Hypocholesterolemic Effect of Indigenous Dadih Lactic Acid Bacteria by Deconjugation of Bile Salts*

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ABSTRACT : Administration of milk and fermented milks produced from indigenous dadih lactic acid bacteria on serum lipids and bile acids, fecal bile acids and microflora was estimated in hypercholesterolemic rats. Anaerobic lactic acid bacteria decreased and coliforms increased in the feces of the control group; however, the number of fecal lactic acid bacteria remained unchanged when rats were administered milk and fermented milks. Only fermented milk made from *Lc. lactis* subsp. *lactis* IS-10285 significantly reduced serum total cholesterol, LDL cholesterol and total bile acids. Milk and fermented milks did not influence the HDL cholesterol. Triglyceride and phospholipid levels were significantly lower in the rats fed fermented milk of *Lc. lactis* subsp. *lactis* IS-10285 than rats fed milk and fermented milk of *Lc. lactis* subsp. *lactis* IS-29862, but not significantly different from the control group. Hypocholesterolemic effect of *Lc. lactis* subsp. *lactis* IS-10285 was attributed to its ability to suppress the reabsorption of bile acids into the enterohepatic circulation and to enhance the excretion of bile acids in feces of hypercholesterolemic rats. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 12 : 1741-1745)***

Key Words : Hypocholesterolemic Effect, Dadih, *Lactococcus lactis*, Coronary Heart Disease

INTRODUCTION

Epidemiological, laboratory and clinical studies have shown a good correlation between high serum cholesterol and increased risk for the incidence of coronary heart disease. Reduction in total cholesterol and LDL cholesterol in hypercholesterolemic men reduces the incidence of cardiovascular disease (Lipid Research Clinics Program, 1984). Modification of diets such as ingestion of probiotic in the form of fermented milk is considered as a more natural way to decrease serum cholesterol in humans (Bazzacre et al., 1983). Mann and Spoerry (Mann, 1977) were the first to report the hypocholesterolemic effect of milk cultured with a wild type of *Lactobacilli ssp.* in Maasai tribes. This finding prompted further studies to elucidate the causative link between fermented milk and cholesterol levels. Grunewald (1982) reported the potential of fermented milk of *Lb. acidophilus* in reducing serum cholesterol in rats. Many other researchers reported hypocholesterolemic activity of fermented milk (Agarbæk et al., 1995; Akalin et al., 1997; Kawase et al., 2000). However others found that fermented milk had no effect on cholesterol levels (Thompson et al., 1982; McNamara et al., 1989; Sessions et al., 1997). The difference in experimental design and intakes of fermented milk or unsuitable cultures used in the experimental study might cause these contradictory results.

Dadih is an Indonesian traditional fermented milk which is produced by pouring fresh raw unheated buffalo milk into a bamboo tube, capped with banana leave and allowed at room temperature for 2 days until the formation of yogurt-like texture. Lactic acid bacteria (LAB) involved during fermentation of this product were *Leuconostoc sp.*, *Streptococcus sp.*, *Lactobacillus sp.* and a small amount of yeast (Hosono et al., 1989; Surono, 2003). *In vitro* study results show that dadih LAB have potential health benefits such as antimutagenic, cholesterol binding and antipathogenic bacterial activities (Hosono et al., 1989; Hosono and Tono-oka, 1995; Surono and Hosono, 1996; Surono, 2000; Surono and Nurani, 2001; Usman, 2003). To be used as candidate probiotics, the cultures must meet some criteria viz. resistance to lysozyme in the oral cavity, acid in the stomach and bile in the small intestine. Among newly 10 dadih isolates screened, *Lc. lactis* subsp. *lactis* IS 10285 and *Lc. lactis* subsp. *lactis* IS 29862 were found to possess such activities as mentioned above and taurocholate-deconjugating abilities (Surono, 2003). Taranto et al. (1998) reported that a culture of *Lb. reuteri* with high bile-salt deconjugating activities is found to have a hypocholesterolemic effect in hypercholesterolemic mice.

The present study was carried out to evaluate the effect of administration of fermented milk made from the two selected strains of dadih LAB, namely *Lc. lactis* subsp. *lactis* IS-10285 and *Lc. lactis* subsp. *lactis* IS-29862 on serum lipids and total bile acids, and fecal microflora in hypercholesterolemic rats.

