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Evaluation of Peanut Flour Fermented with Lactic Acid Bacteria as a Probiotic Food

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The aim of this study was to evaluate the probiotic value of peanut flour fermented with lactic acid bacteria *in vitro* and *in vivo*. Four strains including *Lactobacillus delbrueckii* LD09, *Lactobacillus casei* LC35, *Lactobacillus acidophilus* LA51, and *Lactobacillus plantarum* P9 were screened for their growth and survival in peanut flour. Among all the strains, *L. plantarum* P9 grew to the highest cell population (9.48 log cfu/g) in peanut flour after 72 h fermentation at 37°C. After 28 days storage at 4°C, no marked change in the viable count of this strain was observed. Peanut flour fermented with *L. plantarum* P9 could also increase the content of crude protein and the degree of protein hydrolysis. In an *in vitro* system, the addition of protein from the fermented peanut flour greatly enhanced the survival of *L. plantarum* P9 in simulated gastric and bile juices. *In vivo* studies, supplementation with the fermented peanut flour in the diet of mice increased significantly the number of lactobacilli in the fecal samples compared to the control group. At the same time, the number of enterobacteria decreased significantly. These results indicated that peanut flour fermented with *L. plantarum* P9 strain could be a novel type of probiotic food.

Key Words: lactic acid bacteria, fermentation, peanut flour, probiotics, gastrointestinal tolerance

INTRODUCTION

Probiotics are live microbial feed or food supplements, which beneficially affect the host by improving its intestinal microbial balance (Fuller, 1989). Upon consumption, probiotics can exert many beneficial effects to the host, including inhibition of pathogenic microorganisms and prevention gastrointestinal disorders (Lewis and Freedman, 1998), modulation of the host immune responses (Isolauri et al., 2001) and prevention of cancer and cardiovascular diseases (Bukowska et al., 1998). In recent years, probiotic products have become a primary choice for the consumers because of their health attributes. Therefore, there has been a worldwide increasing interest to select more active probiotics to satisfy the increasing request of the market.

Peanut is a good source of supplementary protein for the human diet. Peanut flour (PF) is widely used in food industries for its relatively high protein content, bland

flavor and light tan color (Prinyawiwatkul et al., 1995). Many studies have reported that soy products fermented with lactic acid bacteria (LAB) can be looked as unique probiotic foods for human nutrition (Shimakawa et al., 2003). Like soy products, lactic acid fermented peanut flour may be a suitable probiotic bacterial strains carrier to the host.

Therefore, the aim of this study was to evaluate the probiotic value of peanut flour fermented with LAB *in vitro* and *in vivo*. Although some studies indicated that peanut products are suitable for the growth of LAB (Beuchat and Nail, 1978; Lee and Beuchat, 1991) to the best of our knowledge, no research on the probiotic value of peanut products fermented with LAB has been found.

MATERIAL AND METHODS

Microorganisms and Materials

The strains *Lactobacillus delbrueckii* LD09, *Lactobacillus casei* LC35, and *Lactobacillus acidophilus* LA51 used in this study were obtained from the Culture Collection of Southern Yangtze University (Wuxi, China). *Lactobacillus plantarum* P9 was kindly provided by Dr Jia L. (Shandong Agricultural University, Taian, China).

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Commercial defatted peanut meal (crude protein concentration = 48.3%) was provided by Eastocean Cereals & Oils Co., Ltd. (Zhangjiagang, China). Prior to further use, peanut meal was cleaned and ground into fine flour to pass a 0.4 mm screen. All other reagents and chemicals used in this study were of analytical grade.

Methods

Experimental Design

The study consisted of a sequence of three experiments. The first experiment was designed to select the best strain of LAB in terms of their growth and survival in peanut flour. *Lactobacillus plantarum* P9 was selected because of its good growth and survival in peanut flour. The second experiment was designed to determine the effect of peanut protein on the survival of *L. plantarum* P9 in simulated gastric and bile juices *in vitro*. Then the effect of fermented-PF (FPF) on the fecal microflora of mice was investigated *in vivo*.

