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ORIGINAL ARTICLE

Fermentation characteristics of six probiotic strains in soymilk

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Abstract Fermented soymilk was produced with the single culture of the six probiotic strains, including *Lactobacillus casei* Zhang, *Bifidobacterium animalis* ssp. *lactis* V9, *Lactobacillus acidophilus* NCFM, *Lactobacillus rhamnosus* GG, *Bifidobacterium animalis* Bb12, and *Lactobacillus casei* Shirota, and their fermentation characteristics were evaluated. The free amino nitrogen of soymilk fermented with the four probiotic strains, *L. casei* Zhang, *B. animalis* V9, *L. acidophilus* NCFM and *L. rhamnosus* GG, increased greatly. Significant increases in viable counts were observed among the six probiotic strains. All the fermented soymilk had a viable count above 8.69 log CFU/g at the end of fermentation, especially the four probiotic strains of *L. casei* Zhang, *L. acidophilus* NCFM, *B. animalis* Bb12, and *L. casei* Shirota, which were all over 9.00 log CFU/g. The content of glucose and fructose decreased for all the six probiotic strains after the fermentation, but along with the reduction in the content of stachyose and raffinose only for *B. animalis* V9, *L. rhamnosus* GG, and *B. animalis* Bb12. Our results indicated that the six probiotic strains can grow well and increase the contents of bioactive substances in soymilk, including γ-aminobutyric acid, vitamin B6, and total isoflavone aglycone, and deserves further research on utilization as potential starters for soymilk.

Keywords Soymilk · Probiotic · Fermentation characteristics · Lactic acid bacteria

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Introduction

Soymilk, a traditional oriental food beverage, is the water extract of soybean that provides a rich yet economical supply of protein and calories, contains no cholesterol or lactose, and only a small quantity of saturated fatty acid compared with cows' milk (Scalabrini et al. 1998). Soymilk could provide unique health benefits to the consumers because of its hypolipidemic, anticholesterolemic, antioxidant, and antiatherogenic properties; it could also reduce the risk of hormone-associated health disorders (Favaro Trindade et al. 2001). However, the unfavorable beany flavor, flatulence factors, and the high content of indigestible alpha-galactosyl oligosaccharides such as raffinose and stachyose limit the consumption of soybeans as raw food material (Thananunkul et al. 1976). Fermentation of soymilk with various organisms, especially lactic acid bacteria, has been reported to overcome the problem of the beany flavor and flatulence to increase both its acceptability and nutritional value (Mattick and Hand 1969; Thananunkul et al. 1976).

Probiotics, defined as "live microorganisms that when administered in adequate amounts confer a health benefit on the host" by the Food and Agricultural Organisation (FAO 2001), have become a major topic of lactic acid bacteria (LAB) research over the past 10 years (Kailasapathy and Chin 2000). Probiotics mainly include the genera *Lactobacillus* and *Bifidobacterium*. These organisms can be the

predominating members of the endogenous intestinal flora in humans, which are reported to exert beneficial effects including the activation of the immune system, reduction of serum cholesterol, and inhibition of the growth of potential pathogens that may cause infections in the host (Holzapfel et al. 2001, Ishibashi and Yamazaki 2001). Therefore, incorporation of these probiotic bacteria into soymilk to increase its therapeutic value has become a popular trend (Ishibashi and Shimamura 1993; Blanchette and Roy 1995; Pestka et al. 2001; Wang et al. 2006; Rekha et al. 2008; Kitawaki et al. 2009). Furthermore, probiotic bacteria generally do not grow rapidly in cows' milk, and therefore cannot attain a high enough viability as starter cultures in yoghurt manufacture (Champagne et al. 2005). However, many studies indicate that soy is a good substrate for probiotic bacteria, especially the probiotic *Bifidobacterium* (Mital et al. 1974; Scalabrini et al. 1998).

To develop the probiotic soymilk drink, we fermented soymilk with six single cultures of probiotic bacteria, including *Lactobacillus casei* Zhang, *Bifidobacterium animalis* ssp. *lactis* V9, *Lactobacillus acidophilus* NCFM, *Lactobacillus rhamnosus* GG, *Bifidobacterium animalis* Bb12, and *Lactobacillus casei* Shirota, and investigated their fermentation characteristics, including acidity, proteolysis, and contents of lactic and acetic acid, γ-aminobutyric acid, vitamins B, carbohydrate, and isoflavones. *Lactobacillus casei* Zhang (EF536364) is a probiotic isolated from Koumiss, a traditional fermented alcoholic beverage prepared from mare's milk in Inner Mongolia, with high acid resistance, bile salt resistance, persistence of gastrointestinal transportation, and cholesterol-reducing and antimicrobial activities (Wu et al. 2009). *Bifidobacterium animalis* V9 (CP001892) was isolated from the feces of a healthy Mongolian child in China with high acid resistance, bile salt resistance, persistence of gastrointestinal transportation, and antimicrobial activities against *Shigella dysenteriae*, *Pseudomonas aeruginosa*, *Escherichia coli* O157 882364, and *Salmonella typhimurium* S50333 (Gao et al. 2009a, b). The other four probiotics of *L. acidophilus* NCFM, *L. rhamnosus* GG, *L. casei* Shirota, and *B. animalis* Bb12 are all very well-known probiotics worldwide.

Materials and methods

Bacterial strains

Both *L. casei* Zhang and *B. animalis* V9 were provided by the Bacterial Bank of the Key Laboratory of Dairy Biotechnology and Bioengineering, Education Ministry of China, Inner Mongolia Agricultural University. *Lactobacillus acidophilus* NCFM was provided by Danisco (Copenhagen, Denmark), and *L. rhamnosus* GG by Valio (Finland), and *B. animalis* BB12 by Chr. Hansen (Hørsholm, Denmark). *Lactobacillus casei* Shirota was isolated from the fermented milk beverage produced by Yakult Honsha (Tokyo, Japan).

