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Effect of *Lactobacillus brevis* 119-2 isolated from Tsuda kabu red turnips on cholesterol levels in cholesterol-administered rats

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In a previous *in vitro* study, we reported that the potential mechanism of the cholesterol-lowering effect of *Lactobacillus brevis* 119-2 isolated from turnip Tsuda kabu was the incorporation of cholesterol to cell membrane. In this study, we analyzed serum cholesterol and hepatic gene expression of Sprague-Dawley (SD) rats kept on a cholesterol diet with or without *L. brevis* 119-2 for 2 weeks, to evaluate the cholesterol-lowering effect *in vivo*. Serum cholesterol levels were significantly reduced in SD rats kept on a diet including *L. brevis* 119-2 compared with that in SD rats kept on a diet without *L. brevis* 119-2, and both viable and dead *L. brevis* 119-2 induced this effect. Hepatic gene analysis by DNA microarray suggested that the potential mechanism of the cholesterol-lowering effect of *L. brevis* 119-2 *in vivo* was inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase activity by insulin induced gene (Insig) protein, and induction of catabolism of cholesterol to bile acid by Cyp7a1 (cytochrome P450 a1). In addition, we found that inclusion of *L. brevis* 119-2 in the diet decreased serum low density lipoprotein (LDL) cholesterol levels by inducing overexpression of the LDL receptor gene. In contrast, *Lactobacillus acidophilus* ATCC 43121 increased high density lipoprotein cholesterol levels by inducing overexpression of the ATP-binding cassette sub-family. A member 1 (Abca1) and Angiopoietin-like 3 (Angptl3) genes. These results suggest that *L. brevis* 119-2 decreases the risk of atherosclerosis by lowering serum cholesterol, ameliorating the effect of fatty liver.

[**Key words:** Cholesterol-lowering effect; Lactic acid bacteria; DNA microarray; Quantitative real-time PCR; L/H ratio]

Metabolic syndrome combined with at least two other factors (hyperlipidemia, hypertension, or hyperglycemia) leads to a synergistic increase in the risk of atherosclerosis (1). About twenty million people are applicable person and reserve army of the metabolic syndrome in Japan (National Health and Nutrition Examination Survey, 2004). Globally, 1 in approximately every 4 or 5 adults develops metabolic syndrome depending on the environmental conditions and daily lifestyle habits of the citizens of the country where he or she resides. The incidence of this syndrome has been estimated to increase with age for individuals over 50. Metabolic syndrome affects 27% of the population in India, nearly 30% in Europe (2), and more than 40% in the USA (3). Elevated serum cholesterol levels, a risk factor for ischemic heart disease and other lethal disease, have been correlated with the risk of developing atherosclerosis. Manson et al. noted that a 1% decrease in serum cholesterol levels reduced the risk of atherosclerosis by 2–3% in humans (4). Although, many researchers are studying diseases associated with metabolic syndrome, successful treatments for these diseases have not been discovered to date. Thus, early interventions to treat metabolic syndrome are needed.

In a previous report, we investigated the cholesterol-lowering effect of lactic acid bacteria screened from several vegetables farmed in the Shimane prefecture in Japan. We defined the cholesterol-lowering effect of *Lactobacillus brevis* 119-2 isolated from Tsuda kabu red turnips *in vitro*, and it was considered that the mechanism was incorporation of cholesterol into the *L. brevis* 119-2 cell (5).

In this study, to verify our *in vitro* results and investigate the effectiveness of *L. brevis* 119-2 ingestion in decreasing the risk of atherosclerosis, we investigated the cholesterol-lowering effect of *L. brevis* 119-2 using cholesterol-administered Sprague–Dawley (SD) rats, and examined hepatic gene expression induced by ingestion of *L. brevis* 119-2 using DNA microarray analysis.