Fermentation and Storage

Peanut flour and distilled water in the amount to adjust moisture content of the mixtures to 55% were mixed in a food processor mixer (DW-25, Better Boiler Ltd., Shanghai, China). Each 150 g of the moistened peanut flour was transferred into a 250 mL conical flask and autoclaved at 121°C for 30 min. Then they were inoculated with freeze-dried cells (de Valdez et al., 1983) of each strain to give approx. 7 log cfu/g in PF after inoculation. After thorough mixing, the flasks incubated at 37°C for 72 h with N₂ as headspace gas. After fermentation, the fermented products were stored at 4°C for 28 days. During the storage period, viable counts of LAB in FPF were determined.

Microbial and Chemical Analysis

Samples of FPF collected during the fermentation and storage were used for bacterial enumeration. Each sample (5.0 g) was mixed with 45 mL of 0.1% sterile peptone water followed by mixing homogeneously using a Labblender 400 stomacher (Seward Laboratory, London, UK). Then 0.1 mL of appropriate dilutions was surface spread on MRS agar. The plates were incubated at 37°C in an anaerobic cabinet (YQX-II, Cimo Medical Instrument Ltd., Shanghai, China) containing an anoxic atmosphere (10% H₂, 10% CO₂, 80% N₂). Bacterial counts were determined after 48 h of incubation.

Samples were collected during the fermentation with *L. plantarum* P9 and used for chemical analysis. The pH and titratable acidity (TA) expressed as percent lactic acid were determined according to the method described by Ikenebomeh (1989). The content of crude protein

(N × 6.25) was determined by the AOAC (1980) method. The degree of protein hydrolysis (DH), defined as the percentage of peptide chain cleaved, was determined by an amino nitrogen analysis using trinitrobenzenesulfonic acid (Adler-Nissen, 1979).

In Vitro Studies of Gastrointestinal Tolerance

Preparation of Peanut Protein and Washed Bacterial Cell Suspension

Peanut protein was extracted from FPF according to the method described by Li et al. (2004). FPF was mixed with distilled water in the ratio of 1 : 10 (w/v), and pH of the mixture was adjusted to 10.0 with 1.0 M NaOH. The mixture was stirred at room temperature for 1 h, and then centrifuged at 3000 × g for 20 min. The supernatant was adjusted to pH 4.5 with 1.0 M HCl and centrifuged at 3000 × g for 20 min. After washing with water three times, the protein precipitate was re-suspended in water and freeze-dried. Before use, peanut protein was suspended into 0.5% (m/v) NaCl solution at a concentration of 2.5 or 5% (m/v). Then the peanut protein solutions were filter sterilized using a 0.22 µm membrane filter (Millipore, Billerica, MA, USA).

After serial transfer of *L. plantarum* P9 in fresh MRS broth at 37°C for 18 h, an aliquot (1 mL) was centrifuged (5000 rpm, 10 min) and washed three times with sterile 0.5% NaCl solution, and the cells re-suspended in the same solution. The total viable count of the washed cell suspension was determined prior to assay of gastrointestinal tolerance.

Preparation of Simulated Gastric and Bile Juices

Simulated gastric juices were prepared by suspending pepsin (Sigma Chemical Co., St. Louis, USA) in sterile 0.5% NaCl solution to a final concentration of 3 g/L and adjusting the pH to 2.0 with 12 M HCl. Simulated bile juices were prepared by suspending pancreatin (Sigma Chemical Co., St. Louis, USA) in sterile 0.5% NaCl solution to a final concentration of 1 g/L, with 4.5% bile salts (Difco Laboratories, Detroit, MI, USA) and adjusting the pH to 8.0 with sterile 0.1 M NaOH.

