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Full Length Research Paper

Function and safety assessment of *Lactococus lactis* subsp*. lactis* LB12 as potential probiotic strain

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The effect of yogurt fermented by *Lactococus lactis* subsp. *lactis* LB12 isolated from traditional Chinese pickled cabbage on serum cholesterol and triacylglycerol levels were investigated in mice. In the same, the characterizations of the strain, such as acid-tolerance, bile-tolerance, antimicrobial activity, antibiotic sensitivity and safety were also examined. The serum total cholesterol, triglyceride and bile acid levels significantly decreased of mice given a high-cholesterol diet supplemented with yogurt fermented by *Lactococus lactis* subsp. *lactis* LB12. Characterization of *L. lactis* subsp. *lactis* LB12 showed that it was bile- and acid-tolerant, resistant to five commercial antibiotics tested, and possessed antimicrobial activity to pathogenic *Escherchia coli* and *Staphylococcus aureus*. None of morphological changes was noted as a result of *L. lactis* subsp. *lactis* LB12 treatment, nor were there significant differences in the visceral weight indices of the lymph nodes, spleen, bacterial translocation, or aberration rate of sperm of mice compared to control. The results indicated that *L. lactis* subsp. *lactis* LB12 might be effective as a probiotic with cholesterol-lowering activities.

Key words*:* Yogurt, *Lactococus lactis* subsp. *lactis* LB12, acid and bile tolerance, antimicrobial and antibiotic sensitivity, cholesterol-lowering effect.

INTRODUCTION

Cardiovascular disease is the leading cause of death in many countries and it is strongly associated with hypercholesterolemia (Law et al., 1994). It is therefore important to develop new ways of reducing serum cholesterol. Recently, lactic acid bacteria have attracted attention as potential cholesterol-lowering milk additives (Chandan, 1999; Roos and Matin, 2000). The reduction of cholesterol by lactobacilli and bifidobacteria that can survive in the intestine has been demonstrated in human, mouse, and pig studies (Kawase et al., 2000; Haberer et al., 2003; Lim et al., 2004; Usman and Hosono, 2000; Nguyen et al., 2007). In contrast, few studies exist on the probiotic activity of lactococci since they are traditionally not considered to be natural inhabitants of the human gastrointestinal tract (Teuber et al., 1995). However, several works showed the possibility of the presence of lactococci in the flora of the human or animal gastrointesrinal tract (Gruzza et al., 1992; Grahn et al., 1994; Klijin et al., 1995). Lactococci are widely used as starter bacteria in manufacturing cheese and other fermented dairy products. Thus, isolation and establishing the effective probiotic properties of lactococci could lead to development of new probiotic foods.

Potherb mustard (Brassica juncea, Coss.), a member of the *Cruciferae*, often called pickled cabbage, is always brined and stored as pickle in China. Traditionally, the potherb mustard pickle is homemade in the beginning of winter or spring in most of the rural areas and some households in urban areas of China. After harvesting, the mustard leaves are washed and drained, wilted in the sun, then mixed with salt, packed into earthenware pots in layers, and then was pressed tightly by a wooden ladle; spices may be added. The pots are sealed and the leaves allowed fermentation for couple of months.

In the present study, we identified and characterized a

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lactococci strain screened from traditional Chinese pickled cabbage and evaluated its potential probiotic function.

MATERILS AND METHODS

Materials

Strains of *Lactococus lactis* subsp. *lactis* NBRC 12007 (obtained from Biological Resource Center of National Institute of Technology and Evaluation, Chiba, Japan), *Escherchia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 (obtained from the Micro-biological Laboratory of Life Science College of Nanjing Normal University, Nanjing, China) were serially transferred at least three times prior to use in present study. Oxgall (Sigma, USA, pH 7.0) and cholesterol were purchased from Shanghai Chemical Co. Ltd. (China); The kits of total cholesterol (TC), high density lipoprotein-cholesterol (HDL-c) and total bile acids (TBA) were purchased from Shanghai Rongsheng Biotech Co. Ltd.(China); Kit of triacylglycerol (TG) from Zhejiang Dongou Biotechnology Co. Ltd. (China).

Isolation of bacterial strains

Serial dilutions of collected Chinese pickled cabbage juice were made in quarter-strength Ringer's solution, and 50 ml of each dilution was spread-plated onto MRS (Sigma) and Rogosa (Sigma) agar. The plates were incubated under microaerobic conditions with 10% CO_2 at 37°C for 48 h. Colonies were selected from plates of showing highest growth, then subcultured in MRS broth and restreaked onto MRS agar to ensure purity.

