

Screening Lactic Acid Bacteria Isolates with Potential Probiotic Properties of Cholesterol lowering from Chinese Traditional Fermented Dairy Products

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Abstract: The objectives of this study were to screen Lactic Acid Bacteria (LAB) isolates with potential probiotic property of assimilating cholesterol and to confirm whether consumption of selected strains would significantly prevent an increase of serum cholesterol in mice fed a high cholesterol diet. Six hundred and seventeen LAB strains isolated from variety of traditional dairy products in Xinjiang, China, were examined *in vitro* for their acid and bile tolerance and 14 LAB isolates including three *S. thermophilus*, two *E. faecium*, three *L. lactis subsp. lactis*, one *L. casei*, *L. plantarum* and *L. delbrueckii. subsp. bulgaricus* and three *L. rhamnosus* were found to remain at the levels of $<10^4$ CFU mL⁻¹ after 3 h of incubation at pH 2.5 or 0.3% (w/v) oxgall, which indicated that these strains may survive upper gastrointestinal tract conditions. These 14 acid and bile tolerant strains exhibited variable cholesterol lowering abilities *in vitro* and the cholesterol reducing rate ranged from 5.46-50.35% per 10^9 CFU, in which strains *S. thermophilus* X1, *E. faecium* X5, *E. faecium* Y5 and *L. rhamnosus* g x 10 showed significantly higher ($p<0.05$) ability to remove cholesterol in media than that of the other strains examined. The effect of fermented milk with *L. rhamnosus* g x 10 or *S. thermophilus* X1 or *E. faecium* X5 and *E. faecium* Y5 on serum total cholesterol, high-density lipoprotein, low-density lipoprotein and triglycerides were determined in rats fed on a cholesterol enriched diet. The results indicated that *L. rhamnosus* g x 10 fermented milk significantly decreased serum total cholesterol level of rats in experimental group compared with that of model group ($p<0.05$). The serum triglycerides level in the rats fed the LAB isolates fermented milk was also tended to decrease but the decrease was not significant. The liver tissue sections were carried out to evaluate effects of probiotic strain *in vivo*. it was observed that histopathological changes in livers from those rats given cholesterol enriched diet supplemented with yoghurt enriched *S. thermophilus* X1 (Exp.1 group), *S. thermophilus* X5 (Exp. 2 group), *E. faecium* Y5 (Exp. 3 group) and *L. rhamnosus* g x 10 (Exp. 4 group) was less extensive than those of mice receiving cholesterol enriched diet alone in model group, furthermore, *L. rhamnosus* g x 10 group was found to be almost the same as the rats fed cholesterol-free diet from blank group in liver tissues. The present study demonstrated some lactic acid bacteria isolates from Chinese traditional dairy products were effective in decrease serum total cholesterol and had potential of treating hypercholesterolemia.

Key words: Lactic acid bacteria, Fermented milk, Probiotics, Cholesterol-lowering effect, serum triglycerides, diet supplementy

INTRODUCTION

Probiotic bacteria, mainly Lactic Acid Bacteria (LAB), are used as adjuncts in food to provide a wide variety of health benefits. The colonization of the gut by probiotic bacteria prevents the growth of harmful bacteria by

competitive exclusion and by the production of organic acids and antimicrobial compounds (Fuller, 1989). Other therapeutic functions attributed to probiotics are anticholesterol activity, improved lactose utilization and anticarcinogenic activity (Fuller, 1992; Jahreis *et al.*, 2002). In order to beneficially affect the health of the host, a

probiotic culture must be ingested in sufficient quantities. The suggested concentration for probiotic bacteria is in the range 10^6 - 10^7 cfu g^{-1} of the product (Robinson, 1987). Dairy products such as yoghurt are often used as carriers for probiotic cultures. Hypercholesterol is a risk factor for cardiovascular disease, the leading cause of death in many countries (Law *et al.*, 1994). It is therefore important to develop new ways of lowering serum cholesterol. Diet has been identified as a means of controlling serum cholesterol concentrations.

