

Effect of *Lactobacillus casei* strain Shirota-fermented milk on metabolic abnormalities in obese prediabetic Japanese men: a randomised, double-blind, placebo-controlled trial

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An obesity-related prediabetic state is characterised by metabolic abnormalities such as post-glucose load hyperglycaemia and dyslipidaemia and consequently increases the risk for type 2 diabetes and cardiovascular disease. This study aimed to investigate the effects of *Lactobacillus casei* strain Shirota (LcS) on metabolic abnormalities in obese prediabetic subjects in a randomised, double-blind, placebo-controlled trial. Herein, 100 obese subjects (body mass index ≥ 25), who had moderate post-load hyperglycaemia (1-hr post-load plasma glucose (PG) levels ≥ 180 mg/dl during the oral glucose tolerance test), consumed LcS-fermented milk or placebo milk daily for 8 weeks. The post-load PG and fasting blood markers were evaluated. Although post-load PG levels were not significantly different between the groups, 1-hr post-load PG, glycoalbumin, and HbA1c levels decreased at 8 weeks compared with the baseline levels only in the LcS group ($p=0.036$, $p=0.002$, and $p=0.006$, respectively). The reduction in glycoalbumin levels was statistically significantly greater in the LcS group than in the placebo group ($p=0.030$). Stratified analyses revealed significantly improved 1-hr post-load PG and glycoalbumin levels in the LcS group compared with the placebo group among subjects with severe glucose intolerance (2-hr post-load PG levels higher than the median at baseline; $p=0.036$ and $p=0.034$, respectively). In terms of lipidic outcomes, total, low-density lipoprotein, and non-high-density lipoprotein cholesterol levels were significantly lower in the LcS group than in the placebo group ($p=0.023$, $p=0.022$, and $p=0.008$, respectively). These findings suggest that LcS may favourably affect metabolic abnormalities in obese prediabetic subjects, though the effects on glycaemic control may be limited.

Key words: probiotics, oral glucose tolerance test, glycaemic control, glycoalbumin, dyslipidaemia

INTRODUCTION

The incidence of diabetes mellitus (DM) is increasing worldwide in conjunction with the obesity epidemic. Epidemiological studies have revealed that the number of DM patients in 2015 was estimated at 415 million, and this number is expected to reach approximately 642 million in 2040 [1]. Obesity-associated type 2 DM is commonly accompanied by other metabolic abnormalities such as dyslipidaemia and hypertension because of the insulin resistance induced by increased secretion of pro-inflammatory adipokines from adipose tissue [2, 3]. DM and the accumulation of these metabolic abnormalities have been linked to a high

prevalence of cardiovascular disease [2]. Medication and lifestyle changes are beneficial for preventing DM and cardiovascular disease but can be associated with problems such as side effects and poor compliance. Therefore, easy and safe tools for preventing these diseases are required. Post-glucose load and/or post-meal hyperglycaemia in the prediabetic state are characteristic signs of the development of type 2 DM [4]. Elevated 1-hr and 2-hr post-load plasma glucose (PG) levels, which are typical markers of post-glucose load hyperglycaemia, are major risk factors for type 2 DM [5]. Moreover, prediabetic individuals with post-glucose load hyperglycaemia, as well as patients with DM, are at high risk for cardiovascular disease [6, 7]. The STOP-NIDDM trial has demonstrated that preventive treatment for prediabetic subjects using agents targeting post-meal hyperglycaemia is associated with a significant reduction in the incidence of diabetes and risk for hypertension and cardiovascular disease [8]. In addition, prediabetic subjects with elevated post-load PG levels exhibit a pro-atherogenic lipid profile characterised by hypertriglyceridemia, low high-density lipoprotein

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cholesterol (HDL-C) levels, and high non-HDL-cholesterol (non-HDL-C) levels [9, 10]. Thus, pleiotropic control of these metabolic abnormalities is expected to provide benefits to obese prediabetic individuals in terms of preventing type 2 DM and cardiovascular disease.

Accumulating evidence indicates that the gut microbiota play an important role in the development of obesity-associated metabolic disorders [11]. The composition of the gut microbiota is drastically altered in obesity and type 2 DM [11, 12]. This change is considered to lead to increased intestinal permeability and results in endotoxaemia, which in turn triggers chronic low-grade inflammation and insulin resistance [13]. Probiotics are defined as living microorganisms, which, when administered in adequate amounts, confer a health benefit on the host by modulating gut microbiota and immune responses [14]. Previous studies have demonstrated that oral administration of *Lactobacillus casei* strain Shirota (LcS), a typical probiotic strain, improves obesity-associated metabolic abnormalities, including insulin resistance, impaired glucose tolerance [15], type 2 DM [16], and hepatic steatosis [17, 18], in mouse models. Recently, Hulston *et al.* indicated that LcS supplementation suppresses the decline of the insulin sensitivity induced by high-fat overfeeding in healthy adults [19]. Some other clinical trials have found that probiotic treatment improves glycaemic control in type 2 DM patients [20]. However, to our knowledge, no clinical trials have been conducted to investigate the effects of probiotic treatment on these metabolic abnormalities in prediabetic subjects characterised by post-load hyperglycaemia, who are at high risk of DM and cardiovascular disease.

Therefore, the present study aimed to investigate the effects of LcS on glycaemic and lipidic control in obese prediabetic subjects by evaluating post-load PG levels and other glycaemic and lipidic control markers.