Screening of strains with cholesterol-lowing effects

Sterile MRS broth were supplemented with pleuropneumonia-like organism (PPLO) serum fraction obtained from Beijing Sunbio Medical Co. Ltd.(China) and 0.30% (w/v) oxgall, cholic acid, or taurocholic acid (Sigma) for 24 h at 37°C. PPLO served as the source of cholesterol. Oxgall, which is the dehydrated fresh bile, was added to the media in order to mimic conditions that would be encountered in the human gastrointestinal tract. Cholic and taurocholic acid were added as the source of deconjugated and conjugated bile respectively. Aliquots were removed prior to inoculation to determine the initial cholesterol content of the media. Bacterial strains were grown in the absence or presence of 0.30% (w/v) oxgall in MRS broth containing 2.0% PPLO under anaerobic conditions at 37°C. Following incubation for 24 h, cells were centrifuged at 6000 rpm for 10 min and the residual cholesterol in the supernatant was determined as described (Rudel and Morris, 1973). The strain with the largest cholesterol-lowing effect was selected for further characterization

Strain identification

The strain used was identified based on Gram staining, morphology, and catalase activity. Scanning electron microscopy was performed as described (Yamauchi and Snel, 2000). The pattern of carbohydrate fermentation was determined by using the API 50 CH system (Biomerieux S.A., La Balme les Grottes, France) according to the manufacturer's instructions. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell protein lysates was used to compare the isolated strain with reference *Lactococus lactis* subsp. *lactis* strains as described (Pot et al., 1993). For definite identification, 16S rRNA sequencing was performed using total DNA from the isolate.

Preparation of yogurt

For preparation of yogurt, milk (1.0% fat and 11.0% solid no fat) was heated at 95°C for 15 min, then cooled to 43°C, inoculated with a 5% (v/v) liquid culture of *L. lactis* subsp. *lactis* LB12. The inoculated mixes was poured into containers and incubated at 42°C until pH 4.6, cooled to 4°C and stored at that temperature no longer than 2 days before feeding. The numbers of bacteria in freshly fermented yogurt were determined by established procedures to be approximately 10^9 CFU/ml (International Dairy Federation, 1988).

Lowing cholesterol test of *L. lactis* subsp. *lactis* LB12

Four weeks old male Sprague-Dawley mice with an average initial body weight of 220 g (obtained from the Animal Breeding Station of Nanjing Medical University, P. R. China) were used in present experiment. The mice were randomly assigned to four treatments each of twelve. The four dietary treatments were arranged as following: group 1 mice fed commercial diet (purchased from Nanjing Qinglongshan Animal Resources Centre, China), served as model control; group 2 mice fed commercial diet plus 1.0% (w/w) cholesterol, 8.0% (w/w) lard and 0.50% (w/w) oxgall, served as high fat mould; group 3 mice fed commercial diet plus 1.0% (w/w) cholesterol, 8.0% (w/w) lard, 0.50%(w/w) oxgall and 5.0% (v/w) milk (1.0% fat and 11.0% solid no fat, acidifying it to pH 4.6 with 10% lactic acid), and served as experimental control; group 4 mice fed commercial diet plus 1.0% (w/w) cholesterol, 8.0% (w/w) lard, 0.50% (w/w) oxgall and 5.0% (v/w) yogurt fermented by *L. lactis* subsp. *lactis* LB12, and served as experimental. The mice were housed individually in standard cages, maintained at a constant environmental temperature (24 - 26°C) and relative humidity (60 - 64%) with a 12 h light and dark cycle. Diets were given each morning at 120 g/kg body weight and water was freely available. The activity, behavior, and general health of the mice were monitored daily. Food and water intake were measured daily, and body weight was measured weekly.

After feeding for 28 days, the mice were fasted for 12 h and then anesthetized by ether. Blood was obtained from the *arteria cervicalis*, the viscera were excised, weighed and tested for bacterial translocation aseptically. Blood placed in sterile tubes containing EDTA as anticoagulant. Serum samples were isolated from the blood by centrifugation (4000 rpm for 30 min) and analyzed for total cholesterol (TC), triglycerides (TG), and high density lipoprotein cholesterol (HDL-c) using kits as described by Loh et al. (2002), respectively.

