Effect of Lactic Acid Bacteria on Lipid Metabolism and Fat Synthesis in Mice Fed a High-fat Diet

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Visceral fat accumulation is a major risk factor for the development of obesity-related diseases, including diabetes, hyperlipidemia, hypertension, and arteriosclerosis. Stimulation of lipolytic activity in adipose tissue or inhibition of fat synthesis is one way to prevent these serious diseases. Lactic acid bacteria have an anti-obesity effect, but the mechanisms are unclear. Therefore, we evaluated the effect of the administration of lactic acid bacteria (*Lactobacillus gasseri* NT) on lipid metabolism and fat synthesis in a mouse high-fat-diet model, focusing on visceral fat. Balb/c mice were fed a 45 kcal% fat diet for 13 weeks with and without a freeze-dried preparation of *L. gasseri* NT (10^9 CFU/g). An *ex vivo* glycerol assay with periovarian fat revealed that *L. gasseri* NT did not stimulate lipolytic activity. However, *L. gasseri* NT decreased the mRNA expression of sterol regulatory element-binding protein (SREBP) and its target gene fatty acid synthase (FAS) in the liver and decreased free fatty acid (FFA) in the blood. In conclusion, these findings indicated that administration of *L. gasseri* NT did not enhance lipid mobilization but can reduce fat synthesis, suggesting its potential for improving obesity-related diseases.

Key words: visceral fat, *L. gasseri* NT, free fatty acid, SREBP

INTRODUCTION

Obesity is defined as the excess accumulation of visceral adipose tissue [1] because of a lack of exercise and improper food habits. Visceral fat accumulation is a major risk factor for the development of several diseases, including diabetes, hyperlipidemia, hypertension and arteriosclerosis. Therefore, stimulation of lipolytic activity in adipose tissue or inhibition of fat synthesis is one way to prevent these serious diseases. For example, a dietary fiber, gum arabic, helps reduce body fat deposition by enhancing fat utilization in adipose tissues [2]. Higher lipolytic activity may be related to the higher expression level of beta 3 adrenaline receptor (ADRB3) in visceral adipose tissue. ADRB3 is, in fact, believed to contribute to the development of obesity [3]. On the other hand, the lipid composition of animal cells is controlled by SREBPs, transcription factors released from membranes by sterol-regulated proteolysis [4]. SREBPs are also known as a master regulator that participates in insulin resistance [5].

Probiotics, namely, lactobacilli and bifidobacteria, are now well recognized for their health-promoting effects [6]. In many cases, lactic acid bacteria react with the mucosal immunity of the gut to exert a physiological effect [7]. For example, lactic acid bacteria stimulate chloride secretion from the gut to increase the water content of the digesta [8] and adjust the sympathetic and parasympathetic nervous systems to reduce blood pressure and blood sugar levels [9]. The clearest effect was obtained in the improvement of hypercholesterolemia [10]. The anti-obesity effect of lactic acid bacteria has also been reported. For example, Usman and Hosono [11] indicated the capability to remove cholesterol from a culture medium with *Lactobacillus gasseri* strains. Portugal et al. [12] suggested that the effect of *L. delbrueckii* on cholesterol metabolism was through *ApoE*. These health-promoting effects may be related to the anti-obesity effects of lactic acid bacteria. Indeed, the anti-obesity effects of lactic acid bacteria have been reported. Kadooka et al. [13] indicated that administration of the probiotic bacterium *L. gasseri* in fermented milk reduced adiposity and body weight in obese adults, possibly by reducing lipid absorption and inflammatory status.

In this study, we evaluated the effects of the administration of lactic acid bacteria on lipid metabolism and fat synthesis in a mouse high-fat-diet model focusing on visceral fat. In addition, we introduce the use of an *ex-vivo* test to measure ADRB3-dependent fat mobilization to better determine the responsiveness of adipose tissue to adrenergic stimulation, since ADRB3 expression is an

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indirect indicator of fat mobilization.

