+CD28^-$ that inhibited lymphocyte proliferation and activation, resulting in the suppressed cell-mediated immunity and humoral immunity (Poggí & Zocchi, 2008). With the supplementation of SF68 in the present study, the results of $T_c$ cells indirectly supported the activation of the immune function in the probiotic-fed mice.

Several studies revealed that oral administration of probiotics could trigger and stimulate the immune system underlying the intestinal mucosa, which tended to enhance IgA production (Fang, Elina, Heikki, & Seppo, 2000; Kaburagi et al., 2007; Tejada-Simon et al., 1999). As the major class of antibodies present in the mucosal secretions of the gastrointestinal tract, IgA plays an important role in the defense against invasion by ingested food substances. In the present study, we found a slight increase of IgA concentration in the intestinal fluid of mice fed *E. faecium* SF68, although no difference was observed in either the intestine or in the plasma. Several studies with probiotic bifidobacteria revealed that some of these intestinal microflora (Gram-positive lactic acid-producing bacteria) showed no immune-stimulating effects on the secretory immune response in rodents (Scharek et al., 2005). This might indicate that, as an autochthonous inhabitant of the mouse gut flora, *E. faecium* also lacks mucosal immune-stimulating properties (Scharek et al., 2005).

Contrary to IgA, the IgG concentration was significantly increased in the plasma of probiotic-fed mice compared to the control in this study. It is known that IgG plays a minute role and relatively small amounts of IgG are secreted in the mucosa in intestinal mucosal immunity; however, large quantities of IgG antibody in the serum are stimulated when food substances, such as probiotics, are absorbed into the blood through the intestinal epithelial cells. The serum IgG antibody plays an essential role in the systemic immune response, which has been reported in numerous previous publications (Benyacoub et al., 2005; Gizzarelli et al., 2006).

As noted above, T lymphocytes can be classified as $CD4^+$ or $CD8^+$ subsets based on their surface markers. $CD4^+$ T cells defined as helper T cells can be further divided into T-helper 1 (Th1) cells and T-helper 2 (Th2) cells according to secretion patterns of cytokines (Carter & Dutton, 1996). Th1 lymphocytes secrete IL-2, tumour necrosis factor-α, and IFN-γ that are vital to cell-mediated immunity, while Th2 lymphocytes are predominantly involved in humoral immunity and allergic responses by producing IL-4, IL-5, IL-6 and IL-10 (Carter & Dutton, 1996; He et al., 2005).

The IL-2 is produced by antigen-activated T cells in an autocrine manner. It stimulates T cell proliferation, growth *in vitro* and apoptosis in antigen-activated T cells. Therefore, IL-2 possesses dual functions for both induction and regulation of T cell-mediated immune responses and stimulation of proliferation and differentiation of B cells (He et al., 2005). In this study we found no variations in the concentration of IL-2 in the spleen cell culture of *E. faecium*-fed mice and control mice.

IL-4 supports the antigen-presenting functions of monocytes and macrophages by up-regulating the expression of MHC II molecules, and activates monocytes/macrophages to enhance T cell proliferation. Furthermore, IL-4 indirectly stimulates the production of IgA in the mucosa of the intestine by promoting B lymphocytes to proliferate and differentiate into plasma cells, which secrete more IgA antibody.

The IFN-γ produced by activated T cells and NK cells can also be produced by macrophages and dendritic cells (Frucht et al., 2001). Numerous studies have shown that LAB can influence cytokine production by immunocompetent cells in animals and humans (Pereyra, Falcoff, Falcoff, & Lemonnier, 1991; Solis Pereyra & Lemonnier, 1993). The LAB contained in conventional yoghurt has been reported to significantly enhance IFN-γ production (Solis Pereyra & Lemonnier, 1993), theoretically, an enhanced production of IFN-γ could lead to a better response against pathogen challenges. Schierack et al. (2007) reported that IL-4 and IFN-γ production of polyclonally stimulated peripheral blood mononuclear cells was, on average, higher in the *Bacillus cereus* var. toyoi-fed piglets (Schierack et al., 2007). Consistent with these results, we observed higher concentrations of IL-4 and IFN-γ stimulated by spleen cells of *E. faecium*-fed mice. In addition, the IFN-γ was also increased in mice supplemented with SF68.

IL-6 is another Th2 type cytokine that induces the proliferation and differentiation of B lymphocytes to secrete IgA, IgG and IgM antibodies (Carter & Dutton, 1996). The IL-6 stimulates the synthesis of acute phase proteins by hepatocytes, as well as the growth of antibody-producing B lymphocytes. Many *in vitro* and *in vivo* studies have suggested a potential influence of IL-6 on augmenting the IgA antibody response in mucosal B cells or at mucosal surfaces (Pockley & Montgomery, 1991). In the present study, elevated production of IL-6 on day 14 was observed when SF68 was supplemented. Miettinen et al. (1996) reported that live LAB induced the release of IL-6 better than fixed bacteria, which indicated that LAB could be used as vaccine vectors or as probiotics for the purpose of stimulating nonspecific immunity (Miettinen et al., 1996).

Taken together, the current study demonstrates that SF68 fulfilled the selection criteria of a probiotic without any adverse effects. Supplementation with SF68 was found to have an impact on the intestinal microbial flora of the mice and appeared to reduce the enteropathogenic bacterial load. Systemic immune responses to SF68 was observed with increased DP cells in peripheral blood and plasma IgG levels, as well as an increase of IL-4, IL-6 and IFN-γ in splenocytes of the probiotic-fed mice. These results indicated that SF68 has the characteristics of a viable probiotic, which benefits the immune function in BALB/c mice.

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