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The effects of *Lactobacillus*-fermented milk on lipid metabolism in hamsters fed on high-cholesterol diet

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Abstract The objective of this study was to evaluate the effects of local *Lactobacillus* strains (NTU 101 and 102) on cholesterol-lowering effects in vivo. Thirty male hamsters were housed, divided into five groups, and fed on a cholesterol diet (5 g/kg diet) to induce hypercholesterolemia. Milk fermented by *Lactobacillus paracasei* subsp. *paracasei* NTU 101, *Lactobacillus plantarum* NTU 102, and *Lactobacillus acidophilus* BCRC 17010 was administrated for this study. After treatment with different fermented milk, blood was taken and liver was removed for the determination of lipoproteins, including total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglyceride. Lactobacilli and bifidobacteria decreased (10^5) in the control group; when hamsters were fed on fermented milk, the number of lactobacilli (10^7–10^8) and bifidobacteria (10^5–10^7) was increased. Serum and liver total cholesterol levels were significantly reduced by about 26.4, 23.5, and 30.1% and by about 17.7, 15.9, and 13.4% when hamsters were given fermented milk. However, serum HDL-C and LDL-C were also reduced. The results of this study showed that the hypocholesterolemic effect of local *Lactobacillus* strains was attributed to its ability to lower serum and liver total cholesterol levels. Thus, local *Lactobacillus* strains could significantly increase probiotic count.

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Introduction

Dietary habit change toward lipid-rich foods in recent years has resulted in risks for cardiovascular diseases. Serum cholesterol level is commonly recognized as an important factor in disease development. Since Mann and Spoerry (1974) discovered hypocholesterolemic effects arising from the diet of the Massai tribespeople in Africa, who ingested large intakes of milk fermented by *Lactobacillus* strains, the relationship between lactic acid bacteria or other probiotics and cholesterol concentration in serum has become a focus of great interest. Harrison and Peat (1975) reported a reduction in serum cholesterol level in newborns fed on fresh milk when the fecal titer of *Lactobacillus acidophilus* was raised. A reduction in serum cholesterol level was also associated with yogurt intake in rabbits (Thakur and Jha 1981), and humans (Hepner et al. 1979; Keim et al. 1981) demonstrated that intestinal lactic acid bacteria, such as *L. acidophilus*, caused bile salts to deconjugate and coprecipitate with cholesterol under anaerobic conditions.

In addition to *L. acidophilus*, Nielson and Gilliland (1985) also showed the cholesterol-reducing activity of *Lactobacillus casei*. Gilliland et al. (1985) reported that consumption of *L. acidophilus* RP32, which grew on bile and assimilated cholesterol from a laboratory medium, significantly inhibited increases in the serum cholesterol level of pigs fed on a high-cholesterol diet. Fukushima and Nakao (1995) also reported that a probiotic mixture of *Bacillus subtilis*, *Bacillus natto*, *Bacillus megaterium*, *L. acidophilus*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *L. casei*, *Streptococcus faecalis*, *Streptococcus lactic*, *Streptococcus thermophilus*, *Saccharomyces cerevisiae*, and *Candida utilis* at 10^7–10^8 CFU/g rice bran showed lower levels of total cholesterol, low-density lipoprotein, and liver cholesterol in hypercholesterolemic rats. Brashears et al. (1998) found that in vitro cultivation of *L. casei* strains without pH control could reach the maximal amount of removed cholesterol and that *L. acidophilus* removed the most cholesterol by incorporating the compound into cellular membranes. *L. casei* mainly did this by destabilizing

cholesterol micelles and coprecipitating cholesterol with deconjugated bile salts at pH <6.0.

In addition, some researchers investigated the in vitro cholesterol-reducing ability of bifidobacteria strains and found that *Bifidobacterium bifidum* was the only strain so far expressing an obvious outcome of cholesterol elimination equivalent to that of *L. acidophilus*. The main purposes of this study are: (1) to explore the relationship of three *Lactobacillus* strains (*Lactobacillus paracasei* subsp. *paracasei* NTU 101, *L. plantarum* NTU 102, and *L. acidophilus* BCRC 17010) with serum cholesterol level in hamsters and their effects on intestinal microflora; and (2) to reduce serum cholesterol level and improve intestinal microflora in host animals with these probiotics.

