Original Research

Lactobacillus plantarum strain No. 14 reduces adipocyte size in mice fed high-fat diet

Naoki Takemura[1], Takuma Okubo[1] and Kei Sonoyama[2]

[1]Graduate School of Life Science; [2]Laboratory of Food Biochemistry, Research Faculty of Agriculture, Hokkaido University, Kita-9, Nishi-9, Kita-ku, Sapporo, Hokkaido 060-8589, Japan
Corresponding author: Kei Sonoyama. Email: ksnym@chem.agr.hokudai.ac.jp

Abstract

Because gut microbiota has recently attracted much attention as an environmental factor involved in the development of obesity, probiotics may be useful in preventing and/or improving obesity and its related disorders. The present study aimed to investigate the effects of *Lactobacillus plantarum* strain No. 14 (LP14), a bacterial strain reported to decrease body fat percentage in healthy volunteers, on adipocyte size in mice. Female C57BL/6 mice were fed either normal- or high-fat diet and administered intragastrically with LP14 (1×10^8 colony-forming units/mouse) or vehicle daily for 11 weeks. High dietary fat intake increased body weight gain, white adipose tissue weight, mean adipocyte size and serum total cholesterol and leptin concentrations, and decreased serum adiponectin concentration. In mice fed the high-fat diet, LP14 administration significantly reduced the mean adipocyte size and tended to reduce the white adipose tissue weight and serum total cholesterol and leptin concentrations as compared with the vehicle-administered mice. All mice had undetectable serum levels of conjugated linoleic acids that reportedly exert antiobesity action. In a separate experiment, LP14 ingestion had no influence on serum triacylglycerol accumulation following olive oil administration in Triton WR1339-treated mice, suggesting that dietary fat absorption is unaffected by LP14. In conclusion, we propose that LP14 may exert a beneficial effect on the onset of diet-induced obesity by reducing the cell size of white adipose tissues, and it seems unlikely that previously reported mechanisms for other bacterial strains are involved in the action of LP14.

Keywords: *Lactobacillus plantarum*, probiotics, diet-induced obesity, mice

Experimental Biology and Medicine 2010; **235**: 849–856. DOI: 10.1258/ebm.2010.009377

Introduction

Obesity and its associated disorders, including cardiovascular diseases, type 2 diabetes mellitus and certain cancers, have become public health problems worldwide. Although the development of obesity is a process involving genetic and environmental factors, the complex pathways are poorly understood. Recently, gut microbiota has attracted much attention as an environmental factor involved in the development of obesity.[1] In particular, Dr Jeffrey I Gordon and co-workers have nicely illustrated the relationship between gut microbiota and obesity in mice and humans. Bäckhed *et al.*[2] demonstrated that conventionalization of germ-free mice with normal gut microbiota harvested from the cecum of conventionally raised mice leads to obesity and insulin resistance. Ley *et al.*[3] demonstrated by analysis of bacterial 16S rRNA gene sequences that genetically obese *ob/ob* mice had a 50% reduction in the abundance of bacteroidetes and a proportional increase in firmicutes, and analogous differences in gut microbiota were observed in studies of lean versus obese humans.[4] In addition, Turnbaugh *et al.*[5] demonstrated that this trait was transmissible, as colonization of germ-free wild-type mice with gut microbiota harvested from *ob/ob* mice resulted in a significantly greater increase in total body fat when compared with colonization with microbiota from lean donors. Similarly, transplantation of a diet-induced obesity gut microbiota to lean germ-free recipient mice promoted greater fat deposition than transplantation from lean donor mice.[6] These findings shed light on the impact of gut microbiota on genetic and diet-induced obesity.

It has also been demonstrated that low-grade inflammation, which contributes to the development of pathologies associated with obesity,[7] is at least partly attributable to gut microbiota. Cani *et al.*[8] showed that increased uptake of gut microbiota-derived endotoxin/lipopolysaccharide in mice fed high-fat diet (HFD) results in metabolic endotoxemia, which in turn contributes to obesity and insulin resistance. They also demonstrated that

modulation of gut microbiota by prebiotic and antibiotic treatments reduced the metabolic endotoxemia and improved obesity and insulin resistance in mice fed HFD.[9,10] Thus, regulating gut microbiota may be an appropriate strategy to improve obesity and its related disorders.

Probiotics are defined as viable microbial dietary supplements that exert beneficial effects on host health.[11] Probiotics have attracted public attention because of their potential effectiveness for both the prevention and treatment of immune diseases.[12] In addition, recent experimental studies have reported the preventive effects of some bacterial strains on obesity. *Lactobacillus rhamnosus* PL60 and *L. plantarum* PL62, which are capable of producing conjugated linoleic acids (CLAs) that reduce body fat,[13,14] exerted beneficial effects on diet-induced obesity in mice.[15,16] Consumption of fermented skimmed milk containing *L. gasseri* SBT2055 reduced the size of adipocytes in visceral adipose tissues possibly through the inhibition of dietary fat absorption in rats.[17,18] VSL no. 3, a mixture of viable lyophilized bifidobacteria, lactobacilli and *Streptococcus thermophilus*, improved diet-induced obesity and its related hepatic steatosis and insulin resistance by increasing hepatic natural killer T-cells and reducing inflammatory signaling in mice.[19] *L. paracasei* ST11 (NCC2461) reduced diet-induced obesity by enhancing lipolysis in white adipose tissues (WATs) and thermogenesis in brown adipose tissues (BATs) by enhancing sympathetic nerve activity and suppressing parasympathetic nerve activity in rats.[20] These animal data suggest that some bacterial strains may be useful probiotics to prevent diet-induced obesity and its related disorders.

