KK/Ta Mice Administered *Lactobacillus plantarum* Strain No. 14 Have Lower Adiposity and Higher Insulin Sensitivity

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Excess accumulation of white adipose tissue can lead to obesity-related metabolic abnormalities such as insulin resistance. We previously reported that intragastric administration of *Lactobacillus plantarum* No. 14 reduced adipocyte size in diet-induced obese C57BL/6 mice. The present study tested whether *L. plantarum* No. 14 affects adiposity and insulin sensitivity in an animal model of type-2 diabetes mellitus. Male KK/Ta mice were fed a normal-fat diet and intragastrically given *L. plantarum* No. 14 (10^8 CFU/mouse) or vehicle daily for 10 weeks. Interscapular brown adipose tissue and inguinal, mesenteric, and retroperitoneal white adipose tissue weights, serum leptin and insulin concentrations, and insulin resistance index (HOMA-IR) were significantly lower in *L. plantarum* No. 14-fed mice than in vehicle-fed mice. The sum of the inguinal, epididymal, mesenteric and retroperitoneal white adipose tissue weights correlated with serum leptin and non-esterified fatty acid concentrations and HOMA-IR. The mesenteric adipose tissue mRNA levels of monocyte chemoattractant protein-1 and tumor necrosis factor-α were significantly lower in *L. plantarum* No. 14-fed mice than in vehicle-fed mice. Mesenteric adipose tissue weight correlated with interleukin-6, monocyte chemoattractant protein-1, and tumor necrosis factor-α mRNA levels. HOMA-IR correlated with monocyte chemoattractant protein-1 and tumor necrosis factor-α mRNA levels. These data suggest that *L. plantarum* No. 14 prevents the development of insulin resistance, which is at least partly attributable to the prevention of obesity, in KK/Ta mice.

Key words: *Lactobacillus plantarum*, obesity, insulin sensitivity, probiotics, KK/Ta mice

INTRODUCTION

Obesity and its related disorders, including type-2 diabetes mellitus (T2DM), have become global public health problems. The excessive growth of white adipose tissue (WAT) that occurs during the development of obesity results in the activation of inflammatory pathways, a process that is evidenced by increased infiltration of macrophages and upregulation of pro-inflammatory signals such as interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-α (TNF-α) and nonesterified fatty acids (NEFAs) in WAT [1]. Subsequently, such an inflammatory profile leads to the paracrine/autocrine-mediated cellular insulin resistance that is characteristic of most patients with T2DM [1]. Although the development of obesity is a process involving genetic and environmental factors, the exact pathways are complex and poorly understood. Gut microbiota have recently attracted much attention as an environmental factor involved in the development of obesity and its related disorders [2]. Therefore, modulation of gut microbiota may be an appropriate strategy for managing obesity and its related disorders.

Probiotics are defined as live microorganisms that when administered in adequate amounts confer a health benefit on the host [3]. Recent experimental studies have reported the beneficial effects of some bacterial strains on obesity and its related disorders. For example, *Lactobacillus rhamnosus* PL60 and *L. plantarum* PL62, which are capable of producing conjugated linoleic acids (CLAs) that reduce body fat [4, 5], have been found to exert beneficial effects on diet-induced obesity (DIO) in mice [6, 7]. Further, consumption of fermented skimmed milk containing *L. gasseri* SBT2055 reduced the size of visceral adipocytes in rats, possibly through the inhibition of dietary fat absorption [8, 9]. In mice, a

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mixture of viable lyophilized bifidobacteria, lactobacilli, and *Streptococcus thermophilus*, improved DIO, hepatic steatosis, and insulin resistance by increasing hepatic natural killer T cells and reducing inflammatory signaling [10]. By enhancing sympathetic nerve activity and suppressing parasympathetic nerve activity, *L. paracasei* ST11 (NCC2461) was found to reduce DIO in rats via augmented lipolysis and thermogenesis in WAT and brown adipose tissues (BAT), respectively [11]. Additionally, *Bifidobacterium breve* B-3 (MCC1274) reduced DIO and insulin resistance in mice, as a possible consequence of increased expression of fasting-induced adipose factor (Fiaf) and adiponectin [12]. Similarly, increased expression of Fiaf accompanied suppression of DIO in mice by *L. paracasei* ssp *paracasei* F19 [13]. Finally, *L. casei* Shirota (YIT9029) improved insulin resistance and glucose intolerance in DIO mice in association with reduced endotoxemia [14]. Therefore, these bacterial strains may be useful in preventing and/or treating obesity and its related disorders.