Recently, LAB has attracted attention as potential cholesterol lowering milk additives (Roos and Katan, 2000). LAB normally reside in the mouth and intestinal tract where they enhance immune responses (Fernandes and Shahani, 1990), exert antimutagenic and anticarcinogenic activities (Gilliland, 1989) and protect against gastrointestinal diseases (Kasper, 1998). The reduction of serum cholesterol could be an important health benefit of LAB, as a 1% reduction in serum cholesterol is associated with an estimated reduction of 2-3% in the risk of coronary artery disease (Manson *et al.*, 1992). The reduction of cholesterol by LAB has been demonstrated in human, mouse and pig studies (Kawase *et al.*, 2000; Haberer *et al.*, 2003). Cholesterol lowering effects may be due in part to the deconjugation of bile salts by strains of bacteria that produce the enzyme bile salt hydrolase (Pereira *et al.*, 2003). As deconjugated bile salts are more readily excreted in the feces than conjugated bile salts (De Rodas *et al.*, 1996), bacteria with BSH activity may effectively lower 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 and thus lowering its uptake from the gut. The population in Xinjiang area of China consumes large amounts of mutton and dairy products. The aim of this study was to screen acid and bile tolerant LAB isolates with cholesterol lowering effect from traditional fermented

dairy products in Xinjiang, China and evaluated its potential as a cholesterol lowering probiotic in mice model.

MATERIALS AND METHODS

Bacteria and culture conditions: All lactic acid bacteria strains used in this study (Table 1) were isolated from traditional dairy products of Xinjiang, China, by Institute of Dairy Science and Technology, Yangzhou University, China. Strains were routinely stored at $-18^{\circ}C$ after inoculation in sterile skim milk containing 0.5% (wt/vol) yeast extract. The stock cultures were grown in sterile MRS broth (Difco) from 1% inoculums with 48 h anaerobic incubation at $37^{\circ}C$ and transferred successively three times in MRS broth prior to use.

Acid and bile tolerance tests: To evaluate the ability of the strains to tolerate acid and bile concentrations typically found in the upper gastrointestinal tract, survival rates of cultures in medium with acid and bile were tested. Strains propagated in MRS broth for 24 h at $37^{\circ}C$ were inoculated to mMRS broth previously adjusted to pH 2.5 with HCl and MRS broth containing 0.3% (wt/vol) oxgall (Oxoid) to concentration of 10^7 cfu mL^{-1} and the mixtures were incubated anaerobically for 3 h. One milliliter mixture samples were taken and serially 10 fold diluted in anaerobic diluent (half strength peptone water plus 0.5 g of L-cysteine, pH 7.0) and then plated in triplicate onto MRS agar. The plates were incubated at $37^{\circ}C$ for 48 h under anaerobic conditions. The survival rates were expressed as the ratios of viable cells enumerations to initially inoculums. The experiments were repeated three times.

Cholesterol removal *in vitro*: The test medium used for screening cultures for cholesterol removal was sterile

Table 1: Survival of the lactic acid bacteria strains at pH 2.5 and in the presence of 0.3% oxgall

LAB strains	pH2.5-3 h ^a	0.3% oxgall-3 h ^a	Origin
<i>S.thermophilus</i> X1	5.15±0.23	5.46±0.14	kumiss
<i>S.thermophilus</i> X3	4.52±0.31	4.27±0.16	natural fermented camel milk
<i>S.thermophilus</i> X7	4.25±0.24	4.55±0.21	kumiss
<i>E. faecium</i> X5	4.17±0.13	5.32±0.25	kumiss
<i>E. faecium</i> Y5	5.66±0.34	5.68±0.43	natural fermented milk
<i>L. lactis subsp. lactis</i> X12	4.68±0.33	4.35±0.33	natural fermented milk
<i>L. lactis subsp. lactis</i> X16	4.32±0.36	4.59±0.27	natural fermented milk
<i>L. lactis subsp. lactis</i> X17	4.75±0.23	4.67±0.31	natural fermented camel milk
<i>L. casei</i> Y17	4.37±0.26	5.23±0.11	kumiss
<i>L. plantarum</i> Y3	4.38±0.41	4.61±0.20	natural fermented camel milk
<i>L. delbrueckii subsp. bulgaricus</i> Y1	4.86±0.29	4.74±0.18	natural fermented milk
<i>L. rhamnosus</i> g × 10	5.44±0.31	5.12±0.21	natural fermented camel milk
<i>L. rhamnosus</i> X21	5.68±0.24	5.46±0.37	kumiss
<i>L. rhamnosus</i> Y18	5.72±0.35	5.65±0.36	natural fermented milk

^aExperiments were performed in triplicate, the data are expressed as log cfu mL^{-1} mean values + standard deviation

PTYG broth supplemented with 0.1% (w/v) of water soluble filter-sterilized cholesterol (polyoxyethanyl-cholesteryl sebacate, purchased from Sigma), 0.1% (w/v) sucrose esters, 0.15% (w/v) of oxgall, 1.0% (v/v) of Tween 80 and 0.5% (v/v) of acetic acid. Freshly prepared lactic acid bacteria cultures were inoculated to test medium with 1% (v/v) inoculums and incubated anaerobically at 37°C for 24 h. After the incubation, the bacteria cells were centrifuged and the remaining cholesterol concentration in the spent broth was determined according to the procedure described by Xiaona *et al.* (2006). Each determination was conducted in triplicate.