In humans, Nagata *et al.*[21] reported a small-scale, randomized, placebo-controlled, double-blind trial that showed a body-fat-reducing effect of *L. plantarum* strain No. 14 (LP14), which is used as a starter strain for pickled shallots. Of the 28 healthy female volunteers, 13 volunteers received lyophilized powder containing 2×10^{10} colony-forming units (CFU) of LP14 and the rest received placebo (branched dextrin) daily for three weeks. Volunteers receiving LP14 showed a significant decrease in their body fat percentage without any change in body weight, while there was no change in the body fat percentage of the placebo group.[21] This suggests that LP14 may be a candidate for probiotic bacterial strains to prevent obesity and its related disorders. In order to illuminate the obesity-preventive action of LP14, the present study investigated the effect of LP14 on adipose tissue weight, adipocyte size and serum lipid and adipocytokine concentrations in mice fed the normal-fat diet (NFD) and HFD.

Materials and methods

Animals and diets

All study protocols were approved by the Animal Use Committee of the Hokkaido University Research Faculty of Agriculture (approval no. 16), and animals were maintained in accordance with the guidelines for the care and use of laboratory animals of Hokkaido University.

Female C57BL/6 mice (age, 3 weeks) were purchased from Japan SLC (Hamamatsu, Japan) and housed in standard plastic cages in a temperature-controlled ($23 \pm 2^{\circ}$C) room under a 12-h light:dark cycle. They were allowed free access to food and water. Mice were acclimatized with the purified diet prepared according to AIN-93G[22] for one week before starting the experiment. Mice were then allocated into two groups of mice fed NFD and HFD (Table 1). Mice in the NFD and HFD groups were further divided into two groups ($n = 5$ or 6 per group); mice administered intragastrically with 0.2 mL of phosphate-buffered saline (PBS) containing lyophilized powder of LP14 (1×10^8 CFU/mouse; donated by Momoya Co, Ltd, Tokyo, Japan) daily, and mice administered 0.2 mL of PBS containing branched dextrin (control) daily. Mice in each group were housed in a single cage ($n = 5$ or 6 per cage). Body weight of individual mice was measured weekly. After 11 weeks of feeding, mice were fasted for six hours and were thereafter anesthetized by inhalation of diethyl ether, after which whole blood was drawn from the carotid artery. Serum was separated from blood samples and was subjected to the measurement of lipids, adipocytokines and CLAs, as described below. Following a laparotomy, the liver and WATs from periuteral, periovarian, mesenteric, perirenal and subcutaneous (extending from the dorsolumbar area to the inguinal area) regions were excised and weighed. Periovarian WAT was subjected to measurement of adipocyte size as described below.

In a separate experiment, 18 female C57BL/6 mice (6 weeks old) were acclimatized for one week as described above, and were then subjected to an oral fat-loading test, as described below.

Measurement of serum lipids and adipocytokines

Serum concentrations of triacylglycerols (TAG), nonesterified fatty acids (NEFA) and total cholesterol were

Table 1 Composition of test diets

Ingredient	NFD (normal-fat diet) (g/kg)	HFD (high-fat diet) (g/kg)
α-Cornstarch*	529.5	299.5
Casein†	200.0	200.0
Sucrose‡	100.0	100.0
Soybean oil¶	70.0	70.0
Lard¶	0.0	230.0
Cellulose#	50.0	50.0
Mineral mix (AIN-93-MX)**	35.0	35.0
Vitamin mix (AIN-93-VX)**	10.0	10.0
L-Cystine¶	3.0	3.0
Choline bitartrate¶	2.5	2.5

NFD, normal-fat diet; HFD, high-fat diet
*Purchased from Chuo-Shokuryou (Amylalpha CL; Aichi, Japan)
†Purchased from New Zealand Dairy Board (ALACID; Wellington, New Zealand)
‡Gifted from Nippon Beet Sugar Mfg Co (Obihiro, Japan)
¶Purchased from Wako Pure Chemical Industries (Osaka, Japan)
#Purchased from Advantec Toyo Ltd (Cellulose powder type D; Tokyo, Japan)
**Mineral mixture and vitamin mixture are identical to AIN-93-MX and AIN-93-VX, as reported by Reeves *et al.*[22] respectively. These were purchased from Nihon Nosan Kogyo Co (Kanagawa, Japan)

measured using commercial enzymatic reagent kits (Triglyceride G-test Wako, NEFA C-test Wako and Cholesterol E-test Wako, respectively; Wako Pure Chemical Industries, Osaka, Japan). Serum adiponectin and leptin concentrations were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (mouse/rat adiponectin ELISA kit [Otsuka Pharmaceutical, Tokyo, Japan] and ELISA mouse leptin kit [Morinaga Institute of Biochemical Science, Kanagawa, Japan], respectively).

Cultivation of LP14

de Man Rogosa Sharpe (MRS) broth (Becton Dickinson, Franklin Lakes, NJ, USA) was used for the cultivation of LP14. LP14 during logarithmic growth phase in MRS broth were collected by centrifugation at 3000 *g* for 10 min and re-suspended in MRS broth. This washing step was repeated once more. Then, 1×10^7 CFU of LP14 were cultured in 20 mL of MRS broth supplemented with 0.1 mg/mL of linoleic acid and 0.01 mg/mL of Tween-80. After the cultivation at 37°C for 12 h, the culture supernatant was collected by centrifugation at 3000 *g* for 10 min at 4°C and immediately subjected to the analysis of CLA as described below.