Materials and methods

Bacterial strains

The bacteria strains used in this study, which were effective in cholesterol reduction in in vitro trials, were two local strains isolated in our laboratory and in type culture (*L. acidophilus* BCRC 17010). These local strains were *L. paracasei* subsp. *paracasei* NTU 101 (which was isolated from human infant feces and showed good survival at low pH, tolerance to high bile concentration, and ability to reduce serum cholesterol in vitro) (Lin et al. 2004) and *L. plantarum* NTU 102 (which was isolated from home-made Korean-style cabbage pickles, was able to survive in vitro at low pH and in the presence of bile salt, and demonstrated pathogen inhibition activities, especially against *Pseudomonas aeruginosa*) (Pan et al. 2002). Growth media included Lactobacilli MRS Broth (Difco Laboratories, Detroit, MI, USA) for the aforementioned strains and Bifidobacteria Iodoacetate Medium-25 (BIM-25) for *Bifidobacterium* spp.

Animal feeding and grouping

Thirty male Syrian hamsters, 4 weeks of age and weighing about 70 g (mean), were randomly divided into five groups with six members each and fed on high-cholesterol diet and ordinary water supply for 1 month. Then, water supply was replaced with a variety of drinking substitutes: group A, water only; group B, sterilized milk; group C, milk fermented by *L. paracasei* subsp. *paracasei* NTU 101; group D, milk fermented by *L. plantarum* NTU 102; and group E, milk fermented by *L. acidophilus* BCRC 17010. During feeding time, environmental conditions were well controlled; relative humidity was 60% and room temperature was 20–25°C, with 12-h light exposure in a daily cycle from 6 a.m. to 6 p.m. Food and liquid were accessible at all times and were replenished everyday. The animals were fed for 8 weeks, during which time body weight and food intake were recorded. After the feeding period, the animals were not fed overnight and were presented for further tests. The feeding material in this study was mainly AIN-76, supplemented with 5% cholesterol and 0.3% bile salt. The diet formula is shown in Table 1. The experiments were carried out in a qualified animal breeding room in the animal center at our institute. (The protocol complied with guidelines described in the "Animal Protection Law," amended on 17 January 2001, Hua-Zong-(1)-Yi-Tzi-900000 7530, Council of Agriculture, Executive Yuan, Taiwan.)

Preparation of fermented and nonfermented milk

Fermented milk Skim milk powder was weighed and dissolved in water to constitute 4% skim milk (wt/vol), which was then sterilized using an autoclave at a temperature of 121°C and a pressure of 1.2 kg/cm^2 for 15 min and then cooled to room temperature. The milk was inoculated by adding a 1% bacterial solution (vol/vol) in a lamina flow cabinet and by incubating at 37°C for 18 h. After adding 0.2% carboxylmethyl cellulose as a stabilizer, the fermented milk was homogenized by a blender and fed to the experimental hamsters.

Nonfermented milk Four percent skim milk was prepared, sterilized, and cooled to room temperature, as described above, and then fed to the animals.

Enzymatic kits

Enzymatic kits used to quantify the levels of serum cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglyceride (TG) were as follows: cholesterol, 1.14830.0001; HDL-C, 1.14210.0001; LDL-C, 1.14992.0001; and TG glycerol phosphate oxidase–phenylperoxidaseaminophenozonphenol (GPO-PAP), 1.14856.0001 (Merck, Darmstadt, Germany).

