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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 5×10^5 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