All media were purchased from Kanto Chemical (Tokyo, Japan) and Rong Research (Tokyo, Japan). *Lactobacillus casei* Zhang, *L. acidophilus* NCFM, *L. rhamnosus* GG and *L. casei* Shirota were grown in MRS broth at 37°C for 18 h, and *B. animalis* V9 and *B. animalis* Bb12 in TPY broth at 37°C for 18 h. All strains were subcultured twice prior to the experiments.

Fermented soymilk manufacture

In the present study, preparation and fermentation of soymilk were performed according to the procedures as following. Whole dry soybeans (Jilin, China) were first washed and soaked overnight in 0.5% $NaHCO_3$ solution. After decanting the water, the soaked soybeans were mixed with distilled water of 7 times their weight. The resultant slurry was then filtered through a 200-mesh sieve to yield soymilk, homogenized at 90°C at 30 MPa for 30 s, then sterilized at 95°C for 15 min and cooled to 37°C. The sterilized soymilk was inoculated with the probiotic strains at the inoculation levels (5×10^6 CFU/ml). After inoculation, the soymilk was incubated at 37°C until the termination pH fell to 4.5. Samples were assayed prior to fermentation, and every 2 h during the fermentation.

Fermentation characteristics of the six probiotic strains in soymilk

Acidity, proteolysis and viable counts The pH values were measured using a pHSJ-3 F pH meter (Leici, Shanghai, China). The titratable acidity (TA) was determined with 0.1 N NaOH using a 0.5% phenolphthalein as the indicator. Proteolysis was expressed by the production of free amino nitrogen (FAN), determined by spectrophotometric assay using o-phthaldialdehyde according to the method described by Church et al. (1983) The viable counts were enumerated according to the description of Tharmaraj and Shah (2003).

Carbohydrate contents Contents of carbohydrate in the fermented soymilk, including stachyose, raffinose, sucrose, glucose, and fructose, were determined by HPLC analysis. One gram of sample was treated by ultrasonic for 3 min after blended with 3 ml water, then sterilized at 95°C for 15 min, and concentrated at 10,000 *g* for 10 min. The supernatant was filtered through a 0.45-mm Minipore PVDF filter (Millipore, Bedford, MA, USA). The filtrate was used for the HPLC analysis.

The HPLC equipment used was a chromatography (Agilent 1100; USA) equipped with CARBOsep CHO-620 CA column (300× 6.5 mm, 5 μm; Transgenomic, USA), a refractive index detector (GA1362A; Agilent), and an Agilent ChemStore

Chromatography data processor. The mobile phase was double distilled water. The flow rate was set at 0.5 ml/min and the column temperature 90°C. Sample of 10 μl was applied to the column.

Acetic and lactic acid contents Acetic and lactic acid contents in the samples were determined according to Akalin et al. (1996) by HPLC methods. One gram of sample was mixed with 3 ml 1 M hydrochloric acid, and concentrated at 3,500 *g*, for 10 min, then maintained in the water bath at 95°C for 15 min. The supernatant was filtered through a 0.45-mm Minipore PVDF filter (Millipore, Bedford, MA, USA). The filtrate was used for the HPLC analysis.

The HPLC equipment used was a chromatography (Agilent 1100) equipped with a Zorbax SB-Aq column (250× 4.6 mm, 5 μm; Agilent). Operational conditions were as follows: mobile phase, Methanol:PBS (10 mM PBS, pH 2.0) (3:97, v/v); flow rate, 0.5 ml/min; column temperature, 35°C; detector wavelength, 210 nm (UV/vis detector, GA1314A; Agilent). Reference acetic and lactic acids (Supelco, USA) were chromatographed to determine their retention times, integrator response factor, and recovery value.

Vitamins B contents Contents of vitamins B1, B2, and B6 were measured by HPLC methods reported by Schrijver et al. (1981) and Speek et al. (1982) with some changes. Sample pretreatment was same with the determination of acetic and lactic acid contents. The vitamins B1, B2, and B6 standards were all products of Supelco.

The contents of vitamins B1, B2, and B6 was determined by a HPLC system (Agilent 1100) equipped with Zorbax SB-C18 (4.6×150 mm, 5 μm, Agilent, USA) column, and a UV detector detector (GA1314A, Agilent) set at 280 nm. The mobile phase was prepared with 0.005 M sodium hexanesulfonate solvent, methanol and acetic acid (75:24:1, v/v/v). The flow rate was set at 1.0 ml/min and the column temperature 30°C. Sample of 0.5 μl was applied to the column.

γ-Aminobutyric Acid (GABA) contents Content of GABA was determined by a HPLC system (Agilent 1100, USA) equipped with Develosil C30-UG-5 column (4.6× 150 mm, 3.5 μm; Agilent), and a FI detector (GA1321A; Agilent). The pre-column derivatization reagent was 9-Fluorenylmethoxycarbonyl chloride (FMOC-Cl). The fluorescence wavelength was set at excitation 340 nm and emission 450 nm. The mobile phase was prepared with 10 mM PBS buffer, pH 7.8 (A) and methanol:acetonitrile:water (45:45:10, v/v/v) (B). The flow rate and the column temperature were set at 2.0 ml/min and 30°C, respectively, and a 0.5 μl sample was applied to the column. After the 0.5 μl injection of the sample into the column (40°C), solvent B increased from 15 to 20% in 45 min, increased to 100% in 2 min and held at 100% for 5 min, finally reduced to 0% in 5 min.

Isoflavones contents The isoflavones, including daidzin, glycitin, genistin, daidzein, glycitein, and genistein, were measured by HPLC methods. Fermented soymilk samples of approximately 4 g were accurately weighed into a 125-ml screw-capped Erlenmeyer flask. After 10 ml of acetonitrile and 7 ml of double-distilled water were added, the flask was capped and stirred for 2 h at room temperature in a rotary shaker at 300 rpm. The mixture was vacuum-filtered (No. 42 filter paper; Whatman, Hillsboro, OR, USA), and the filtrate was evaporated to dryness under vacuum at ≤30°C. Dry matter was dissolved to a final volume of 10 ml with 80% methanol in water. The sample was filtered through a 0.45-μm polytetrafluoroethylene filter and isoflavones were quantified by HPLC.