Measurement of CLAs

Aliquots of serum samples obtained from five or six mice were pooled in each group. Lipids were extracted from the serum samples and the culture supernatant of LP14 and were methylated according to the method described by Alonso *et al.*[23] Internal standard (heptadecadienoic acid/hexane) was added to serum samples. Amounts of *cis*-9, *trans*-11-octadecadienoic acid (c9, t11-CLA), *trans*-9, *trans*-11-octadecadienoic acid (t9, t11-CLA) and *trans*-10, *cis*-12-octadecadienoic acid (t10, c12-CLA) were measured by gas chromatography-mass spectrometry (GC-MS) using a QP5000 (model GC-17A; Shimadzu, Kyoto, Japan) with a ZB-column (30 m × 0.25 mm; 0.5-μm phase thickness). Helium was used as a carrier gas at a flow rate of 1.3 mL/min. Oven temperature was maintained at 100°C for five minutes, followed by an increase to 280°C at a rate of 20°C/min. A splitless injector was used to introduce sample (1 μL) into the GC. CLA peaks were identified by comparison with the retention times and MS of the authentic samples of each CLA isomer (Cayman Chemical, Ann Arbor, MI, USA). The spectra of methyl esters of heptadecadienoic acid and linoleic acid isomers have molecular ions at $m/z = 284$ and $m/z = 294$, respectively.

Measurement of adipocyte size

Samples from periovarian WAT (~100 mg) were fixed with osmium tetroxide (Sigma, St Louis, MO, USA) according to the method of Hirsch *et al.*[24] After fixation, samples were passed through a 250-μm nylon filter in order to remove fibrous tissue and intact shreds of fixed adipose tissue. Tissue shreds were gently rubbed with a plastic plunger on the filter and were flushed thoroughly with PBS. The filtrate was then passed through a 25-μm nylon filter to trap fixed adipocytes, and the cells were washed thoroughly with PBS. Cell suspensions were applied to glass slides and were then observed under a light microscope. A total of 200 cells per mouse were analyzed using Scion Image software (www.scioncorp.com).

Oral fat-loading test

After fasting overnight, mice were divided into three groups ($n = 6$ per group). Mice were orally administered 0.1 mL of 3% (w/v) carboxymethyl cellulose sodium salt solution containing either branched dextrin, lyophilized powder of LP14 (1×10^8 CFU/mouse) or orlistat (Xenical; 60 mg/kg body weight; Roche Diagnostics, Basel, Switzerland). At 30 min after administration, mice were intraperitoneally injected with 10% (w/v) Triton WR1339 (1 mg/kg body weight; Nacalai Tesque, Kyoto, Japan) in PBS, followed by oral administration of olive oil (5 mL/kg body weight). Blood samples were collected from the retro-orbital venous plexus at zero, one, two and four hours after the oral fat loading. Mice were deprived of food throughout the experimental period. Serum TAG concentration was measured as described above.

Statistical analyses

Results are presented as means ± standard error of the mean. Tukey–Kramer's test following one-way or two-way analysis of variance (ANOVA) was used to compare mean values. Data analysis was performed using StatView for Macintosh (version 5.0; SAS Institute, Cary, NC, USA). Correlations between parameters were assessed by Pearson's correlation test using Microsoft Excel. P values of <0.05 were considered to be statistically significant.

Results

High dietary fat intake significantly increased body weight gain (Table 2). There was no effect of LP14 administration on body weight gain in both mice fed NFD and HFD. High dietary fat intake significantly increased inguinal, perituteral, periovarian, mesenteric and perirenal WAT weights as well as the total WAT weight (Table 2). WAT weights were similar between vehicle- and LP14-administered mice fed NFD. In mice fed HFD, however, WAT weights tended to be lower in LP14-administered mice than in vehicle-administered mice. Neither high dietary fat intake nor LP14 administration altered liver weight. All tissue weight data are also expressed as tissue size relative to body weight, and the relative weight of WATs showed similar tendencies for the absolute values. Relative weight of liver was significantly decreased by high fat intake. Daily food intake could not be measured accurately.

Serum TAG concentration was similar among the groups (Table 2). High dietary fat intake significantly increased serum total cholesterol and leptin concentrations, and decreased serum NEFA and adiponectin concentrations. Serum total cholesterol and leptin concentrations in mice

Table 2 Effects of *Lactobacillus plantarum* strain No. 14 administration on body weight, tissue weight, serum lipids and adipocytokines in C57BL/6 mice fed NFD and HFD*

