Hypocholesterolemic Effects of *Lactobacillus casei* subsp. *casei* TMC 0409 Strain Observed in Rats Fed Cholesterol Contained Diets

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Abstract In order to evaluate the hypocholesterolemic effect of *Lactobacillus casei* subsp. *casei* TMC 0409 strain (TMC 0409), an isolate from 'Dadih', an Indonesian traditional fermented dairy food, Sprague-Dawley rats were fed cholesterol containing diets supplemented with fermented milk cultured with TMC 0409, skim milk and TMC 0409 lyophilized cells. No significant inhibitory effect was observed in the rats fed fermented milk and skim milk after 14 days feeding. But the increase of serum cholesterol was suppressed (36%) in the rats fed TMC 0409 lyophilized cells. Total cholesterol, HDL-cholesterol, triglyceride and phospholipid in serum, neutral steroid and bile acids in feces, cholesterol and triglyceride in liver were analyzed in the rats fed TMC 0409 cells after 14 days feeding. Total cholesterol and phospholipid were significantly decreased compared to those of control group ($P < 0.01$), however HDL-cholesterol was significantly increased ($P < 0.05$). Serum triglyceride was also decreased but the difference was not significant. From these results, the inhibition of increase of serum cholesterol in rats fed TMC 0409 cells was considered to have resulted from the reduction of LDL + VLDL-cholesterol. Meanwhile, the accumulation of triglyceride in the liver was inhibited significantly compared with the control group ($P < 0.05$), and the excretion of bile acid in the feces was also enhanced significantly ($P < 0.01$). These results imply that strain TMC 0409 can alter the serum cholesterol concentration, especially LDL + VLDL-cholesterol by enhancing the excretion of bile acids in the feces.

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There is an apparent association between the high serum cholesterol and the development of coronary heart diseases. Therefore, keeping serum cholesterol in desirable level in the normal diet life is considered a practical preventive method for these diseases.

Fermented milk products has been well known for their benefits in the maintenance of human health and well being²¹⁾. Since Mann and Sperry's¹³⁾ initial in-

vestigation in 1974, many studies have also been conducted to evaluate the hypocholesterolemic effect of fermented milks in animal and human^{1, 4, 8, 18-20)}. However, the results of these studies were not consistent. The hypocholesterolemic effect of the tested fermented milks greatly varied with the difference of lactic acid bacteria flora involved in these fermented milks. In order to obtain the expected

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hypocholesterolemic effect of fermented milk, utilization of the selected lactic acid bacteria, well characterized *in vitro* and *in vivo*, in fermented milk is necessary.

Lactobacillus casei subsp. *casei* TMC 0409 strain (TMC0409) was isolated from Dadih, an Indonesian traditional fermented dairy food. In the previous comparative studies with our stock culture lactic acid bacteria, this bacterium exhibited the highest possibility to alter the serum cholesterol in rats⁶⁾. However, the hypocholesterolemic effect of TMC 0409 should be more characterized, as its related mechanism are still unclear.

In the present study, fermented milk cultured with strain TMC 0409, skim milk and TMC 0409 cells were compared for their abilities to alter the serum cholesterol level of the rats fed with cholesterol containing diet. Among the tested rats, the cholesterol concentration in the serum, liver and stool were analyzed in the rats fed TMC 0409 lyophilized cells.

Materials and Methods

Bacterial Strain

TMC 0409 strain was originally isolated from an Indonesian fermented dairy food 'Dadiah' and identified as *Lactobacillus casei* subsp. *casei* based on the phenotype characters according to Bergey's Manual of Systemic Bacteriology (1986). Strain TMC 0409 is deposited in the culture collection of the laboratory of Takanashi Milk Products, Yokohama, Japan.

Preparation of lyophilized cells

TMC 0409 was inoculated into Lactobacilli MRS broth (Difco) and incubated at 37°C for 18 hours. After incubation, cells were harvested by centrifuging at 10,000×g for 10 min, washed twice in sterile distilled water and then freeze-dried using the method described by Kozaki *et al.* 1986.