Feaces of each group of mice were collected daily on each of the last 5 days of the experimental period and lyophilized. 1.0 g of crushed lyophilized feces were suspended in chloroform-methanol (1:1 v/v), sonicated for 5 min, then extracted at 60°C for 60 h. The extract was evaporated and dissolved in methanol for measurement of total bile acids (TBA) with a commercial kit (Loh et al., 2002). The total cholesterol of feaces was determined as described by Rudel and Morris (1973).

Bacterial translocation

Translocation of bacteria to blood and tissues was assessed as described (Zhou et al., 2000). Briefly, one drop of blood (10 - 15 µl) was inoculated onto the surface of MRS and brain heart infusion (BHI, Oxoid, Shanghai Qianchen Biological Science Co. Ltd, P. R.China) agar plates prior to being emptied into EDTA tubes. The plates were incubated at 37°C anaerobically (MRS) or aerobically (BHI) to detect bacteremia. The excised mesenteric lymph nodes

(MLN), spleen, and liver were ground with a tissue grinder. Half of each tissue suspension was plated on MRS agar plate and the other half was plated on BHI agar plate. The plates were then incubated anaerobically (MRS) or aerobically (BHI) at 37℃ for 48 h.

Acute toxicity test of *L. lactis* subsp. *lactis* LB12

An acute oral toxicity study was performed in accordance with the Organization of Economic Cooperation and Development guidelines (OECD, 1995). Four weeks-old Kunming mice (purchased from Nanjing Qinglongshan Animal Resources Centre, P. R. China) with a body weight ranging from 18 - 22 g were randomly divided into two groups, and each group contains ten females and ten males. Yogurt fermented by *L. lactis* subsp. *lactis* LB12 was administered at dose of 100 ml/kg·bw by gastric intubation to single female and male mice once a day throughout the experimental period of 10 days, and served as treated group, control group received sterilized low fat milk (1.0% fat and 11.0% SNF, acidifying it to pH 4.7 with 10% lactic acid) at dose of 100 ml/kg·bw in the similar manner of treated group. Diet and water were freely available, and the housed conditions of mice were similar to lowing cholesterol test.

All the tested mice were observed shortly after dosing, and then each mouse was observed daily for a period of 10 consecutive days, and examined for general behavior signs twice daily. The general behavior signs included changes in the skin and fur, eyes and mucous membranes, excreta and also food and water taking, as well as behavioral pattern. All the tested mice were killed and the vital organs were separated and processed for routine gross and microscopically examination at the end. The epididymus of all male mice were excised and immersed into plates which contained sterilized 0.90% (w/v) NaCl solution for sperm shape abnormality measured, respectively.

Acid tolerance of *L. lactis* subsp. *lactis* LB12

Acid tolerances were evaluated by growing *L. lactis* subsp. *lactis* LB12 strain in MRS broth adjusted to acidic pH 1.5, 2.5, 3.5, 4.5 by adding concentrated hydrochloric acid and incubated at 37℃ for 6 h. 1 ml culture was attained aseptically from the media at 2 h intervals during 6 h incubation for determining the viable cell numbers, cells were harvested by centrifugation (5000 rpm for 10 min at 4℃), diluted by a sterile saline (0.90% NaCl, w/v), evenly spread onto MRS-agar plates (pH 6.8) to confirm the survival of bacteria and incubated at 37℃ for 48 h. Observed colonies in the plates were considered to be the viable cells. The plates were duplicated in all the experiments.

Bile salt tolerance of *L. lactis* subsp. *lactis* LB12

In order to assess bile salt tolerance of *L. lactis* subsp. *lactis* LB12, the strain was inoculated into MRS broth (pH, 7.0) supplemented with 0.10, 0.20, 0.30 and 0.40% (w/v) oxgall (Sigma, pH 7.0) respectively, and incubated at 37℃ for 6 h. 1 ml culture was attained aseptically from the media at 2 h intervals during 6 h incubation for determining the viable cell numbers, cells were harvested by centrifugation (5000 rpm for 10 min at 4℃), diluted by a sterile saline (0.85% NaCl, w/v), evenly spread onto MRS-agar plates (pH 6.8) to confirm the survival of bacteria and incubated at 37℃ for 48 h. Observed colonies in the plates were considered to be the viable cells. The plates were duplicated in all the experiments.