MATERIALS AND METHODS

Animal experiments

Thirty 12-week-old female Balb/c mice were purchased from Japan SLC (Shizuoka, Japan). They were housed in five plastic cages (each containing six mice) in a room kept at 25±1°C with a 12-h light and dark cycle. The mice were divided into the following four groups: LaboMR (six mice fed a conventional crude diet, Labo MR Stock, Nihon Nosan Kogyo, Tokyo, Japan), 10%FD (six mice fed 10 kcal% fat diet, D12450B, Research Diets, Inc., New Brunswick, NJ, USA), 45%FD (nine mice fed 45 kcal% fat diet, D12451, Research Diets, Inc., New Brunswick, NJ, USA) and 45%FD+Lg (nine mice fed 45 kcal% fat diet, D12451, Research Diets, Inc.) including *Lactobacillus gasseri* NT(10^9 CFU/g). *L. gasseri* NT was originally isolated from human feces. The composition of each diet is shown in Table 1. The mice had free access to their diet and drinking water for 13 weeks. On the last day of feeding, the body weight and periovarian fat weight were measured. Periovarian fat, regarded as visceral adipose tissue, was removed through a midline incision. A portion of the periovarian fat was collected in RNAlater solution (Sigma, Japan) for mRNA analyses and in Hanks' Balanced Salt Solutions for the *ex vivo* test for lipolysis. A portion of the liver was removed and fixed in the RNAlater solution for mRNA analyses. Blood samples were collected from the inferior vena cava to measure the free fatty acid (FFA), triglyceride (TG) and insulin concentrations.

The experiments were approved by the Animal Experiment Committee of Kyoto Prefectural University.

Ex vivo test for lipolysis of visceral adipose tissue

The collected fat tissues were cut into pieces of ca. 20 mg and cultured in 96-well plates with a basal medium. The basal medium was composed of DMEM/Ham's F12 (Nacalai Tesque, Kyoto, Japan) containing 10% (v/v) bovine fetal serum [14] and 1% (w/v) Pen-Strep Solution (10,000 units/mL penicillin and 10,000 ug/mL streptomycin in 0.85% NaCl) (Thermo Fisher Scientific, Kanagawa, Japan). After three hours of incubation, glycerol released into media was measured with the use of a Glycerol Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA).

Blood test

Serum FFA and TG levels were determined using a NEFA C-test (Wako, Japan) and TG E-test (Wako, Japan), respectively. Serum insulin was measured using a Mouse Insulin Kit (Morinaga Institute of Biological Science, Yokohama, Japan).

Table 1. The composition of each diet

Ingredient	Labo MR Stock	D12450B	D12451
Protein	18.80%	19.20%	23.70%
Carbohydrate	54.70%	67.30%	41.40%
Fat	3.90%	4.30%	23.60%
Calories	2.31 kcal/g	3.87 kcal/g	4.73 kcal/g
Calories from fat	0.351 kcal/g	0.385 kcal/g	2.12 kcal/g

Determination of mRNA expression level

Extraction of RNA from the periovarian adipose tissue and the liver and subsequent cDNA synthesis was carried out as reported elsewhere [15]. The expression levels of each gene were evaluated by the real-time PCR approach using a LightCycler 480 Real-Time PCR System (Roche Applied Science, Tokyo, Japan). PCR was performed with a thermal cycle program with an initial denaturation at 95°C for 5 min followed by 50 cycles of 95°C for 10 s and 60°C for 20 s. In this analysis, the β-actin gene was used as the housekeeping gene. The genes and the oligonucleotide primer sets together with TaqMan probes were as follows: beta actin (GeneBank accession number: NM007393), 5′-agagggaaatcgtgcgtgac-3′ (forward) and 5′-caatagtgacctggccgt-3′ (reverse), Roche probe No.101; beta 3 adrenaline receptor (*ADRB3*; GeneBank accession number: NM013462), 5′-cagccagccctgttgaag-3′ (forward) and 5′-ccttcatagccatcaaacctg-3′ (reverse), Roche probe No.13; tumor necrosis factor alpha (*TNF-α*; GeneBank accession number: NM013693), 5′-ttgtcttaacgctgatttggt-3′ (forward) and 5′-gggagcagaggttcagtgat-3′ (reverse), Roche probe No.64; monocyte chemoattractant protein 1 (*MCP-1, CCL2*; GeneBank accession number: NM011333), 5′-catccacgtgttggctca-3′ (forward) and 5′-gatcatcttgctggtgaatgagt-3′ (reverse), Roche probe No.62; insulin receptor (*INSR*; GeneBank accession number: NM010568), 5′-agcacagtttgggagagtgg-3′ (forward) and 5′-ccagctgccacacaatacag-3′ (reverse), Roche probe No.4; sterol regulatory element-binding protein (*SREBP*; GeneBank accession number: NM011480), 5′-ggttttgaacgacatcgaaga-3′ (forward) and 5′-cgggaagtcactgtcttggt-3′ (reverse), Roche probe No.78; and fatty acid synthase (*FAS*; GeneBank accession number: NM007988), 5′-gctgctgttggaagtcagc-3′ (forward) and 5′-agtgttcgttcctcggagtg-3′ (reverse), Roche probe No.58. Delta Ct was calculated by subtraction of the crossing point cycle of the housekeeping gene from those of the other genes analyzed. All data for mRNA