* Supported by the Ministry for Research and Technology of the Republic of Indonesia in the form of Indonesian International Joint Research Program.

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Received February 13, 2004; Accepted July 2, 2004

MATERIALS AND METHODS

Source and maintenance of cultures

Lc. lactis subsp. *lactis* IS-10285 and *Lc. lactis* subsp. *lactis* IS-29862 which have high taurocholate-deconjugating activities used in this study were isolated from dadih by Surono and Nurani (2001), and kept in our stock culture collection (Center the Assessment and Application of Biotechnology, Tangerang, Indonesia). These two cultures were maintained by subculture in MRS broth using 1% inocula and 18 h of incubation at 37°C, freeze-dried and stored at -20°C.

Preparation of fermented milk

Each culture was first subcultured in 10% sterilized skim milk (SKM). Then the reconstituted skim milk was inoculated aseptically with 1% (v/v) inoculum of each of the active cultures, incubated at 37°C for 18 h and transferred immediately to 4°C until experimental use. Number of viable in the fermented milk was around 2.2-2.7×10^8cfu/ml.

Rats and diets

Twenty male Sprague-Dauley (SD) rats were obtained at the age of four weeks. The rats were fed a commercial powdered chow (Clea Japan Inc., Tokyo, Japan) for two days. After this adaptation period, rats were divided into four groups of five each. Rats were individually housed in metal cages in a room with controlled temperature (22±2°C) and humidity (56±5%) and maintained in a cycle of 12 h of light and 12 h of dark. The composition of cholesterol-enriched diet was (g/100 g): casein 20, safflower oil 10, vitamin mixture (AIN-76; American Institute of Nutrition, 1977) 1, mineral mixture (AIN-76; American Institute of Nutrition, 1977) 4, choline chloride 0.2, sodium cholate 0.12, cellulose powder 2, sucrose 62.17 and cholesterol 0.5.

Group 1 received cholesterol-enriched diet plus water, group 2 received cholesterol-enriched diet plus skim milk, group 3 received cholesterol-enriched diet plus fermented milk of *Lactococcus lactis* subsp. *lactis* IS-29862, and group 4 received cholesterol-enriched diet plus plus of *Lactococcus lactis subsp. lactis* IS-10285. Skim milk and fermented milks in the form of drink type and water were freely available and the rats receive their assigned diets for *ad libitum* intake for 12 d. Food intake is recorded daily, and body weight is recorded at the beginning and end of the study. For the assay of fecal LAB and coliforms, fresh samples were collected at d 0 and 12 by gentle squeezing the rectal part of rats and put into sterile test tubes in anaerobic jars, then analyzed within 30 min.

For determination of fecal bile acids, fecal samples were collected for the last 2 days, freeze-dried and then stored at -20°C until analysis. At the 12 d feeding period, the rats were deprived of food for 12 h and then anesthetized by diethyl ether. Blood samples were collected from the ventral artery of the rat tail, placed in sterile tubes, and centrifuged for 20 min at 3,000 rpm. The obtained serum samples were analyzed for the total cholesterol, HDL cholesterol, triglycerides and total bile acids.

Assay for fecal microflora

To determine the total anaerobic lactic acid bacteria, the obtained samples were homogenized in 0.067 M phosphate buffer saline (PBS), pH 6.8 on a Vortex mixer for 4 min. Then the homogenized samples were diluted in PBS and plated on MRS agar (Sreekumar and Hosono, 2000). The plates were incubated anaerobically for 48 h in a Gaspak hydrogen-carbondioxide anaerobic system. The number of fecal coliforms was determined on violet red bile agar (Usman and Hosono, 2000). The plates were incubated at 37°C for 24 h and the colonies were counted with a colony counter. The results were reported as log 10 of count per gram of wet weight of feces.

Assay for serum lipids

Serum total cholesterol was measured enzymically with Total Cholesterol Kit (Determiner TC5555; Kyowa Medics, Tokyo, Japan). HDL cholesterol were assayed by HDL Cholesterol Kit, triglycerides by Triglycerides Kit and total bile acids by Total Bile Acids Kit (HDL Cholesterol Test Wako, Wako Junyaku; Triglycerides G Test Wako, Wako Junyaku and Total bile acids Tests Wako, Wako Junyaku, Osaka, Japan respectively). LDL cholesterol was calculated by difference between total cholesterol and HDL cholesterol.