MATERIALS AND METHODS

Test beverage

The test beverage was milk fermented with LcS YIT 9029, which was obtained from the Culture Collection Research Laboratory of Yakult Central Institute, Tokyo, Japan. The placebo was non-fermented milk with the same nutritional content (protein, 1.4 g/100 ml; fat, 0.1 g/100 ml; carbohydrates, 13.9 g/100 ml; and calories, 62.0 kcal/100 ml), colour, flavour, taste, and pH made using the same ingredients as the LcS-fermented milk, with the addition of lactic acid [21]. The beverages were distributed to each subject weekly via a refrigerated parcel-delivery service and stored at 0–10°C. LcS-fermented milk contained $>1.0 \times 10^{11}$ colony forming units of LcS per 100 ml during the intervention.

Subjects

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Institutional

Review Board of the incorporated medical institution of Aiseikai Aisei Hospital Ueno Clinic, Tokyo, Japan. Written informed consent was obtained from all subjects. Based on the Guidelines for the Diagnosis of Diabetes Mellitus [22], obese prediabetic Japanese men were recruited for this study. The inclusion criteria were as follows: age between 20 and 64 years, body mass index (BMI) ≥ 25 kg/m², and 1-hr post-load PG level ≥ 180 mg/dl. The exclusion criteria were as follows: regular use of foods or beverages containing lactic acid bacteria (>3 times a week); taking medicines or health foods that could influence the outcome of the study; history of serious disease such as liver disease, kidney disease, hypertension, or ischemic heart disease; allergies to dairy products; and participation in another clinical trial during the past 1 month. Women were excluded because of the lower prevalence of obesity in women in Japan.

Study design

A randomised, double-blind, placebo controlled trial was conducted from June to December 2013 by a contract research organization (TTC Co., Ltd., Tokyo, Japan). The subject flow throughout the study is shown in Fig. 1. A total of 847 individuals were recruited via the Internet and telephone and assessed for participation eligibility. Screening was conducted on 248 subjects with obesity (BMI ≥ 25 kg/m²) and post-load PG levels close to the prediabetic range. Obese prediabetic subjects (n=100) who met the criteria were enrolled and randomly allocated to the LcS group (n=50) or placebo group (n=50) using a computer-generated random number sequence. The allocation list was prepared by an investigator with no clinical involvement in the study and concealed from both clinical staff and subjects throughout the study. One 100-ml bottle of either fermented milk containing LcS or placebo milk was consumed daily for 8 weeks. The trial consisted of a pre-intervention period of 2–3 weeks, an 8-week intervention period, and a post-intervention period of 4 weeks. Each subject visited the clinic (Nishi-Shinjuku Kisaragi Clinic, Tokyo, Japan) at baseline during the pre-intervention period, at 4 and 8 weeks during the intervention period, and at the end of the post-intervention period. All subjects were required to record their intake of the test sample and any adverse events throughout the study; the subjects were instructed to maintain their usual physical activity and dietary habits.

Outcomes

The aim of the present study was to investigate the effects of LcS on glycaemic control and lipid profiles in obese prediabetic subjects. Therefore, post-load PG levels, glycoalbumin (GA) levels, HbA1c levels, insulinogenic index, homeostasis model assessment-insulin resistance (HOMA-IR), HOMA- β , and the Matsuda index [23] were evaluated as indices of glycaemic control. Lipidic outcomes were total cholesterol (TC), non-HDL-C, low-density lipoprotein cholesterol (LDL-C), HDL-C, and triacylglycerol (TAG) levels.

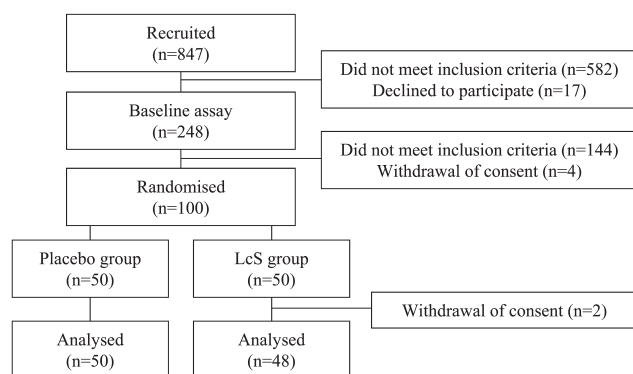


Fig. 1. Subject flow throughout the trial.

Oral glucose tolerance test (OGTT)

Each subject visited the clinic after an overnight (≥ 10 hr) fast. Fasting blood samples were collected from the antecubital vein, and subjects were subsequently administered 225 ml of a 75-g anhydrous glucose solution (Toleran-G75[®], Ajinomoto Pharma Co., Ltd., Tokyo, Japan). Blood was collected at 30, 60, 90, and 120 min after glucose loading to measure the PG and serum insulin levels. The areas under the curve for PG during the OGTT were calculated using the trapezoidal rule. The HOMA-IR, HOMA- β , insulinogenic index, and Matsuda index values were calculated using the following formulas: HOMA-IR = (PG0 [mg/dl] \times Ins0 [μ U/ml])/405, where PG0 and Ins0 represent fasting PG and serum insulin levels, respectively; HOMA- β = (Ins0 [μ U/ml] \times 360)/(PG0 [mg/dl] - 63); Insulinogenic index = (Ins30 [μ U/ml] - Ins0 [μ U/ml])/(PG30 [mg/dl] - PG0 [mg/dl]), where PG30 and Ins30 represent the 30-min post-load PG and serum insulin levels, respectively; and Matsuda index = $10,000/\sqrt{(PG0 \times Ins0 \times \text{mean PG} \times \text{mean Ins})}$, where mean PG and mean Ins represent the mean PG and serum insulin level, respectively, during the OGTT [23].

