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Human originated bacteria, *Lactobacillus rhamnosus* PL60, produce conjugated linoleic acid and show anti-obesity effects in diet-induced obese mice

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Abstract

Many previous studies have reported that conjugated linoleic acid could be produced by starter culture bacteria, but the effects of the bacteria were not investigated. Moreover, there was no evidence of the conjugated linoleic acid-producing bacteria having potential health or nutritional effects related to conjugated linoleic acid, including reducing body fat. Here, we investigated the anti-obesity effect of *Lactobacillus rhamnosus* PL60, a human originated bacterium that produces t10, c12-conjugated linoleic acid, on diet-induced obese mice. After 8 weeks of feeding, *L. rhamnosus* PL60 reduced body weight without reducing energy intake, and caused a significant, specific reduction of white adipose tissue (epididymal and perirenal). Although the size of epididymal adipocytes was not reduced by *L. rhamnosus* PL60, apoptotic signals and UCP-2 mRNA levels increased in adipose tissue. Liver steatosis, a well known side effect of CLA, was not observed by *L. rhamnosus* PL60 treatment; on the contrary it seemed to be normalized. Results showed that the amount of conjugated linoleic acid produced by *Lactobacillus rhamnosus* PL60 was enough to produce an anti-obesity effect.

Keywords: Conjugated linoleic acid; *Lactobacillus rhamnosus*; Diet induced obese; Anti-obesity; Mouse

1. Introduction

Conjugated linoleic acid (CLA) is a generic name for a mixture of linoleic acid isomers with conjugated double bonds. Double bonds of CLA are mainly found at positions 9 and 11, or 10 and 12 [1]. Food products from beef and dairy products such as milk fat, natural and processed cheeses, yogurt, and plant oil, are major dietary sources of CLA for humans. Based on studies primarily in animal models, several scientists have suggested that CLA has potential health or nutritional effects, including anti-carcinogenic activity [2], anti-artherogenic activity [3,4], the ability to reduce the catabolic effects of immune stimulation [5], and the ability to reduce body fat [4].

It is generally accepted that CLA in ruminants originate from the incomplete biohydrogenation of the unsaturated fatty acid linoleic acid by rumen bacteria [6]. In addition to the ability of some rumen bacteria to form CLA from dietary linoleic acid, it has been demonstrated that certain cultures used in food fermentation possess the ability to generate CLA. Strains of intestinal microbiotia from rats [7], two strains of *Propionibacterium freudenreichii* spp. *freudenreichii*, one strain of *P. freudenreichii* spp. *Shermanii* [8,9], and six lactic cultures (three *lactobacilli*, two *lactococci,* and one *Streptococcus*) [10] are among those shown to possess this capability. Unlike ruminants, human production of CLA from linoleic acid does not appear to occur at any significant level. The amount of CLA in human adipose tissue is thought to be directly related to dietary intake [11]. Therefore, it is reasonable to suggest that, in order to increase CLA in humans, it

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Table 1
Feeding schedules used in the present study

Groups	Food	Treatment (oral administration for 8 weeks)
STD	Standard diet	0.5 ml of sterilized PBS
HFD-Veh	45% high fat diet	0.5 ml of 5% skim milk in sterilized PBS
HFD-PL60H	45% high fat diet	0.5 ml of 1×10^9 CFU *L. rhamnosus* PL60 in sterilized PBS
HFD-PL60L	45% high fat diet	0.5 ml of 1×10^7 CFU *L. rhamnosus* PL60 in sterilized PBS

has to be ingested as food products, alternatively produced by the intestinal microbiotia, or by an ingested probiotic that could use dietary linoleic acid to generate CLA.

In this study, a human derived lactobacillus strain was assessed for CLA production from linoleic acid, and the anti-obesity effects of this strain was investigated in C57BL/6J diet-induced obese (DIO) mice.