Yoghurt preparation: Skim milks (10% w/w) inoculated with 3% (v/v) of LAB were incubated at 37°C to reach cell numbers of 5×10^8 cfu mL⁻¹ and the fermented milks were stored at 4°C.

Animal assay: ICR mice (body weight 20-24 g) were obtained for Nanjing Experimental Animal Center (china). The mice were housed in cages with wire mesh floors in a room at 22±1°C and 60-75% relative humidity. All animals were fed on a basal diet for one week. After this adaptation period, these rats were divided randomly into 7 experimental groups (Table 2), each group containing 10 mice. Group 1 received a basal diet (cholesterol-free diet) throughout the experimental period of four weeks and served as a blank group. Mice in group 2-6 were fed on the basal diet with 0.5% (w/w) cholesterol enriched diet (CED) for 1-2 weeks to create hypercholesterolaemic model, in which group 2 was continually fed on CED in following period and served as negative control and group 3 was administrated simvastain and served as positive control. Groups 4-7 were fed for three weeks on a cholesterol enriched diet supplemented with yoghurt enriched *S. thermophilus* X1 and X5, *E. faecium* Y5 and *L. rhamnosus* g x 10. The rats were allowed free access to experimental diet and water and their body weights were monitored. At the end of the 4 week experimental period, blood samples were collected from the eye vein under diethyl ether anesthesia. The samples were collected in tubes, with EDTA as an anti-coagulating agent. The tubes were centrifuged at 3000 rpm for 15 min to obtain the plasma, which was kept at -20°C until analysis. The liver

tissues were washed with ice cold saline solution (0.9%, w/v, NaCl) and homonized for centrifugation and the superants were stored at -20°C until analysis. Determination of serum cholesterol, high-density lipoprotein, low-density lipoprotein and triglycerides. Total plasma cholesterol, High-density Lipoprotein (HDL), Low-density Lipoprotein (LDL) and triglycerides were determined using the kits (Boehringer Mannheim Gm bH, Mannheim, Germany). Super Oxide Dismutase (SOD) and Malondialdehyde (MDA) were extracted from the liver and analyzed using the diagnostic kits (Nanjing Jiancheng Biothech. Institute, China).

Pathological evaluation of liver tissues: The left lateral lobe of the livers (three slices per rat) were fixed with 10% neutral formalin and embedded in paraplast. Tissue sections (5 µm) were cut and stained by hematoxylin and eosin to analyze pathophysiological changes in liver tissues.

Statistical analysis: Data are presented as means and standard deviation. The significance differences between groups/treatments were analyzed using statistical analysis systems (SAS Institute, Inc., Cary, NC, USA) by Least Significant Difference (LSD) at P = 0.05 and 0.01.

RESULTS

Acid resistance and bile tolerance of LAB isolates: A total of 617 lactic acid bacteria, strains were isolated from the traditional dairy products in Xinjiang area, China, of which 106 strains were isolated from natural fermented milk, 102 strains were isolated from Kefir, 102 strains were isolated from Kumiss, 108 strains were isolated from natural fermented goat milk, 101 strains were isolated from natural fermented camel milk and 98 strains were isolated from sour milk cake. Probiotic bacteria must be resistant to the high acidity of the stomach and high concentration of bile components in the proximal intestine in animal nutrition and in therapy. These characteristics may be observed *in vitro* and can be used for selection of strains (Salminen *et al.*, 1996). Thus, MRS broth adjusted to pH 2.5 or containing 0.3% (wt/vol) oxgall were used to select acid resistant and bile tolerant LAB isolates in this study, it was observed that most of the LAB tested is sensitive to acid and no survival was observed after exposure to acidified MRS broth of pH 2.5 for 3 h. However, there were 14 strains of LABs including three *S. thermophilus*, two *E. faecium*, three *L. lactis* subsp. *lactis*, one *L. casei*, *L. plantarum* and *L. delbrueckii* subsp. *bulgaricuss* and three *L. rhamnosus* showed moderate survival rates (from 0.15-5.25%) and the final viable bacteria counts at pH