Antimicrobial test of *L. lactis* subsp. *lactis* LB12

The antimicrobial tests of the culture, clear supernatant of culture (5000 rpm for 10 min at 4℃) and sterilized culture (85℃ for 15 min) of *L. lactis* subsp. *lactis* LB12 were tested on the pathogenic *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 using the agar-gel diffusion inhibition test. In the agar-gel diffusion inhibition test as described by Opara and Ansa (1993), 0.2 ml of a 24 h broth culture of each of the test microorganisms was aseptically introduced and evenly spread using bent sterile glass rod on the surface of gelled sterile Mueller-Hinton agar plates. Four wells of about 3.0 mm diameter were aseptically punched on each agar plate using a sterile cork borer, allowing at least 30 mm between adjacent wells and between peripheral wells and the edge of the petri dish. 0.05 ml of the culture, clear supernatant of culture and sterilized culture of *L. lactis* subsp. *lactis* LB12 were then introduced into the wells in the plates, respectively. 0.05 ml of sterilized MRS broth, the culture medium of *L. lactis* subsp. *lactis* LB12, was introduced into the remainder well as the control. pH value of all above culture was adjusted to 7.0 before filling into the well. The plates incubated at 37℃ for 24 h for the test bacteria, and were duplicated in all the experiments.

Antibiotic resistance testing of *L. lactis* subsp. *lactis* LB12

Antibiotic resistance of *L. lactis subsp. lactis* LB12 was assessed by disc diffusion assay using antibiotic disks (Hangzhou Microbiology Reagent Factory, Hangzhou, China) (Bauer et al., 1966). The antibiotics tested were ampicillin (10 µg), tetracycline (30 µg), gentamicin (10 µg), roxithromycin (15 µg), and cefoxitin (30 µg). The commercial antibiotic discs were placed on nutrient agar plates previously seeded with 18 h broth culture of the strain in duplicate. The plates were incubated at 37℃ for 48 h, after which zones of inhibition were examined. Earlier, the potencies of all the antibiotics used in the study were confirmed using susceptible strain of *S. aureus* ATCC 25923.

Statistical analysis

Results were presented as mean ± standard error of mean for all groups. Student t-test was used for test of significance between two groups.

RESULTS

Strain isolation and identification

Ten strains isolated on MRS and Rogosa agar from pickled vegetable juice were preliminarily indentified as lactic acid bacteria. The strain with the largest cholesterol-lowing effect was a sphere-shaped, Gram-positive, and able to grow at temperatures between 10 and 45℃. The patterns of carbohydrate fermentation and SDS-PAGE analysis of the isolate were similar to those of the *L. lactis* group. Partial sequencing of 16S rRNA indicated 98% identity with *L. lactis* subsp. *lactis* NBRC 12007. Therefore the isolate was confirmed as a strain of *L. lactis subsp. lactis*, and was designated *L. lactis* subsp. *lactis* LB12.

Lactococus lactis subsp. *lactis LB12* on serum lipids and bile acid levels of mice

The results of assigned diets on serum lipids and bile acid

Table 1. Different diets fed for 28 days on serum lipids and bile acid levels of the mice.

Treatment	Serum TC (mmol/L)	Serum TG (mmol/L)	Serum HDL-c (mmol/L)	Serum TBA (μmol/L)
Group 1	1.35 ± 0.24	1.08 ± 0.21	0.92 ± 0.13	8.11 ± 3.15
Group 2	4.34 ± 1.32♦	1.46 ± 0.35♦	0.87 ± 0.12	12.27 ± 3.59♦
Group 3	4.43 ± 0.99	1.37 ± 0.28	0.86 ± 0.12	12.41± 4.51
Group 4	3.25 ± 0.56*	1.15 ± 0.20*	0.88 ± 0.12	8.64 ± 2.30*

Treatments: Group 1 (model control) mice were fed commercial diet (purchased from Nanjing Qinglongshan Animal Resources Centre, China); group 2 (high fat mould) mice were fed commercial diet plus 1.0% (w/w) cholesterol, 8.0% (w/w) lard and 0.50% (w/w) oxgall; group 3 (experimental control) mice were fed commercial diet plus 1.0% (w/w) cholesterol, 8.0% (w/w) lard, 0.50%(w/w) oxgall and 5.0% (v/w) low fat milk; group 4 (experimental) mice were fed commercial diet plus 1.0% (w/w) cholesterol, 8.0% (w/w) lard, 0.50% (w/w) oxgall and 5.0% (v/w) yogurt fermented by *L. lactis subsp. lactis* LB12.
Data are mean ± SD of measurements from 12 mice.
*Significant ($p < 0.05$) compared to experimental control (group 3); ♦greatest differences ($p < 0.01$) compared to mould control (group 1).