Laboratory measurements

Blood samples were centrifuged at 1,200 g for 10 min at room temperature to separate the serum components. Measurement of blood parameters was outsourced to LSI Medience Corporation (Tokyo, Japan). HbA1c level in whole blood was measured enzymatically using a clinical chemistry analyser (JCA-BM9130, JEOL Ltd., Tokyo, Japan). PG, GA, TAG, TC, LDL-C, and HDL-C levels were determined enzymatically, and serum insulin levels were determined using a chemiluminescent immunoassay. Non-HDL-C levels were determined using the following equation: non-HDL-C = TC - HDL-C.

Anthropometric measurements

Height was measured at baseline. Body weight was measured at each visit with subjects dressed in light clothing and barefoot. BMI was calculated using height and body

weight. Blood pressure was measured at each visit using a standard digital sphygmomanometer (HEM705IT, Omron Healthcare Co., Ltd., Kyoto, Japan). Percentage of body fat was measured with the bioelectrical impedance method (Karada Scan HBF-354IT, Omron Healthcare Co., Ltd.).

Assessment of dietary intake and physical exertion

Dietary intake was assessed on the basis of the contents of a food diary maintained by subjects for 3 days before each visit. Dietitians calculated daily energy, protein, fat, and carbohydrate intakes, and mean intakes were calculated for the 3-day period. Physical activity was assessed for 7 days before each visit. Subjects wore an activity monitor (Active style Pro HJA-350IT, Omron Healthcare Co., Ltd.) during each period, and the mean values of physical activity per day were calculated for each period.

Sample size

The required sample size was calculated based on the estimated changes in post-load PG levels. Considering that LcS would reduce PG levels by 20 mg/dl and that the standard deviation of the changes would be 35 mg/dl, we calculated that 48 subjects were needed in each group to detect any differences between the groups with a power of 80% and a significance level of 5%. This number was increased to 50 per group to accommodate for anticipated dropouts.

Statistical analysis

Continuous data are shown as the means \pm standard error. Data were analysed using PASW Statistics 18 (SPSS Inc., Chicago, IL, USA). The unpaired Student's t-test was performed to compare differences between the groups. The paired Student's t-test with Bonferroni-Holm adjustment for multiple testing was used for within-group comparisons. A two-sided p value < 0.05 was considered to indicate statistical significance.

RESULTS

Baseline characteristics of the subjects

Figure 1 shows the subject flow throughout the trial. Of the 100 randomised subjects who started taking the test beverages, two dropped out due to withdrawal of informed consent. No seriously adverse events were reported in both groups during the study. Therefore, 98 subjects completed the study and were included in the analyses. The baseline characteristics of the subjects are shown in Table 1. There were no significant differences in any of the parameters among the groups. HbA1c levels were below 6.5% in all subjects, showing that glycaemic control was within the non-diabetic range.

Dietary intake, physical activity, body composition, and blood pressure

Dietary intake and physical activity levels during the study are shown in Table 2. There were no significant changes from baseline among any of the assessed parameters in either

Table 1. Baseline characteristics of the subjects

	Placebo (n=50)		LcS (n=48)		p ^a
	MEAN	SEM	MEAN	SEM	
Age (years)	47.4	1.0	46.6	1.1	0.588
Height (cm)	169.6	0.8	171.4	0.8	0.124
Body weight (kg)	83.6	1.4	86.7	1.5	0.145
BMI (kg/m ²)	29.0	0.4	29.5	0.4	0.454
Body fat (%)	27.6	0.4	28.0	0.5	0.515
FPG (mg/dl)	110.3	1.1	109.6	1.3	0.669
Post-OGTT glucose					
1-hr (mg/dl)	217.9	3.3	218.8	3.7	0.853
2-hr (mg/dl)	165.8	4.6	161.5	3.5	0.461
AUC (mg/dl·h)	368.9	5.0	365.5	5.2	0.642
Glycoalbumin (%)	13.5	0.1	13.4	0.2	0.838
HbA1c (%)	5.79	0.04	5.74	0.04	0.454
Fasting insulin (μU/ml)	7.9	0.5	8.0	0.5	0.917
TC (mg/dl)	218.3	4.4	212.4	4.1	0.330
LDL-C (mg/dl)	142.6	4.0	136.1	3.6	0.234
HDL-C (mg/dl)	50.8	1.3	53.1	1.5	0.255
Non-HDL-C (mg/dl)	167.6	4.4	159.3	4.1	0.173
TAG (mg/dl)	160.3	9.9	153.4	9.4	0.614

LcS: *L. casei* strain Shirota YIT 9029; FPG: fasting plasma glucose; OGTT: oral glucose tolerance test; AUC: area under the curve; TC: total cholesterol; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; non-HDL-C: non-high-density lipoprotein cholesterol; TAG: triacylglycerol.