Assay for fecal bile acids

The total bile acids were determined following the methods of Hashimoto et al. (1999). Freeze-dried feces (0.1 g) were extracted with 2.5 ml ethanol at 80°C for 1 h. After two extractions, the ethanol was evaporated under N_2 gas at 50°C, and residue was dissolved in 2.5 ml ethanol. The total bile acids in feces were analyzed by commercial test kit (Enzabile II, Daiichi Kagaku Yakuhin, Tokyo, Japan).

Statistical analysis

The data were analyzed by the ANOVA procedure from StatView (Haycock et al., 1992). The least significant difference procedure was used to determine if statistically significant differences occurred among means.

RESULTS

The effect of dietary milk and fermented milks of dadih LAB on weight gain and food intake in hypercholesterolemic rats is shown in Table 1. Rats fed

Table 1. Effect of dietary milk and milk cultured with dadih lactic acid bacteria on weight gain and feed intake in rats fed cholesterol-enriched diets

Treatment groups	Weight gain (g)	Feed intake (g/d)	Feed efficiency[4]
Control	94.3^{ab}	14.1^{a}	6.65^{b}
SKM[1]	97.5^{a}	12.4^{b}	7.87^{a}
FM-IS 29862[2]	82.4^{ab}	12.7^{ab}	6.45^{b}
FM-IS 10285[3]	79.8^{b}	12.9^{ab}	6.16^{b}

a, b Mean in the same column with different superscript letters differ ($p<0.05$).
[1] SKM: Skim milk.
[2] FM-IS 29862: Fermented milk made from *Lactococcus lactis* subsp. *lactis* IS 29862.
[3] FM-IS 10285: Fermented milk made from *Lactococcus lactis* subsp. *lactis* IS 10285.
[4] Feed efficiency: Weight gain/feed intake.

Table 2. Effect of dietary milk and milk cultured with dadih lactic acid bacteria on fecal Coliforms in rats fed cholesterol-enriched diets

Treatment groups	Before treatment (log cfu/g)	After treatment (log cfu/g)
Control	7.08^{a}	9.43^{b}
SKM[1]	7.55^{a}	9.41^{b}
FM-IS 29862[2]	7.33^{a}	9.39^{b}
FM-IS 10285[3]	7.57^{a}	9.63^{b}

a, b Mean in the same column with different superscript letters differ ($p<0.05$).
[1] SKM: Skim milk.
[2] FM-IS 29862: Fermented milk made from *Lactococcus lactis* subsp. *lactis* IS 29862.
[3] FM-IS 10285: Fermented milk made from *Lactococcus lactis* subsp. *lactis* IS 10285.

milk and dadih fermented milks showed no significant ($p>0.05$) different in weight gain with those in the control group. Rats received fermented milk of strain IS-10285 had significantly ($p<0.05$) lower body weight gain than rats received the milk group. The feed intake was greater in the control group than the milk group, but not significantly ($p>0.05$) higher than the fermented milk groups. The feed efficiency in rats fed milk was greater than the other groups.

Table 2 and 3 show the effect of milk and fermented milks of dadih LAB on fecal coliforms and lactic acid bacteria, respectively. At d 12 the number of coliforms increased significantly ($p<0.05$) by 2 log cycles in rats fed cholesterol-enriched diets with or without milk and fermented milks (Table 2). The number of fecal anaerobic LAB decreased significantly ($p<0.05$) by about 1 log cycle for rats in the control group. Fecal LAB count remained unchanged when rats were fed milk and dadih fermented milks (Table 3).