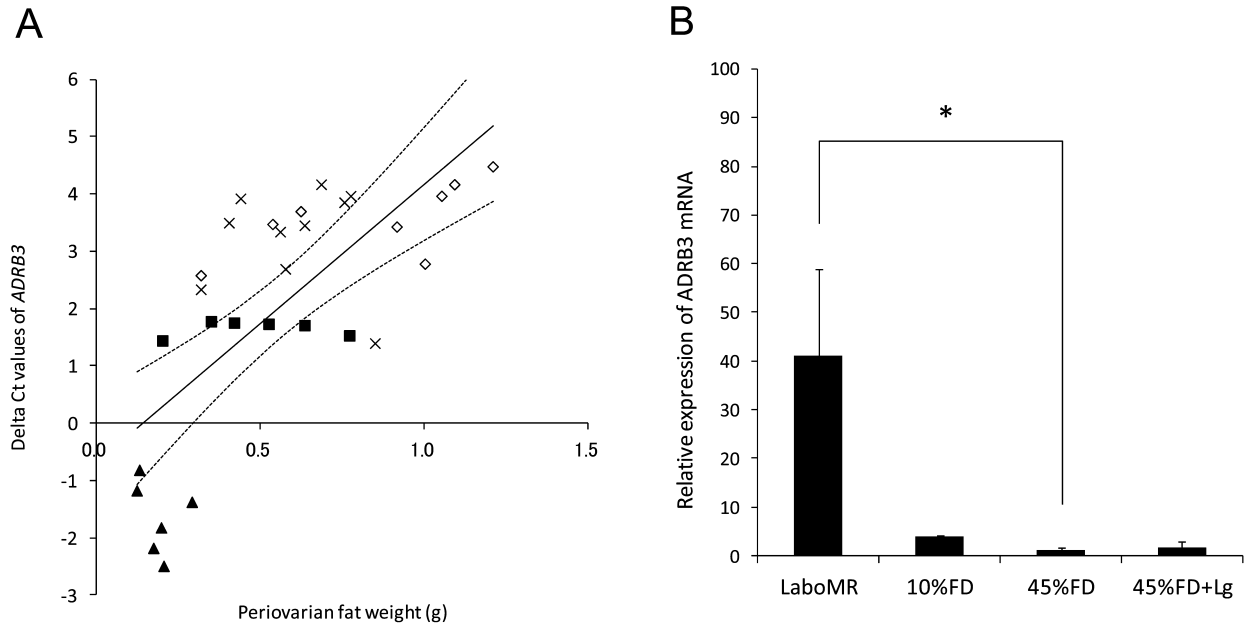

Fig. 1. ADRB3 mRNA expression of periovarian fat.

A, Relation between the delta Ct values of *ADRB3* of periovarian fat and periovarian fat weight. y=4.87x-0.70 (r=0.71) (y, delta Ct values of *ADRB3* of periovarian fat; x, periovarian fat weight; r, correlation coefficient) was obtained. Delta Ct was calculated by subtraction of the crossing point cycle of the β-actin gene from the ADRB3 gene. The broken lines represent 95% confidence lines. A significant positive correlation ($p<0.01$) is shown between the Delta Ct values of *ADRB3* and periovarian fat weight. This means that negative correlation is shown between the expression levels of *ADRB3* and periovarian fat weight. B, Relative expression of ADRB3 mRNA for each group. The data were assessed by Dunnett's multiple comparison tests. The mean value of the 45%FD group was set to 1.
▲, LaboMR; ■, 10%FD; ◇, 45%FD; ×, 45%FD+Lg * $p<0.01$

expression level, except for Fig. 1A and Fig. 3, are presented as a ratio relative to the control.

STATISTICAL ANALYSIS

Statistical analyses were performed with JMP 10 (SAS Institute Japan) and Excel Toukei 2010 (Social Survey Research Information, Tokyo, Japan).