Preparation of fermented milk

The reconstituted skim milk (no fat solid : 8.0% ; sugar 8.0%) containing 0.1% of soy peptide (Hainyuto-SMP, Fujiseiyu, Osaka, Japan) was pasteurized at 80°C and cooled to 37°C. After inocu-

Table 1. Composition of experimental diets

Constituent	Diets (g/100 g)			
	Control	Diet 1 ³⁾	Diet 2 ⁴⁾	Diet 3 ⁵⁾
Casein	20	20	20	20
Safflower oil	10	10	10	10
Vitamin mixture ¹⁾	1	1	1	1
Mineral mixture ²⁾	4	4	4	4
Choline chloride	0.2	0.2	0.2	0.2
Cellulose powder	2	2	2	2
Cholesterol	0.5	0.5	0.5	0.5
Sodium cholate	0.125	0.125	0.125	0.125
Sucrose	62.175	57.175	57.175	57.175
Fermented milk	0	5	0	0
Skim milk	0	0	5	0
Bacterial cells	0	0	0	5
Total	100	100	100	100

^{1),2)} ; AIN-76

³⁾ Diet 1 : TMC 0409 fermented milk

⁴⁾ Diet 2 : Skim milk

⁵⁾ Diet 3 : TMC 0409 lyophilized cells

lation with 3% TMC 0409 culture, the skim milk was incubated at 37°C for 18–20 hours until pH became 4.5. The fermented milk was kept at 4°C, until use.

Diets

Composition of diets is shown in Table 1. Cholesterol containing diet which included 0.5% cholesterol and 0.125% sodium cholate, was used in this study based on the method of Nagata *et al.*¹⁶⁾. Fermented milk, skim milk and bacterial cells were added to the control diet at a level of 5%, replacing an equal amount of sucrose.

Animals

Experimental animals consisted of 4-week-old male Sprague-Dawley rats (Japan Clea, Tokyo, Japan). After feeding standard diets (CA-1, Japan Clea, Tokyo, Japan) for one week, the rats were grouped according to their body weights (ten rats in each group) and housed in individual cages in an air conditioned room at $24 \pm 1^\circ\text{C}$ with $55 \pm 5\%$ humidity under a 12 h light cycle. The animals were given their respective diets and allowed tap water *ad libitum*. During the test period, body weight and intake of diets were recorded everyday. Rats were bled from orbital vein at 0 and abdominal aorta at 14 days for serum lipid analysis after fasting overnight. The liver was

excised immediately, weighed and stored at -20°C until lipid analysis. Feces were collected on the last 4 days of the experimental period and freeze-dried for 18 hours.

Analyses of serum and liver lipids

Total serum cholesterol concentration, triglycerides, HDL-cholesterol and phospholipid were analyzed using Cholesterol E Test Wako, Triglyceride G Test Wako, HDL-cholesterol Test Wako and Phospholipid C Test Wako (Wako pure chemical, Tokyo, Japan), respectively. Lipids from the liver was extracted as described by Folch *et al.*²⁾, and analyzed as noted above.

Analysis of fecal steroids

Aliquots of freeze-dried feces (0.1 g) were suspended in 2.5 ml hot ethanol in a sealed tube and extracted at 80°C for one hour. After two extractions, the supernatant fraction was evaporated under nitrogen gas at 50°C and redissolved in 2 ml ethanol. Concentrations of cholesterol and coprostanol in the feces were measured by gas-liquid chromatography (GLC). All GLC analyses were performed on an instrument equipped with a hydrogen flame ionization detector (G-3000, Hitachi seisakusyo, Tokyo, Japan). The columns were 30 m glass tubes, 0.25 mm *i.d.*

Fig. 1. Inhibitory effect of lactic acid bacteria containing diet on the increase of serum total cholesterol levels in Rats.

^{a)} Diet 1 : TMC 0409 fermented milk, ^{b)} Diet 2 : Skim milk, ^{c)} Diet 3 : TMC 0409 cells.