The results of serum lipid levels were shown in Table 4. The serum total cholesterol in rats fed fermented milks of strain IS-10285 was significantly ($p<0.05$) than that of the control group. Feeding rats with milk and fermented milk of strain IS-29862 slightly reduced the serum total cholesterol,

Table 3. Effect of dietary milk and milk cultured with dadih lactic acid bacteria on fecal lactic acid bacteria in rats fed cholesterol-enriched diets

Treatment groups	Before treatment (log cfu/g)	After treatment (log cfu/g)
Control	^{x}9.88	^{y}8.82^{b}
SKM[1]	^{x}9.74	^{x}9.21^{ab}
FM-IS 29862[2]	^{x}10.13	^{x}10.12^{a}
FM-IS 10285[3]	^{x}10.03	^{x}9.98^{a}

a, b Mean in the same column with different superscript letters differ ($p<0.05$).
x, y Mean in the same row with different superscript letters differ ($p<0.05$).
[1] SKM: Skim milk.
[2] FM-IS 29862: Fermented milk made from *Lactococcus lactis* subsp. *lactis* IS 29862.
[3] FM-IS 10285: Fermented milk made from *Lactococcus lactis* subsp. *lactis* IS 10285.

but the reduction was statistically not different ($p>0.05$). Also no significant ($p>0.05$) difference in HDL among rats fed milk, fermented milks and the control group was observed. LDL cholesterol of rats that were given fermented milk of strain IS-10285 had a significantly ($p<0.05$) lower than that of the control group, but was not significantly ($p>0.05$) lower than that of the milk and strain IS-29862 fermented milk groups.

Triglyceride levels in rats fed fermented milk of strain IS-10285 decreased significantly ($p<0.05$) as compared to the milk and strain IS-29862 fermented milk groups. Fermented milk of strain IS-10285 reduced the concentration of LDL cholesterol and consequently decreased the atherogenic index value.

The effect of milk and fermented milks on phospholipid levels is shown in Table 4. Phospholipid levels were significantly ($p<0.05$) lower in the strain IS-10285 fermented milk group than the milk and strain IS-29862 fermented milk groups. Rats fed fermented milk of strain IS-10285 had lower serum phospholipids than that of the control group but the decrease was not significant ($p<0.05$).

Fermented milk made from strain IS-10285 significantly ($p<0.05$) reduced the serum total bile acids. Feeding milk and fermented milk of strain IS-29862 had no effect on serum total bile acids of hypercholesterolemic rats. Total bile acid secretion was significantly ($p<0.05$) higher in rats fed fermented milk of strain IS-10285 than the milk group, but not significantly ($p>0.05$) different than that of the strain IS-29862 fermented milk and control group (Table 4).

DISCUSSION

The present study evaluated the effect of feeding fermented milk made from dadih indigenous LAB on the serum lipids in hypercholesterolemic rats. Reduction in serum total cholesterol and LDL cholesterol levels after 12 d of feeding trials was observed in rats fed fermented milk

Table 4. Effect of dietary milk and milk cultured with dadih lactic acid bacteria on serum lipids, phospholipids, total serum and fecal bile acids in rats fed cholesterol-enriched diets

Treatment groups	Total cholesterol (mg/dl)	HDL cholesterol (mg/dl)	LDL[1] cholesterol (mg/dl)	Triglycerides (mg/dl)	AI[2]	Phospholipids (mg/dl)	Total serum bile acids (µmol/l)	Fecal total bile acids (mol/rat/day)
Control	276.62[a]	26.97	232.24[a]	79.15[b]	9.88[a]	133.20[ab]	91.04[a]	3.34[ab]
SKM[3]	204.05[ab]	25.49	150.52[ab]	140.17[a]	6.95[ab]	154.68[a]	70.25[a]	2.92[b]
FM-IS 29862[4]	223.97[ab]	24.49	154.60[ab]	138.13[a]	8.18[ab]	174.78[a]	67.19[a]	3.43[ab]
FM-IS 10285[5]	132.49[b]	23.94	91.75[b]	57.02[b]	3.38[b]	105.42[b]	36.27[b]	4.18[a]

[a, b] Mean in the same column with different superscript letters differ ($p<0.05$).
[1] LDL cholesterol: Total cholesterol-HDL cholesterol-triglycerides/5.
[2] AI (Atherogenic index): LDL cholesterol/HDL cholesterol. [3] SKM: Skim milk.
[4] FM-IS 29862: Fermented milk made from *Lactococcus lactis* subsp. *lactis* IS 29862.
[5] FM-IS 10285: Fermented milk made from *Lactococcus lactis* subsp. *lactis* IS 10285.