Blood lipid analysis

The hamsters were euthanized using CO_2. Blood specimens were taken with syringe from the celiac vein and transferred to nonheparinized vacuum blood collection tubes. The tubes were held stationary until the blood

Table 1 The composition of high-cholesterol diets

Ingredient	Content (g/kg)
Casein	200
Safflower oil	100
Vitamin[a]	10
Mineral[a]	35
Choline chloride	2
Sodium cholate	1
Cellulose	20
Sucrose	579
Methionine	3
Cholesterol	50

[a]Based on AIN-76 formula (American Institute of Nutrition 1977)

obviously appeared in two layers and were then centrifuged at 1,750×*g* for 15 min. The supernatant was taken and stored in a refrigerator at 4°C for later tests.

Measurement of serum cholesterol, HDL-C, and LDL-C

Cholesterol oxidase–phenylperoxidaseaminophenozonphenol (CHOD-PAP) method was used to measure cholesterol levels in blood specimens (Richmond 1973), whereas GPO-PAP was used to measure TG levels in blood specimens (Bucolo and David 1973; Fossati and Prencipe 1982; McGowan et al. 1983).

Measurement of liver cholesterol

After an animal had been killed, the viscera were opened and the liver was removed, rinsed with saline, blotted dry with filter paper, and weighed. A piece of liver tissue weighing about 1 g was placed in a sample bottle, to which Folch solution (chloroform:methanol=2:1; vol/vol) 20 times the tissue volume was then added. After the liver tissue in the sample bottle had been homogenized, the mixture was agitated for 30 min in an orbital shaker at room temperature to facilitate lipid extraction. The homogenate was then filtered with Whatman No. 2 filter paper, quantified, and stored in a freezer at −20°C for later use. The CHOD-PAP method was used to analyze samples. One hundred microliters of liver extractant in Folch solution was taken and dried with nitrogen, and 1 ml of chromogemic reagent was added in a 37°C water bath. The reaction of cholesterol in the specimen with cholesterol lipase and cholesterol oxidase produced 4-(*p*-benzo-quinone-monoimine) in red. The observed A_{550} was referred to a standard curve to calculate specimen cholesterol concentration.

Analysis of intestinal microflora

After the feeding period had been completed, the animals were fasted for 12 h and then killed. Once the viscera had been opened and the blood had been collected, the cecum (including a small portion of adjacent colon tissue) of each animal was removed and placed in a capped test tube, which was taken to a lamina flow cabinet where 1 g of cecum tissue was weighed, transferred into a tube with 9 ml of anaerobic diluent, and homogenized by vortexing. The homogenate was taken into a nitrogen-filled glove box where 1 ml of sample was transferred to a tube with 9 ml of anaerobic diluent, and the same procedure was repeated several times to perform a serial dilution. The sample diluents in appropriate dilution factors were added to, and pour-plated with, MRS agar and BIM-25. The plates were placed in anaerobic containers and incubated at 37°C for 48 h. The number of colonies counted after incubation represented the cell counts of *Lactobacillus* and *Bifidobacterium*, respectively (Juang et al. 2000).

Measurement of fecal water content

On the last day of the fourth and eighth weeks, feces were collected, packed in airtight bags, weighed, and stored in a freezer at −20°C during the feeding period. For fecal water content measurement, fecal samples that had been weighed were freeze-dried until constant weights have been reached within about 24 h (Juang et al. 2000). The calculation of fecal water content was as follows: fecal water content (%)= [(weight before freeze drying−weight after freeze drying)/ weight before freeze drying]×100%.

Statistical analysis

All data underwent duplicate analysis using one-way analysis of variance in a statistical analysis system. Duncan's multiple range test was performed to compare any significant differences ($p<0.05$) in variables between groups.

Results

Growth of hamsters

In Table 2, all groups of hamsters show no significant differences in body weight gain, total food intake, and food efficiency ($p>0.05$). This indicates that the animals grow in similar patterns.