Table 2: Diet treatments of mice in different experimental groups

Group	Diet treatment
Blank	Cholesterol free diet
Model	Cholesterol Enriched Diet(CED)
Control	CED+simvastain
Exp. 1	CED+ yoghurt enriched <i>S.thermophilus</i> X1
Exp. 2	CED+ yoghurt enriched <i>S.thermophilus</i> X5
Exp. 3	CED+ yoghurt enriched <i>E.faecium</i> Y5
Exp. 4	CED+ yoghurt enriched <i>L.rhamnosus</i> g x 10

2.5 or in presence 0.3% oxgall after 3 h of incubation remained at the levels of $>10^4$ CFU mL⁻¹. The results are shown in Table 1. The data indicated that these strains might survive the low pH conditions in the stomach (pH 2.0 in extreme cases). Unconjugated bile acids, even at low concentrations, can inhibit the *in vitro* growth of microorganisms (Fuller, 1992). According to Gilliland *et al.* (1984), 0.3% is considered to be a critical concentration for screening for resistant strains. Table 1 showed the survival viable bacteria counts (mean value of log CFU/mL ± standard deviation) of 14 strains of LABs in broth containing 0.3% oxgall. Three *L. rhamnosus* (g x 10, X21 and Y18) and one *S. thermophilus* (X1) remained at the levels of 10^5 CFU mL⁻¹ after 3 h of incubation in presence of oxgall, which showed higher ability to withstand bile concentration of 0.3% than the other strains. This indicates that these four strains may be better adapted to tolerate the intestinal bile conditions.

The capacity of cholesterol reduction of LAB isolates *in vitro*: All tested strains could growth well (the final total viable bacteria of the culture varied between 10^9 and 10^{10} CFU in plate counting) in test medium supplemented with cholesterol, in order to take growth variation among strains into account, the cholesterol reducing rate of each strain was standardized with the final viable bacteria of the culture, thus the cholesterol lowering capacities were expressed as cholesterol reducing rate per 10^9 CFU. The rate of cholesterol assimilated *in vitro* during 24 h of anaerobic growth at 37°C (Fig. 1) revealed a wide variation

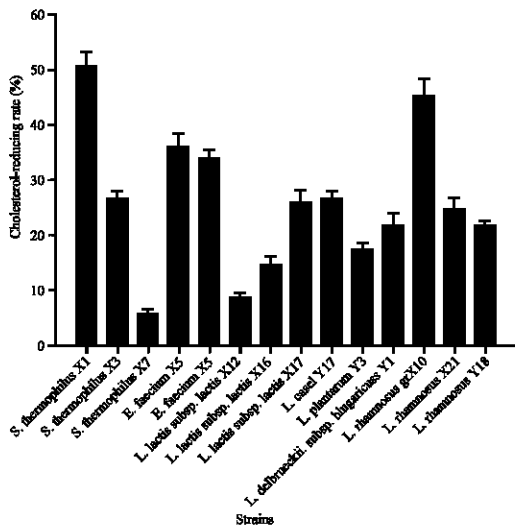


Fig. 1: The capacity of cholesterol lowering of LAB *in vitro*. Experiments were performed in triplicate the data are expressed as percent of the cholesterol reduction in the media (%) per 10^9 CFU (mean values ± standard deviation)

among LAB strains. All strains showed varying degree of cholesterol lowering abilities and the cholesterol reducing rate ranged from 5.46-50.35% of the cholesterol in the media per 10^9 CFU. Strains *S. thermophilus* X1, *E. faecium* X5, *E. faecium* Y5 and *L. rhamnosus* g x 10 showed superior ability to remove cholesterol in media and their cholesterol reducing rate were 50.35, 35.49, 33.63 and 45.24% per 10^9 CFU, respectively, which were significantly higher ($p < 0.05$) than that of the other strains examined.

***In vivo* cholesterol lowering effect of LAB isolates:** Rats fed yoghurt with different LAB had varying degree of total serum cholesterol lowering and triglycerides lowering effects compared to the model control rats (Table 3). No significant differences ($p > 0.05$) in body weight, food intake or visceral weight index or differences in behavior between the groups were noted.