	NFD		HFD		Two-way ANOVA *P* value		
	Veh	LP14	Veh	LP14	Fat	LP14	Fat × LP14
Body weight gain (g)	4.1 ± 0.5a	4.7 ± 0.4a	7.5 ± 1.3b	7.7 ± 0.7b	<0.001	0.607	0.733
Tissue weight (mg)							
Liver	744 ± 31	737 ± 25	779 ± 67	728 ± 35	0.737	0.475	0.589
Inguinal fat pad	217 ± 27a	217 ± 4a	812 ± 187b	526 ± 35ab	<0.001	0.151	0.151
Periuteral fat pad	245 ± 25a	284 ± 43a	687 ± 116b	502 ± 90ab	<0.001	0.332	0.146
Periovarian fat pad	71 ± 13a	79 ± 13a	189 ± 45b	147 ± 23ab	0.002	0.503	0.338
Mesenteric fat pad	98 ± 5a	95 ± 14a	218 ± 41b	147 ± 25ab	0.002	0.144	0.177
Perirenal fat pad	44 ± 9a	47 ± 9a	191 ± 46b	129 ± 32ab	0.001	0.298	0.253
Total fat pad	676 ± 63a	722 ± 109a	2056 ± 477b	1450 ± 265ab	0.004	0.262	0.195
Relative tissue weight (mg/g body weight)							
Liver	36.8 ± 1.5	35.4 ± 1.2	32.7 ± 2.8	31.7 ± 1.5	0.006	0.396	0.848
Inguinal fat pad	10.7 ± 1.3a	10.4 ± 0.2a	34.1 ± 7.9b	22.9 ± 1.5ab	<0.001	0.103	0.129
Periuteral fat pad	12.1 ± 1.2a	13.7 ± 2.1a	28.9 ± 4.9b	21.8 ± 3.9ab	<0.001	0.320	0.129
Periovarian fat pad	3.5 ± 0.6a	3.8 ± 0.6a	7.9 ± 1.9b	6.4 ± 1.0ab	0.002	0.531	0.388
Mesenteric fat pad	4.9 ± 0.2a	4.6 ± 0.7a	9.2 ± 1.7b	6.4 ± 1.1ab	0.004	0.121	0.192
Perirenal fat pad	2.2 ± 0.4a	2.3 ± 0.4a	8.0 ± 1.9b	5.6 ± 1.4ab	<0.001	0.293	0.271
Total fat pad	33.4 ± 3.0a	34.5 ± 4.7a	84.2 ± 15.7b	63.2 ± 9.4ab	<0.001	0.220	0.178
TAG (mg/dL)	54 ± 6	53 ± 3	50 ± 7	41 ± 3	0.116	0.269	0.376
NEFA (mEq/dL)	1.15 ± 0.07	1.30 ± 0.13	1.02 ± 0.08	1.04 ± 0.04	0.031	0.332	0.465
Total cholesterol (mg/dL)	112 ± 5a	109 ± 5a	142 ± 11b	126 ± 4ab	0.002	0.171	0.362
Leptin (ng/mL)	3.1 ± 0.6a	3.5 ± 1.0a	11.5 ± 3.2b	7.1 ± 1.4ab	<0.001	0.224	0.147
Adiponectin (μg/mL)	57.5 ± 4.9ab	58.6 ± 1.4a	48.7 ± 3.1ab	48.3 ± 1.9b	0.002	0.887	0.777

NFD, normal-fat diet; HFD, high-fat diet; ANOVA, analysis of variance; Veh, vehicle; LP14, *Lactobacillus plantarum* No. 14; TAG, triacylglycerols; NEFA, non-esterified fatty acids; SEM, standard error of the mean

*Values are expressed as means with SEM. Mean values not sharing common letters are significantly different as estimated by Tukey–Kramer's test ($P < 0.05$)

fed NFD were the same between LP14- and vehicle-administered mice. In mice fed HFD, however, LP14-administered mice tended to have lower concentrations of total cholesterol and leptin in comparison to vehicle-administered mice fed NFD. There were significant correlations between serum total cholesterol concentrations and total weight of WATs ($r^2 = 0.549$, $P < 0.05$, Figure 1a) and between serum leptin concentrations and total weight of WATs ($r^2 = 0.867$, $P < 0.01$, Figure 1b).

Figure 2a shows the light microscopic appearance of osmium tetroxide-fixed adipocytes prepared from periovarian WAT. High dietary fat intake significantly increased average adipocyte size (Figure 2b, ANOVA, $P < 0.001$). In mice fed NFD, there were no significant differences in average adipocyte size between LP14- and vehicle-administered mice. In mice fed HFD, however, LP-14 administered mice exhibited significantly lower adipocyte size as compared with vehicle-administered mice. There was a significant correlation between periovarian WAT weight and average adipocyte size ($r^2 = 0.865$, $P < 0.01$, Figure 2c).

Linoleic acid was detected in serum samples from all groups and the culture supernatant of LP14 (Supplementary Figures 1–6). However, no detectable levels of c9, t11-CLA, t9, t11-CLA and t10, c12-CLA were observed in serum in any mice and in the culture supernatant of LP14.

In mice injected with Triton WR1339, a lipoprotein lipase (LPL) inhibitor, the plasma concentrations of TAG increased following oral administration of olive oil (Figure 3). At two hours after olive oil administration, plasma TAG concentrations were significantly lower in mice fed orlistat than in mice fed LP14. At four hours after the olive oil administration, plasma TAG concentrations were significantly lower in mice fed orlistat than in mice fed LP14 or vehicle. There were no significant differences in plasma TAG concentrations between mice fed LP14 and mice fed vehicle throughout the experimental period.