Blood lipid analysis

Figure 1 shows the effects of fermented milk diet containing *Lactobacillus* strains on serum cholesterol level. Fermented-milk-feeding groups (groups C, D, and E) displayed significantly lower serum cholesterol levels than those of group A (control) and group B (milk-feeding). In summary, the diet of *L. acidophilus* BCRC 17010 (group E) achieved the maximal cholesterol reduction of 30.1%, followed by *L. paracasei* subsp. *paracasei* NTU 101 (group C;

Table 2 Body weight gain, total food intake, and food efficiency of hamsters fed on high-cholesterol diet for 8 weeks

Group	Body weight gain (g)	Total food intake (g)	Food efficiency[a] (%)
A	40.56±7.19*	338.20±9.69*	11.98±1.53*
B	41.86±9.16*	337.01±23.88*	12.51±2.44*
C	37.38±3.25*	323.84±42.05*	11.58±2.90*
D	45.86±9.97*	352.41±36.72*	13.20±2.37*
E	39.56±8.86*	329.24±30.96*	12.12±2.09*

A Control group; *B* high-cholesterol diet + milk; *C* high-cholesterol diet + fermented milk containing *L. paracasei* subsp. *paracasei* NTU 101; *D* high-cholesterol diet + fermented milk containing *L. plantarum* NTU 102; *E* high-cholesterol diet + fermented milk containing *L. acidophilus* BCRC 17010

*Significantly different at $p<0.05$

[a]Food efficiency (%)=(body weight gain/food intake)×100

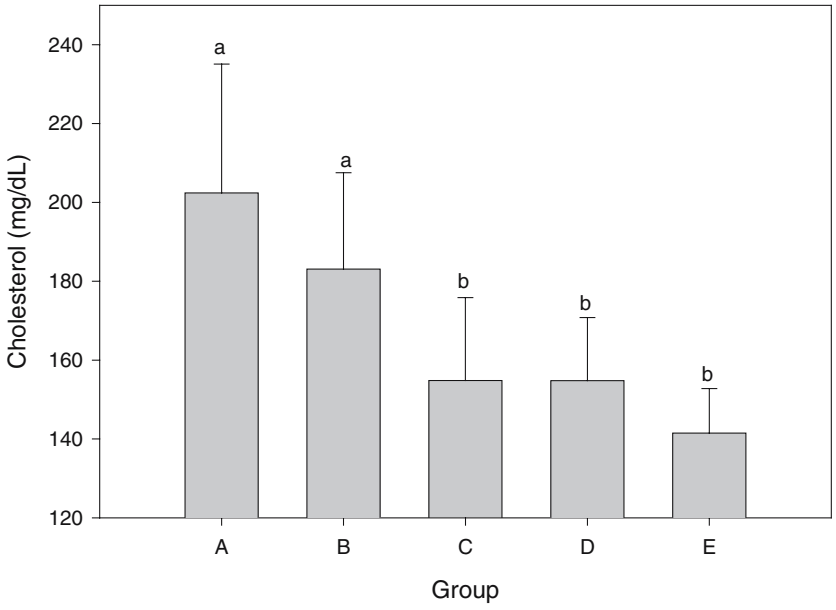

Fig. 1 Serum total cholesterol concentration of hamsters fed on high-cholesterol diet. Data are presented as mean±SEM (n=6). *A* Control group; *B* high-cholesterol diet + milk; *C* high-cholesterol diet + fermented milk containing *L. paracasei* subsp. *paracasei* NTU 101; *D* high-cholesterol diet + fermented milk containing *L. plantarum* NTU 102; *E* high-cholesterol diet + fermented milk containing *L. acidophilus* BCRC 17010.
[a,b]Values with different superscripts are significantly different at $p<0.05$

26.4%) and *L. plantarum* NTU 102 (group D; 23.5%). Although the diet of fresh milk (group B) also produced 8% cholesterol reduction, it was regarded as insignificant after statistical analysis.

The contents of other blood lipids (HDL-C, LDL-C, and TG) are shown in Table 3. Group B expressed the highest HDL-C concentration, followed by group A and then by groups C, D, and E. The difference among the last three groups was not significant. LDL-C contents in fermented-milk-feeding animals (groups C, D, and E) were significantly lower than those in animals fed on a diet without lactic acid bacteria (groups A and B). Group E (fed on diet containing *L. acidophilus* BCRC 17010) expressed the lowest LDL-C content with a reduction of 47.4% compared to the control group, followed by group C (*L. paracasei* subsp. *paracasei* NTU 101) with 32.9% reduction and then by group D (*L. plantarum* NTU 102) with 27.8% reduction. The group ranking in blood TG level was: E < C < D < A < B. The difference between groups C, D, and E and groups A and D appeared small in statistical significance. However, compared to group A, both groups C and E had an obvious reduction ($p<0.05$).