^ap values analysed by the unpaired Student's t-test.

group throughout the trial, and no significant differences were found between the groups at any time point. Changes in body composition and blood pressure during the study are shown in Table 3. In each group, body weight, BMI, and percentage of body fat significantly increased from that at baseline at each visit after the start of the intervention. Diastolic blood pressure varied significantly during the trial including the washout period in the placebo group; however, no statistically significant differences were found between the groups at any time.

Glycaemic control

Changes in PG levels during the OGTT are shown in Table 4. Fasting and post-load PG levels did not differ between the groups at any visit. However, 1-hr post-load PG levels significantly decreased at 8 weeks compared with at baseline in the LcS group ($p=0.036$) but not in the placebo group. The reduction in 1-hr post-load PG levels was not observed after the washout period (12 weeks). Changes in other glycaemic parameters are shown in Table 5. Plasma GA levels significantly decreased at 8 weeks compared with at baseline in the LcS group only ($p=0.002$), and consequently, a significantly higher reduction in GA levels was observed in the LcS group when compared with the placebo group ($p=0.030$) (Fig. 2). The reduction in GA levels were not maintained after the washout period. HbA1c levels at 8 and 12 weeks were significantly reduced compared with at baseline in the LcS group only ($p=0.006$ and $p=0.000$, respectively). There were no significant differences among the indices for insulin sensitivity and insulin secretion both within and between groups.

Table 2. Dietary intake and physical activity of each group during the study

	Baseline			4 weeks			8 weeks			12 weeks (after washout period)		
	MEAN	SEM	p ^a	MEAN	SEM	p ^a	MEAN	SEM	p ^a	MEAN	SEM	p ^a
Dietary intake												
Total energy (kcal/day)												
Placebo group	2,029	77	0.835	1,978	65	0.636	1,906	63	0.651	1,980	68	0.603
LcS group	2,050	65		1,933	66		1,947	65		2,037	84	
Proteins (g/day)												
Placebo group	67	2	0.960	67	2	0.904	65	2	0.464	68	3	0.691
LcS group	67	2		67	2		68	2		69	3	
Lipids (g/day)												
Placebo group	66	4	0.981	62	3	0.698	63	3	0.730	67	3	0.777
LcS group	66	3		61	3		65	3		68	4	
Carbohydrates (g/day)												
Placebo group	263	10	0.826	261	10	0.600	243	8	0.725	251	9	0.703
LcS group	266	9		254	9		248	9		256	10	
Physical activity (METs-hr/day)												
Placebo group	4.0	0.3	0.470	3.9	0.3	0.372	4.4	0.3	0.916	4.3	0.3	0.918
LcS group	4.3	0.4		4.3	0.3		4.3	0.4		4.2	0.4	

LcS: *L. casei* strain Shirota YIT 9029; METs: metabolic equivalents.

^ap values analysed by the unpaired Student's t-test.

Table 3. Body composition and blood pressure of each group during the study

	Baseline			4 weeks			8 weeks			12 weeks (after washout period)		
	MEAN	SEM	p ^a	MEAN	SEM	p ^a	MEAN	SEM	p ^a	MEAN	SEM	p ^a
Body weight (kg)												
Placebo group	83.6	1.4	0.145	84.3 ^b	1.4	0.176	84.7 ^b	1.4	0.209	84.6 ^b	1.5	0.237
LcS group	86.7	1.5		87.2 ^c	1.5		87.3 ^d	1.6		87.5 ^e	1.5	
BMI (kg/m ²)												
Placebo group	29.0	0.4	0.454	29.3 ^b	0.4	0.538	29.4 ^b	0.4	0.624	29.4 ^b	0.4	0.679
LcS group	29.5	0.4		29.6 ^f	0.4		29.7 ^e	0.4		29.8 ^g	0.4	
Body fat (%)												
Placebo group	27.6	0.4	0.515	28.1 ^b	0.4	0.663	28.5 ^b	0.4	0.730	28.7 ^b	0.4	0.735
LcS group	28.0	0.5		28.3 ^c	0.5		28.8 ^b	0.5		29.1 ^b	0.5	
SBP (mmHg)												
Placebo group	126.5	1.7	0.023	129.8	1.9	0.590	128.3	2.1	0.848	132.5 ^h	1.8	0.672
LcS group	132.6	2.0		131.2	1.9		128.9	2.1		130.2	2.1	
DBP (mmHg)												
Placebo group	83.5	1.2	0.119	86.5 ⁱ	1.6	0.856	85.1	1.5	0.653	87.7 ^j	1.5	0.568
LcS group	86.8	1.7		86.1	1.8		84.2	1.5		86.2	1.6	

LcS: *L. casei* strain Shirota YIT 9029; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure.

^ap values analysed by the unpaired Student's t-test.

^{b-j}Mean values were significantly different from those at baseline (paired Student's t-test with Bonferroni-Holm adjustment). The p values were as follows: ^bp=0.000; ^cp=0.019; ^dp=0.014; ^ep=0.020; ^fp=0.024; ^gp=0.025; ^hp=0.015; ⁱp=0.002; ^jp=0.001.