Measurement of Gastrointestinal Tolerance

The tolerance of washed cell suspension of *L. plantarum* P9 to simulated gastric and bile juices was determined following the method of Charteris et al. (1998). Aliquots (0.2 mL) of the free-cell suspension were separately transferred to a 2.0 mL Eppendorf tube, mixed with 0.3 mL of sterile 0.5% NaCl solution, or peanut protein solution (2.5 or 5%), and finally mixed with 1.0 mL of simulated gastric or bile juices. The final concentrations of peanut protein in the Eppendorf tube

were 0.5% and 1% (m/v). The mixture was then incubated at 37°C. In gastric transit tolerance measurements, aliquots of 0.1 mL were taken after 0, 30, 60, 90, 120, 150, and 180 min for determination of total viable count. In bile tolerance measurements, aliquots of 0.1 mL were taken after 0, 60, 120, 180, and 240 min. The viable counts of *L. plantarum* P9 were determined by the method mentioned above.

In Vivo Studies

Animals and Diets

Ninety 3-week-old male mice (KunMing strain) were purchased from Shanhe pharmacy Co., Ltd. (Wuxi China) and housed in stainless steel cages under controlled conditions (temperature $22 \pm 1^\circ\text{C}$, humidity 60–65%, lights on from 6:00 to 18:00). After a 5-days acclimatization period (day 0), all the mice were weighed and randomly assigned to 3 groups of 30 mice and fed with three different diets containing different contents of FPF. The composition of the diets is shown in Table 1. After feeding on the three experimental diets for 4 weeks (day 28), all animals remained on the control diet for a further 7 days (day 35). Feed and water were available *ad libitum* throughout this study. The experimental protocol was approved by the Animal Care and Use Committee of Southern Yangtze University.

Bacteriological Analysis of Fecal Samples

Fresh fecal samples from mice were collected at 14, 28, and 35 days and used for bacterial enumeration. Bacteriological procedures were according to the method described by Xia et al. (2005). Lactobacilli were enumerated on MRS agar after incubation in the anaerobic cabinet mentioned above at 37°C for 48 h. Enterobacteria were enumerated on MacConkey agar

after incubation at 37°C for 24 h. For the enumeration of bifidobacteria, the diluted samples were spread on reinforced clostridial agar plus supplements (Munoz and Pares, 1988), and incubated in the anaerobic cabinet at 37°C for 72 h. Subsequently, the bacteria were identified to genus level on the basis of colonial appearance, Gram reaction, spore production, cell morphology, and fermentation end-product formation.

Statistical Analysis

Unless otherwise indicated, all results in this article were means of three independent trials $\pm$ SD. Data were analyzed by one-way analysis of variance (ANOVA) using SPSS version 11.5 (SPSS, Chicago, IL, USA). Differences among the means were tested using Duncan's multiple-range tests. Values of $p < 0.05$ were considered significant.

RESULTS

Growth and Survival of LAB in PF

All strains attained high cell populations of 9.19–9.48 log cfu/g in PF after 72 h of fermentation, except for *L. acidophilus* LA51 (Figure 1). After 28 days of storage at 4°C, there was no marked change in the cell number of *L. plantarum* P9. In contrast, the number of other strains were much reduced at the end of storage. The number (9.15 log cfu/g) of *L. plantarum* P9 at the end of storage was much higher than 7 log cfu/g, which is necessary for positive effects on the host.

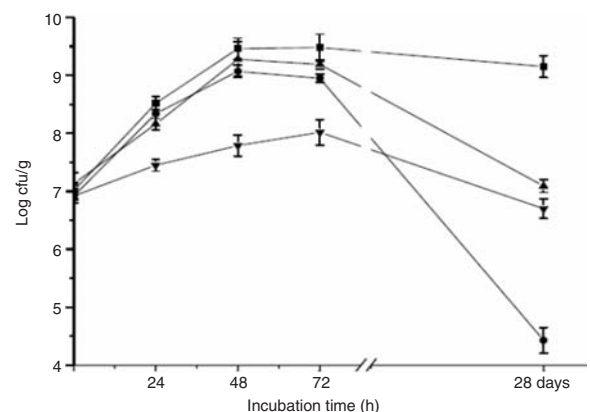


Figure 1. Growth of *L. plantarum* P9 (■), *L. delbrueckii* LD09 (●), *L. casei* LC35 (▲), *L. acidophilus* LA51 (▼) in peanut flour during 72 h fermentation at 37°C and their survival in the fermented peanut flour stored at 4°C for 28 days. Data were calculated on wet weight basis.