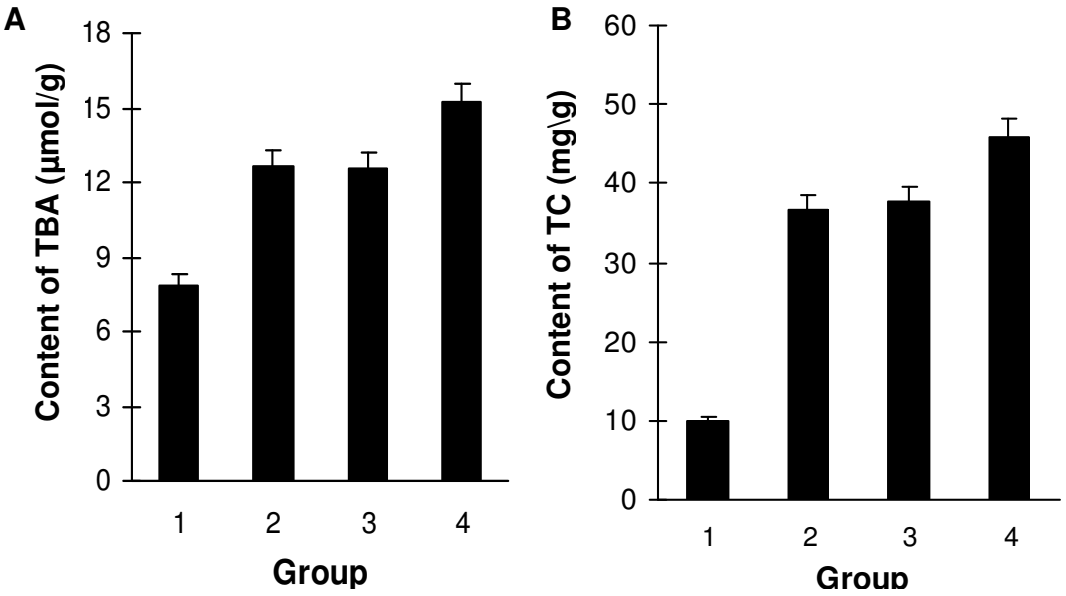

Figure 1. Effect of feeding of commercial diet (group 1), mixture of commercial diet plus cholesterol plus lard plus oxgall (group 2), commercial diet plus cholesterol plus lard plus oxgall plus low fat milk (group 3), commercial diet plus cholesterol plus lard plus oxgall plus yogurt fermented by *L. lactis* subsp. *lactis* LB12 (group 4) on the total bile acids (TBA) concentration (A) and cholesterol (TC) concentration (B) of feces of mice. Values are means ± SD of measurements from 12 mice.

levels of mice are presented in Table 1. Levels of serum total cholesterol (TC) and triacylglycerols (TG) in group 2 mice were significant increased compared to the model control mice (group 1) when the high-cholesterol diets were fed to the mice. It meant that the high fat mould mice had set up. Mice fed yogurt (group 4) fermented by *L. lactis* subsp. *lactis* LB12 had significantly lower total cholesterol, triacylglycerols and total bile acid compared to the experimental control mice (group 3). It suggested that yogurt fermented by *L. lactis* subsp. *lactis* LB12 had cholesterol-lowering effect.

Bile acid and cholesterol concentration in feaces

The effects of assigned diets on feaces bile acid and cholesterol content of mice are illustrated in Figure 1(a, b). The bile acid and cholesterol content of feaces in mice given high-cholesterol diets (group 2, 3 and 4) were significantly higher than those in model control mice (group 1). The bile acid and cholesterol level in feaces of the mice fed with yoghurt (group 4) were significant higher than those of experimental control (group 4) and high fat mould mice (group 2). It suggested that *L. lactis* subsp.

Table 2. Different diets fed for 28 day on body and viscera weight indexs of the mice.