Table 4. Plasma glucose levels during OGTT at each visit and changes from baseline at the end of the intervention period (8 weeks)

	Baseline			4 weeks			8 weeks			12 weeks (after washout period)			Changes from baseline to 8 weeks		
	MEAN	SEM	p ^a	MEAN	SEM	p ^a	MEAN	SEM	p ^a	MEAN	SEM	p ^a	MEAN	SEM	p ^a
Fasting (mg/dl)															
Placebo group	110.3	1.1	0.669	111.0	1.1	0.460	109.7	1.2	0.804	112.4	1.4	0.262	-0.6	0.9	0.833
LcS group	109.6	1.3		109.7	1.3		109.3	1.4		110.2	1.4		-0.3	0.8	
30-min (mg/dl)															
Placebo group	181.9	3.2	0.932	184.0	3.4	0.541	184.6	3.2	0.676	188.4	3.7	0.356	2.7	3.1	0.594
LcS group	181.5	3.0		187.1	3.8		186.6	3.6		183.9	3.2		5.1	3.2	
1-hr (mg/dl)															
Placebo group	217.9	3.3	0.853	213.3	3.9	0.939	210.2	6.1	0.713	213.1	4.7	0.608	-7.7	5.5	0.582
LcS group	218.8	3.7		213.8	5.2		207.1 ^b	5.3		209.4	5.4		-11.6	4.4	
90-min (mg/dl)															
Placebo group	199.9	4.6	0.456	198.6	4.7	0.372	194.7	6.3	0.724	187.1 ^c	6.0	0.989	-5.2	4.2	0.770
LcS group	195.1	4.4		192.0	5.7		191.8	5.3		187.0	6.3		-3.3	4.9	
2-hr (mg/dl)															
Placebo group	165.8	4.6	0.461	167.5	5.1	0.373	162.0	5.5	0.694	158.9	5.8	0.952	-3.9	4.5	0.250
LcS group	161.5	3.5		161.3	4.7		164.7	4.3		158.4	4.9		3.2	4.2	
AUC (mg/dl·h)															
Placebo group	368.9	5.1	0.642	367.6	5.7	0.705	362.7	8.0	0.893	362.1	6.9	0.634	-6.2	5.4	0.791
LcS group	365.5	5.2		364.2	6.8		361.3	6.4		357.3	7.4		-4.2	5.3	

OGTT: oral glucose tolerance test; LcS: *L. casei* strain Shirota YIT 9029.

^ap values analysed by the unpaired Student's t-test.

^{b, c}Mean values were significantly different from those at baseline (paired Student's t-test with Bonferroni-Holm adjustment). The p values were as follows: ^bp=0.036; ^cp=0.019.

Lipid profiles

Lipid profiles are shown in Table 6. TC, LDL-C, HDL-C, and non-HDL-C levels significantly increased compared

with at baseline at 8 weeks (TC, p=0.001; LDL-C, p=0.000; HDL-C, p=0.042; non-HDL-C, p=0.004) in the placebo group but not in the LcS group. TC, LDL-C, and non-HDL-C

Table 5. Glycoalbumin, HbA1c, and indices of insulin secretion and insulin resistance at each visit

	Baseline			4 weeks			8 weeks			12 weeks (after washout period)		
	MEAN	SEM	p ^a	MEAN	SEM	p ^a	MEAN	SEM	p ^a	MEAN	SEM	p ^a
Glycoalbumin (%)												
Placebo group	13.5	0.1	0.838	13.6	0.1	0.670	13.4	0.1	0.288	13.5	0.1	0.520
LcS group	13.4	0.2		13.5	0.2		13.2 ^b	0.2		13.3	0.2	
HbA1c (%)												
Placebo group	5.79	0.04	0.454	5.76	0.04	0.603	5.75	0.04	0.306	5.75	0.04	0.208
LcS group	5.74	0.04		5.73	0.05		5.69 ^c	0.05		5.67 ^d	0.05	
Fasting insulin (μU/ml)												
Placebo group	7.9	0.5	0.917	7.8	0.4	0.819	7.7	0.5	0.806	7.5	0.6	0.771
LcS group	8.0	0.5		7.9	0.5		7.5	0.4		7.3	0.4	
HOMA-IR												
Placebo group	2.17	0.15	0.973	2.14	0.12	0.906	2.09	0.14	0.802	2.12	0.18	0.588
LcS group	2.18	0.13		2.16	0.13		2.04	0.11		2.00	0.12	
HOMA-β												
Placebo group	61.3	4.2	0.736	59.6	3.4	0.583	60.2	3.8	0.941	55.0	3.3	0.684
LcS group	63.2	3.5		62.3	3.7		59.8	3.0		56.9	3.4	
Matsuda Index												
Placebo group	4.12	0.36	0.735	4.01	0.24	0.915	4.35	0.30	0.794	4.33	0.27	0.638
LcS group	3.97	0.22		3.97	0.24		4.24	0.25		4.55	0.38	
Insulinogenic Index												
Placebo group	0.43	0.03	0.998	0.47	0.04	0.713	0.44	0.04	0.687	0.46	0.04	0.917
LcS group	0.43	0.03		0.49	0.04		0.47	0.04		0.47	0.04	

LcS: *L. casei* strain Shirota YIT 9029; HOMA: homeostatic model assessment.

^ap values analysed by the unpaired Student's t-test.

^{b-d}Mean values were significantly different from those at baseline (paired Student's t-test with Bonferroni-Holm adjustment). The p values were as follows: ^bp=0.002; ^cp=0.006; ^dp=0.000.