^{d)} Inhibition (%) = $[(\text{Control-Diet})/\text{Control}] \times 100$

Asterisks indicate significant difference from the control of table-1 (* $P < 0.01$).

(DBTM-17, J & W Scientific, CA, USA). The column temperature were 240°C, the temperature of the flash heater was 250°C and of the detector was 300°C. He-gas was used as carrier gas at the flow rate of 43 cm/sec. Fecal bile acid was measured by commercial test kit (Enzabale II, Daiich Kagaku Yakuhin, Tokyo, Japan).

Statistical analysis

Experimental data is expressed as mean $\pm$ S.E. Difference among groups was conducted using Student's *t* test.

Results

Inhibitory effects of TMC 0409 fermented milk, skim milk and TMC 0409 cells on the increase of serum cholesterol level

In the present study, the body weight did not differ significantly among each group, although the three experimental groups exhibited higher weight gain than the control group.

After 14 days feeding, only slightly inhibitory effects on the increase of serum cholesterol level were observed in the rats fed fermented milk (4.4×10^8 cfu/ml) prepared with TMC 0409 and skim milk. However, the increase in total cholesterol was suppressed to 36% in the rats fed cholesterol containing diet supplemented with TMC 0409 cells (3.4×10^{10} cfu/g) compared to the control (Fig. 1).

Serum cholesterol of rats fed TMC 0409 cells

After 14 days feeding, serum total cholesterol, HDL-cholesterol, triglyceride, phospholipid concentration were analyzed in the rats fed cholesterol containing diets supplemented with or without TMC 0409 cells (Table 2). The total cholesterol level and phospholipid of the rats fed TMC 0409 cells were significantly ($P < 0.01$) lower than those of the control group. The HDL-cholesterol levels were significantly ($P < 0.05$) increased compared to control group. The triglyceride levels were decreased but the difference was not significant between the two groups.

Table 2. Food intake, body weights, serum total cholesterol, HDL-cholesterol, triglyceride and phospholipid concentrations of rats fed TMC 0409 cells

	Groups	
	Control (n=10)	Diet 3 (n=10)
Food intake 14 days (g/d)	19.85 $\pm$ 0.79 ¹⁾	20.60 $\pm$ 0.65
Body weight gain (g/14 d)	114.40 $\pm$ 2.61	118.70 $\pm$ 2.53
Total cholesterol (mg/dl)		
0 day	82.89 $\pm$ 3.14	76.27 $\pm$ 4.43
14 day	242.91 $\pm$ 10.53	155.80 $\pm$ 11.15**
HDL-cholesterol (mg/dl)		
0 day	32.16 $\pm$ 0.95	34.04 $\pm$ 1.79
14 day	22.78 $\pm$ 0.76	26.25 $\pm$ 0.66*
Triglyceride (mg/dl)		
0 day	119.22 $\pm$ 14.74	100.88 $\pm$ 10.84
14 day	191.80 $\pm$ 11.44	172.15 $\pm$ 15.86
Phospholipid (mg/dl)		
0 day	137.94 $\pm$ 5.52	123.64 $\pm$ 3.97
14 day	184.17 $\pm$ 5.09	133.04 $\pm$ 5.55**

¹⁾ Values are means $\pm$ SEM.

Asterisks indicate significant difference from the control (* $P < 0.05$,

** $P < 0.01$).

Table 3. Liver weight and lipid levels in rats fed TMC 0409 cells

	Groups	
	Control (n = 10)	Diet 3 (n = 10)
Liver weight (g/100 g B.W.)	4.384 ± 0.082 ¹⁾	4.747 ± 0.104*
Cholesterol (mg/wet g)	32.86 ± 19.70	24.80 ± 17.26
Triglyceride (mg/wet g)	82.40 ± 18.61	61.34 ± 12.01*

¹⁾ Values are means ± SEM.

Asterisks indicate significant difference from the control (* P < 0.05).