RESULTS

Body weight and visceral fat

Body weight differed little between the 45%FD and 45%FD+Lg groups throughout the experiments. However, the weight of the periovarian fat was significantly lower in group 45%FD+Lg than in group 45%FD ($p<0.05$). The relative weight (% in body weight) of the periovarian fat of group 45%FD+Lg was nearly the same as that of group 10%FD (Table 2).

Gene expression in the periovarian adipose tissue and the liver

The relative expression levels of *ADRB3* in the periovarian fat decreased with the increase in periovarian fat weight ($p<0.01$) (Fig. 1A). However, there were no differences between group 45%FD+Lg and group 45%FD (Fig. 1B), contrary to our expectations. The relative expression of *TNF-α* mRNA in the periovarian fat was lower in group 45%FD+Lg than in group 45%FD ($p<0.05$) (Fig. 4A). The MCP-1 expression level in the periovarian fat was significantly enhanced by feeding a high fat diet (45%FD). However, there was no significant difference between groups 45%FD and 45%FD+Lg (Fig. 4B).

The relative expression of hepatic *SREBP* mRNA was lower in group 45%FD+Lg than in group 45%FD ($p<0.05$). The relative expression of hepatic *FAS* mRNA was lower in group 45%FD+Lg than in group 45%FD ($p<0.05$). The relative expression of hepatic *INSR* mRNA was higher in group 45%FD+Lg than in group 45%FD ($p<0.01$) (Fig. 5).

Glycerol release from the periovarian fat

A portion (ca. 20 mg) of the periovarian adipose tissue released around 0.30–1.31 umol of glycerol in 3 hours without an ADBR3 agonist. These values are considered

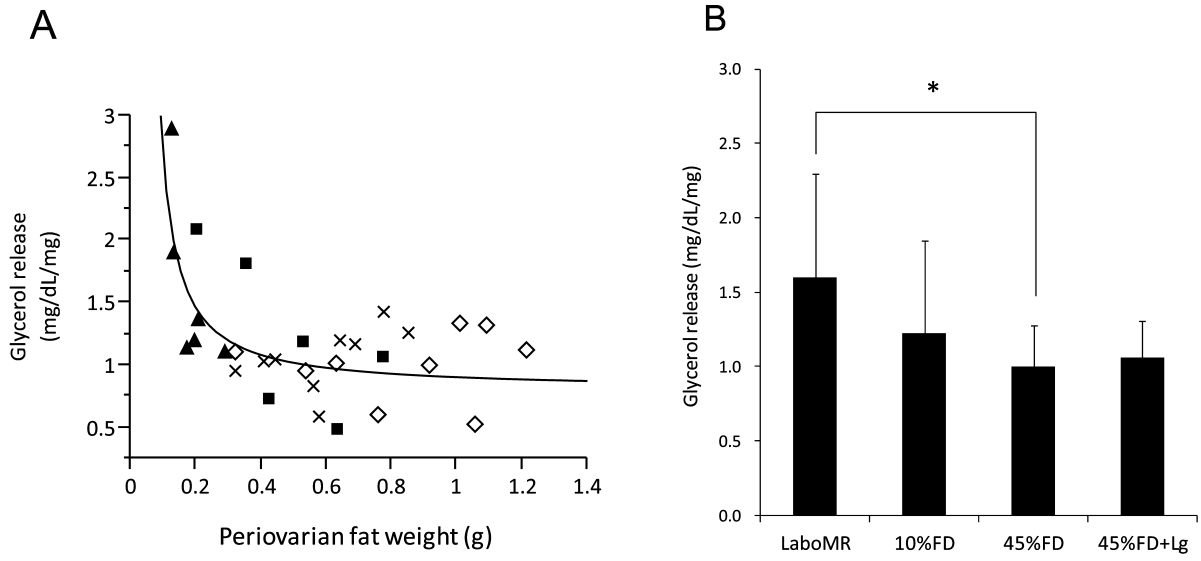

Fig. 2. Glycerol release from periovarian fat.

A, Relation between glycerol release from periovarian fat and periovarian fat weight. Log(y)=-0.22+0.12/x (y, glycerol release; x, periovarian fat weight) was obtained. A negative correlation ($p<0.05$) is shown between the glycerol release and periovarian fat weight. B, Glycerol release from periovarian fat in each group. The data were assessed by Dunnett's multiple comparison tests. The mean value of the 45%FD group was set to 1.