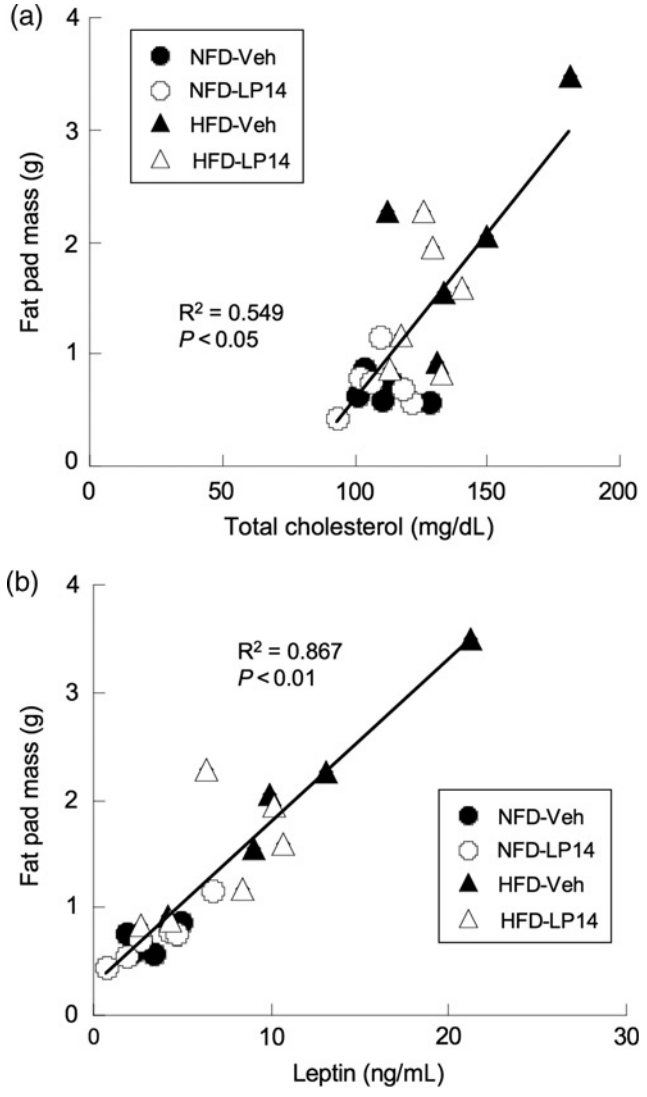

Figure 1 Correlation between serum total cholesterol and leptin concentrations and white adipose tissue weight in C57BL/6 mice. Mice were fed either normal-fat diet (NFD) or high-fat diet (HFD) and administered *Lactobacillus plantarum* strain No. 14 (LP14, 1×10^8 CFU/mouse) or vehicle (Veh) daily for 11 weeks. Each symbol represents individual mice

Discussion

The present study clearly showed that high fat intake increased body weight gain, WAT weights and adipocyte size in periovarian WAT, suggesting successful induction of diet-induced obesity in C57BL/6 mice. In mice fed HFD, average adipocyte size was significantly lower in LP14-administered mice than in vehicle-administered mice. Clearly, WAT mass is accompanied by the size and/or the number of adipocytes. In fact, LP14 administration tended to reduce WAT weights in mice fed HFD, and there was a significant correlation between tissue weight and average adipocyte size in the periovarian WAT. However, the body weight gain did not reflect the WAT weights, probably due to statistically non-significant decrease in WAT weights in LP14-administered mice. Nevertheless, the present data raise the possibility that LP14 may prevent diet-induced obesity through reduction of adipocyte hypertrophy, and the LP14 action might be associated with the observation that LP14 administration reduced the body fat percentage in healthy female volunteers.[21]

In the present study, high fat intake increased serum total cholesterol and leptin concentrations, and LP14 administration tended to reduce the HFD-induced increase in total cholesterol and leptin in sera. Previous studies described that obesity is accompanied by increased concentrations of serum total cholesterol and leptin.[25,26] In fact, we observed significant correlations between serum total cholesterol concentrations and total weight of WATs and between serum leptin concentrations and total weight of WATs. Therefore, it appears likely that LP14 may prevent obesity-related increase in total cholesterol and leptin in sera. Because leptin production is reportedly dependent on adipocyte size,[27,28] reduction of HFD-induced increase in adipocyte size by LP14 administration might be responsible for the lower concentration of leptin in sera.

Previous studies have reported antiobesity actions for some bacterial strains in animal experiments. Lee *et al.*[15,16]

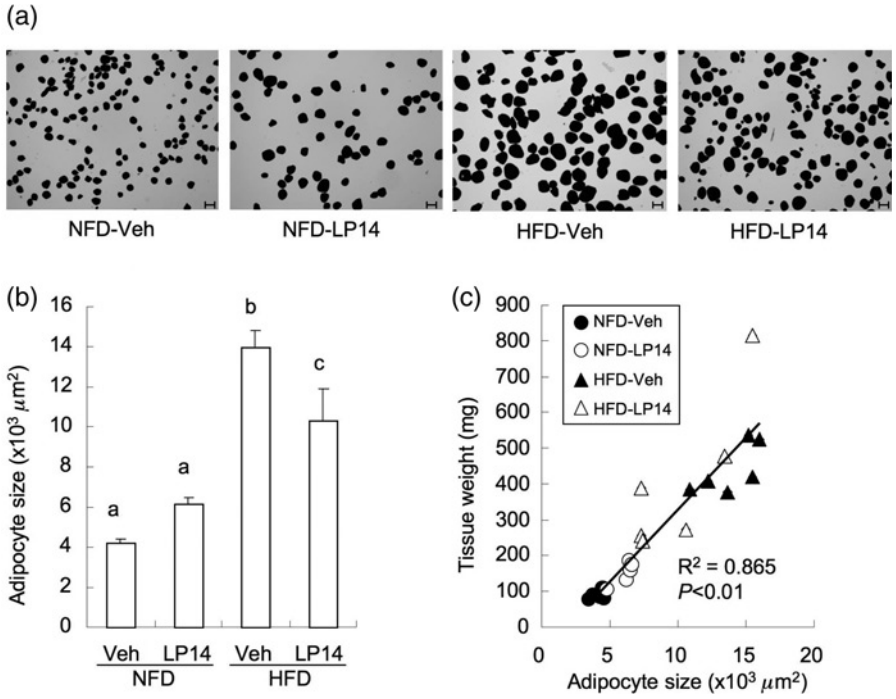

Figure 2 Changes in adipocyte size in periovarian white adipose tissue of C57BL/6 mice. Mice were fed either normal-fat diet (NFD) or high-fat diet (HFD) and administered *Lactobacillus plantarum* strain No. 14 (LP14, 1×10^8 CFU/mouse) or vehicle (Veh) daily for 11 weeks. Chart A, light microscopic appearance of osmium tetroxide-fixed adipocytes (scale bar: 100 μm); chart B, average adipocyte size; and chart C, correlation between average adipocyte size and adipose tissue weight. Values are expressed as means with standard error of the mean. Mean values with different letters are significantly different ($P < 0.05$), as estimated by Tukey–Kramer's test in chart B

reported that administration of *L. rhamnosus* PL60 and *L. plantarum* PL62 reduced fat storage in diet-induced obese mice, and they found that CLAs produced by