2. Materials and methods

2.1. Isolation and preparation of CLA producing lactic-acid bacteria (LABs)

LABs isolated from baby feces and fermented foods were grown in 10% skim milk containing 0.01% linoleic acid (LA). LABs that grew well in the presence of LA were selected for further study. After selected bacteria were tested for Gram-positive and catalase-negative rod form, production of c9,t11- and t10,c12-CLA, one isolate was selected and named as PL60. This isolate was identified with various biochemical and physiological tests using API50CH (Bio-Merieux, Marcy-l'Etoile, France), and their identification was confirmed with DNA sequencing of 16S rRNA. This isolate was cultured in whey and freeze-dried skim milk.

2.2. In vitro assay of c9, t11-CLA and t10, c12-CLA with gas chromatography

LABs were grown in 10% skim milk containing 1 mg/ml LA. Cells were collected and washed with phosphate buffered saline (PBS) and broken with glass beads in a bead beater (Biospec). Cell lysate was prepared after removing unbroken cells with centrifugation. Cell lysate (200 μl) was added to the reaction mixture (2.7 ml, 0.1 M potassium phosphate buffer; 0.2 ml, 1,3-propanediol; 20 μg linoleic acid) and incubated at 37 °C. The amounts of c9,t11-CLA and t10, c12-CLA in the reaction mixture before and after the reaction were measured with gas chromatography using the method described by Alonso et al. [12]. To assay only the amounts of CLA isomers produced by the enzyme reaction, the amounts of CLA isomers before the enzyme reaction were subtracted from those after the enzyme reaction. Gas chromatography was carried out on a GC (Hewlett Packard GC model 5890, Palo Alto, CA) equipped with a DB-FFAP capillary column (30 m by 0.25 mm inner diameter, 0.25 μm film thickness). Helium was used as a carrier gas at a flow rate of one ml/min. The oven temperature was raised from 50 °C to 210 °C at a rate of 48 °C /min. Samples (2 μl) were injected into the GC, which was operating with a split ratio of 50:1. *Lactobacillus paracasei* IFO 12004 was used as c9,t11-CLA producing strain and *L. pentosus* IFO 12011 were used as both c9,t11- and t10,c12-CLA producing strains [13]. The amount of protein was assayed using the BCA method (Sigma).

2.3. Attaching ability of PL60 to mouse intestines

Three mice received 1×10^9 CFU of *L. rhamnosus* PL60 in PBS and feces were collected at 24-h intervals, serially diluted, and plated onto MRS agar plates containing 0.002% bromophenol blue. After 48 h at 37 °C, colonies with characteristics of *L. rhamnosus* was recovered and identified with Gram-staining.

2.4. Animals and treatment

Male C57BL/6J mice were obtained from Charles River Laboratory of Animal Science (Orient Co., Korea) at five week of age and fed a normal diet (Purina) for 1 week to stabilize all metabolic conditions. Each cage contained one mouse. Mice were exposed to a 12-h light:dark cycle and maintained at a constant temperature of 22±1 °C and humidity of 55±10%. All animal experimentation was performed according to the guidelines for the care and use of laboratory animals approved by IACUC of Seoul National University. One week after arrival, mice were divided into four groups and three groups were fed a 45% high-fat diet (D12451, Research Diets, CA, USA) and test-materials simultaneously for 8 weeks. As shown in Table 1, high-fat diet (HFD)-fed mice were divided three groups, two groups received a daily dose of PL60 (1×10^7 or 1×10^9, CFU per mouse) for 8 weeks, while the vehicle control group received (HFD-Veh) PBS for the same period. Others received continuous feeding of a standard diet (STD, #5057, Purina, Kyung-gi, Korea) during the experiment. On a caloric basis, the high-fat diet consisted of 45% fat, 25.6% carbohydrate, and 16.4% protein (5.252 cal/g), whereas the standard diet contained 11.4% fat, 62.8% carbohydrate, and 25.8% protein (3.28 cal/g). Food intake and body weight were measured twice a week.

At the end of the experimental period (eight weeks treatment), animals were sacrificed by bleeding from the inferior vena cava under diethyl ether anesthesia. Epididymal, perirenal, mesenteric, and inguinal white adipose tissues, liver, spleen, kidney, and brain were quickly excised and weighed. All organs were carefully cleaned from the surrounding muscular tissue and other adipose tissue prior to being weighted.