▲, LaboMR;■, 10%FD; ◇, 45%FD; ×, 45%FD+Lg * $p<0.01$

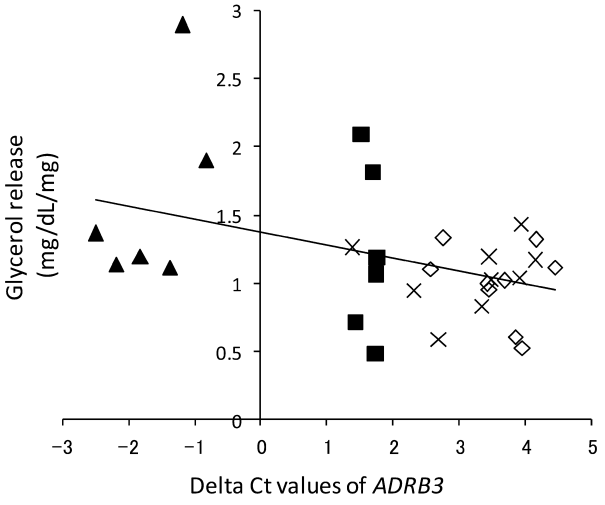

Fig. 3. Relation between glycerol release from periovarian fat and the delta Ct values of *ADRB3* of periovarian fat.

y=–0.10x–1.37 (r=0.41) (y, glycerol release from periovarian fat; x, delta Ct values of *ADRB3* of periovarian fat; r, correlation coefficient) was obtained. Delta Ct was calculated by subtraction of the crossing point cycle of the β-actin gene from that of the ADRB3 gene. A significant negative correlation ($p<0.05$) is shown between glycerol release from periovarian fat and the delta Ct values of *ADRB3* of periovarian fat. This means that positive correlation is shown between between glycerol release from periovarian fat and the expression levels of *ADRB3*.

to represent the range of the basal lipolytic rate. Diet significantly affected the lipolytic rate, as shown in Fig. 2A and Fig. 2B.

The relationship between the glycerol release and periovarian fat weight is also shown in Fig. 2A. Glycerol release decreased with the increase in periovarian fat weight ($p<0.05$), but there was no difference between group 45%FD and group 45%FD+Lg (Fig. 2B). A similar relationship was observed for the effect of *L. gasseri* NT, as shown in Fig. 1B.

Blood FFA, TG, and insulin

As shown in Table 3, a significant difference in FFA between group 45%FD and group 45%FD+Lg was observed ($p<0.05$).

DISCUSSION

We evaluated the effects of the administration of lactic acid bacteria on lipid metabolism and fat synthesis in a mouse high-fat-diet model, focusing on visceral fat.

Lipid mobilization from the adipose tissue releases FFA and glycerol. This mobilization is mediated by the sympathetic nervous system; norepinephrine is the most potent regulator of lipid mobilization in adipocytes [16]. This stimulation is mediated by the β3 adrenaline receptor [17]. For this reason, mRNA expression of *ADRB3* was

Table 2. Effects of L. gasseri NT on body weight and periovarian fat weight

	LaboMR	10%FD	45%FD	45%FD+Lg
Body weight (g)	24.2 ± 1.19**	24.9 ± 1.77**	29.1 ± 2.06	28.7 ± 1.86
Periovarian fat (g)	0.189 ± 0.061**	0.487 ± 0.204**	0.837 ± 0.294	0.585 ± 0.175*
Periovarian fat (%)	0.775 ± 0.217**	1.921 ± 0.719*	2.832 ± 0.881	2.030 ± 0.559*

Each value is the mean ± SD.
The data were assessed for statistical significance compared with the 45%FD group by Dunnett's multiple comparison tests.
A significant difference in periovarian fat between the 45%FD group and 45%FD+Lg group was observed ($p<0.05$).
**$p<0.01$; *$p<0.05$