Table 4. Fecal neutral steroid and bile acid levels in rats fed TMC 0409 cells

	Groups	
	Control (n = 10)	Diet 3 (n = 10)
Cholesterol (μmol/rat/day)		
Initial	16.763 ± 0.570 ¹⁾	18.037 ± 0.938
Final	31.879 ± 3.293	38.380 ± 2.281
Coprostanol (μmol/rat/day)		
Initial	17.445 ± 1.008	19.819 ± 1.335
Final	13.472 ± 1.277	7.979 ± 1.370**
Neutral steroid (μmol/rat/day)		
Initial	34.208 ± 1.335	37.856 ± 1.877
Final	45.352 ± 3.415	46.359 ± 2.788
Bile acid (μmol/rat/day)		
Initial	12.004 ± 1.080	13.221 ± 0.941
Final	13.547 ± 1.243	23.167 ± 1.581**
Total steroid (μmol/rat/day)		
Initial	46.212 ± 2.137	51.077 ± 1.971
Final	58.900 ± 4.551	69.525 ± 2.830

¹⁾ Values are means ± SEM.

Asterisks indicate significant difference from the control (** P < 0.01).

Liver weight and cholesterol of rats fed TMC 0409 cells

The results of liver weight and lipid levels of the rats fed TMC 0409 cells are presented in Table 3. The liver weight per body weight was significantly higher in the rats fed TMC 0409 cells than the control group (P < 0.05). There was no significant difference in the levels of cholesterol between the two groups, whereas triglyceride level was significantly lower in the rats fed TMC 0409 cells than the control group (P < 0.05).

Fecal steroid of rats fed TMC 0409 cells

After 14 days feeding, the fecal neutral steroid and bile acid concentration of the rats fed TMC 0409 cells were analyzed (Table 4). No significant difference was observed in fecal neutral steroid between rats fed TMC 0409 cells and control group. However, bile acid in the feces was significantly higher in the rats fed TMC 0409 cells compared to that of the control group (P < 0.01).

Discussion

'Dadih' is a popular fermented dairy product in West Sumatra of Indonesia, which has been consumed by local people and are generally believed to possess health promoting effects on human being. *Lactobacillus casei* subsp. *casei* TMC 0409 strain (TMC 0409) was isolated from Dadih. Our previous studies on the potent ability of lactic acid bacteria to influence serum cholesterol metabolism revealed that TMC 0409 cells suppressed the increase of serum total cholesterol more effectively than other tested lactic acid bacteria⁶⁾. In the present study, TMC 0409 fermented milk and skim milk were tested for their abilities to alter serum cholesterol of the rats fed cholesterol containing diet. The fermented milk prepared with TMC 0409 strain and skim milk did not show significant inhibitory effects on the increase of serum cholesterol of the rats fed cholesterol containing diet, whereas the TMC 0409 cells preparation significantly suppressed the increase of serum total cholesterol of rats fed cholesterol containing diet after 14 days of administration, which are consistent with our previous study⁶⁾. These results indicated that the hypocholesterolemic effect is mainly contributed by strain TMC 0409 cells. In the present study, the impact of TMC 0409 cells on serum cholesterol metabolism of the rats fed cholesterol containing diet was evaluated after 14 days of intervention. Serum HDL-cholesterol was significantly increased, meaning that the decrease of total serum cholesterol by TMC 0409 cells is considered to result from the reduction of serum LDL + VLDL-cholesterol. These results indicate that TMC 0409 strain can alter serum LDL + VLDL-cholesterol, as do other health promoting lactic acid bacteria¹⁾. Moreover, the risk of arteriosclerosis is inversely proportional to the level of HDL. Thus, this strain may have a significant role in human health care.