The HPLC equipment used was a chromatography (Agilent 1120) equipped with a Zorbax SB-C18 column (250× 4.6 mm, 5 μm; Agilent), a UV-Vis detector (1260 Infinity, Agilent) set at 254 nm, and an Agilent ChemStore Chromatography data processor.

A linear HPLC gradient was composed of (A) 2% glacial acetic acid and 10% methanol in H_2O and (B) 2% glacial acetic acid in methanol. After the 20 μl injection of sample onto the column (30°C), solvent B increased from 10 to 40% in 40 min, increased to 41% in 10 min, then further increased to 50% in 10 min and held at 50% for 10 min, finally reduced to 10% in 30 min. The solvent flow rate was 1.0 ml/min. The contents were expressed as μg/g of soymilk. The authentic isoflavone standards were all products of Sigma-Aldrich (USA).

Statistical analysis

Statistical analysis of data was carried out using SAS 9.0 (SAS Institute, Cary, USA). The comparisons of differences between the means of the treatments were tested by ANOVA at the significance level of $P<0.05$. All analyses were conducted in triplicate.

Results and discussion

TA and pH of the fermented soymilk inoculated with the six probiotic strains during fermentation are shown in Fig. 1a, b. Soymilk was found to support the growth of all the six probiotic strains. Significant increases in TA and decreases in pH in soymilk during fermentation were observed among the six probiotic strains. It took the shortest time of 9.5 h for *L. rhamnosus* GG to ferment the soymilk to final pH 4.5, the longest time 21 h for *L. acidophilus* NCFM, and 12–16 h as for the other four probiotic strains. The final TA of soymilk reached 76.31ºT (TepHep) for *B. animalis* V9 as the maximum, 56.65ºT for *L. rhamnosus* GG as the minimum, whereas the TA values of the other four strains were 68.74–73.56ºT.

The changes in FAN of soymilk during fermentation are shown in Fig. 1c. During the fermentation period, the FAN

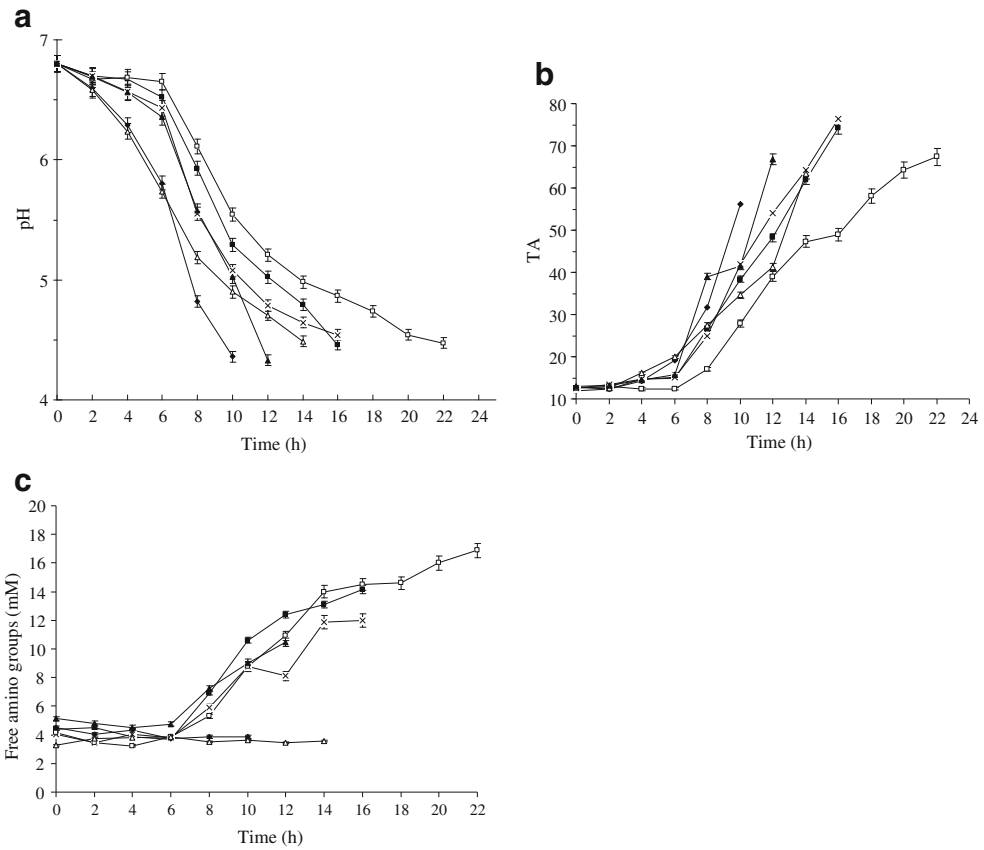

Fig. 1 Fermentation characteristics of the six probiotic strains in soymilk during the course of fermentation; The value of each strains in the presence of ■, *L. casei* Zhang; ×, *B. animalis* V9; ▲, *L. casei* Shirota; □, *L. acidophilus* NCFM; ♦, *L. rhamnosus* GG; Δ, *B. animalis* Bb12. **a** pH value. **b** Titratable acidity (TA). **c** Free amino nitrogen (FAN)

of the soymilk fermented with the four probiotic strains, *L. casei* Zhang, *B. animalis* V9, *L. acidophilus* NCFM, and *L. rhamnosus* GG, increased greatly as the fermentation time was extended. The FAN of soymilk fermented with *L. acidophilus* NCFM increased the most markedly, and achieved as much as 16.9 mM at the end of the fermentation. The FAN changes of soymilk fermented with *B. animalis* Bb12 and *L. rhamnosus* GG were not significant.