Table 3 Serum HDL-C, LDL-C, and TG contents of hamsters fed on high-cholesterol diet

Group	HDL cholesterol (mg/dl)	LDL cholesterol (mg/dl)	TG (mg/dl)
A	94.36±22.29[b]	80.34±20.50[a]	136.52±3.37[b]
B	106.42±5.05[a]	77.56±17.67[a]	162.50±28.36[a]
C	81.12±4.33[c]	53.93±13.17[b]	113.49±20.22[c]
D	73.45±9.75[c]	58.00±10.21[b]	129.70±18.64[b,c]
E	70.70±2.84[c]	42.26±6.97[b]	102.09±23.60[c]

A, B, C, D, and E conditions are the same as in Table 2
[a,b,c]$p<0.05$ is significantly different from A

Liver lipid analysis

Table 4 shows data on liver weight and liver lipid content. Liver weight remained within a range with little significant change in the hamsters either fed or not fed on high-cholesterol diet. The milk-feeding and fermented-milk-feeding animals (groups B, C, D, and E) had a great reduction in liver cholesterol content compared to the control group (group A). The fermented-milk-feeding groups C, D, and E expressed lower cholesterol contents than milk-feeding group B. Group C had the lowest cholesterol content. The group ranking in liver cholesterol content was: A > B > E > D > C. The difference between two particular groups was significant ($p<0.05$). In liver TG content, group A expressed a level higher than those of groups B, C, D, and E. However, there existed no significant difference among the four experimental groups ($p<0.05$).

Analysis of intestinal microflora

Figure 2 illustrates the cell counts of bacteria in the ceca of hamsters. In the cecum microflora, the cell numbers of lactic acid bacteria in groups C, D, and E (fed on

Table 4 Hepatic cholesterol and TG in experimental hamsters

Group	Liver weight (g)	Cholesterol (mg/g)	TG (mg/g)
A	6.23±0.79[a]	303.81±16.02[a]	109.50±17.14[a]
B	6.86±0.68[a,b]	295.44±13.48[a,b]	86.26±7.54[b]
C	6.63±0.59[a,b]	249.99±11.82[c]	87.81±18.81[b]
D	7.12±1.34[a,b]	255.61±12.73[c]	93.22±16.51[b]
E	6.31±0.72[a]	262.97±10.72[b,c]	99.62±23.93[a,b]

A, B, C, D, and E conditions are the same as in Table 2
[a,b,c]$p<0.05$ significantly is different from A