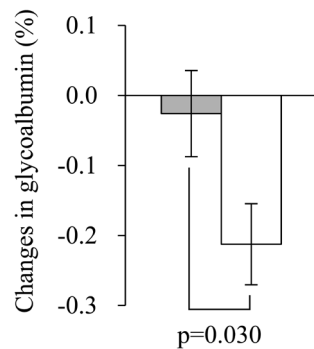


Fig. 2. Changes from baseline in glycoalbumin levels at the end of the consumption period.

Data are expressed as means \pm SEM. Closed bar, placebo group; open bar, LcS group.

Data were analysed by the unpaired Student's t-test.

levels were significantly lower in the LcS group than in the placebo group at 8 weeks (TC, $p=0.023$; LDL-C, $p=0.022$; non-HDL-C, $p=0.008$). At 12 weeks, these differences were not observed. When the changes from baseline among these parameters were compared between groups, the LcS group showed improvements in non-HDL-C ($p=0.0496$), TC

($p=0.079$), and LDL-C ($p=0.084$) levels (Fig. 3). TAG levels did not change during the trial in either group.

Stratified analysis

A stratified analysis based on the median 2-hr post-load PG value (164 mg/dl) at baseline was conducted to clarify which subjects showed a good response to probiotic treatment (Fig. 4). Among subjects with 2-hr post-load PG levels above the median at baseline ($n=50$), the reduction in the 1-hr post-load PG and GA levels at 8 weeks was significantly greater in the LcS group than in the placebo group ($p=0.036$ and $p=0.034$, respectively) (Fig. 4a and 4b). Interestingly, in the subgroup with higher baseline 2-hr post-load PG levels, the increase in the insulinogenic index was significantly greater in the LcS group than in the placebo group ($p=0.038$) (Fig. 4c), but similar changes were not observed in the Matsuda index (Fig. 4d). In the subgroup with lower baseline 2-hr post-load PG levels, there were no significant differences among these values between groups (data not shown).

DISCUSSION

A prediabetic state with elevated post-load PG levels increases the risk of type 2 DM and cardiovascular disease. For prevention of these diseases, pleiotropic control of

Table 6. Lipid profile at each visit

	Baseline			4 weeks			8 weeks			12 weeks (after washout period)		
	MEAN	SEM	p ^a	MEAN	SEM	p ^a	MEAN	SEM	p ^a	MEAN	SEM	p ^a
TC (mg/dl)												
Placebo group	218.3	4.4	0.330	223.0	4.0	0.103	228.4 ^b	4.3	0.023	222.8	4.2	0.218
LcS group	212.4	4.1		213.6	4.0		215.0	3.9		215.7	3.8	
HDL-C (mg/dl)												
Placebo group	50.8	1.3	0.255	52.3	1.6	0.680	52.6 ^c	1.5	0.305	53.9 ^d	1.6	0.745
LcS group	53.1	1.5		53.2	1.5		55.0	1.8		54.7	1.7	
LDL-C (mg/dl)												
Placebo group	142.6	4.0	0.234	147.3	3.9	0.101	151.7 ^e	4.1	0.022	147.6	4.0	0.083
LcS group	136.1	3.6		138.2	3.8		139.2	3.3		137.9	3.8	
Non-HDL-C (mg/dl)												
Placebo group	167.6	4.4	0.173	170.7	4.3	0.087	175.8 ^f	4.5	0.008	168.8	4.6	0.201
LcS group	159.3	4.1		160.4	4.1		160.0	3.7		161.0	3.9	
TAG (mg/dl)												
Placebo group	160.3	9.9	0.614	159.4	11.3	0.577	172.5	12.9	0.120	144.3	10.0	0.275
LcS group	153.4	9.4		151.1	9.3		147.0	9.8		179.8	31.2	

LcS: *L. casei* strain Shirota YIT 9029; TC: total cholesterol; LCL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; non-HDL-C: non-high-density lipoprotein cholesterol; TAG: triacylglycerol.

^ap values analysed by the unpaired Student's t-test.

^{b-f}Mean values were significantly different from those at baseline (paired Student's t-test with Bonferroni-Holm adjustment). The p values were as follows: ^bp=0.001; ^cp=0.042; ^dp=0.016; ^ep=0.000; ^fp=0.004.

metabolic abnormalities such as failure of glycaemic control and dyslipidaemia is required. Although some clinical trials have suggested that probiotic supplementation improves glycaemic control in type 2 DM patients [20], no clinical trials have been conducted in prediabetic subjects. Therefore, the present study aimed to investigate the effects of LcS on parameters of glycaemic and lipid control in prediabetic obese subjects characterised by post-load hyperglycaemia.

In the present study, there were no significant differences in post-load PG levels between the groups. However, a reduction in the GA level, which is one of the typical glycaemic control markers, was statistically significantly higher in the LcS group than in the placebo group at 8 weeks. The GA levels indicate an average blood glucose level over the preceding 2–4 weeks and reflect glucose fluctuations and postprandial glucose excursions [24]. Thus, GA is considered a suitable marker in terms of monitoring glycaemic control [24]. In the LcS group, but not in the control group, 1-hr post-load PG, GA, and HbA1c levels were decreased at 8 weeks compared with at baseline. Moreover, the reductions in GA and 1-hr post-load PG levels disappeared after the washout period. These results suggest that glycaemic control in the LcS group changed for the better. On the other hand, the reduction in HbA1c levels was maintained in the LcS group after the washout period. HbA1c is the most common marker for diagnosis of diabetes, and it reflects the average blood glucose levels during a relatively longer period (past 1–2 months) than GA [24]. Therefore, the reduction in HbA1c levels at 12 weeks could also be attributed to the consumption of LcS.