We previously reported that DIO mice administered *L. plantarum* No. 14 (LP14) exhibit smaller adipocytes [15]. The same bacterial strain was also reported to reduce body fat in healthy human volunteers [16]. The present study examined the effects of LP14 on the development of obesity and its related abnormalities in KK/Ta mice, an animal model of T2DM. This mouse strain exhibits moderate obesity, insulin resistance, hyperinsulinemia, and glucose intolerance.

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. 08-0139). Animals were maintained in accordance with the Hokkaido University guidelines for the care and use of laboratory animals.

Male specific pathogen-free KK/Ta mice (age, 4 weeks) were purchased from CLEA Japan (Tokyo, Japan) and housed individually in standard plastic cages inside a temperature-controlled (23 ± 2 °C) room under a 12-hr light:dark cycle. Mice were given free access to food and water, and were acclimatized to the purified normal-fat diet prepared according to AIN-93G [17] for 1 week before the start of the experiment. Mice were then allocated into two groups: those intragastrically administered 0.2 mL of PBS supplemented with lyophilized powder of LP14 (1×10^8 CFU per mouse; donated by Momoya Co., Ltd., Tokyo, Japan) per day (n=6), and those administered 0.2 mL of PBS daily (control; n=5). Body weight and

food intake of individual mice were measured daily. After 9 weeks of feeding, mice were subjected to an intraperitoneal glucose tolerance test (IPGTT) as described below. Thereafter, mice continued to be fed for one week. Following16 hr of fasting on the last day of the experimental period, mice were anesthetized by inhalation of diethyl ether. Whole blood was drawn from the carotid artery, and serum was separated from blood samples and subjected to the measurement of biochemical parameters. Following a laparotomy, the liver, BAT from the interscapular region, and WAT from the inguinal, mesenteric, retroperitoneal, and epididymal regions were excised and weighed. Mesenteric WAT was snap-frozen in liquid nitrogen and stored at –80°C for RNA isolation.

IPGTT

Glucose intolerance was evaluated by IPGTT. Following an18-hr fast, mice were intraperitoneally injected with 150 g/l glucose in PBS at a dose of 1.5 g/kg body weight. Blood samples were collected from the tail vein just before and 30, 60, 90, and 120 min after glucose injection. Plasma was separated by centrifugation, and glucose concentrations were measured by Glucose CII-test Wako (Wako Pure Chemical Industries, Osaka, Japan).

Biochemical analyses

Serum glucose, triacylglycerols (TAG), NEFA, and total cholesterol concentrations were measured using commercial enzymatic reagent kits (Glucose CII-test Wako, Triglyceride E-test Wako, NEFA C-test Wako, and Cholesterol E-test Wako, respectively; Wako Pure Chemical Industries). Commercial ELISA kits were used to measure serum insulin (U-type Mouse Insulin ELISA Kit; Shibayagi, Gunma, Japan), adiponectin (Mouse/Rat Adiponectin ELISA Kit; Otsuka Pharmaceutical, Tokyo, Japan), and leptin concentrations (Mouse Leptin ELISA Kit; Morinaga Institute of Biochemical Science, Kanagawa, Japan). The homeostasis model assessment insulin resistance (HOMA-IR), an insulin resistance index, was calculated using the following equation:

HOMA-IR = fasting glucose (mg/dL) × fasting insulin (ng/mL)/22.5 [18].