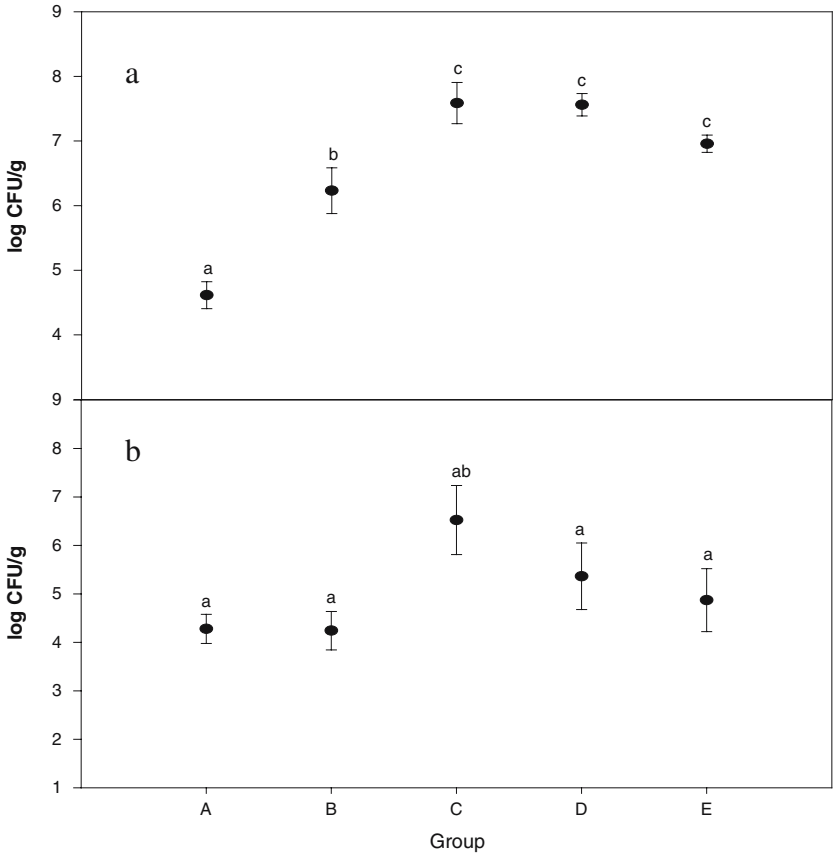

Fig. 2 Effect of nonfermented milk, fermented milk produced from *Lactobacillus*, and control group on lactobacilli and bifidus. **a** Count of *Lactobacillus* colony in cecum. **b** Count of *Bifidus* colony in cecum. *A*, *B*, *C*, *D*, and *E* conditions are the same as in Fig. 1. $^{a,b,c}p<0.05$ is significantly different from *A*

Lactobacillus-fermented milk) were greater than those in group B (fed on milk) and group A (fed on water) ($p<0.05$). Approximately, the cell numbers of lactic acid bacteria in the animals fed on fermented milk and in those fed on milk were 10^3 and 10^1 times the count in the control group, respectively. The difference was significant ($p<0.05$). The cell counts of *Bifidobacterium* in the cecum, ranging from 10^4 to 10^6, showed no significant difference in the animals fed and not fed on fermented milk ($p>0.05$). However, the colony number of group C (fed on fermented milk containing *L. paracasei* subsp. *paracasei* NTU 101) was slightly greater than those of the other groups. The group ranking in colony numbers was: C > D > E > A > B.

Fecal water content

In the middle (after 1 month) and at the end (after 2 months) of the feeding period, animal feces were collected, freeze-dried, and weighed to calculate the fecal water content. The result is shown in Fig. 3. Fecal water content can be used as an index of fecal elimination. Since the animals were fed on the same diet and water supply in the first feeding month, all five groups showed no significant difference in fecal water content ($p>0.05$). The fecal water content ranged between 15 and 30%, and the standard deviation (SD) of animals within the same group was low. At the end of the feeding period, as the supply of water was changed to a variety of substitutes, the fecal water content varied in the five groups. The group ranking in fecal water content was: D > E > B > C > A. The difference between group D (fed on milk fermented by *L. plantarum* NTU 102) and control group A was statistically significant. Although the fecal water content still ranged between 15 and 30%, the SD of animals within one group increased. This indicated that the variation of the group members was enlarged.

Discussion

High concentrations of cholesterol and LDL-C in the blood account for increasing risk for cardiovascular diseases. According to Frick et al. (1987), every 1% reduction in body cholesterol content lowers the risk for cardiovascular diseases by 2%. A change in dietary habit, such as eating fermented products containing lactic acid bacteria, can lead to cholesterol reduction. In this study, hamsters in which high blood cholesterol levels were induced with a cholesterol-rich diet were fed on milk fermented by *L. paracasei* subsp. *paracasei* NTU 101, *L. plantarum* NTU 102, and *L. acidophilus* BCRC 17010. The results showed that all the three *Lactobacillus* strains were effective in reducing cholesterol and LDL-C levels. This was in agreement with other studies (Harrison and Peat 1975; Grunewald 1982; Gilliland et al. 1985; Danielson et al. 1989). In the hamsters fed on skim milk, although blood cholesterol and LDL-C were also lowered, the difference was not significant