While the dietary intake and physical activity levels

remained constant throughout the trial in both groups, significant increases in body weight, BMI, and body fat percentage were observed in both groups when compared with those at baseline. These increases were also observed in both groups after the washout period, indicating that these increases were not attributed to the possible increase in calorie intake by consumption of test beverages (each 62 kcal/day) but other factors such as seasonal variation. There were no significant differences in these parameters between the groups. The glycaemic control markers remained constant throughout the study in the placebo group, suggesting that the changes in glycaemic control markers observed in the LcS group were associated with the consumption of LcS. Stratified analyses according to the median baseline 2-hr post-load PG levels revealed that the consumption of LcS resulted in significant improvements in the 1-hr post-load PG and GA levels in the subgroup with higher baseline 2-hr post-load PG levels (indicating relatively severe glucose intolerance) but not in the subgroup with lower baseline 2-hr post-load PG levels. These findings suggest that consumption of LcS has the potential to improve glycaemic control, especially in subjects with relatively advanced glucose intolerance, who are at high risk of both DM and cardiovascular disease.

Previous studies demonstrated that LcS improved post-load hyperglycaemia and insulin resistance in an obese mouse model induced by feeding a high-fat diet [15] and prevented the exacerbation of glycaemic control and decline of insulin action induced by overfeeding a high-fat diet in a clinical trial involving Caucasian subjects [19]. In contrast, the consumption of LcS did not affect the markers of insulin

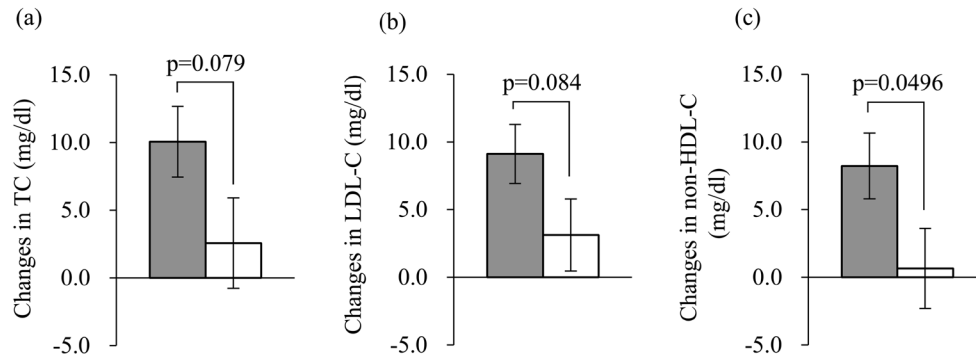


Fig. 3. Changes from baseline in blood cholesterol levels at the end of the consumption period.

Changes from baseline in (a) total cholesterol, (b) LDL-cholesterol, and (c) non-HDL-cholesterol levels at the end of the consumption period. Data are expressed as means \pm SEM. Closed bar, placebo group; open bar, LcS group. Data were analysed by the unpaired Student's t-test.

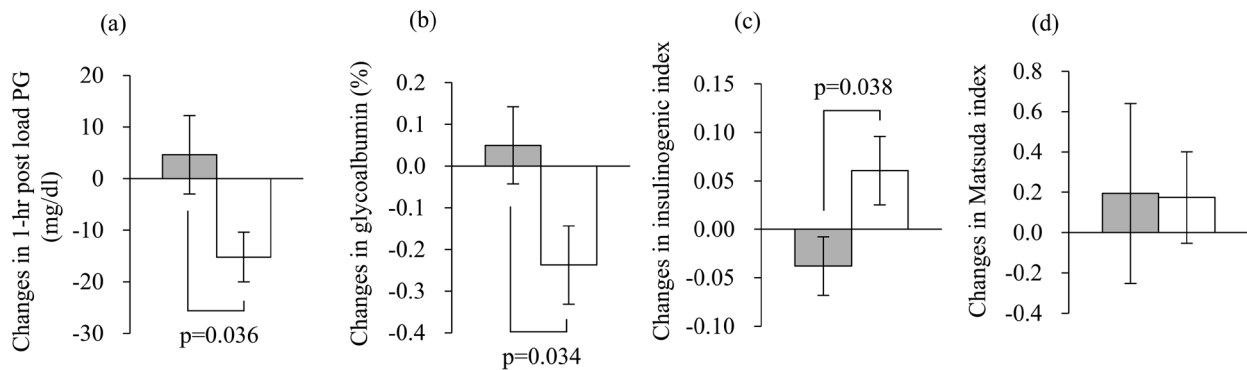


Fig. 4. Stratified analysis of glycaemic control based on median 2-hr post-load PG values at baseline.

Changes from baseline in (a) 1-hr post-load PG, (b) glycoalbumin, (c) insulinogenic index, and (d) Matsuda index at the end of the consumption period in subjects with high 2-hr post-load PG levels at baseline (164–245 mg/dl; placebo group, $n=26$; LcS group, $n=24$). Data are expressed as means \pm SEM. Closed bar, placebo group; open bar, LcS group. Data were analysed by the unpaired Student's t-test.

resistance such as the HOMA-IR and the Matsuda index in the present study. There are some differences in experimental settings between these studies. First, the degree of insulin resistance in East Asian populations including Japanese people is considerably lower than that in Caucasians [25]. In fact, the insulin resistance observed in the subjects in the present study was not as severe as that observed in Caucasian prediabetic individuals. Second, the previous studies were performed in the presence of acutely exacerbated insulin resistance induced by high-fat diet feeding. Therefore, the present study could not evaluate the effect of LcS on insulin resistance appropriately.