The cholesterol concentration in serum is greatly influenced by the metabolism of bile acid and cholesterol in the intestine and liver. Dietary fiber and anion exchange resins can alter the serum cholesterol of human and animal with their capacity to bind bile acids and excrete them through feces^{10-12, 17, 22)}. The

hypocholesterolemic effects of some lactic acid bacteria were also found to be related to their capacity to bind bile acids and cholesterol in their cells^{5, 9, 14)}. Furthermore, the cholesterol-lowering effect of dietary fiber is also related to fermentation of the fed dietary fiber in the intestine by intestinal bacteria, which lead to the lowering of lumen pH and insulation of bile acid⁷⁾. Some lactic acid bacteria have been found to survive in human intestine and alter intestinal environment such as pH and enzyme profile by their metabolism³⁾. These knowledge and findings lead to a hypothesis that lactic acid bacteria could exhibit practical hypocholesterolemic effects if they have a high ability to colonize in human intestine and bind with bile acid and cholesterol. In the previous study on screening lactic acid bacteria, TMC 0409 strain was found to possess the highest ability to bind bile acid *in vitro* than other stock cultures⁶⁾. Furthermore, TMC 0409 strain can also resist acid environment and adhere to human Caco-2 cell line better than other tested strains⁶⁾. Caco-2 cell line is a human cell culture, which is widely used to evaluate the capacity of lactic acid bacteria to colonize in human intestine³⁾. The hypocholesterolemic effect of TMC 0409 cells observed in this study might be attributed to the activities of this bacterium in the intestine of the tested rats, colonizing in intestine, binding bile acid to their cells and elevating the excretion of this steroids into feces, like dietary fibers and ion exchange resins. This explanation was well supported by the fact that the fecal bile acids of rats fed TMC 0409 cells preparation were enhanced (Table 3). In the following studies, the fecal bacterial composition of rats and human subjects fed TMC 0409 cells preparation will be analyzed to confirm the colonization of TMC 0409 strain in the intestines of animal and human so that more reasonable conclusion could be made.

Mann and Spoerry¹³⁾ first reported the positive effects of the fermented milk in modifying the serum cholesterol in the epidemic study of 1970s. However, the results obtained from late studies aimed to clarify the hypocholesterolemic effect of fermented milk were controversial^{11, 4, 8, 18-20)}. In the recent study, whey protein exhibited stronger hypocholesterolemic effect in the rats fed cholesterol containing diet compared to

the casein protein and soy bean protein¹⁵⁾. No whey protein was added in the fermented milk and skim milk used in this study. As shown in our previous study⁶⁾, the hypocholesterolemic effect of TMC 0409 strain was dose-dependent. In the present study, the number of bacterial cell were 4.4×10^8 cfu/ml (fermented milk) and 3.4×10^{10} cfu/g (TMC 0409 cells), respectively. Lactic acid bacteria cells in Diet 1 were 2.2×10^9 cfu/100 g and Diet 3 were 1.7×10^{11} cfu/ml. These indicated that the cells preparation of TMC0409 showed the strongest inhibitory effect on the increase of serum cholesterol compared with the fermented milk prepared with TMC0409 strain and skim milk, indicating that the hypocholesterolemic effect to fermented milk are mainly determined by the activities of lactic acid bacteria involved in the fermented milk rather than the component of milk and metabolism produced in fermentation process. From these observation, we concluded that the wide variation of hypocholesterolemic effect of the fermented milk among the reported studies may be partly related to the differences of lactic acid bacteria involved in these fermented milk.