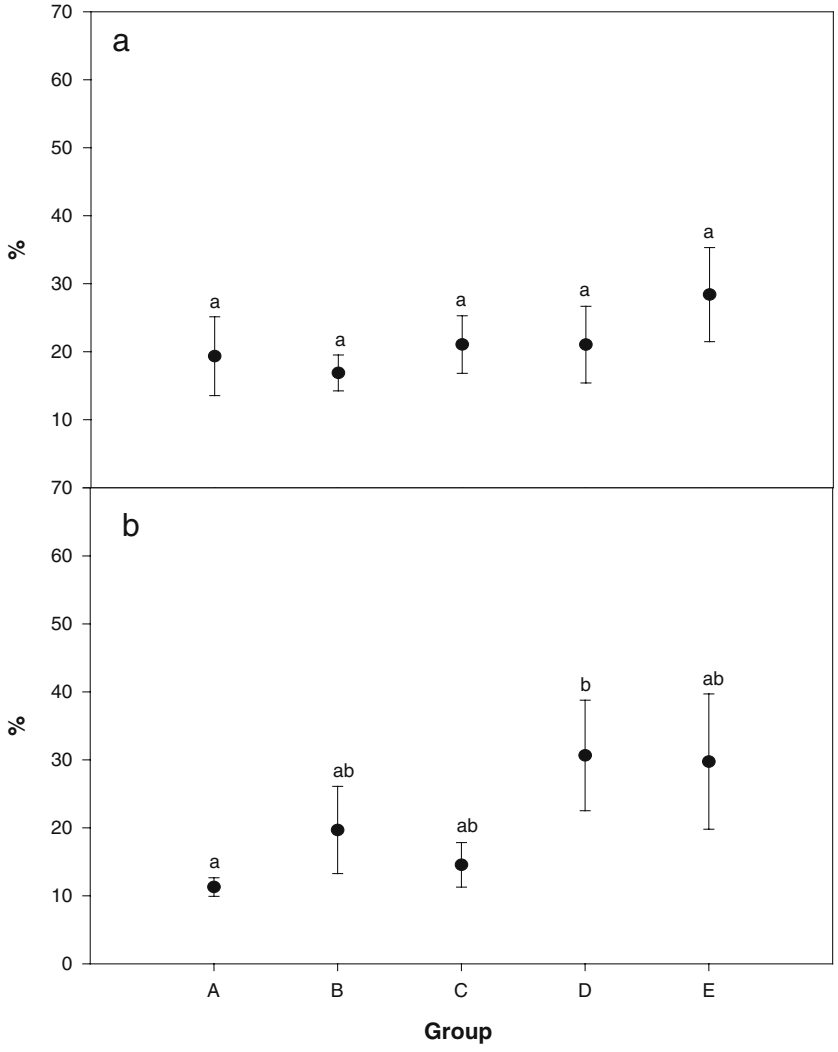

Fig. 3 Effect of nonfermented milk, fermented milk produced from *Lactobacillus* strains, and control group on fecal water content. **a** Water contained in feces during half of the feeding period. **b** Water contained in feces at the end of the feeding period. *A*, *B*, *C*, *D*, and *E* conditions are the same as in Table 2. $^{a,b}p<0.05$ is significantly different from *A*

compared to that of the control group. A similar result was also observed by Grunewald (1982) and Mann (1977).

In this study, HDL-C and TG contents were reduced in the hamsters fed on diet containing lactic acid bacteria. Similar results in humans and swines were also reported by Keim et al. (1981) and Rossouw et al. (1981). However, in a study performed by Hashimoto et al. (1999), a diet containing *L. casei* TMC 0409 was found to raise the concentration of HDL-C in the blood. Besides, Fukushima and Nakao (1996) indicated that no significant difference was found in the HDL-C content corresponding to supplement of probiotics, including *Lactobacillus* and *Streptococcus*, in lipid-rich and cholesterol-rich diets.