Total cholesterol in the serum of rats fed the cholesterol enriched diet (model group) was significantly higher ($p < 0.01$) than that of rats fed the cholesterol free diet (blank group). Comparing with model group, the lower serum total cholesterol level was observed in the groups of the rats fed a cholesterol enriched diet supplemented with simvastain (control group) and yoghurt enriched *S. thermophilus* X1 (Exp. 1 group), *S. thermophilus* X5 (Exp. 2 group), *E. faecium* Y5 (Exp. 3 group) and *L. rhamnosus* g x 10 (Exp. 4 group). It can be seen from the Table 3 that *L. rhamnosus* g x 10 fermented milk significantly decreased serum total cholesterol level of rats in Exp. 4 group compared with that of model group ($p < 0.05$). It was also observed that there was higher serum HDL-cholesterol and lower LDL-cholesterol concentrations in the groups fed yogurt and simvastain with cholesterol enriched diet than those fed only cholesterol enriched diet (model group), however, there was no significant difference among control group and the experimental dietary groups. The serum triglycerides level in the rats fed the LAB isolates fermented milk was also tended to decrease but the decrease was not significant. In addition, we found the MDA concentration in the liver homogenate of rats in Ex 1-4 group is significantly lower than that of rats in model group ($p < 0.05$) and no significant difference with that of rats in blank and control group. Contrary to this, the SOD activity of liver homogenate in the experimental groups is higher than that of the model group ($p < 0.01$).

Pathological evaluation: The liver tissue sections were carried out to evaluate pathological effects of probiotic strain *in vivo*. The results were shown in Fig 2. For rats from blank group fed cholesterol free diet, there were no pathological changes in liver tissues (Fig. 2A). As expected, liver sections from mice fed cholesterol enriched

Table 3: Levels of serum cholesterol, triglycerides and liver MDA and SOD in rats fed on experimental diets for four weeks

Diet treatment ^a	Serum ^d				Liver ^d	
	Total cholesterol (mg 100 mL ⁻¹)	Triglycerides (mg 100 mL ⁻¹)	HDL-C ^b (mg 100 mL ⁻¹)	LDL-C ^c (mg 100 mL ⁻¹)	MDA (nmol mg prot ⁻¹)	SOD (U mg prot ⁻¹)
Blank	2.391±0.497	0.835±0.072	1.757±0.126	0.204±0.061	0.966±0.378	56.07±6.38
Model	4.106±0.670	0.985±0.102	1.522±0.363	0.304±0.073	1.314±0.409	40.79±8.30
Control	3.274±0.377	0.884±0.133	1.801±0.430	0.265±0.093	0.887±0.386*	53.81±8.69*
Exp. 1	3.263±0.444	0.844±0.101	1.775±0.392	0.252±0.078	0.973±0.368	50.70±9.74
Exp. 2	3.251±0.400*	0.858±0.074	1.600±0.186	0.239±0.062	0.970±0.203	52.32±7.71
Exp. 3	3.475±0.666	0.846±0.084	1.712±0.284	0.237±0.083	0.944±0.183	54.46±7.85*
Exp. 4	3.188±0.386*	0.839±0.086	1.828±0.396	0.231±0.058*	0.924±0.155*	54.75±6.61*

*means significant difference compared with model group (p<0.05), ^adetails of diet treatment (Table 2), ^bHDL-C: High Density Lipoprotein-cholesterol, ^cLDL-C: Low Density Lipoprotein-cholesterol, ^dthe data are expressed as mean values ±SD (n = 6)