Reference

- 1) Akalin SA, Gong S, Duel S. Influence of yoghurt and acidophilus yoghurt on serum cholesterol levels in mice. *Journal of Dairy Science*, 80 : 2721-2725. 1997.
- 2) Folch J, Lees M, Sloane-Stanley GH. A simple method for the isolation and purification of total lipids from animal tissue. *Journal of Biological Chemistry*, 226 : 497-509. 1957.
- 3) Goldin BR, Gorbach SL, Saxelin M, Baraskat S, Gualtieri L, Salminen S. Survival of *Lactobacillus* Specie (Strain GG) in human gastrointestinal tract. *Digestive Diseases and Sciences*, 37 : 121-128. 1992.
- 4) Gruenewald K K. Serum cholesterol levels in rats fed skim milk fermented by *Lactobacillus acidophilus*. *Journal of Food Science*, 47 : 2078-2079. 1982.
- 5) Hartman RE, Holmlund C E. Binding of steroid by microorganisms. *Journal of Bacteriology*, 84 : 1254-1259. 1962.
- 6) Hashimoto H, Yamazaki K, He F, Yasuko A, Kawase M, Hosoda M, Hosono A. Effect of lactic acid bacteria on serum cholesterol level in rats fed cholesterol diet. *Animal Science and Technology*, 69 : 702-707. 1998.
- 7) Hassan Y, Lerrat MA, Pemigne C, Remesy C. Resistant starch is more effective than cholestyramine as lipid lowering agent in the rat. *Lipids*, 30 : 847-853. 1995.
- 8) Hepner G, Fried R, Jeor S, Fusetti L, Morin R. Hypocholesterolemic effect of yogurt and milk. *American Journal of Clinical Nutrition*, 32 : 19-24. 1979.
- 9) Hosono A, Tono-oka T. Binding of cholesterol with lactic acid bacterial cells. *Milchwissenschaft*, 50 : 556-560. 1995.
- 10) Kihara K, Toda H, Mori M, Hashimoto M, Mizogami S. The bile acid binding and hypocholesterolemic activity of amino-exchange resins bearing the imidazolium salt group. *European Journal of Medicinal Chemistry*, 23 : 411-415. 1988.
- 11) Kiriya S, Okazaki Y, Yoshida A. Hypocholesterolemic effect of polysaccharides and polysaccharide-rich foodstuffs in cholesterol-fed rats. *Journal of Nutrition*, 97 : 382-388. 1969.
- 12) Lindenbaum S, Higuchi T. Binding of bile acids to cholestyramine at gastric pH conditions. *Journal of Pharmaceutical Sciences*, 64 : 1887-1889. 1975.
- 13) Mann GV, Spoerry A. Studies of a surfactant and cholesteremia in the Massai. *American Journal of Clinical Nutrition*, 27 : 464-469. 1974.
- 14) Midtvedt T, Morman A. Adsorption of bile acids to intestinal microorganisms. *Acta Pathologica et Microbiologica Scandinavica [Section B]*, 80 : 202-210. 1972.
- 15) Nagaoka S, Kanamaru Y, Kuzuya Y, Kojima T, Kuwata T. Comparative studies on the serum cholesterol lowering action of whey protein and soybean protein in rats. *Bioscience Biotechnology and Biochemistry*, 56 : 1484-1485. 1992.
- 16) Nagata Y, Tanaka K, Sugano M. Serum cholesterol levels of rat and mice fed soy-been protein or casei. *Journal of Nutritional Science and Vitaminology*, 27 : 583-593. 1981.
- 17) Okenfull DG, Fenwick DE. Adsorption of bile salts from aqueous solution by plant fiber and cholestyramine. *British Journal of Nutrition*, 40 : 299-309. 1978.
- 18) Pulusani SR, Rao DR. Whole body, liver and plasma cholesterol levels in rats fed *Thermophilus*, *Bulgaricus* and *Acidophilus* milks. *Journal of Food Science*, 48 : 280-281. 1983.
- 19) Rao DR, Chawan CB, Pulusani SR. Influence of milk and *Thermophilus* milk on plasma cholesterol levels and hepatic cholesterogenesis. *Journal of Food*

Hypocholesterolemic Effects of Lactic Acid Bacteria

- Science*, 46 : 1339-1341. 1981.
- 20) Rodas BZ, Gilliland SE, Maxwell CV. Hypocholesterolemic action of *Lactobacillus acidophilus* ATCC 43121 and calcium in swine with hypercholesterolemia induced by diets. *Journal of Dairy Science*, 79 : 2121-2128. 1996.
- 21) Salminen S, Deighton M A, Benno Y, Gorbach SL. Lactic acid bacteria in health and disease. In : Lactic Acid Bacteria. (Salminen S, von Wright eds.) 369-386. A. Marcel Dekker, Inc. New York. 1997.
- 22) Vahouny GV, Tombes R, Cassidy MM, Kritchevsky D, Gallo LL. Dietary fibers : V. Binding of bile salts, phospholipids and cholesterol from mixed micelles by bile acid sequestrates and dietary fibers. *Lipids*, 15 : 1012-1018. 1980.