The cholesterol reduction produced by lactic acid bacteria can be explained by five mechanisms (Rao et al. 1981; Grunewald 1982; Suzuki et al. 1991; Fukushima and Nakao 1995; Beena and Prasad 1997; Hashimoto et al. 1998), as follows: (1) fermentation products of lactic acid bacteria inhibit the activity of enzymes for cholesterol synthesis and thus reduce cholesterol production; (2) the bacteria facilitate the elimination of body cholesterol in feces; (3) the bacteria inhibit the absorption of cholesterol back into the body by binding with cholesterol; (4) the bacteria interfere with the recycling of bile salt (a metabolic product of cholesterol) and facilitate its elimination, which raises the demand for bile salt made from cholesterol and thus results in body cholesterol consumption; and, (5) due to the assimilation of lactic acid bacteria, cholesterol in the host body is incorporated into the cell membrane or cell wall of bacteria to increase the resistance of bacterial cell membrane to environmental challenge; thus, the host cholesterol content is reduced.

The *Lactobacillus* strains used in this study (*L. paracasei* subsp. *paracasei* NTU 101, *L. plantarum* NTU 102, and *L. acidophilus* BCRC 17010) lowered the cholesterol content in the growth medium and increased incorporated cholesterol in the cell membrane when bile salt was added in an in vitro trial (data not shown). This indicated that these bacteria were effective in cholesterol reduction in the presence of bile salt. According to the study of Noh et al. (1997), the reduction in bile salt content was due to the activity of bile salt hydrolase in lactic acid bacteria. Bile salt is first hydrolyzed as bile acid, which is then incorporated into lactic acid bacteria, where bile acid is converted to cholesterol. Therefore, the mechanism of cholesterol reduction in the three *Lactobacillus* strains used in this study is likely to convert bile salt to free bile acid

through an enzymatic activity that deconjugates conjugated bile acid in the intestinal lumen. As bile acid is ready to bind cellulose or intestinal bacteria, it can be eliminated quickly (Chikai et al. 1987). This lowers the bile acid content and causes the liver to use cholesterol to produce bile acid, which is later converted to bile salt to meet the demands of lipid digestion and absorption. Thus, the outcome is reduction in body cholesterol. The three *Lactobacillus* strains used in this study reduced blood and liver cholesterol contents in the in vivo trial.

At the end of the feeding period, the cell counts of lactic acid bacteria in the cecum in groups C, D, and E were 10^3 times greater than that in the control group. This indicated that the *Lactobacillus* strains fed to the animals in this study could successfully tolerate gastric acid and bile salt, adhere to the intestinal wall, grow, and proliferate. The result was in agreement with a study of Usman and Hosono (2000). There was no significant difference in the colony numbers of *Bifidobacterium* among the five groups when *Lactobacillus* strains were not fed to the animals. Many studies have shown that lactic acid bacteria inhibit the proliferation of pathogenic bacteria, improve intestinal microflora, reduce the risk for digestive diseases such as diarrhea and ulcer, and promote the health of host animals.

From the effects of cholesterol reduction shown by the three *Lactobacillus* strains (*L. paracasei* subsp. *paracasei* NTU 101, *L. plantarum* NTU 102, and *L. acidophilus* BCRC 17010) in hamsters, it is deduced that the activity of bile salt hydrolase might be involved. The lactic acid bacteria could grow and proliferate in the cecum; thus, they might interfere with pathogenic bacteria in the stomach. Group D (fed on *L. plantarum* NTU 102) showed fecal water content higher than that of the control group, suggesting that the *Lactobacillus* strain might facilitate fecal elimination. Therefore, lactic acid bacteria may improve food digestion, food absorption, and fecal elimination in the host.

Conclusion

In this study, both *Lactobacillus* strains isolated from the human gut and pickled vegetables, respectively, were effective in reducing cholesterol in the blood and in the liver. We further plan to initiate a toxicity trial and a clinical trial to confirm the hypocholesterolemic effects of these *Lactobacillus* strains.

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