MRNA expression analysis

Total RNA was isolated from tissue homogenates using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After digestion of genomic DNA with RQ1 RNase-free DNase (Promega, Madison, WI, USA), approximately 10 ng of total RNA was annealed with Oligo (dT) 12–18 primer
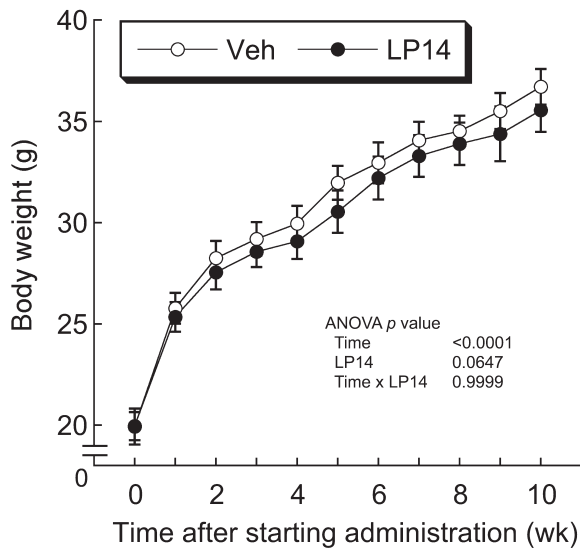
Fig. 1. Time-course changes in body weight of KK/Ta mice. Mice were fed a normal-fat diet and administered *Lactobacillus plantarum* strain No. 14 (LP14, 1 × 10⁸ CFU per mouse) or vehicle (Veh) daily for 10 weeks. Data are expressed as means with SEM. Effects of time and LP14 were analyzed by two-way repeated measures ANOVA.

Table 1. Effects of *Lactobacillus plantarum* strain No. 14 administration on tissue weights in KK/Ta mice

	Veh [1]	LP14
Tissue weight (mg)		
Liver	1.215 ± 0.094	1.050 ± 0.048
Brown fat pad	0.289 ± 0.028	0.223 ± 0.010*
Inguinal fat pad	1.433 ± 0.114	1.094 ± 0.092*
Mesenteric fat pad	0.680 ± 0.060	0.540 ± 0.019*
Retroperitoneal fat pad	0.554 ± 0.014	0.419 ± 0.030*
Epididymal fat pad	1.291 ± 0.063	1.210 ± 0.057
Total white fat pad [2]	3.957 ± 0.211	3.264 ± 0.142*
Relative tissue weight (mg/g body weight)		
Liver	34.8 ± 1.8	31.8 ± 0.9
Brown fat pad	8.25 ± 0.57	6.77 ± 0.28*
Inguinal fat pad	41.1 ± 2.7	33.0 ± 2.3*
Mesenteric fat pad	19.4 ± 1.2	16.4 ± 0.4*
Retroperitoneal fat pad	16.0 ± 0.7	12.8 ± 1.0*
Epididymal fat pad	37.1 ± 1.3	36.7 ± 1.4
Total white fat pad	113.6 ± 3.9	98.9 ± 3.1*

[1] Veh, vehicle; LP14, *Lactobacillus plantarum* No. 14.
[2] Total white fat pad is the sum of the inguinal, mesenteric, retroperitoneal and epididymal fat pads.
[3] Values are expressed as means with SEM. Comparison of mean values was done by Student's t-test. * p<0.05 *vs.* Veh.

(Invitrogen) for 10 min at 70°C. First strand cDNA was then synthesized using M-MLV reverse transcriptase (Invitrogen), followed by RNA digestion with DNase-free RNase H (Invitrogen). RT-qPCR was performed using a TP800 Thermal Cycler Dice Real Time System (Takara, Ohtsu, Japan). Primer sequences for IL-6, MCP-1, and TNF-α were identical to those reported by Giulietti et al. [19]. Amplification was carried out in a 25-μl reaction volume containing 12.5 μL 1 x SYBR Premix Ex Taq (Takara), 200 nM of each primer, and 1 μL of template cDNA. The reaction conditions were as follows: 95°C for 10 sec followed by 40 cycles at 95°C for 15 sec and 40 cycles at 60°C for 30 sec, with a dissociation curve at 95°C for 15 sec, 60°C for 30 sec and 95°C for 15 sec. Relative gene expression levels for each sample were normalized to the levels of 18S rRNA. Primer sequences for 18S rRNA were identical to those reported by Hewitt et al. [20].