Table 1. Composition of the experimental diets.

Ingredients (%)	Diets		
	Control	LFPF ¹	HFPP ¹
Soybean meal	50	47	44
Fermented peanut flour ²		3	6
soy oil	5	5	5
Corn starch	36	36	36
Cellulose	3	3	3
Vitamin mixture ³	5	5	5
Mineral mixture ⁴	1	1	1

¹LFPF, low fermented peanut flour; HFPP, high fermented peanut flour.

²The fermented peanut flour was prepared by 72 h fermentation at 37°C. The fermented product was freeze dried and contained 9.11 log cfu/g *L. plantarum* P9.

³AIN-VX mixture (Shiau et al., 1987).

⁴AIN-93 M mixture (Shiau et al., 1987).

Table 2. Chemical changes during the fermentation *L. plantarum* P9.

Fermentation Time (h)	pH	Titrateable Acidity (%)	Crude Protein (% wb)	Degree of Protein hydrolysis (%)
0	6.59 ± 0.05	0.28 ± 0.02	24.01 ± 0.05	1.38 ± 0.03
12	5.16 ± 0.23	1.17 ± 0.31	24.59 ± 0.17	2.51 ± 0.05
24	4.17 ± 0.12	2.62 ± 0.18	25.17 ± 0.12	4.82 ± 0.01
36	4.04 ± 0.07	3.01 ± 0.03	25.89 ± 0.07	6.45 ± 0.02
48	4.01 ± 0.02	3.04 ± 0.02	26.05 ± 0.14	8.31 ± 0.04
60	3.99 ± 0.02	3.06 ± 0.04	26.13 ± 0.15	10.57 ± 0.02
72	3.98 ± 0.01	3.08 ± 0.01	26.09 ± 0.09	10.80 ± 0.04

Chemical Changes during the Fermentation with *L. plantarum* P9

Table 2 shows the pH, TA, DH, and crude protein of PF during the fermentation with *L. plantarum* P9. An appreciable decrease in pH and increase in TA were noted in PF after 24 h of fermentation. At the end of 72 h fermentation, the pH declined from 6.59 to 3.98, while TA increased from 0.28 to 3.08%. At the same time, there was a slight increase in the content of crude protein. The degree of hydrolysis of the protein in peanut flour increased significantly after 72 h of fermentation from 1.38 to 10.80% (Table 2).

In Vitro Studies of Gastrointestinal Tolerance

In the control experiment, without addition of peanut protein, the viable population of *L. plantarum* P9 decreased dramatically from 8.95 to 3.01 log cfu/mL during 30 min exposure to gastric juices (Figure 2). After 60 min exposure, no viable cells were detectable in the gastric juices, however, in the parallel experiments, the addition of peanut protein significantly improved the gastric tolerance. At the end of incubation, the cell population reduction of *L. plantarum* P9 was 2.19 or 0.54 log cfu/mL in the presence of 0.5 or 1% peanut protein, respectively. A progressive resistance to gastric juices was observed as the peanut protein concentration increased.

The effect of peanut protein on the survival of *L. plantarum* P9 during 240 min exposure in the bile juices is shown in Figure 3. The cell population of *L. plantarum* P9 decreased gradually from 8.48 to 7.21 log cfu/mL during exposure for 240 min. The addition of 0.5 or 1% of peanut protein had a protective effect on the survival of *L. plantarum* P9, the cell population changed from 8.47 to 8.62 and from 8.49 to 9.03 log cfu/mL during 240 min exposure, respectively. A slight increase was observed.