cultured with *Lc. lactis subsp. lactis* IS-10285. Some researchers (Danielson et al., 1989; Grunewald, 1982) have previously reported similar results on the hypocholesterolemic effect of fermented milk and unfermented milk. The rats receiving fermented milk made from *Lc. lactis subsp. lactis* IS-29862 showed a trend to decrease serum total cholesterol and LDL cholesterol, however the reduction was not significant. The same phenomena were reported by Thompson et al. (1982). These complicting results may be due to the difference in strains or unsuitable cultures used in the experimental studies. Dietary milk had no effect on total cholesterol and LDL cholesterol in agreement with the findings by Mann (1977) and Usman and Hosono (Usman and Hosono, 1999; Usman and Hosono, 2000). Dietary milk and fermented milks made from dadih LAB did not influence the serum HDL cholesterol levels. This is in agreement with the findings reported by Fukushima and Nakao (1996) and Kawase et al. (2000).

Total count of fecal LAB decreased while fecal coliforms increased in rats fed cholesterol-enriched diets. We observed the same phenomenon in our previous study (Usman and Hosono, 2000; Usman and Hosono, 2001). However, supplementation of cholesterol-enriched diets with milk and fermented milk could maintain the high levels of fecal anaerobic LAB till the end of experimental period. This may be attributed to certain compounds in milk that can stimulate the growth of intestinal LAB or due to the acid and bile tolerance of ingested dadih LAB.

Despite the acid and bile tolerance of dadih isolates, the results from present study showed that strain IS-10285 exhibited hypocholesterolemic effect in rats, however, no effect was found in the rats receiving fermented milk made from strain IS-29862. The inability of the latter strain to reduce serum cholesterol and bile acids was due to its low capability to colonize the intestinal tract (Lee, 2002). The great number of LAB observed in the feces of rats fed fermented milk made from strain IS-29862 may resulted from its resistance to acid and bile (Surono, 2003) that enable this strain to survive passage through the intestinal tract.

Meanwhile, strain IS-10285 might be able to adhere to the intestinal cell lines of rats, grow and perform its beneficial health effects such as deconjugation of bile salts. *In vitro* study showed that this strain produce enzyme called bile salt hydrolase or BSH (Surono, 2003). Deconjugation of bile salts by BSH produced by this strain resulted in an increased production of deconjugated bile acids. Deconjugated bile acids are less well absorbed from the small intestine than the conjugated bile acids (Schiff et al., 1972). Thus the amount of bile acids returned to the liver during enterohepatic circulation decreased. This fact is in agreement with the present finding. Deconjugated bile acids are also excreted more rapidly than conjugated bile acids and they bind more easily to the dietary fiber and intestinal bacteria than conjugated bile acids (Chikai et al., 1987). This fact is also supported our present finding. Fecal loss of bile acids may indeed result in an increased requirement of cholesterol as a precursor for the synthesis of new bile acids. As a consequence, the total cholesterol levels in the body were decreased.

In this study, it is found that rats received fermented milk made from strain IS-10285 had significantly lower body weight gain than rats received milk. The similar result was also obtained in our previous finding (Usman and Hosono, 2000). The present results also exhibited no significant difference in feed intake among groups fed received milk, fermented milk of IS-29862 and fermented milk of IS-10285. However, feed efficiency in rats fed milk was significantly higher than that in rats fed fermented milks of strain IS-29862 and IS-10285. It is presumed that there may be a certain compound in milk that can stimulate the growt of rats, and that compound was degraded by dadih LAB during fermentation. This presumption and its relation to serum profile should be further studied.

In conclusion, fermented milk made from *Lc. lactis* subsp. *lactis* IS 10285 could exert hypocholesterolemic effect in rats fed a cholesterol-enriched diet. The cholesterol-lowering activity was attributed to its active deconjugation of bile salts in the small intestine, which resulted in reduction of bile acids returned to the liver and increase total bile acids excreted through the feces.

ACKNOWLEDGEMENTS

This study was financed by grants from the Ministry for Research and Technology of the Republic of Indonesia in the form of Indonesian International Joint Research Program. and supported by Shinshu University of Japan by means of laboratory facilities.

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