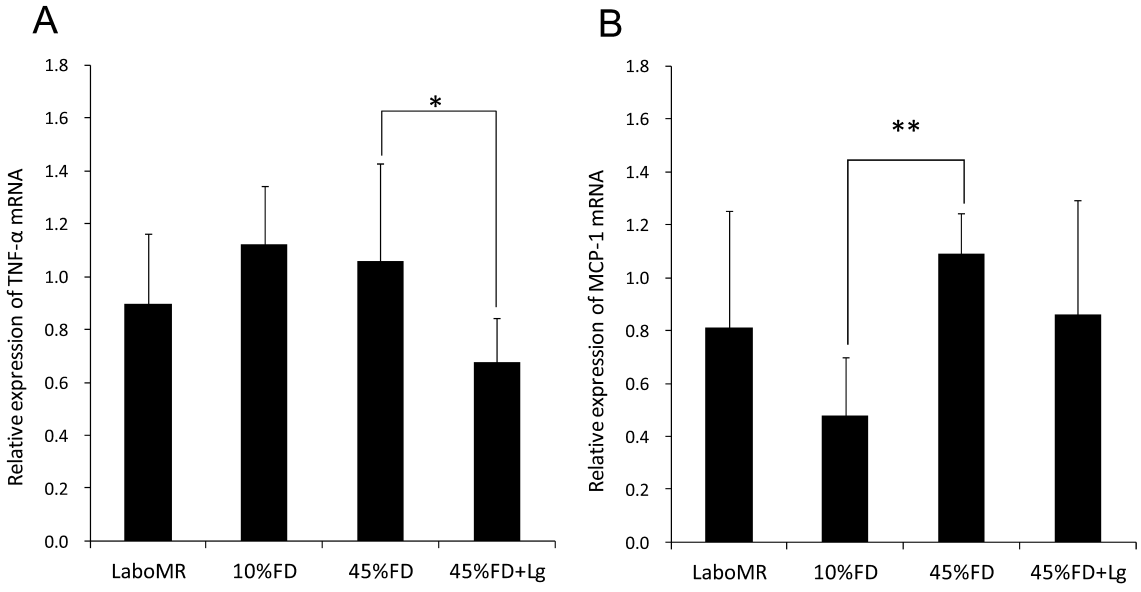

Fig. 4. Relative expression of proinflammatory marker gene in periovarian fat.
A, Relative expression of TNF-α mRNA in periovarian fat. The data were assessed by Dunnett's multiple comparison tests. The mean value of the 45%FD group was set to 1. B, Relative expression of MCP-1 mRNA in periovarian fat. The data were assessed by Dunnett's multiple comparison tests. The mean value of the 45%FD group was set to 1.
**$p<0.01$; *$p<0.05$

used to evaluate lipolysis [2, 18, 19]. In this context, an *ex vivo* test is a beneficial way to evaluate directly lipid mobilization because *ADRB3* expression is an indirect indicator of fat mobilization. It was shown in this experiment that the heavier visceral adipose tissue had a lower expression of *ADRB3*. Therefore, the negative correlation between adipose tissue weight and *ADRB3* expression level suggests the involvement of *ADRB3* in the development of obesity, as indicated by earlier studies [20]. Declines in adrenergic receptor expression may result in lower lipid mobilization ($p<0.05$) (Fig. 3), which leads to the development of obesity. However, this was not the case when we compared groups 45%FD+Lg and 45%FD. The periovarian fat weight in the former group was significantly lower than that in the 45%FD group without a significant difference in *ADRB3* expression and glycerol release. This suggests that the anti-obesity effects

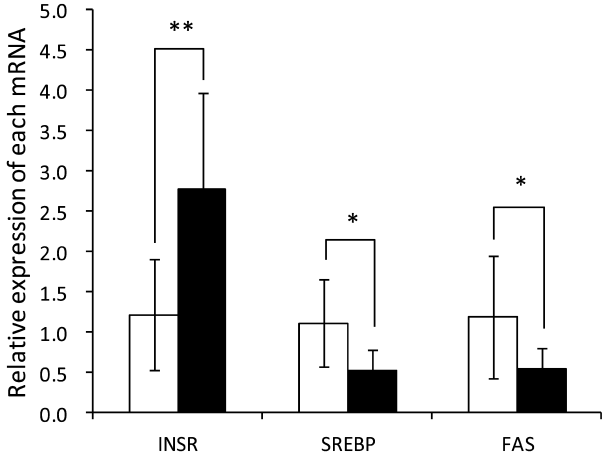

Fig. 5. Relative expression of insulin receptor (*INSR*), sterol regulatory element-binding protein (*SREBP*) and fatty acid synthase (*FAS*) mRNA in the liver. Differences were evaluated using the Student's t-test.
□, 45%FD; ■, 45%FD+Lg **$p<0.01$; *$p<0.05$