Growth of some probiotic strains in soymilk might also be dependent on the ability of proteolysis, resulting in the liberation of amino acids (Shihata and Shah 2000). Supplements rich in FAN, such as whey protein concentrates or acid casein hydrolysates, are added to enhance the growth of some LABs, resulting in reducing the time required for fermentation (Dave and Shah 1998). Our experiment showed that the growth of *L. rhamnosus* GG and *B. animalis* Bb12 in the soymilk might depend the least on amino acids because the pH value of the soymilk decreased the most rapidly and, correspondingly, TA increased the most rapidly (Fig. 1a, b), along with almost no amino acids produced in the fermented soymilk at the end of fermentation (Fig. 1c). In contrast, the growth of *L. acidophilus* NCFM might depend the most on amino acids because it was delayed for 2 h after the start of the fermentation, compared with the growth of *L. rhamnosus* GG and *B. animalis* Bb12, along with the production of the greatest amount of amino acids (Fig. 1c). Delay time might be needed to generate enough amino acids to support growth. However, the growth delay could also be affected by some other factors, such as a reduction of the free oxygen level and a drop in pH to a level more favorable for the probiotics. Further research is thus needed to explain the growth behaviors of the six probiotics in soymilk.

Viable counts before and after fermentation are documented in Table 1, and there are significant differences in the viable counts among the samples at the end of the fermentation. Generally, a significant increase in viable counts was observed among the six probiotic strains. Moreover, the viable counts of *L. casei* Zhang reached the maximum, amounting to 9.38±0.02 log CFU/g after the fermentation, whereas that of *L. rhamnosus* GG reached the minimum, amounting to 8.69±0.07 log CFU/g. To exert the health benefits in vivo probiotics must arrive at the intestinal tract alive, which require them to retain their viability in the food during shelf-life and after consumption, and to resist the acidic conditions of the stomach as well as the bile salts in the intestine (Kailasapathy and Rybka 1997). Therefore, a viable cell count above 10^6–10^7 CFU/g is necessary in order to supply a sufficient "daily dose" of 10^6–10^9 viable bacteria (Lee and Salminen 1995). As for this experiment, all the fermented soymilk samples had a viable count above 8.69 log CFU/g at the end of fermentation, especially the four probiotic strains of *L. casei*

Table 1 Viable counts (log CFU/g) of the fermented soymilk before and after fermentation

Probiotic strains	Viable counts		
	Before fermentation	After fermentation	Fermentation in milk
L. casei Zhang	6.70±0.01 a	9.38±0.02 b	8.13±0.48 (Wang et al. 2006)
B. animalis V9	6.71±0.01 a	8.95±0.03 e	8.06±0.04[a]
L. acidophilus NCFM	6.69±0.01 a	9.21±0.00 c	6.8-6.9 (log CFU/ml) (Sanders et al., 1996)
L. rhamnosus GG	6.69±0.02 a	8.69±0.07 f	8.0-8.4 (log CFU/g) (Holzapfel et al., 1998)
B. animalis Bb12	6.68±0.01 a	9.03±0.05 d	8.9 (log CFU/ml) (Hilde et al., 2003)
L. casei Shirota	6.71±0.00 a	9.26±0.04 c	7.9-8.9 (log CFU/g) (Holzapfel et al., 1998)

Presented values are means of triplicate determinations; ± indicates standard deviation from the mean

Values within the same column followed by different letters differ significantly ($P<0.05$)

[a]Data about *B. animalis* V9, obtained in our laboratory, has not been published (Heping Zhang, unpublished data)

Zhang, *L. acidophilus* NCFM, *B. animalis* Bb12, and *L. casei* Shirota, which were all over 9.00 log CFU/g. By comparison, the viable counts of the six probiotic strains fermented in cow's milk were lower than those in soymilk (Table 1), which proved that soymilk was the better substrate for these probiotic strains. It took 15.8, 16.0, 21.0, 9.5, 13.8, and 12.0 h for the fermentation of the six probiotic strains in cow's milk, whereas it took 21.4, 30.4, 15.3, 23.8, 21.2, and 23.2 h in soymilk for *L. casei* Zhang, *B. animalis* V9, *L. acidophilus* NCFM, *L. rhamnosus* GG, *B. animalis* Bb12, and *L. casei* Shirota, respectively (Heping Zhang, unpublished data). Obviously, the fermentation time of the five probiotic strains among them was much longer in cow's milk than those in soymilk, except for *L. acidophilus* NCFM, which suggested that the selected five probiotic strains grow more quickly in soymilk than in cow's milk. However, Mital et al. (1974) reported a higher growth rate of lactic acid bacteria in cow's milk than in soymilk. It is presumed that the viability is associated with the ability of proteolysis, as *L. casei* Zhang, *B. animalis* V9, *L. acidophilus* NCFM, and *L. casei* Shirota could all produce much FAN and high viability, but *L. rhamnosus* GG is the exact opposite (Table 1; Fig. 1c). *Bifidobacterium animalis* Bb12 was the exception. Further experiments are being performed to clarify the relationships between viability and proteolysis in our laboratory.

Changes in the content of stachyose, raffinose, sucrose, glucose, and fructose in soymilk fermented with the six probiotic strains are shown in Table 2. A larger magnitude of reduction in the content of stachyose and raffinose, 7.41 and 44.45%, respectively, was noted in the soymilk fermented with *L. rhamnosus* GG. After 24 h of fermentation, the soymilk fermented with the single culture of *L. rhamnosus* GG contained 3.272±0.028 and 0.861±0.051 mg/l of stachyose and raffinose, respectively. Content of both stachyose and raffinose decreased significantly ($P<0.05$) in the soymilk fermented with the single culture of *B. animalis* V9 or *B. animalis* Bb12, but exploited these substrates less efficiently than *L. rhamnosus* GG. The single culture of *L. acidophilus* NCFM reduced the content of stachyose by 6.20% and increased the content of raffinose by 7.18% after 24 h of fermentation. However, the single culture of both *L. casei* Zhang and *L. casei* Shirota increased the contents of stachyose and raffinose, 9.88 and 6.69% for *L. casei* Zhang, and 1.44 and 16.56% for *L. casei* Shirota, respectively, which suggested that *L. casei* Zhang and *L. casei* Shirota could synthesize stachyose and raffinose. Stachyose and raffinose, the principal oligosaccharides in soymilk, are believed to cause flatulence in humans after eating soybean foods. These oligosaccharides can be hydrolysed by the enzyme, α-galactosidase, the production of which by LAB