Stratified analyses according to the median 2-hr post-load PG values also found that consumption of LcS could increase the insulinogenic index—a marker of early-phase insulin-secretion capacity—in subjects with higher baseline 2-hr post-load PG levels. Failure of glucose-stimulated early-phase insulin secretion and insulin resistance are major

causes of impaired glucose tolerance [4]. Moreover, insulin secretion capacity is poorer in East Asian populations than in Caucasians [25], and a decreased insulinogenic index is the major factor involved in elevated 1-hr post-load PG values in Japanese males [26]. Therefore, LcS may affect glycaemic control via enhancement of pancreatic β cell function in subjects with relatively advanced glucose intolerance.

Recent animal studies have suggested that the inflammatory process is involved in pancreatic β cell dysfunction [27] as well as insulin resistance. Obesity-related inflammation is considered to be triggered, at least partly, by changes in the composition of the gut microbiota, which leads to increased intestinal permeability and consequential endotoxaemia. Moreover, Sato *et al.* have reported that dysbiosis of the gut microbiota and high rates of live gut bacteria in the blood are observed in Japanese type 2 DM patients, thus indicating the existence of translocation of bacteria from the gut to the bloodstream in this population [12]. Recently, Okubo *et al.*

demonstrated that LcS reduced the plasma levels of bacterial lipopolysaccharide and changed the composition of the gut microbiota in an animal model of hepatic steatosis [17]. On the other hand, LcS has been reported to exhibit beneficial effects via anti-inflammatory actions against inflammatory bowel disease [28], arthritis [29], and type 1 diabetes [30] in some animal models and alcoholic liver cirrhosis patients [31]. Thus, the reduction of intestinal permeability and the anti-inflammatory activity may influence pancreatic β cell function.

Hypercholesterolaemia is another major risk factor for the development of cardiovascular disease. Moreover, prediabetic subjects with elevated post-load PG levels exhibit a pro-atherogenic lipid profile including high non-HDL-C levels and low HDL-C levels [9, 10]. Thus, control of blood cholesterol levels in obese prediabetic subjects will contribute to lowering the risk of cardiovascular disease. The hypocholesterolaemic potential of probiotics has been verified in hypercholesterolaemic patients [32] and type 2 DM patients [33] but not in prediabetic subjects. In the present study, a significant increase was observed in the serum TC, LDL-C, and non-HDL-C levels in the placebo group, whereas the LcS group maintained constant levels. The increases in the TC, LDL-C, and non-HDL-C levels were suppressed in the LcS group when compared with the placebo group at 8 weeks. Because the serum cholesterol levels such as TC, LDL-C, and HDL-C were high and did not return to baseline in the placebo group after the washout period, the increases are considered to be due to seasonal variations in serum cholesterol levels, which are higher in winter than in summer in the Japanese population [34]. These findings suggest that consumption of LcS suppresses increases in blood cholesterol levels. Epidemiological studies have demonstrated that post-glucose load hyperglycaemia and type 2 DM are strongly associated with high non-HDL-C and low serum HDL-C but are weakly or not associated with serum levels of TC and LDL-C [9, 10]. Therefore, underlying mechanism(s) of the cholesterol-lowering effect of LcS may be independent of improved glycaemic control.

Although the hypocholesterolaemic effect and mechanisms of action of probiotics differ among strains and species, the following mechanisms have been proposed [32]: 1. assimilation of cholesterol; 2. binding/incorporating of cholesterol to cellular components, such as the cell surface or membrane; 3. enzymatic de-conjugation of bile acids by bile-salt hydrolase; and 4. suppression of the *de novo* synthesis of cholesterol by short chain fatty acids produced by probiotics. LcS is able to assimilate cholesterol *in vitro* [35], and oral administration of the cell wall components of LcS was found to increase faecal sterol secretion and suppress increases in the serum TC levels in cholesterol-fed rats [36]. Thus, LcS has a potential to prevent hypercholesterolaemia via reduction of dietary cholesterol absorption by binding and/or assimilating sterols in obese prediabetic subjects.

There are some limitations of the present study. First, no significant differences were observed in post-load PG levels

between the groups; the beneficial effect of LcS on post-load hyperglycaemia could not be clearly demonstrated. However, the statistically significant reduction in GA levels in the LcS group compared with that in the placebo group and the findings of the stratified analyses suggest that LcS has the potential to exert favourable effects on glycaemic control. Second, we did not investigate the influence of LcS on markers of inflammation and gut barrier function and gut microbiota composition; therefore, the exact mechanism of action of LcS remains unknown. Hence, further studies are required to clarify the beneficial effects of LcS on glycaemic control in a larger number of subjects with advanced glucose intolerance and/or insulin resistance and to clarify the mechanism of action of LcS using animal models and/or clinical trials.

In conclusion, the findings of the present study suggest that LcS may favourably affect metabolic abnormalities in obese prediabetic subjects, though the effects on glycaemic control may be limited.

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