Treatment	28day body weight (g)	liver/body (w/w, %)	nate/body (w/w, %)	spleen/body (w/w, %)
Group 1	359 ± 34	3.53 ± 0.34	0.68 ± 0.05	0.43 ± 0.10
Group 2	385 ± 42	5.12 ± 0.43$^{\lozenge}$	0.60 ± 0.06	0.46 ± 0.12
Group 3	363 ± 34	5.10 ± 0.56$^{\lozenge}$	0.59 ± 0.05	0.48 ± 0.07
Group 4	354 ± 32	4.60 ± 0.45$^{*,\lozenge}$	0.59 ± 0.06	0.43 ± 0.08

Treatments: Group 1 (model control) mice were fed commercial diet (purchased from Nanjing Qinglongshan Animal Resources Centre, China); group 2 (high fat mould) mice were fed commercial diet plus 1.0% (w/w) cholesterol, 8.0% (w/w) lard and 0.50% (w/w) oxgall; group 3 (experimental control) mice were fed commercial diet plus 1.0% (w/w) cholesterol, 8.0% (w/w) lard, 0.50%(w/w) oxgall and 5.0% (v/w) low fat milk; group 4 (experimental) mice were fed commercial diet plus 1.0% (w/w) cholesterol, 8.0% (w/w) lard, 0.50% (w/w) oxgall and 5.0% (v/w) yogurt fermented by *L. lactis subsp. lactis* LB12.
Data are mean ± SD of measurements from 12 mice.
Significant and greatest differences: $p < 0.05$ and $p < 0.01$. $^{*}p < 0.05$ compared to high fat mould and experimental control (group 2 and 3); $^{\lozenge}p < 0.01$ compared to mould control (group 1).

Table 3. Different diets fed on body weight gain of the mice.

Treatment	0 day body weight (g)	10 day body weight (g)	Body weight gain (g)
Control	18.90 ± 0.70	25.80 ± 1.95	6.90
Experimental	19.30 ± 1.10	26.60 ± 2.05	7.30

Treatments: Control and experimental group mice were administered sterilized low fat milk (1.0% fat and 11.0% SNF, acidifying it to pH 4.7 with 10% lactic acid) and yogurt fermented by *L. lactis* subsp. *lactis* LB12 at dose of 100 ml/kg·bw by gastric intubation once a day throughout the experimental period of 10 days, respectively. Commercial diet (purchased from Nanjing Qinglongshan Animal Resources Centre, China) and water were freely available to all mice.
Data are mean ± SD of measurements from 20 mice.

lactis LB12 could promote the excretion of bile acid and cholesterol.

Effects of *L. lactis* subsp. *lactis* LB12 on body and viscera weight of the mice

The body weight and viscera weight index is showed in Table 2. There are no significant difference in body weight, weight percentage of nates and spleens to body weight among all groups. The weight percentage of liver to body was significant ($p < 0.01$) increased in high fat mould mice (group 2), experimental control mice (group 3) and yogurt treated mice (group 4) compared to that of model control mice (group 1), suggesting that there are more fat stored in liver of high fat mould, experimental control and yogurt treated mice than that of mould control mice. However, the weight percentage of liver to body was significantly ($p < 0.05$) decreased in yogurt treated mice than those in high fat mould and experimental control mice. This suggests that there was fewer fat stored in liver of group 4 than those of groups 3 and 4.

Safety assessment of *L. lactis* subsp. *lactis* LB12

In lowing cholesterol test, there are no significant difference in body weight, weight percentage of nates and spleens to body weight among all groups (Table 2). No deaths or differences in behavior among groups were noted.

During acute toxicity test, no deaths were also observed, no significant difference in body weight or differences in behavior between experimental and control group mice were noted. All tested mice did not show any significant abnormality in the skin, fur, eyes and mucous membranes, as well as behavioral pattern, and also had no obvious signs of toxicity or change in other physiological activities. The body weight, body weight gain (Table 3) and excreta of the mice treated with yogurt had no significant change, as well as no significant difference in food and water tacking compared with those of the control group. At necropsy, the pathological examination of the internal organs related showed that no gross histopathological alterations were found in all the tested animals and the control group, and the aberration rate of sperm had no significant difference between yoghurt treated mice and control mice.

A translocation-positive animal is defined as an animal that had at least one tissue sample (including blood) containing one or more viable bacterial cells. Positive translocation tissue is defined as the tissue from which at least one viable bacterial cell was recovered (one colony).