Table 2 Contents of carbohydrates (mg/l) in soymilk fermented by the six probiotic strains

Probiotic strains	Stachyose	Raffinose	Sucrose	Glucose	Fructose
Unfermented soymilk	3.53±0.07 a	1.23±0.07 a	11.39±3.05 a	214.16±1.17 a	1.97±0.09 a
L. casei Zhang	3.88±0.05 b	1.31±0.58 b	11.62±1.44 b	54.20±4.845 b	0.19±0.01 b
B. animalis V9	3.43±0.05 c	1.18±0.45 c	6.30±0.57 c	61.92±1.10 c	0.40±0.02 c
L. acidophilus NCFM	3.32±0.04 d	1.34±0.02 b	9.37±0.17 d	74.82±0.91 d	0.20±0.02 d
L. rhamnosus GG	3.27±0.03 e	0.86±0.05 d	10.25±0.10 e	173.93±1.03 e	0.20±0.03 b
B. animalis Bb12	3.35±0.03 f	0.94±0.07 e	6.58±0.22 c	85.90±0.30 f	0.28±0.01 e
L. casei Shirota	3.59±0.04 a	1.43±0.02 f	11.07±0.13 f	53.34±2.30 b	0.18±0.01 b

Presented values are means of triplicate determinations; ± indicates standard deviation from the mean

Values within the same column followed by different letters differ significantly ($P<0.05$)

has been reported (Desjardins and Roy 1990; Hughes and Hoover 1995; Mital et al. 1973).

During fermentation, capability to utilize sucrose, the main disaccharide in soymilk, varied with different kinds of LABs (Mital and Steinkraus 1975; Rogosa and Sharpe 1959). As shown in Table 2, after 24 h of fermentation, sucrose content decreased from its initial value by 44.69, 17.72, 14.10, 42.28 and 2.84% in soymilk inoculated with the single culture of *B. animalis* V9, *L. acidophilus* NCFM, *L. rhamnosus* GG, *B. animalis* Bb12, and *L. casei* Shirota, respectively, but increased by 17.72% for *L. casei* Zhang. This demonstrated that the five probiotic strains tested, *B. animalis* V9, *L. acidophilus* NCFM, *L. rhamnosus* GG, *B. animalis* Bb12, and *L. casei* Shirota, could utilize sucrose. Our previous experiment proved that *L. casei* Zhang could utilize sucrose. Therefore, *L. casei* Zhang could not only utilize sucrose but also produce sucrose.

Regardless of the starter culture used, content of glucose and fructose greatly decreased for all the six probiotic strains after the fermentation, suggesting that all the six probiotic strains could readily utilize glucose and fructose (Table 2). Moreover, along with the reduction in the content of stachyose, raffinose, and sucrose, a decrease in the content of fructose and glucose was noted for the four probiotic strains except for *L. casei* Zhang and *L. casei* Shirota (Table 2), which might be attributed to the hydrolysis of stachyose, raffinose, and sucrose, and the decomposition of fructose and glucose during fermentation. This observed phenomenon was contrary to the report of Chumchuere and Robinson (1999), in which the content of stachyose and raffinose decreased along with the content increase of fructose and glucose. It could be expected that the combined utilization of *L. casei* Zhang or *L. casei* Shirota with bifidobacteria in soymilk will encourage each other's growth because *L. casei* Zhang or *L. casei* Shirota could produce stachyose and raffinose, whereas stachyose and raffinose have been reported to stimulate the growth of bifidobacteria (Gibson and Wang 1994).

Hou et al. (2000) reported that fermentation of soymilk with *B. longum* or *B. infantis* resulted in the reduction in the content of stachyose and raffinose. Owing to the catalytic action of α- and β-galactosidase produced by bifidobacteria, fermentation with bifidobacteria has been shown to reduce the content of oligosaccharide, but to increase the content of monosaccharide in soymilk (Matsuyama et al. 1992; Hou et al. 2000). However, *B. animalis* V9 and *B. animalis* Bb12 in this experiment exhibited the relatively strong ability to decompose the monosaccharide among the six probiotic strains.

Content of acetic and lactic acid are showed in Fig. 2 and Table 3. Being homofermentative lactics, the single culture of the six probiotic strains all produced lactic acid at 6.27, 3.43, 4.58, 4.85, 2.73, 5.19 g/l for *L. casei* Zhang, *B. animalis* V9, *L. acidophilus* NCFM, *L. rhamnosus* GG, *B. animalis* Bb12, and *L. casei* Shirota, respectively. In conjunction with the higher TA and low pH value observed (Fig. 1a, b), *L. casei* Zhang produced the highest amount of lactic acid among the six probiotic strains, but almost no acetic acid (Fig. 2; Table 3). At 24 h of fermentation, the population of *L. casei* Zhang (9.38 log CFU/g) was more than that of other five probiotic strains, which may account for its highest amount of lactic acid produced among the six probiotic strains. LABs, being saccharolytic, derived the energy for growth from substrate-level phosphorylation, by converting carbon source to lactic acid and other compounds.