MATERIALS AND METHODS

Strains and culture *L. brevis* 119-2 was isolated from Tsuda kabu red turnips, which were harvested in Matsue city, Shimane Prefecture, Japan, and was stored at the Shimane Institute for Industrial Technology (5). *Lactobacillus acidophilus* ATCC 43121 was purchased from American Type Culture Collection (USA) supplied by

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Summit Pharmaceuticals International Corporation (Tokyo, Japan). These strains were cultured in MRS medium (Becton, Dickinson and Company, MD, USA) at 37°C for 20 h with static culture. Cultured strains were harvested by centrifugation at 5000 ×g for 3 min and washed with distilled water. Then, *L. brevis* 119-2 and *L. acidophilus* ATCC 43121 were harvested. Viable *L. brevis* 119-2 and *L. acidophilus* ATCC 43121 were freeze-dried (Eyla FDV-830, Tokyo Rikakikai Co. Ltd., Tokyo, Japan), and viable cells count of these strains were 3.6×10^{14} cfu/g dry weight and 7.1×10^{14} cfu/g dry weight respectively. Dead *L. brevis* 119-2 was freeze-dried after autoclaving. These dried strains were stored in a desiccator until use. Through this study, *L. acidophilus* ATCC 43121 were used only viable cells.

Animal and study design Male Jcl: SD rats (4 weeks of age) were obtained from CLEA Japan, Inc. (Tokyo, Japan). Rats were maintained under controlled environmental conditions (temperature 23 ± 3°C, relative humidity 55% ± 25%, 12/12 h light/dark cycle) and given food and water ad libitum. All rats were acclimated 1 week prior to the experiments. Three groups of 11 rats each were fed the respective group diets for 2 weeks. A control group was kept on a high-cholesterol diet containing 10 g cholesterol kg^{-1}, 5 g cholic acid kg^{-1}, 985 g mouse & rat & rabbit diet (CRF-1) kg^{-1} obtained from Oriental Yeast Co. Ltd. (Tokyo, Japan). The other two groups were fed the same high-cholesterol diet viable *L. brevis* 119-2, dead *L. brevis* 119-2, or *L. acidophilus* ATCC 43121 kg^{-1}. Compositions of the experimental diets are shown in Table 1. Body weights were measured at days 0, 3, 8, 10, 14, and 15 and final body weight was defined as the weight at day 15. Diet consumption was measured at days 0, 3, 8, and 10 and an average caloric base was determined (Table 1).

This study and all procedures satisfied the approved regulations and code of ethics for experimental animals of the Chitose Japan Food Research Laboratories.

Biochemical analysis After an 18-h fasting period, heparin sodium treated blood was obtained from abdominal aortas of rats under sevoflurane (Maruishi Pharmaceutical Co. Ltd., Osaka, Japan) inhalation anesthesia, and then heparin treated blood was centrifuged at 3000 rpm (1200 ×g) for 10 min at 4°C to obtain plasma. The items in Table 2 were measured using plasma. Concentrations of total cholesterol, high density lipoprotein (HDL) cholesterol, triglycerides (TG), glucose (GLU), urea (UA), urea nitrogen (UN), creatinine (CRE), total bilirubin (TBIL), creatine phosphokinase, aspartic acid aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltranspeptidase (γ-GTP), alkaline phosphatase (ALP), and amylase (AMY) were measured using enzymatic methods (automatic biochemical analyzer, Fujifilm Medical Co. Ltd., Tokyo, Japan). Low density lipoprotein (LDL) cholesterol levels were calculated using the Friedewald formula (6). Lipids were extracted by Folch method (7). Briefly, 1.5 g of the left outer lobe of the liver was homogenized with 9 mL chloroform–methanol (2:1, v/v) mixture. The filtrate of the homogenate obtained using filter paper was diluted to 20 ml with a chloroform–methanol (2:1, v/v) mixture, shocked with 3 mL 0.5% sodium chloride, and stored at 4°C for 2 h. The organic layer (1 ml) was air-dried at room temperature and dissolved in 1 mL isopropanol. A commercial kit was used to measure the concentration of total cholesterol and triglycerides in liver lipid extracts and plasma.