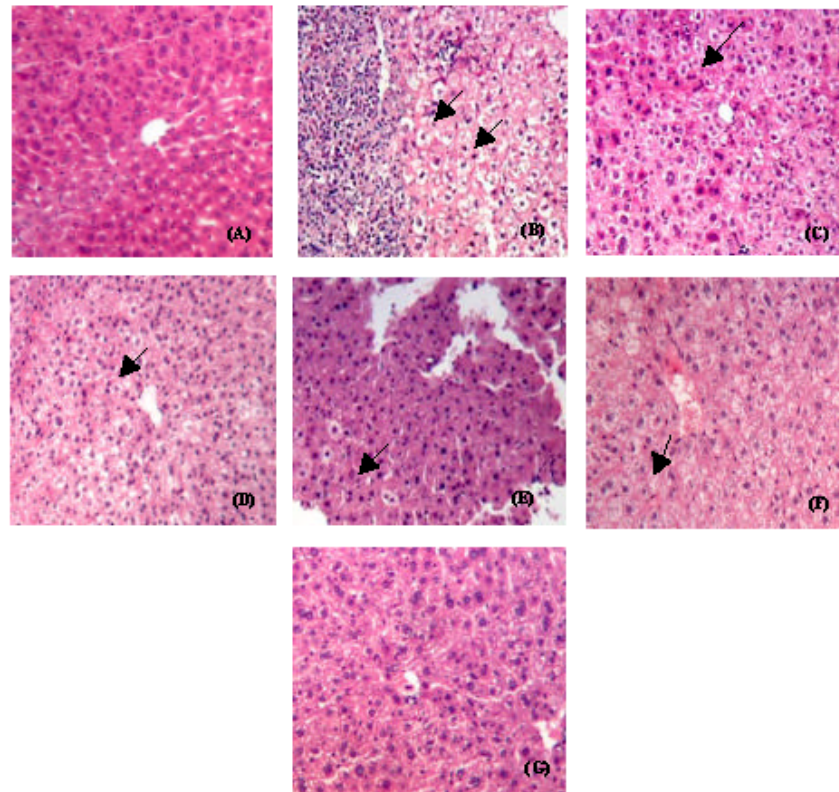


Fig. 2: Representative photomicrograph of liver section from rats in dietary groups (H and E×65). (A) Representative photomicrograph of liver section from rat given Cholesterol free diet (blank group). (B) Representative photomicrograph of liver section from rat given Cholesterol enriched diet (model group). (C) Representative photomicrograph of liver section from rat given Cholesterol enriched diet supplemented with simvastain (control group). (D), (E) and (F) Representative photomicrograph of liver section from rat given Cholesterol enriched diet supplemented with yoghurt enriched *S. thermophilus* X1 (Exp. 1 group), *S. thermophilus* X5 (Exp. 2 group), *E. faecium* Y5 (Exp. 3 group) and *L. rhamnosus* g x 10 (Exp. 4 group), respectively

diet (model group) showed prominent microvesicular steatosis along with necrosis and the necrotic hepatocytes were characterized by cell enlargement and nuclear dissolution. (Fig. 2B). Pretreatment of the mice with drug (simvastain) attenuated the above histopathological changes and were restored towards normal (Fig. 2C). For those rats given cholesterol enriched

diet supplemented with yoghurt enriched *S. thermophilus* X1 (Exp. 1 group), *S. thermophilus* X5 (Exp. 2 group), *E. faecium* Y5 (Exp. 3 group) and *L. rhamnosus* g x 10 (Exp. 4 group), the histopathological changes in livers was less extensive than those of mice receiving cholesterol enriched diet alone in model group (Fig. 2D-G). It was observed that *L. rhamnosus* g x 10 groups were almost the

same as the blank groups in liver tissues which indicated that *L. rhamnosus* g x 10 might be effective as a probiotic with hypolipemic effect.

DISCUSSION

The characteristics of bile and acid-tolerant are important in potential probiotics as bile tolerance is required for bacterial growth and survival in the small intestine (Lee and Salminen, 1995) and acid tolerance is required for the bacteria to survive passage through the stomach (Henriksson *et al.*, 1999) as well as to survive in food (Lee and Salminen, 1995). In this study, 14 strains of acid-resistant and bile-tolerance isolates were selected out from 617 LAB of traditional fermented dairy products origins according to their survival rate in media of pH 2.5 or 0.3% (w/v) oxgall. The capacity of cholesterol lowering of acid-resistant and bile-tolerance isolates were determined *in vitro*. Strains *S. thermophilus* X1, *E. faecium* X5, *E. faecium* Y5, *L. rhamnosus* g x 10 showed higher ability to lower cholesterol, their cholesterol lowering rates were 50.35, 35.49, 33.63 and 45.24%, respectively. Oral administration of yoghurt with different LAB lowered total serum cholesterol levels in mice without any pathogenic side effects. Assessment of pathogenicity is one important component of probiotic safety studies (Marteau *et al.*, 1997; Zhou *et al.*, 2000), the indicators for which include splenomegaly and hepatomegaly. None of these morphological changes was noted as a result of *L. rhamnosus* g x 10 treatment, nor were these significant differences in the visceral weight indices of the liver. Serum triglycerides were also lowered as a result of the *L. rhamnosus* g x 10 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.*, 1997).

CONCLUSION

The results of present study indicate that *L. rhamnosus* g x 10 (Chinese Patent Application no. 200810023014.X, CGMCC no.2526), is a safe probiotic with the potential to lower 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 bacteria over longer times, to assess the long-term probiotic potential of *L. rhamnosus* g x 10. These hypercholesterolaemia effects of fermented skim milk containing *L. rhamnosus* g x 10, which have been demonstrated in the rats in the present study could make

an effective and economic contribution in treating hypercholesterolemia, if these effects could be confirmed in human volunteers.

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