Bifidobacterium animalis V9 or *B. animalis* Bb12 produced lactic acid as the major organic acid accompanied with a relatively higher amount of acetic acid during the fermentation of soymilk as shown in Fig. 2 and Table 3. For

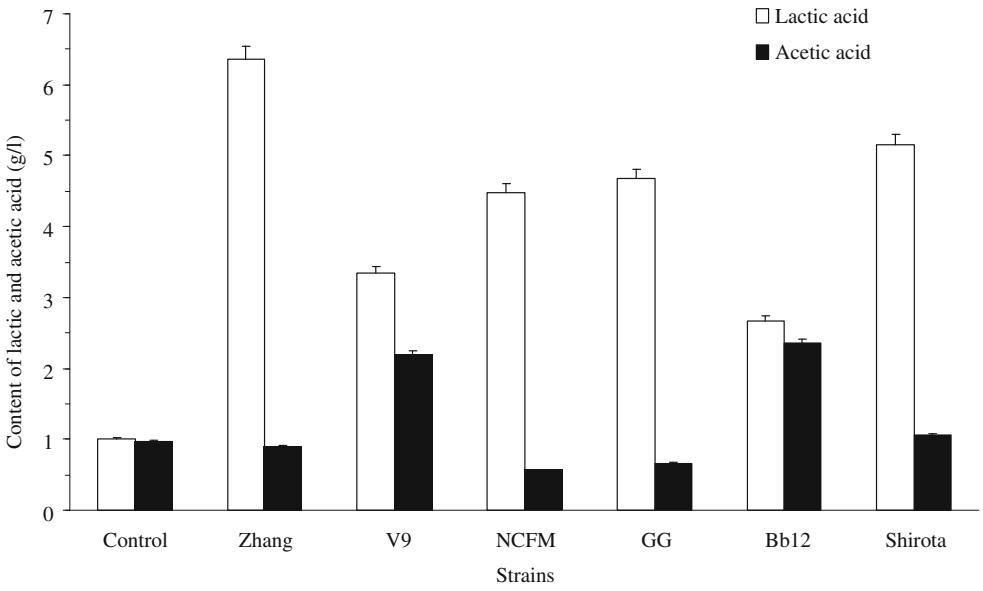

Fig. 2 Content of acetic and lactic acids (g/l) in soymilk fermented by the six probiotic strains: *L. casei* Zhang; *B. animalis* V9; *L. acidophilus* NCFM; *L. rhamnosus* GG; *B. animalis* Bb12 and *L. casei* Shirota

Table 3 Contents of lactic and acetic acids (g/l) in soymilk fermented the six probiotic strains

Probiotic strains	Lactic acid	Acetic acid
L. casei Zhang	6.21±0.12 a	0.91±0.07 a
B. animalis V9	3.45±0.19 b	2.27±0.36 b
L. acidophilus NCFM	4.55±0.23 c	0.75±0.07 c
L. rhamnosus GG	4.95±0.16 d	0.82±0.03 d
B. animalis Bb12	2.80±0.15 e	2.32±0.14 e
L. casei Shirota	5.17±0.31 f	1.02±0.09 f

Presented values are means of triplicate determinations; ± indicates standard deviation from the mean

Values within the same column followed by different letters differ significantly ($P<0.05$)

example, soymilk inoculated with *B. animalis* V9 or *B. animalis* Bb12 contained 2.12 or 2.14 g/l of acetic acid, and 3.48 or 2.65 g/l of lactic acid, respectively, after 24 h of fermentation. Molar ratios of acetic and lactic acid were 1:1 and 6:5 for *B. animalis* V9 and *B. animalis* Bb12, respectively. However, metabolism of carbohydrates by bifidobacteria may lead mainly to the production of acetic and lactic acid, theoretically in a molar ratio of 3:2, if pyruvate is converted to lactate (Modler et al. 1990). The over-production of acetic acid would lead to unfavorable taste and flavor. The change of acetic acid content was not significant for the other four probiotics, *L. casei* Zhang, *L. casei* Shirota, *L. acidophilus* NCFM, and *L. rhamnosus* GG.

After fermentation, the content of both vitamins B1 and B2 decreased significantly for all the six probiotic strains, and none were detected by the HPLC analysis, in comparison with 1.3 and 0.8 mg/l respectively, before fermentation. Content of Vitamin B6 all increased by 354.3, 293.5, 289.1, 208.7, 191.3, and 202.2%, amounting to 20.9, 18.1, 17.9, 14.2, 13.4, and 13.9 mg/l for *L. casei* Zhang, *B. animalis* V9, *L. acidophilus* NCFM, *L. rhamnosus* GG, *B. animalis* Bb12 and *L. casei* Shirota, respectively, as shown in Fig. 3. Among them, *L. casei* Zhang produced the highest amount of vitamin B6 among the six probiotic strains.

GABA, a non-protein amino acid, possesses well-known physiological functions such as neurotransmission, induction of hypotension, and diuretic and tranquilizer effects (Jakobs et al., 1993; Wong et al. 2003). Treatments of sleeplessness, depression, and autonomic disorders (Okada et al. 2003), chronic alcohol-related symptoms (Oh and Oh 2003), and stimulation of immune cells (Oh et al. 2003) have also been related to the administration of GABA. A recent study showed that GABA is a strong secretagogue of insulin from the pancreas (Adeghate and Ponery 2002) and effectively prevents diabetic conditions (Hagiwara et al. 2004).

Several GABA-producing LABs have been reported, including *Lactobacillus brevis* isolated from kimuchi (Ueno et al. 1997) and from alcohol distillery lees (Yokoyama et al. 2002), *Lactococcus lactis* from cheese starters (Nomura et al. 1998), and *Lactobacillus paracasei* NFRI 7415 from a Japanese traditional fermented fish (funa-sushi) (Komatsuzaki et al. 2005).

Content of GABA is shown in Fig. 4. Compared with the raw soymilk, GABA contents increased by 79.8, 86.8, 52.5, 54.5, 57.9, and 85.6%, for *L. casei* Zhang, *B. animalis* V9, *L. acidophilus* NCFM, *L. rhamnosus* GG, *B. animalis* Bb12, and *L. casei* Shirota, respectively, at the end of the fermentation. *B. animalis* V9 produced the greatest amount of GABA, amounting to 45.6 μg/l, whereas *L. acidophilus* NCFM produced the smallest amount of GABA, amounting to 36.0 μg/l.