In Vivo Studies

The results of the microbial analysis of fecal samples are displayed in Figure 4. After 14 days of feeding,

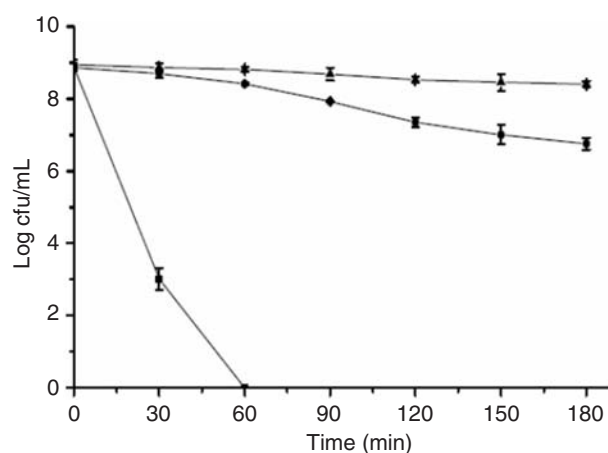


Figure 2. Evolution of *L. plantarum* P9 cell population in the presence of peanut protein during exposure to gastric juices for 180 min. (■) control, (●) 0.5%, (▲) 1% peanut protein.

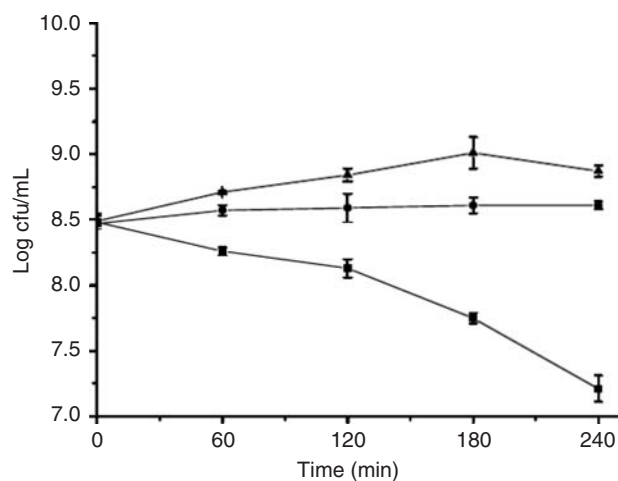


Figure 3. Evolution of *L. plantarum* P9 cell population in the presence of peanut protein during exposure to bile juices for 240 min. (■) control, (●) 0.5%, (▲) 1% peanut protein.

a significant increase ($p < 0.05$) in the number of lactobacilli was observed in the fecal samples of mice fed HFPF group compared to the control group. At the same time, the number of enterobacteria decreased