Table 3. Effects of L. gasseri NT on serum lipid and insulin

	45%FD	45%FD+Lg
TG (mg/dL)	108.8 ± 41.0	91.4 ± 6.3
FFA (mEq/L)	1.103 ± 0.315	0.835 ± 0.121*
Insulin (pg/mL)	3800 ± 1968	2773 ± 841

Each value is the mean ± SD.
Differences were evaluated using the Student's t-test.
A significant difference in FFA between the 45%FD group and 45%FD+Lg group was observed ($p<0.05$).
*$p<0.05$

of *L. gasseri* NT may have other mechanisms. Indeed, stimulation of autonomic nerve systems by intraduodenal administration of lactic acid bacteria was observed in the study of Nagai et al. [21], in which decline of renal sympathetic nerve activity was suggested.

Obesity leads to infiltration of macrophages in adipose tissues with a concomitant increase in pro-inflammatory cytokines, such as TNF-α [22]. Since the level of TNF-α in adipose tissue correlates with the degree of adiposity and insulin resistance [22], TNF-α, therefore, is a key molecule that exacerbates the lifestyle-related diseases caused by obesity. In this experiment, a high-fat diet did not enhance *TNF-α* expression significantly. Therefore, it was suggested that the level of inflammation in adipose tissue in the 45%FD group was not severe. Administration of *L. gasseri* NT significantly decreased *TNF-α* expression in the periovarian fat tissue in this experiment. It is known that *L. rhamnosus* GG decreased TNF-α production in a murine macrophage cell line, RAW 264.7 [23]. Inactivation of macrophages by lactobacilli may be a common phenomenon for probiotic lactobacilli [24]. For example, *L. casei* Shirota suppressed the production of TNF-α by macrophages [25].

If macrophages are the main target of lactic acid bacteria to reduce TNF-α expression in adipose tissue, their infiltration may be affected by *L. gasseri* NT. Monocyte chemoattractant protein 1 (MCP-1) and its receptor, CCR2, play pivotal roles in the development of inflammatory responses. Macrophage infiltration in adipose tissue induced by feeding on a high-fat diet for normal wild-type mice was not observed in CCR2-KO mice, and there was a significant reduction in TNF-α mRNA expression [26–28]. Accordingly, it is reasonable to conclude that most TNF-α in adipose tissues is induced by infiltrated macrophages. The increase in MCP-1 expression in adipose tissue in the 45%FD group suggests the pro-inflammatory status of these mice. *L. gasseri* NT did not significantly alter the expression of MCP-1. Together with the reduction in TNF-α expression in the adipose tissue, it is suggested that *L. gasseri* NT has at least a preventive effect against inflammation induced by fat accumulation.

The intestine is an important immune organ, and the intestinal microbiota plays a crucial role in the development of local and systemic immunity. Stimulation of mucosal immune tissue by probiotic lactobacilli, indeed, inactivates macrophages [24].

Hyperinsulinemia induces SREBP-1c expression, leading to the transcriptional activation of all lipogenic genes in the liver [29]. SREBP has also been known to play a critical role in the development of beta-cell dysfunction in the pancreas caused by elevated FFA [30]. In our report, the mRNA expression levels of *SREBP* and *FAS* were significantly lower in group 45%FD+Lg than in group 45%FD. Blood serum FFA was significantly lower in group 45%FD+Lg than in group 45%FD. These results indicate that suppression of *SREBP* and *FAS* in the liver leads to a decrease in fatty acid synthesis and FFA. In a previous study, lactic acid bacteria reduced hepatic lipogenesis and particularly reduced in the expression of SREBP and FAS [31]. Administration of lactic acid bacteria, therefore, demonstrates anti-lipogenic effects at least in the liver.

In this experiment, the blood insulin level was not high enough to be judged as demonstrating insulin resistance (Table 3). In addition to this, there was no significant difference in insulin level between groups 45%FD+Lg and 45%FD. Therefore, the involvement of insulin in the effects of *L. gasseri* NT on lipid metabolism of mice is still hard to prove.

In conclusion, oral administration of *L. gasseri* NT did not enhance lipid mobilization but could reduce fat synthesis in adipose tissue, which suggests its potential to prevent the development of obesity and obesity-related disorders. It is not clear in which pathways the stimulatory signal caused by orally administered freeze-dried *L. gasseri* NT was transmitted to the effective sites in the liver and the adipose tissue. Further studies are required to clarify this point.

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