STATISTICS

Results are presented as means ± SEM. Student's t-tests, with or without Welch's correction, were used to compare mean values. A two-way, repeated-measures ANOVA was used to compare changes in body weights and plasma glucose levels during the IPGTT. Correlations between parameters were assessed by Pearson's correlation test.

Data analysis was performed using GraphPad Prism for Macintosh (version 5; GraphPad Software, San Diego, CA, USA). p values of <0.05 were considered to be statistically significant.

RESULTS

Daily food intake was similar between LP14- and vehicle-administered mice (3.27 ± 0.07 and 3.34 ± 0.15 g, respectively, p=0.66). Body weight significantly increased in both LP14- and vehicle-administered mice during the experimental period (p<0.0001, Fig. 1) and tended to be lower in LP14-administered mice than in vehicle-administered mice (p=0.0647). After 10 weeks of LP14 and vehicle administration, liver weight was similar between vehicle- and LP14-administered mice (Table 1). Interscapular BAT and inguinal, mesenteric, and retroperitoneal WAT weights were significantly lower in LP14-administered mice than in vehicle-administered mice. However, no significant difference was observed between the groups in epididymal WAT weight. These results remained essentially the same when tissue weight data were also expressed relative to body weight (Table 1).

Serum TAG, total cholesterol, and NEFA concentrations were similar between vehicle- and LP14-administered mice (Table 2). Although mean

Table 2. Effects of *Lactobacillus plantarum* strain No. 14 administration on biochemical parameters in sera of KK/Ta mice

	Veh [1]	LP14
TAG (mg/dl)	240 ± 33	186 ± 18
NEFA (mEq/dl)	1.93 ± 0.17	1.60 ± 0.11
Total cholesterol (mg/dl)	184 ± 24	152 ± 12
Leptin (ng/ml)	40.1 ± 2.9	25.4 ± 3.5*
Adiponectin (µg/ml)	13.0 ± 1.5	13.5 ± 1.1
Glucose (mg/dl)	113 ± 14	114 ± 9
Insulin (ng/ml)	2.30 ± 0.44	0.98 ± 0.12*
HOMA-IR	11.1 ± 1.7	5.1 ± 0.8*
AUC of IPGTT (mM/min)	1579 ± 85	1476 ± 93

[1] Veh, vehicle; LP14, *Lactobacillus plantarum* No. 14.
[2] Values are expressed as means with SEM. Comparison of mean values was done by Student's t-test. * p<0.05 *vs.* Veh.

serum leptin concentrations were significantly lower in LP14-administered mice than in vehicle-administered mice, no differences in serum adiponectin concentration were observed between the groups. Fasting glucose concentrations were similar between groups, while the mean serum insulin concentrations were significantly lower in LP14-administered mice than in vehicle-administered mice. HOMA-IR, an index of insulin resistance, was also significantly lower in LP14-administered mice. In the IPGTT performed after 9 weeks of LP14 or vehicle administration, LP14 administration did not have an apparent effect on the time-course changes in blood glucose levels (data not shown). The area under the curve (AUC) of the blood glucose levels did not differ between the groups (Table 2).

Pearson's correlation analysis revealed that the sum of inguinal, epididymal, retroperitoneal, and mesenteric WAT weights was significantly correlated with serum leptin concentrations, NEFA concentrations, and HOMA-IR (Fig. 2A, 2B, and 2D, respectively). As depicted in Fig. 2C, the serum NEFA concentration was also correlated with HOMA-IR.