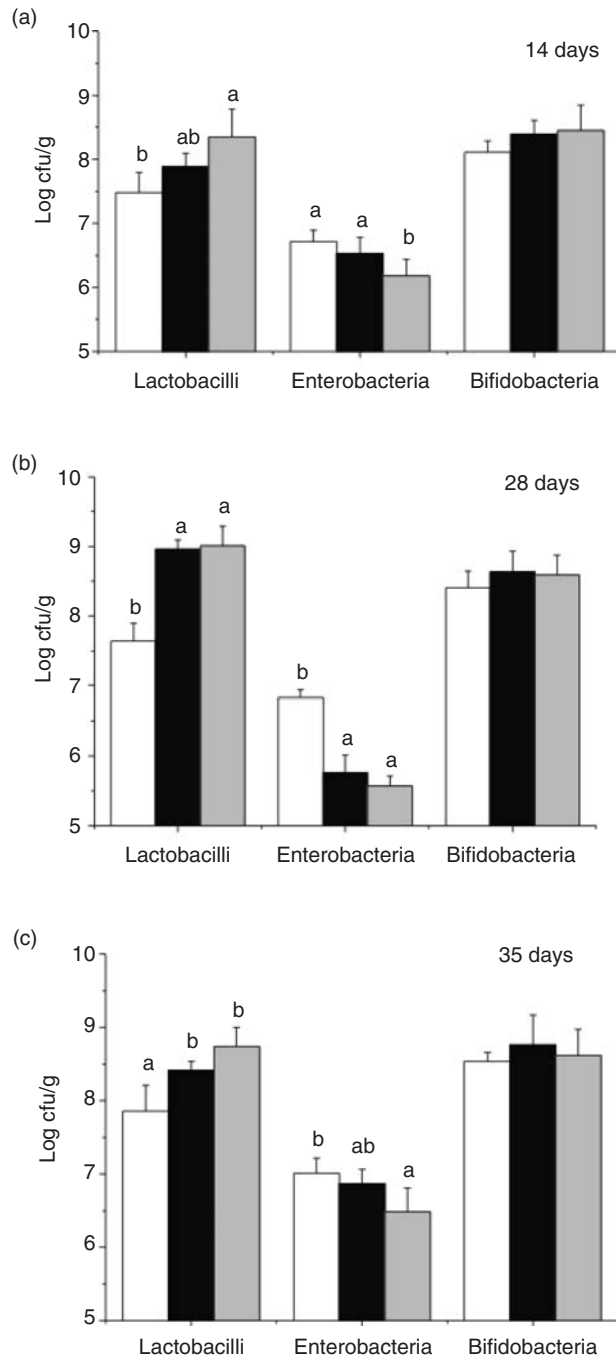


Figure 4. Effect of diets (□ control; ■ LFPF; ■ HFPF) on the composition of faecal microflora of mice ($n=10$). Bacterial numbers were calculated on wet basis. Treatments with different superscript letters on bars differ significantly ($p<0.05$).

($p<0.05$) significantly. After 28 days of feeding, the number of fecal lactobacilli were significantly higher ($p<0.05$) in both groups fed with LFPF and HFPF diets than the control group, while the number of enterobacteria showed a significant decrease ($p<0.05$). No statistically significant differences in the number of fecal bifidobacteria were observed between the three

groups after 14 and 28 days of feeding, although a tendency to increase ($p>0.05$) was observed in the mice fed with the LFPF and HFPF diets. On day 35, 7 days after the termination of FPF intake, the number of lactobacilli were still higher in the fecal samples of LFPF and HFPF groups compared to the control group.

DISCUSSION

More than 400 species of bacteria are estimated to survive in the human gastrointestinal tract and these endogenous bacteria comprise the intestinal microflora (Brück et al., 2002). Normally, the intestinal microbiota is balanced, but some factors such as aging, unbalanced diet, and antibiotics can alter the composition of the intestinal microflora. The intestinal microbiota loses its equilibrium and the host tends to be vulnerable to the attacks of external pathogens and to the proliferation of its own pathogens that form its regular microflora. Probiotics can prevent such disorders by restoring the intestinal microflora to its natural state and maintain the intestinal microbial balance. Various media have been studied as carriers for probiotic bacteria, such as cheese (Bergamini et al., 2005), yoghurts (Vinderola et al., 2000), oat (Angelov et al., 2005) and soymilk (Shimakawa et al., 2003).

To exert their beneficial effects on the host, it is essential that LAB be alive in sufficient numbers in the products at the time of consumption. It is not sufficient to establish the exact viable count of probiotic bacteria in a functional food in order to insure their health benefits, because this number varies with the strain and food. However, 7 log cfu per gram or mL of food, at the time of consumption, is frequently suggested as the minimal probiotic population required to impact favorably on the consumer's health (Bergamini et al., 2005). *Lactobacillus plantarum* P9 could grow to a high cell population in PF and had a high viable count during the storage time (Figure 1), fulfilled the criterion (7 log cfu/g) as a probiotic, indicating that the FPF would be an excellent vehicle for live LAB.