Research in several areas of healthcare has shown that consumption of isoflavones may play a role in lowering risk for disease. They can fight disease on several fronts, such as to ease menopause symptoms, to reduce heart disease risk, to

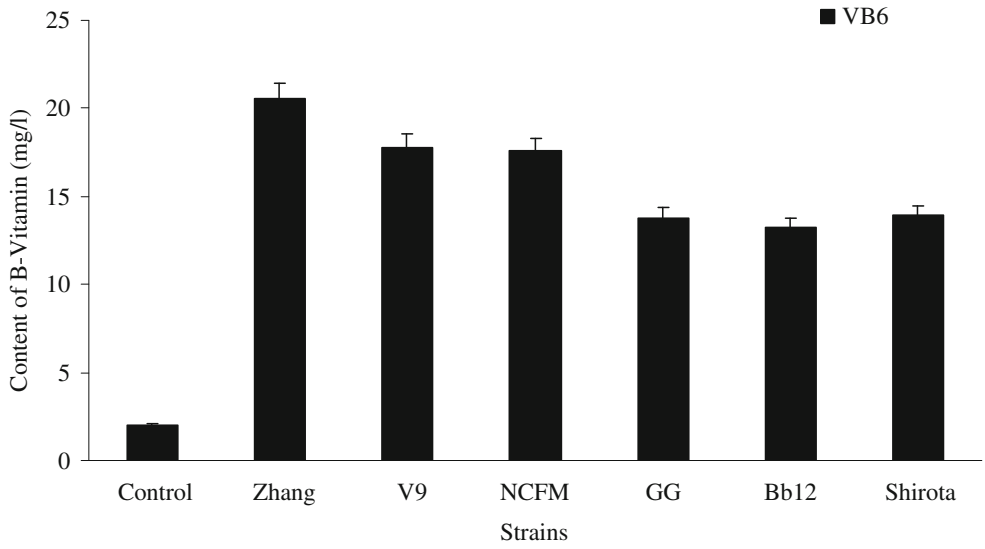

Fig. 3 Content of B-Vitamin (mg/l) in soymilk fermented by the six probiotic strains: *L. casei* Zhang; *B. animalis* V9; *L. acidophilus* NCFM; *L. rhamnosus* GG; *B. animalis* Bb12 and *L. casei* Shirota

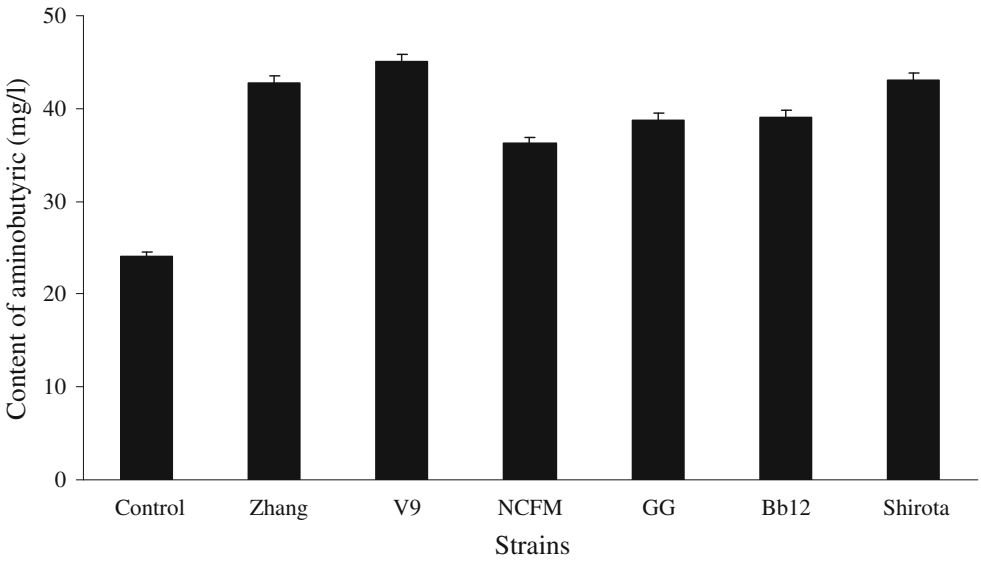

Fig. 4 Content of aminobutyric acid (mg/l) in soymilk fermented by the six probiotic strains: *L. casei* Zhang; *B. animalis* V9; *L. acidophilus* NCFM; *L. rhamnosus* GG; *B. animalis* Bb12 and *L. casei* Shirota

protect against prostate problems, to improve bone health, and to reduce cancer risk (Xiao 2008). Table 4 shows the contents of the main individual isoflavones in soybean, including daidzin, daidzein, genistin, genistein, glycitin, and glycitein, which resulted in raw soymilk and soymilk fermented with the six probiotic strains. Content of individual isoflavones in soymilk fermented with *L. casei* Zhang or *L. casei* Shirota exhibited the same trend, with the content of daidzin, genistin, daidzein, glycitein, and genistein increased ($P<0.05$) and the content of glycitin decreased ($P<0.05$). For the soymilk fermented with *L. rhamnosus* GG, the content of daidzein and genistein increased ($P<0.05$) and the content of daidzin, glycitin ,and genistin decreased ($P<0.05$), the content of glycitein has no significant change ($P>0.05$). The soymilk fermented with *B. animalis* V9 and *B. animalis* Bb12 presented the same trends, with the content of daidzin, glycitin, and genistin decreased ($P<0.05$) and the content of daidzein, glycitein, and genistein increased ($P<0.05$). Content of daidzin, glycitin, genistin, and glycitein decreased ($P<0.05$) and content of daidzein and genistein increased, which was observed in the soymilk fermented with *L. acidophilus* NCFM. The content change of the individual isoflavones associated well with the species of the probiotic strains, while the same species of the probiotic strains almost exhibited the same trend of content change.