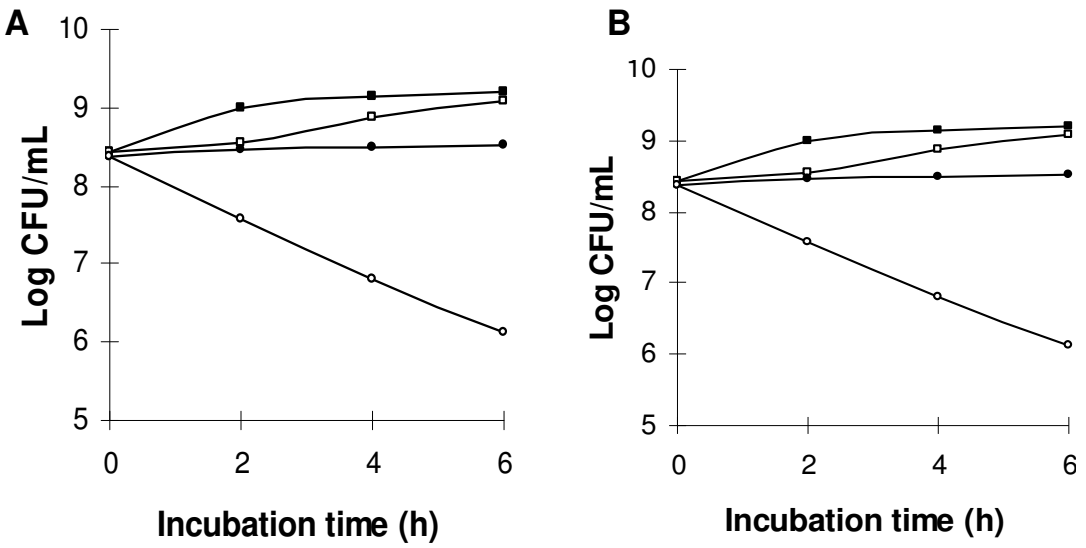

Figure 2. Effect of pH (A) (-■-, -□-, -●- and -○-, the culture pH of 4.5, 3.5, 2.5 and 1.5) and bile salt (B) (-■- 0.10%, - □- 0.20%, -●- 0.30%, -○- 0.40% bile salt) on viability of *L. lactis* subsp. *lactis* LB12. Values are means ± SD of measurements from 3 experiments.

Statistical analysis of results did not reveal any significant differences in the percentage of animals and tissues between the different groups ($p > 0.05$). No bacteria isolated from the tissue samples matched with the test strain fed to the mice.

Low pH tolerance of *L. lactis* subsp. *lactis* LB12

Low pH tolerance of *L. lactis* subsp. *lactis* LB12 was assessed in pH 1.5, 2.5, 3.5 and 4.5, respectively. As shown in Figure 2a, the strain grew obviously for 6 h incubation in pH 3.5 and 4.5, and slightly for 6 h incubation in pH 2.5, suggesting that this strain had the ability to survive at pH 2.5~4.5, and the viable cells could still reach 10^6 CFU/mL after incubated in pH 1.5 for 6 h. It indicated that *L. lactis* subsp. *lactis* LB12 was low pH tolerant.

Bile salt tolerance of *L. lactis* subsp. *lactis* LB12

Results of bile salt tolerance were shown in Figure 2b. It showed that the viable cells increased obviously in the condition of 0.10% bile salt, and decreased obviously in the condition of 0.20, 0.30 or 0.40% bile salt. However, the viable cells were determined above 10^7 CFU/mL when incubated in 0.20% bile salt for 6 h, and 10^6 CFU/mL in 0.30% and 0.40% bile salt for 4 h.

Antimicrobial activity of *L. lactis* subsp. *lactis* LB12

The antimicrobial activities results of *L. lactis* subsp. *lactis* LB12 to the pathogenic *E. coli* and *S. aureus* are showed in Table 4. The diameter of inhibition zones caused by the culture (A), clear supernatant of culture (B) and the sterilized culture (C) of *L. lactis subsp. lactis* LB12 were all more than 17 mm and significant larger than that of control (D). However there are no significant difference among A, B and C, indicating that there could exist some inhibitory substance in the culture produced by the strain during fermentation.

Antibiotic resistance of *L. lactis* subsp. *lactis* LB12

The diameters of inhibition zones caused by ampicillin, tetracycline, gentamicin, roxithromycin and cefoxitin to *L. lactis* subsp. *lactis* LB12 were 13, 14.5, 7.2, 16.1 and 7.0 mm, significant lower than the 15, 17, 12, 18 and 10 mm were caused by these antibiotics to the contrast strain of *S. aureus* ATCC 25923, respectively. This indicates that the strain was resistance to the antibiotics tested.