Protein hydrolysates are superior to intact protein as protein source for human nutrition, because their digestion or absorption in the digestive canal is more rapid than that of an equivalent amount of intact protein (Siemensma et al., 1993). Therefore, protein hydrolysates have been widely used in producing physiologically functional foods for some specific needs, such as patients with malnutrition and children with milk protein allergies (Mahmoud, 1994). Some *L. plantarum* strains were reported to possess high proteolytic activity (Magboul et al., 1997). Addition of *L. plantarum* to cheese or sausage could increase the DH of proteins in them (Fadda et al., 1998; Hynes et al., 2001). In the present study, the DH of peanut protein was increased during fermentation. A part of protein in

FPF was degraded into peptides, even free amino acids, which could be easily digested and absorbed by the consumers. The nutritive value of the protein in FPF was increased after fermentation.

Probiotic bacteria have to firstly survive during the transit through the upper gastrointestinal tract, and then persist in the gut to exert beneficial effects on the host. Besides the intrinsic acid and bile tolerance of probiotic bacteria, the food and food ingredients, such as proteins, polysaccharides, and free amino acids, have been reported to have a protective effect on them and enhance their survival in gastrointestinal tract (Charteris et al., 1998; Charalampopoulos et al., 2003; Michida et al., 2006). In this study, regarding the survival of *L. plantarum* P9 in gastric and bile juices, peanut protein played a key role. The addition of peanut protein significantly improved the viability of *L. plantarum* P9 in simulated gastric and bile juices, the viable cell count remained almost unaltered at the end of incubation. The concentration of peanut protein also had an effect on the gastric and bile tolerance (Figures 2 and 3). These data indicate that the strain of *L. plantarum* P9 can survive through the gastrointestinal tract of host when ingested with the product based on PF.

In the gastric tolerance of *L. plantarum* P9 strain, the buffering capacity peanut protein may play a significant role (Conway et al., 1987; Charteris et al., 1998). In bile tolerance, according to Sugano et al. (1990), soy protein could bind bile acids and aggregate them firmly, then protect the live bacterium from bile acid. Hsieh et al. (1999) reported that soy protein hydrolyzates could be used by many lactobacilli and stimulated their growth. In our study, the protein extracted from FPF was hydrolyzed during the fermentation, which may have an effect on the recovery of the bacterium in gastric and bile juices. However, further studies are required to understand the mechanism of peanut protein hydrolyzates to protect probiotic strains in gastric and bile juices.

In vivo studies, supplementing FPF in the diet could improve the intestinal microbial balance of mice. According to definition of probiotics (Fuller, 1989), FPF can be looked as a probiotic food. The observed rise in the number of lactobacilli in the fecal samples of mice fed with FPF diets may be attributed, to a large extent, to the administration of *L. plantarum* P9. At the same time, there was a slight increase in the population of bifidobacteria in present study. The large number of LAB and high concentration of lactic acid in FPF could decrease the pH of gastrointestinal tract. Being a lactic acid producing bacteria, lactobacilli and bifidobacteria can grow well at an acidic pH and this may have provided a competitive advantage over other bacterial population groups. Besides the pH diminution, according to Plant et al. (2003), the increase of lactobacilli and bifidobacteria could reduce the number of enterobacteria and other pathogenic bacteria in the

gastrointestinal tract by other mechanisms including space occupation, competition for substrates and receptors at mucosal surfaces, secretion of bacteriocins and other regulatory factors.

CONCLUSIONS

The results obtained in this study indicate that *L. plantarum* P9 showed good growth and survival in PF. The addition of protein from FPF greatly enhanced the survival of *L. plantarum* P9 in simulated gastric and bile juices. *In vivo* studies, supplementation with FPF in the diet had a positive effect on the intestinal microbial balance of mice. In conclusion, the PF fermented with *L. plantarum* P9 could be used as an excellent probiotic food.

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