The MCP-1 and TNF-α mRNA levels within mesenteric WAT were significantly lower in LP14-adminsitered mice than in vehicle-administered mice (Table 3). IL-6 mRNA levels also tended to be lower in LP14-administered mice (p=0.1045). Visceral (sum of mesenteric and retroperitoneal) WAT weight was significantly correlated with the mRNA levels of IL-6, MCP-1, and TNF-α (Fig. 3A, 3B, and 3C, respectively). In addition, MCP-1 and TNF-α mRNA levels were significantly correlated with HOMA-IR (Fig. 3E and 3F, respectively). IL-6 mRNA levels also tended to correlate with HOMA-IR, albeit insignificantly (p=0.0873; Fig. 3D).

DISCUSSION

Our previous study showed that DIO female C57BL/6 mice administered LP14 exhibit smaller WAT adipocytes [15]. Additionally, the present study demonstrated that male KK/Ta mice administered LP14 have lower WAT weights. Also, both DIO C57BL/6 mice and KK/Ta mice administered LP14 have lower mean serum leptin concentrations. The circulating leptin level is reportedly proportional to WAT mass [21, 22]. Indeed, a significant correlation between serum leptin concentration and the sum of the inguinal, epididymal, mesenteric, and retroperitoneal WAT weights was observed in the present study. Furthermore, a small-scale, double-blind, randomized, placebo-controlled trial showed that LP14 can lead to a reduction in body fat among humans [16]. Of the 28 healthy female volunteers in that study, 13 received lyophilized powder containing 2×10^{10} CFU of LP14 per day, while the remaining participants received a daily placebo (branched dextrin) for 3 weeks. Volunteers receiving LP14 showed a significant decrease in their body fat percentage without any change in body weight, while the placebo group sustained their baseline levels [16]. These findings suggest that LP14 may be a probiotic bacterial strain that can prevent and/or treat obesity.

Insulin plays an important role in the regulation of glucose homeostasis, lipid metabolism and blood pressure. The failure of target organs to respond normally to the action of insulin defines insulin resistance. When combined with the resultant compensatory hyperinsulinemia, insulin resistance can lead to a cluster of metabolic abnormalities, such as glucose intolerance, hyperlipidemia, hepatic steatosis, and hypertension [1, 23, 24]. In the present study, KK/Ta mice administered LP14 showed significantly lower HOMA-IR and serum insulin concentrations, suggesting that LP14 promotes insulin sensitivity. However, according to the IPGTT results, LP14 failed to reduce plasma glucose levels. It is likely that, despite the lack of differences in plasma glucose levels, higher levels of plasma insulin were required during the IPGTT in vehicle-administered mice compared with LP14-administered mice to establish comparable blood glucose clearance. Thus, our data suggest that LP14 has a primary effect on plasma insulin levels, which might consequently lead to alterations in glucose homeostasis.