Histopathological examination Liver fragments (left outer lobe) were fixed with 10% formalin neutral buffer solution. Fixed tissue was paraffin blocked and cut into thin sections using a microtome. Thin sections were stained with hematoxylin and eosin and observed using an optical microscope.

RNA extraction DNA microarray analysis was performed for four selected rats from the control group and viable *L. brevis* 119-2 group, to determine gene expression in the liver of rats fed a cholesterol diet with viable *L. brevis* 119-2. Rat selection was based on the concentration of total cholesterol in the liver. Four rats were selected with cholesterol levels close to the average value. Total RNA was extracted from each liver by an ISOGEN (Nippon Gene Co. Ltd., Tokyo, Japan) and purified using an RNeasy Mini Kit (Qiagen KK, Tokyo, Japan). Quality and quantity were checked by agarose gel electrophoresis and spectrophotometry.

DNA microarray analysis DNA microarray analysis was performed according to manufacturer's protocol. cDNA was synthesized from 100 ng of purified total RNA and biotinylated cRNA was synthesized using T7 RNA polymerase, fragmented, and hybridized to an Affymetrix Rat Genome230 2.0 array (Affymetrix, Santa Clara, CA, USA), which contains probe sets for more than 31,000 rat genes.

TABLE 1. Components of experimental diets.

Materials	Control	119-2/ATCC 43121
Crude protein (g kg^{-1})	221	209
Crude fat (g kg^{-1})	55	53
Crude carbohydrate (g kg^{-1})	540	514
Crude fiber (g kg^{-1})	31	29
Crude ash (g kg^{-1})	65	62
Moisture (g kg^{-1})	77	73
LAB (g kg^{-1})	0	50
Cholesterol (g kg^{-1})	10	10
Choic acid (g kg^{-1})	5	5
Calories (kcal kg^{-1})	3762	3767

TABLE 2. Growth parameters and plasma chemistries of SD rat fed diet containing cholesterol with or without viable, dead119-2 and ATCC 43121 for 2 weeks.

Parameters	Diet			
	Control	Viable 119-2	Dead 119-2	ATCC 43121
Growth parameters				
Food intake (kcal rat^{-1})	105.8 ± 6	100.4 ± 7	93.1 ± 10	99 ± 6
Final body weight (g)	233.1 ± 11.7	234.3 ± 14.5	225.4 ± 13.5	221.5 ± 15.0
Liver weight (g)	11.0 ± 0.8	11.6 ± 1.0	10.7 ± 0.9	11.3 ± 1.5
Liver TG (mg g^{-1})	53.9 ± 11.4	50.1 ± 16	47.4 ± 11.6	35.5 ± 11.4*
Liver cholesterol (mg g^{-1})	62.5 ± 12.9	50.1 ± 12.6*	52.4 ± 7.6	46.4 ± 10.0*
Plasma chemistries				
Cholesterol (mg dL^{-1})	106 ± 35	79 ± 12*	79 ± 9*	125 ± 37
HDL cholesterol (mg dL^{-1})	24 ± 5	28 ± 8	26 ± 5	36 ± 6*
LDL cholesterol (mg dL^{-1})	61.5 ± 30	23.4 ± 15*	32 ± 12*	66.9 ± 37
Triglyceride (mg dL^{-1})	47 ± 22	55 ± 16	44 ± 21	45 ± 13
Creatine phosphokinase (U L^{-1})	260 ± 28	272 ± 53	253 ± 37	254 ± 64
Aspartate aminotransferase (U L^{-1})	70 ± 8	77 ± 31	73 ± 9	61 ± 8
Alanine aminotransferase (U L^{-1})	20 ± 3	23 ± 16	21 ± 5	18±3
Lactic dehydrogenase (U L^{-1})	265 ± 46	221 ± 34	233 ± 30	230 ± 35
γ-glutamyl transpeptidase (U L^{-1})	10 ± 0	10 ± 0	<10	10 ± 0
Alkaline phosphatase (U L^{-1})	1227 ± 159	1205 ± 236	1111 ± 299	1167 ± 168
Amylase (U L^{-1})	1870 ± 259	2064 ± 201	2019 ± 279	1897 ± 263
Glucose (mg dL^{-1})	104 ± 11	107 ± 8	103 ± 9	95 ± 12
Urea (mg dL^{-1})	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
Blood urea nitrogen (U dL^{-1})	13.2 ± 1.9	13.4 ± 3.1	12.8 ± 1.4	12.8 ± 2.1
Creatinine (mg dL^{-1})	<0.2	<0.2	<0.2	<0.2
Total bilirubin (mg dL^{-1})	<0.2	<0.2	<0.2	0.2 ± 0.0
Total protein (g dL^{-1})	5.9 ± 0.2	5.9 ± 0.2	5.9 ± 0.2	5.9 ± 0.2
Albumin (g dL^{-1})	3.7 ± 0.2	3.8 ± 0.1	3.7 ± 0.1	3.8 ± 0.1