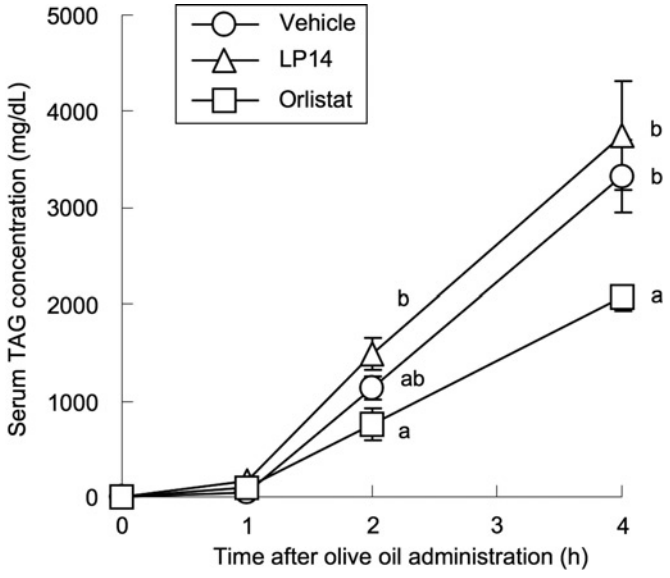

Figure 3 Time course changes in serum triacylglycerol (TAG) concentrations following olive oil administration in C57BL/6 mice. Mice fed normal-fat diet were fasted overnight and administered either vehicle, *Lactobacillus plantarum* strain No. 14 (LP14, 1×10^8 CFU/mouse), or orlistat (60 mg/kg body weight). Thereafter, mice were injected intraperitoneally with Triton WR1339 (1 mg/kg body weight) and intragastrically administered olive oil (5 mL/kg). Values are expressed as means with standard error of the mean. Mean values with different letters were significantly different ($P < 0.05$) at each time point, as estimated by Tukey–Kramer's test

administered lactobacilli mediate the antiobesity effects. Indeed, they detected t10, c12-CLA in sera of mice fed *L. rhamnosus* PL60, but not in mice fed *L. rhamnosus* GG or PBS.[15] In contrast, the present study detected no CLAs in serum in mice fed LP14 and in the culture medium of LP14, suggesting that LP14 has no ability to produce CLAs. Hence, it appears unlikely that CLAs are involved in the obesity-preventing action of LP14.

One may speculate that orally administered LP14 would affect fat digestion and/or absorption in the small intestine during passage through the gastrointestinal tract, which in turn reduces the adiposity. Sato *et al.*[17] reported that fermented skimmed milk containing *L. gasseri* SBT2055 reduced adipocyte size in WATs of Sprague–Dawley rats. In addition, Hamad *et al.*[18] showed that reduction of adipocyte size by fermented skimmed milk containing *L. gasseri* SBT2055 was accompanied by the increased fecal excretion of fatty acids and by the reduced maximal transport rate of TAG and phospholipids in the thoracic duct lymph in rats. The authors therefore concluded that the skimmed milk fermented by *L. gasseri* SBT2055 reduces fat storage through inhibition of dietary fat absorption.[18] In the present study, however, LP14 exerted no reduction in serum TAG accumulation following olive oil administration in Triton WR1339-treated mice. Thus, it appears unlikely that obesity-preventing action of LP14 is mediated by inhibition of dietary fat absorption in the small intestine.

Tanida *et al.*[20] demonstrated that the antiobesity effects of *L. paracasei* ST11 (NCC2461) were associated with increased WAT lipolysis and BAT thermogenesis, as demonstrated by plasma NEFA concentrations and BAT temperature, via

modulation of autonomic nerve activity in diet-induced obese mice. Although we did not examine autonomic nerve activity, LP14 administration exerted no changes in serum NEFA concentrations. In addition, our preliminary realtime quantitative PCR analysis showed that LP14 administration did not alter WAT gene expression of the lipolytic enzyme (i.e. hormone-sensitive lipase) and BAT gene expression of uncoupling protein-1 (unpublished observation), which is capable of uncoupling mitochondrial respiration and plays an essential role in thermogenesis.[29] Furthermore, Bäckhed *et al.*[2] demonstrated that reduced expression of Fiaf, a circulating LPL inhibitor, in the intestine is essential for the commensal gut microbiota-related fat storage in mice. However, no changes in Fiaf mRNA levels in the intestinal mucosa were observed in mice fed LP14 in our preliminary experiment (unpublished observation). Therefore, our data do not support the idea that WAT lipolysis, BAT thermogenesis and intestinal Fiaf expression are responsible for the obesity-preventing action of LP14.

Estrogen, a primary female sex hormone, increases energy expenditure directly through modulating lipid metabolisms in WAT.[30,31] In addition, leptin is a well-known hormone playing a key role in regulating energy intake and expenditure. Importantly, plasma levels of these hormones fluctuate during estrous cycle. In this context, one may speculate that the effect of LP14 on the adiposity observed in female mice is mediated, at least in part, by modulation of the estrous cycle. In our preliminary experiments, however, LP14 administration reduced the increase in WAT weights of male KK/Ta mice, a polygenic mouse model for type 2 diabetes (unpublished data). Although it remains unknown whether LP14 administration alters the estrous cycle, it appears unlikely that the adiposity-reducing action of LP14 is attributed to the modification of estrous cycle.