One major determinant of insulin resistance is excess accumulation of visceral fat, which causes chronic low-grade inflammation characterized by increased macrophage infiltration and pro-inflammatory adipokine production [1, 24]. Pro-inflammatory adipokines, such
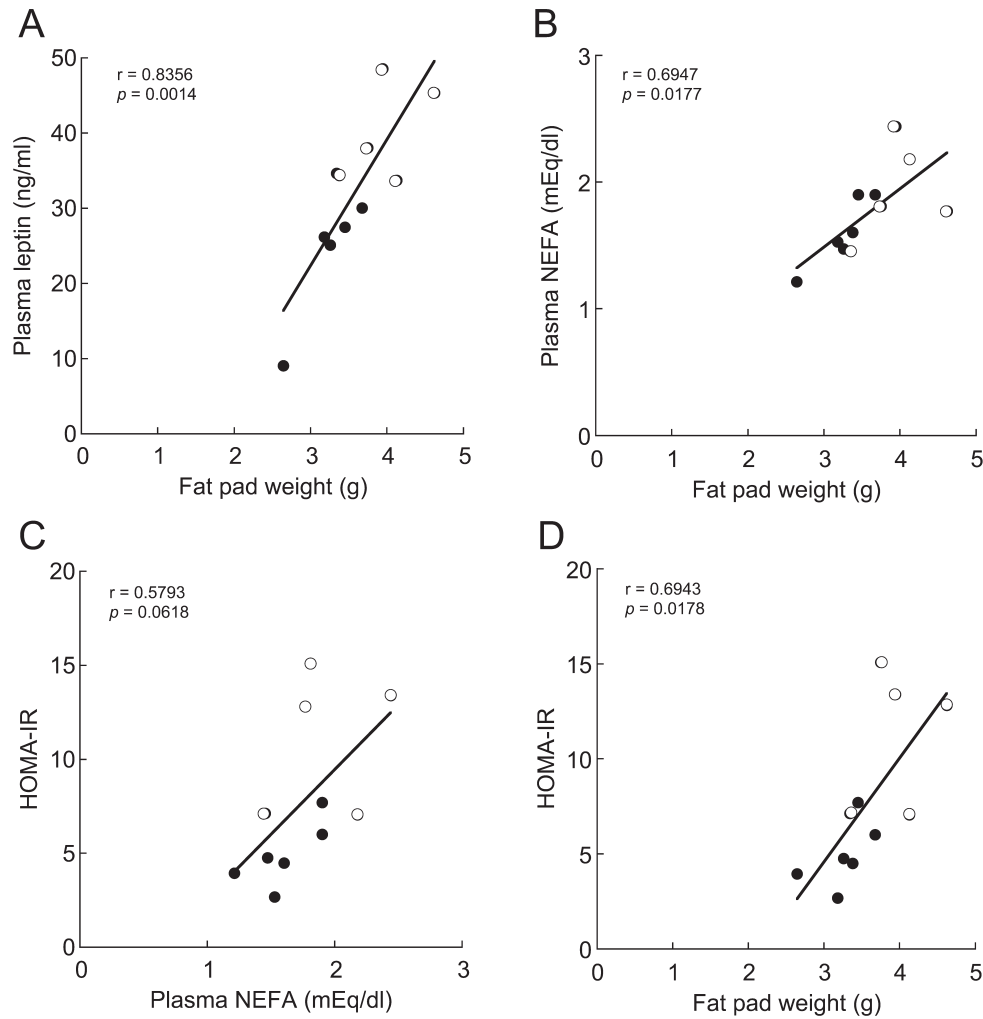
Fig. 2. Correlation between fat pad weight and serum biochemical parameters after 10-week administration of *Lactobacillus plantarum* strain No. 14 in KK/Ta mice. Mice were fed a normal-fat diet and administered *L. plantarum* strain No. 14 (1×10^8 CFU per mouse, closed circles) or vehicle (Veh, open circles) daily for 10 weeks. Fat pad weight represents the sum of the inguinal, retroperitoneal, epididymal and mesenteric WAT weights. Each symbol represents individual mice. Correlations between parameters were assessed by Pearson's correlation test.

Table 3. Effects of *Lactobacillus plantarum* strain No. 14 administration on the gene expression of adipocytokines in the mesenteric fat pad of KK/Ta mice

	Veh [1]	LP14
	Arbitrary unit	
IL-6 mRNA	1.00 ± 0.09	0.79 ± 0.03
MCP-1 mRNA	1.00 ± 0.07	$0.73 \pm 0.03*$
TNF-α mRNA	1.00 ± 0.06	$0.61 \pm 0.03*$

[1] Veh, vehicle; LP14, *Lactobacillus plantarum* No. 14.
[2] Values are expressed as means with SEM. Values of LP14-administered mice are shown relative to the levels in vehicle-administered mice, which are set to 1.00. Comparison of mean values was done by Student's t-test. * p<0.05 *vs.* Veh.