Data are means ± SD ($n = 11$). Asterisks indicate significantly difference compared to control ($p < 0.05$).

Fluorescence signals were scanned by the Affymetrix Gene Chip system. Affymetrix Gene Chip Command Console Software was used to reduce the array images to the intensity of each probe (CEL files). The Cell files were quantified with the qFRAMS algorithm (8), and differentially expressed genes were extracted by comparing the two groups using Rank products method (9) (p–value < 0.05). Gene Ontology (GO) of annotation of differentially expressed genes was categorized using The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (10) and the enriched GO category (modified Fisher exact p-value < 0.005) was applied to QuickGO (11), which is a fast web-based browser for GO terms and annotations to determine the hierarchical chart of the GO terms.

Quantitative real-time (qRT) PCR To determine the mechanism of the cholesterol-lowering effect in all samples in the test section containing the viable *L. brevis* 119-2 feeding and *L. acidophilus* ATCC 43121 feeding groups, target genes expression was analyzed using qRT-PCR. Genes related to HDL cholesterol biosynthesis (Abca1, Angptl3), the LDL cholesterol receptor gene (Ldlr), and genes related to cholesterol homeostasis (Cyp7a1, Insig1) were used as target genes. Actb was used as a housekeeping gene, to normalize qRT-PCR values. RNA (500 ng) was added to 10 μL of reaction mixture containing random hexamers in the Prime Script RT reagent kit (Takara Bio Inc., Otsu, Japan), synthesis of cDNA was performed at 37°C for 15 min, the reverse transcription reaction was stopped by heating at 85°C for 5 s, and sample were cooled at 4°C. cDNA was stored at −80°C until use.

cDNA (1.0 μL) diluted 10 times with water was added to the RT-PCR mixture containing 12.5 μL SYBR Premix Ex Taq II (Takara Bio), 0.5 μL ROX reference Dye, 9.0 μL distilled water DNase/RNase free, and 2.0 μL 10 μM housekeeping gene solution or individual target primer. The primer sequences were the followings: Abca1 forward 5′CAGCAACTACAGTGGCGGTAACA, reverse 5′AATGCTTAGGGCACAATTCCACA; Angptl3 forward 5′CACGCCACTTGTATGTTC ACCTC, reverse 5′ACTGGCATTCAAGAAGAACCCACTG; Cyp7a1 forward 5′GGCA CAGTCAAGGCTGGAGAATG, reverse 5′ATGGTGGTGAAGACGCCAGTA; Insig1 forward 5′CTTACTGACAGCCCAGGACAACAC, reverse 5′TTCGGCTCATTACCACTGAAACA; Ldlr