Gut microbiota helps the host to harvest energy from food by processing indigestible polysaccharides to short chain fatty acids, which are an important energy source for our body. In addition, short chain fatty acids contribute to host energy harvest through influencing the gut motility and intestinal transit rate.[32] Gordon and colleagues[2–6] have demonstrated the impact of gut microbiota composition on genetic and diet-induced obesity. In this context, one may speculate that LP14 exerts obesity-preventing action through modulation of gut microbiota. We recently observed that viable LP14 is recovered in feces of mice following oral administration of LP14, suggesting that LP14 is able to survive in the mouse gastrointestinal tract.[33] However, it remains unexplored whether orally administered LP14 modulates the composition of gut microbiota.

Toll-like receptors (TLRs) are expressed in many types of immune and non-immune cells and play a critical role in the activation of innate immune responses in mammals by recognizing conserved pathogen-associated molecular patterns.[34] TLR4 can be activated by lipopolysaccharide, a major cell wall component of Gram-negative bacteria, and also by non-bacterial ligands such as saturated fatty acids.[34,35] Recent studies have reported that mice deficient in TLR4 or its co-receptor CD14 are protected against diet-induced obesity, suggesting that TLR4-dependent mechanism contributes to the onset and progression of obesity.[8,36,37] Thus, suppression of TLR4 activation may contribute to the prevention and treatment of obesity. Some bacterial strains reportedly inhibit TLR4 expression and/or activation in the intestinal epithelial cells.[38–41] It will be of interest to test whether LP14 affects TLR4 expression and/or activation.

In conclusion, we propose that LP14 may exert a beneficial effect on the onset of diet-induced obesity by reducing the cell size of WATs. Further studies are needed to elucidate the underlying mechanisms.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. NT and TO conducted the experiments.

ACKNOWLEDGEMENTS

We are grateful to Dr Mutsuko Yoshida of the Research Center of Momoya Co, Ltd, for providing LP14 and to Dr Eisuke Kato and Dr Hideyuki Matsuura of Research Faculty of Agriculture, Hokkaido University for their technical advice. This study was partly supported by the Nestlé Nutrition Council, Japan.

Conflict of interest: None of the authors have any conflicts of interest.

REFERENCES

1 Tilg H, Moschen A, Kaser A. Obesity and the microbiota. *Gastroenterology* 2009;**136**:1476–83
2 Bäckhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, Semenkovich CF, Gordon JI. The gut microbiota as an environmental factor that regulates fat storage. *Proc Natl Acad Sci USA* 2004;**101**:15718–23
3 Ley RE, Bäckhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. *Proc Natl Acad Sci USA* 2005;**102**:11070–5
4 Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. *Nature* 2006;**444**:1022–3
5 Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 2006;**444**:1027–31
6 Turnbaugh PJ, Bäckhed F, Fulton L, Gordon JI. Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. *Cell Host Microbe* 2008;**3**:213–23
7 Heilbronn LK, Campbell LV. Adipose tissue macrophages, low grade inflammation and insulin resistance in human obesity. *Curr Pharm Des* 2008;**14**:1225–30
8 Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, Neyrinck AM, Fava F, Tuohy KM, Chabo C, Waget A, Delmée E, Cousin B, Sulpice T, Chamontin B, Ferrières J, Tanti JF, Gibson GR, Casteilla L, Delzenne NM, Alessi MC, Burcelin R. Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* 2007;**56**:1761–72
9 Cani PD, Neyrinck AM, Fava F, Knauf C, Burcelin RG, Tuohy KM, Gibson GR, Delzenne NM. Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. *Diabetologia* 2007;**50**:2374–83
10 Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, Burcelin R. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabetes* 2008;**57**:1470–81
11 Fuller R. Probiotics in man and animals. *J Appl Bacteriol* 1989;**66**:365–78