as IL-6, MCP-1 and TNF-α, interfere with the insulin-signaling pathway of peripheral tissues and facilitate the development of insulin resistance. In addition, NEFAs are recognized by Toll-like receptor 4 and can subsequently trigger inflammatory responses. In the present study, KK/Ta mice administered LP14 exhibited lower levels of MCP-1 and TNF-α mRNA in mesenteric WAT. In addition, we observed a correlation between WAT weight and inflammatory signals (i.e., NEFAs, IL-6, MCP-1 and TNF-α), as well as a correlation between inflammatory signals and HOMA-IR. These data suggest that LP14 prevents the development of insulin resistance, which is
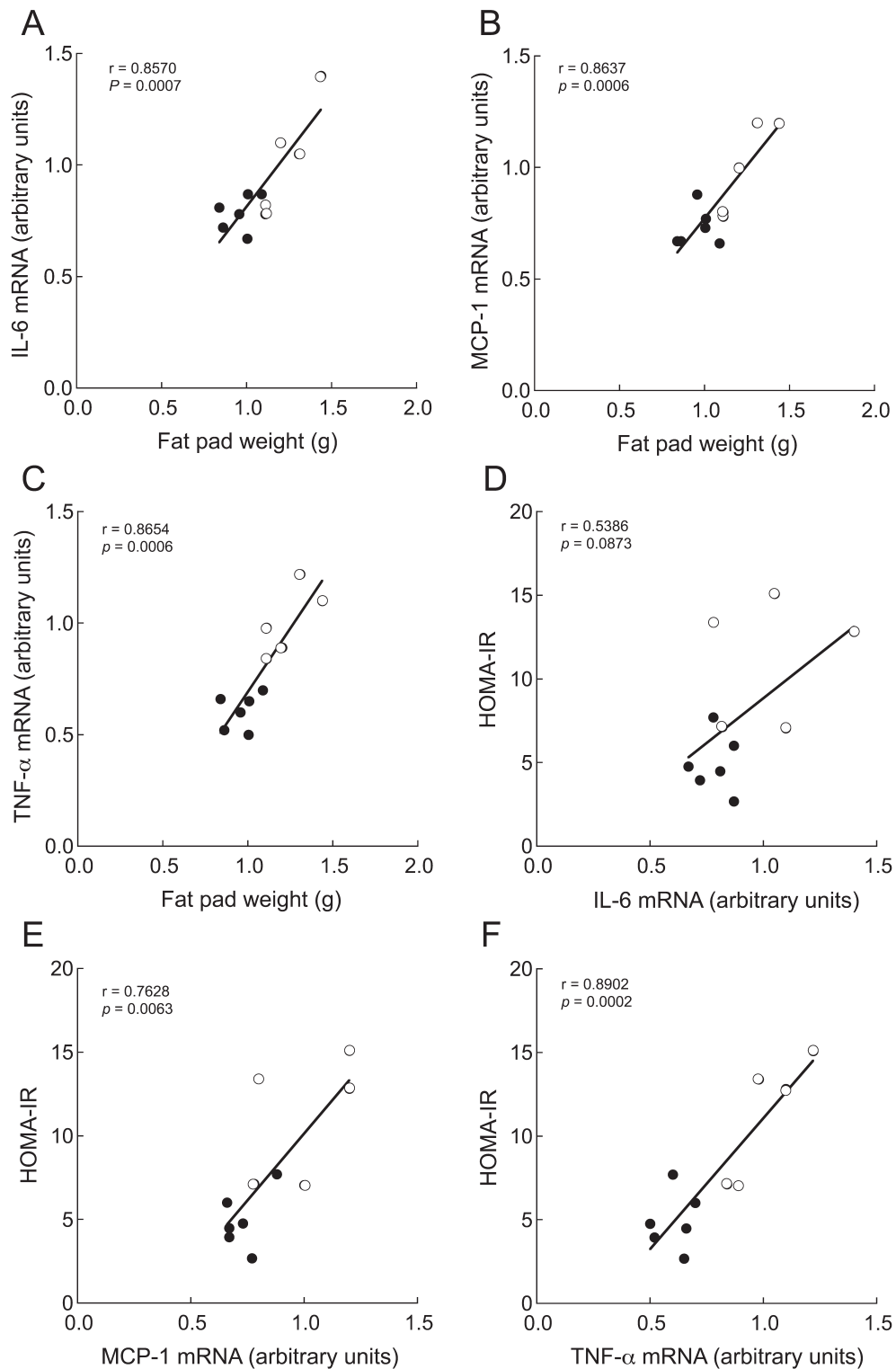
Fig. 3. Correlation between visceral fat pad weight, HOMA-IR, and the mRNA levels of interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor-α (TNF-α) in the mesenteric fat pad after 10-week administration of *Lactobacillus plantarum* strain No. 14 in KK/Ta mice. Mice were fed a normal-fat diet and administered *L. plantarum* strain No. 14 (1×10^8 CFU per mouse, closed circles) or vehicle (Veh, open circles) daily for 10 weeks. Fat pad weight represents the sum of the retroperitoneal and mesenteric WAT weights. Each symbol represents individual mice. Correlations between parameters were assessed by Pearson's correlation test.