DISCUSSION

Lactic acid bacteria are normal components of the intestinal microflora in both humans and animals and have been associated with various health-promoting properties. One beneficial effect is a reduction in serum cholesterol levels. The results from the present study strongly suggest that *L. lactis* subsp. *lactis* LB12 screened from traditional Chinese pickled cabbage juice significantly reduced serum cholesterol of mice.

Different hypotheses have been advanced to explain how the hypocholesterolemic effect of lactic acid bacteria

Table 4. Antimicrobial activities of *L. lactis* subsp. *lactis* LB12 on the test organisms using agar-gel diffusion test.

Treatment	Diameter of inhibition zones (mm)			
	A	B	C	D
E. coli	18.02 ± 2.15*	17.53 ± 1.98*	17.51± 1.47*	7.83 ± 1.06
S. aureus	17.62 ± 2.11*	17.42 ± 2.32*	17.42 ± 1.96*	7.81 ± 1.24

A, B, C and D expressed the groups of culture, clear supernatant of culture, sterilized culture of *L. lactis* subsp. *lactis* LB12 and MRS broth samples, respectively.
Data are mean ± SD of measurements from 3 experiments.
Significant difference: * $p < 0.01$ compared to control group (D).

is possible. In this study, the cholesterol and bile acid levels in the serum of mice fed with yogurt fermented by *L. lactis* subsp. *lactis* LB12 decreased significantly, while the cholesterol and bile acid content increased in mice feces. These effects may be due in part to the deconjugation of bile salts by strains of bacteria that produce the enzyme bile salt hydrolase (BSH) (Taranto et al., 1997; Brashears et al., 1998; Pereira et al., 2003). As deconjugated bile salts are more readily excreted in the feaces than conjugated bile salts (Gilliland and Walker, 1990; De Smet et al., 1994; De Rodas et al., 1996), bacteria with BSH activity may effectively reduce serum cholesterol by enhancing the excretion of bile salts, with a consequent increase in the synthesis of bile salts from serum cholesterol; or by decreasing the solubility of cholesterol, since bile acid is essential for gastrointestional absorption of cholesterol (Sugao and Imaizumi, 1986), and thus reducing its uptake from the gut.

The gastric tract contains gastric juice of low pH due to the high hydrochloric acid concentration of the secreted gastric acid, and the intestinal tract contains bile juice containing bile salts (Holzapfel et al., 1998). As such, probiotic bacteria should have the ability to survive the passage through the gastrointestinal tract, resisting the acidic conditions in the stomach and the bile acids at the beginning of the small intestine (Taranto et al., 1998; Hyronimus et al., 2000; Park et al., 2002). The present results indicated that the strain of *L. lactis* subsp. *lactis* LB12 screened from traditional Chinese pickled cabbage juice had a high level of bible tolerance and acid resistance.

Assessment of pathogenicity is one important component of probiotic safety studies (Zhou et al., 2000; Marteau et al., 1997), the indicators for which include splenomegaly and hepatomegaly. None of these morphological changes was noted as a result of *L. lactis* subsp. *lactis* LB12 treatment, nor were there significant differences in the visceral weight indices of the lymph nodes, spleen, or liver. In addition, bacterial translocation, the process by which intestinal bacteria pass through the mucosal epithelium and invade other organs, is another important safety consideration, as it may cause bacteremia, septicemia, and even multiple organ failure (Borriello et al., 2003). The bacterial translocation in the present study was not significantly different between experimental and control groups. Moreover, *L. lactis* subsp. *lactis* LB12 is resistant to the tested common commercial antibiotics, and possesses antimicrobial activity to pathogenic *E. coli* and *S. aureus*, suggesting that the organism would not be affected by therapies using these antibiotics and might help maintain the natural balance of intestinal microflora during antibiotic treatments.

Serum triglycerides and relative weight of the liver were also lowered as a result of the *L. lactis* subsp. *lactis* LB12 treatment. This suggests that the hypolipemic effect of the bacteria may not be due to a redistribution of lipids from the plasma to the liver, but rather to decreased intestinal absorption of lipids or increased lipid catabolism (Taranto et al., 1998).

The results of this study indicate that *L. lactis* subsp. *lactis* LB12 is a safe probiotic with the potential to reduce serum cholesterol and triglyceride levels. Further studies will be required to determine the mechanism underlying the cholesterol-lowering effect. It will also be necessary to test more animals, using varying doses of this strain over longer times, to assess the long-term probiotic potential of *L. lactis* subsp. *lactis* LB12.

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