As Table 4 shows, the major compositions of isoflavone aglycones were genistein, glycitein, and daidzein in the fermented soymilk. The content of genistein in fermented soymilk produced the greatest increase from 2.54 to 11.37 to

Table 4 Contents of isoflavone (μg/g) in soymilk fermented by the six probiotic strains

Probiotic strains	Daidzin	Glycitin	Genistin	Daidzein	Glycitein	Genistein
Unfermented soymilk	73.75±3.44 a	18.20±0.74 a	105.57±4.20 a	3.33±0.01 a	1.27±0.01 a	2.54±0.26 a
L. casei Zhang	77.16±2.13 b (4.62%)	13.03±0.48 b (-2.84%)	109.55±4.14 b (3.77%)	7.86±0.45 b (136.04%)	1.49±0.02 b (17.32%)	14.76±3.12 b (481.10%)
B. animalis V9	69.37±2.83 c (-5.94%)	12.45±1.56 b (-3.16%)	103.43±2.04 c (-2.03%)	10.47±2.14 c (214.41%)	1.56±0.04 c (22.83%)	14.17±2.43 b (457.87%)
L. acidophilus NCFM	62.67±1.12 d (-15.02%)	10.75±0.25 c (-4.09%)	92.10±2.14 d (-12.76%)	5.43±0.17 d (63.06%)	0.92±0.03 d (-27.56%)	11.37±1.47 c (347.64%)
L. rhamnosus GG	60.84±1.42 e (-17.51%)	10.15±0.27 c (-4.42%)	82.43±1.23 e (-21.92%)	6.31±0.42 e (89.49%)	1.29±0.11 a (0)	18.83±0.12 d (641.34%)
B. animalis Bb12	41.65±1.16 f (-43.53%)	9.13±0.20 d (-4.98%)	69.61±1.92 f (-34.06%)	16.41±0.95 f (392.79%)	1.98±0.11 e (55.91%)	26.63±4.08 e (948.42%)
L. casei Shirota	77.10±1.01 b (4.54%)	12.67±0.16 e (-3.04%)	112.35±1.94 g (6.42%)	7.99±0.95 b (139.94%)	1.61±0.11 f (26.77%)	13.41±1.62 f (427.95%)

Presented values are means of triplicate determinations, and the values in the parenthesis are the increasing percentage; ± indicates standard deviation from the mean

Values within the same column followed by different letters differ significantly ($P<0.05$)

26.63 μg/g, daidzein increased from 3.33 to 5.43 to 16.41 μg/g, and glycitein increased from 1.27 to 1.28 to 1.61 μg/g; however, the glycitein content in the soymilk fermented with *L. acidophilus* NCFM decreased to 0.93 μg/g. The isoflavone aglycone of genistein, glycitein, and daidzein was 3.49% of total isoflavones in the non-fermented soymilk, whereas the soymilk fermented with the six probiotic strains had significant increments in isoflavone aglycone concentrations, with the total content of genistein, glycitein, and daidzein increased to 10.77, 12.39, 9.67, 14.69, 27.21, and 10.22% of total isoflavones in the soymilk fermented with *L. casei* Zhang, *B. animalis* V9, *L. acidophilus* NCFM, *L. rhamnosus* GG, *B. animalis* Bb12, and *L. casei* Shirota, respectively. Correspondingly, the isoflavone glucoside of daidzin, glycitin, and genistin decreased from 96.51% of total isoflavones in the raw soymilk to 89.23, 87.61, 90.33, 85.31, 72.79, and 89.78% in the soymilk fermented with *L. casei* Zhang, *B. animalis* V9, *L. acidophilus* NCFM, *L. rhamnosus* GG, *B. animalis* Bb12, and *L. casei* Shirota, respectively. *Bifidobacterium animalis* Bb12 produced the greatest amount of genistein, glycitein, and daidzein in the fermented soymilk among the six probiotic strains, whereas *L. acidophilus* NCFM produced the least.

β-glucosidase was reported to hydrolyze glucoside isoflavones with the formation of aglycones (Esaki et al. 2004). Tochikura et al. (1986) have reported that the probiotic microorganisms possess β-glucosidase, which plays an important role in hydrolyzing isoflavone glucosides to bio-available aglycones formed in fermented soymilk. The results suggested that all the six probiotic strains possessed β-glucosidase and that there might be a change of the enzyme activity among them.

Izumi et al. (2000) reported that the absorption speed of isoflavone aglycone is faster than that of isoflavone β-glucoside and that the amount of absorbed isoflavones is higher in aglycone form than in β-glucoside form in humans. The structure is a limiting factor for absorption from gastrointestinal tract (Piskula et al. 1999; Hendrich 2002), with aglycones more readily absorbed and more bio-available than highly polar conjugated species (Setchell 2000; Turner et al. 2003). Following absorption of the aglycones, these compounds and their metabolites are readily conjugated in the liver, circulate enterohepatically with potential metabolism and reabsorption in the intestine, and are excreted in the unconjugated form in feces (Adlercreutz et al. 1995). In this experiment, the total isoflavone aglycone level of the soymilk all increased after fermentation by the single culture of the six probiotic strains, whereas the total isoflavone β-glucoside level decreased. Fermentation by the six probiotic strains in soymilk took an important role in converting β-glucoside into aglycone.

The six individual isoflavones, daidzin, daidzein, genistin, genistein, glycitin, and glycitein have been well proved as bioactive substances. After fermentation with *L. casei* Zhang or *L. casei* Shirota, the contents of the six individual isoflavones all increased in soymilk, which demonstrated their novel ability in production of bioactive isoflavones.

Conclusions

The results obtained in this work showed that the six probiotic strains were promising candidates as starter cultures for soymilk fermentation. They were able to grow well in soymilk, to produce lactic acid, and to increase the content of bioactive substances, including GABA, vitamin B6, and total isoflavone aglycone. Moreover, all the fermented soymilk samples had a viable count above 8.5 log CFU/g at the end of fermentation, especially the four probiotic strains of *L. casei* Zhang, *L. acidophilus* NCFM, *B. animalis* Bb12, and *L. casei* Shirota, with viability all over 9.00 log CFU/g, suggesting that soymilk could serve as a potential vehicle for delivery of these probiotic strains. These advantages deserve further research on the development of the six probiotic strains as starters for soymilk.

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