12 Borchers AT, Selmi C, Meyers FJ, Keen CL, Gershwin ME. Probiotics and immunity. *J Gastroenterol* 2009;**44**:26–46
13 Ohnuki K, Haramizu S, Ishihara K, Fushiki T. Increased energy metabolism and suppressed body fat accumulation in mice by a low concentration of conjugated linoleic acid. *Biosci Biotechnol Biochem* 2001;**65**:2200–4
14 Park Y, Albright KJ, Liu W, Storkson JM, Cook ME, Pariza MW. Effect of conjugated linoleic acid on body composition in mice. *Lipids* 1997;**32**:853–8
15 Lee HY, Park JH, Seok SH, Baek MW, Kim DJ, Lee KE, Paek KS, Lee Y, Park JH. Human originated bacteria, *Lactobacillus rhamnosus* PL60, produce conjugated linoleic acid and show anti-obesity effects in diet-induced obese mice. *Biochim Biophys Acta* 2006;**1761**:736–44
16 Lee K, Paek K, Lee HY, Park JH, Lee Y. Antiobesity effect of *trans*-10, *cis*-12-conjugated linoleic acid-producing *Lactobacillus plantarum* PL62 on diet-induced obese mice. *J Appl Microbiol* 2007;**103**:1140–6
17 Sato M, Uzu K, Yoshida T, Hamad EM, Kawakami H, Matsuyama H, Abd El-Gawad IA, Imaizumi K. Effects of milk fermented by *Lactobacillus gasseri* SBT2055 on adipocyte size in rats. *Br J Nutr* 2008;**99**:1013–7
18 Hamad EM, Sato M, Uzu K, Yoshida T, Higashi S, Kawakami H, Kadooka Y, Matsuyama H, Abd El-Gawad IA, Imaizumi K. Milk fermented by *Lactobacillus gasseri* SBT2055 influences adipocyte size via inhibition of dietary fat absorption in Zucker rats. *Br J Nutr* 2009;**101**:716–24
19 Ma X, Hua J, Li Z. Probiotics improve high fat diet-induced hepatic steatosis and insulin resistance by increasing hepatic NKT cells. *J Hepatol* 2008;**49**:821–30
20 Tanida M, Shen J, Maeda K, Horii Y, Yamano T, Fukushima Y, Nagai K. High-fat diet-induced obesity is attenuated by probiotic strain *Lactobacillus paracasei* ST11 (NCC2461) in rats. *Obesity Res Clin Pract* 2008;**2**:159–69
21 Nagata Y, Kamimura Y, Saka T, Yoshida M, Saito M, Kudo H, Kunisaki N, Gomyo T. *Lactobacillus plantarum* strain No. 14 suppresses human allergic reaction. *J Jpn Soc Food Sci Technol* 2008;**55**:625–31 (in Japanese)
22 Reeves PG, Nielsen FH, Fahey GC Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* 1993;**123**:1939–51
23 Alonso L, Cuesta EP, Gilliland SE. Production of free conjugated linoleic acid by *Lactobacillus acidophilus* and *Lactobacillus casei* of human intestinal origin. *J Dairy Sci* 2003;**86**:1941–6
24 Hirsch J, Gallian E. Methods for the determination of adipose cell size in man and animals. *J Lipid Res* 1968;**9**:110–9
25 Dourmashkin JT, Chang GQ, Gayles EC, Hill JO, Fried SK, Julien C, Leibowitz SF. Different forms of obesity as a function of diet composition. *Int J Obes* 2005;**29**:1368–78
26 Ghibaudi L, Cook J, Farley C, van Heek M, Hwa JJ. Fat intake affects adiposity, comorbidity factors, and energy metabolism of Sprague–Dawley rats. *Obes Res* 2002;**10**:956–63
27 Guo KY, Halo P, Leibel RL, Zhang Y. Effects of obesity on the relationship of leptin mRNA expression and adipocyte size in anatomically distinct fat depots in mice. *Am J Physiol Regul Integr Comp Physiol* 2004;**287**:R112–9
28 Lönnqvist F, Nordfors L, Jansson M, Thörne A, Schalling M, Arner P. Leptin secretion from adipose tissue in women: relationship to plasma levels and gene expression. *J Clin Invest* 1997;**99**:2398–404
29 Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. *Physiol Rev* 2004;**84**:277–359
30 Kim BH, Won YS, Kim DY, Kim B, Kim EY, Yoon M, Oh GT. Signal crosstalk between estrogen and peroxisome proliferator-activated receptor alpha on adiposity. *BMB Rep* 2009;**42**:91–5
31 Bryzgalova G, Lundholm L, Portwood N, Gustafsson JA, Khan A, Efendic S, Dahlman-Wright K. Mechanisms of antidiabetogenic and body weight-lowering effects of estrogen in high-fat diet-fed mice. *Am J Physiol Endocrinol Metab* 2008;**295**:E904–12
32 Samuel BS, Shaito A, Motoike T, Rey FE, Backhed F, Manchester JK, Hammer RE, Williams SC, Crowley J, Yanagisawa M, Gordon JI. Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor, Gpr41. *Proc Natl Acad Sci USA* 2008;**105**:16767–72
33 Takemura N, Ozawa K, Kimura N, Watanabe J, Sonoyama K. Inulin-type fructans stimulated the growth of exogenously administered *Lactobacillus plantarum* No. 14 in the mouse gastrointestinal tract. *Biosci Biotechnol Biochem* 2010;**74**:375–81
34 Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006;**124**:783–801
35 Lee JY, Ye J, Gao Z, Youn HS, Lee WH, Zhao L, Sizemore N, Hwang DH. Reciprocal modulation of Toll-like receptor-4 signaling pathways involving MyD88 and phosphatidylinositol 3-kinase/AKT by saturated and polyunsaturated fatty acids. *J Biol Chem* 2003;**278**:37041–51
36 Tsukumo DM, Carvalho-Filho MA, Carvalheira JB, Prada PO, Hirabara SM, Schenka AA, Araújo EP, Vassallo J, Curi R, Velloso LA, Saad MJ. Loss-of-function mutation in Toll-like receptor 4 prevents diet-induced obesity and insulin resistance. *Diabetes* 2007;**56**:1986–98
37 Davis JE, Gabler NK, Walker-Daniels J, Spurlock ME. Tlr-4 deficiency selectively protects against obesity induced by diets high in saturated fat. *Obesity* 2008;**16**:1248–55
38 Watanabe T, Nishio H, Tanigawa T, Yamagami H, Okazaki H, Watanabe K, Tominaga K, Fujiwara Y, Oshitani N, Asahara T, Nomoto K, Higuchi K, Takeuchi K, Arakawa T. Probiotic *Lactobacillus casei* strain Shirota prevents indomethacin-induced small intestinal injury: involvement of lactic acid. *Am J Physiol Gastrointest Liver Physiol* 2009;**297**:G506–13
39 Zhou C, Ma FZ, Deng XJ, Yuan H, Ma HS. *Lactobacilli* inhibit interleukin-8 production induced by *Helicobacter pylori* lipopolysaccharide-activated Toll-like receptor 4. *World J Gastroenterol* 2008;**14**:5090–5
40 Voltan S, Castagliuolo I, Elli M, Longo S, Brun P, D'Incà R, Porzionato A, Macchi V, Palù G, Sturniolo GC, Morelli L, Martines D. Aggregating phenotype in *Lactobacillus crispatus* determines intestinal colonization and TLR2 and TLR4 modulation in murine colonic mucosa. *Clin Vaccine Immunol* 2007;**14**:1138–48
41 Isono A, Katsuno T, Sato T, Nakagawa T, Kato Y, Sato N, Seo G, Suzuki Y, Saito Y. *Clostridium butyricum* TO-A culture supernatant downregulates TLR4 in human colonic epithelial cells. *Dig Dis Sci* 2007;**52**:2963–71

(Received December 16, 2009, Accepted April 5, 2010)