at least partly attributable to the reduction of obesity and subsequent inflammation.

The cellular and molecular mechanisms by which LP14 prevents obesity development remain to be elucidated. Although we cannot rule out the possibility that LP14 exerts its activity through modulating gut microbiota, it is also possible that LP14 directly influences the host physiology like in the examples below. Lee et al. reported that administration of *L. rhamnosus* PL60 and *L. plantarum* PL62 reduced fat storage in DIO mice, an effect that appears to be mediated by the CLAs produced by the administration of lactobacilli [6, 7]. Indeed, the authors detected *t*10, *c*12-CLA in the sera of mice fed *L. rhamnosus* PL60 [6]. However, our previous study failed to detect CLAs in the serum of LP14-fed mice and in the culture medium of LP14, suggesting that CLAs are not involved in the anti-obesity effect of LP14 [15]. In addition, Hamad et al. showed that the reduction of adipocyte size in rats following feeding with fermented skimmed milk containing *L. gasseri* SBT2055 was accompanied by increased fecal excretion of fatty acids and a reduced maximal transport rate of TAG and phospholipids in the thoracic duct lymph [8]. These findings suggest skimmed milk fermented by *L. gasseri* SBT2055 reduces fat storage through the inhibition of dietary fat absorption. In contrast, our previous study showed that LP14 exerted no reduction in serum TAG accumulation following olive oil administration in Triton WR-1339-treated mice, suggesting that the anti-obesity effect of LP14 is also not mediated by inhibition of dietary fat absorption in the small intestine [15]. Furthermore, Kondo et al. and Aronsson et al. indicated that an increased expression of Fiaf is involved in the suppression of DIO by *B. breve* B-3 (MCC1274) and *L. paracasei* ssp *paracasei* F19, respectively [12, 13]. However, we observed no changes in Fiaf mRNA levels in the intestinal mucosa of mice fed LP14 (unpublished observation).

Some lactobacilli secrete exopolysaccharides (EPSs) that confer beneficial health effects including immunostimulatory, antitumor and hypocholesterolemic actions [25]. Hashiguchi et al. reported that LP14 secretes two types of acidic EPS and two types of neutral EPS and that these EPSs exert immunostimulatory actions on Peyer's patch cells and mesenteric lymph node cells of swine [26]. In our preliminary experiments, we observed that LP14 secretes a huge amount of EPSs as compared with the type strain of *L. plantarum* (JCM1149[T]) (unpublished observation). In addition, oral administration of *L. plantarum* (JCM1149[T]) had no effects on the mRNA expression of IL-6, MCP-1, and TNF-α in mesenteric WAT of KK/Ta mice, suggesting

that the anti-inflammatory action of LP14 is strain specific (unpublished observation). We are currently investigating whether EPSs play a role in the LP14 prevention of obesity and its related metabolic diseases.

In conclusion, we propose that LP14 may prevent the development of insulin resistance, which is at least partly attributable to the prevention of obesity, in KK/Ta mice. Therefore, LP14 may be an ideal probiotic bacterial strain to prevent obesity and its related metabolic diseases. Further studies are needed to clarify the cellular and molecular mechanisms involved.

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