2.5. Measuring the amount of CLA isomers and linoleic acid in PL60-fed mice

As a supplementary study, the amount of each CLA isomer and linoleic acid in the sera of HF diet fed mice was measured. In brief, 14 mice were divided into

Table 2
Oligodeoxyribonucleotide primer sequences used for RT-PCR in the present study

Primer sequence	cDNA	GenBank no.	Size (bp)
Forward (5′–3′): GAGCTGCGGCTACGTGGCTA Reverse (5′–3′): GCCGCCGTGAGGTTGCTGTT	FAS	NM017332	340
Forward (5′–3′): CTCTTCAAGGGACAAGGCTG Reverse (5′–3′): CGGACTCCGCAAAGTCTAAG	TNF-α		253
Forward (5′–3′): CAGTTCTACACCAAGGGCTCAG Reverse (5′–3′): TCTGTCATGAGGTTGGCTTTC	UCP-2	AB006613	323
Forward (5′–3′): GCAAAGAGGTGGCCATCCGC Reverse (5′–3′): ATGGCCAAGTCACTGTCATC	PPAR-γ	AB611365	337
Forward (5′–3′): ATGGATGACGATATCGCT Reverse (5′–3′): ATGAGGTAGTCTGTCAGGT	β-actin		569

Primer pairs of FAS, UCP-2, and PPAR-γ were designed by the primer selection program BCM Search Launcher (Baylor College of Medicine, Human Genome Sequencing Center, Houston, TX, USA). Primer pairs of TNF-α and β-actin were used as previously reported [43].

Table 3
CLA produced by the crude enzyme prepared from washed cells

Strain	Amount of CLA produced by washed cells[a] (μg/mg protein)	
	c9,t11-CLA[b]	t10,c12-CLA[c]
Lact. rhamnosus PL60	2836.5±260.92	1602.50±391.03
Lact. paracasei IFO 12004	3454.0±38.18	–[d]
Lact. pentosus IFO 12011	3713.0±199.40	2124.5±294.86

[a] Amount of CLA produced by enzyme reaction.
[b] Amount of CLA measured with gas chromatography.
[c] Amount of CLA measured with gas chromatography.
[d] Not detected.

3 groups and fed 45% fat diet. Five mice received 1×10^9 CFU of *L. rhamnosus* PL60 in PBS per day and another 5 mice received 1×10^9 CFU of *L. rhamnosus* GG per day for 3 weeks. Control group (4 mice) received 0.5 ml of PBS during the same period. Blood was collected from venous sinus at every week. At the third week, the sera obtained from each group were pooled and each CLA was assayed by GC as described above.

2.6. Serological analysis

Serum levels of glucose, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), and total cholesterol (TC) were measured with a wet-type automatic analyzer 767 (Hitachi, Tokyo, Japan), and leptin was measured with a mouse leptin EIA kit (Assay Designs, Ann Arbor, MI, USA).

2.7. Histological analysis

Liver and epididymal white adipose tissues from each group were fixed with 10% formalin, dehydrated in an alcohol-xylene series, and embedded in paraffin wax. Thin slices of liver tissue were stained with hematoxylin and eosin. Ten×200 light-microscopic fields were assessed by an image analyzer (Image-Pro® Plus version 4.5, Media Cybernetics, MD, USA) on each section and scored for the severity of steatosis according to the following criteria with a slight modification to a previous [14]. The following grades of liver steatosis were given: grade 0, no fat; grade 1, fatty hepatocytes with micro fat vacuoles, no macro fat; grade 2, fatty hepatocytes with macro fat occupying under 25% of the hepatic parenchyma; grade 3, fatty hepatocytes with macro fat occupying 25% to 50% of the hepatic parenchyma; grade 4, fatty hepatocytes with macro fat occupying more than 50% of hepatic parenchyma. Results for hepatic scoring were presented as the mean±SD for each group.

2.8. In situ detection of apoptotic cells in adipose tissue

In situ detection of apoptosis was performed on paraffin-embedded tissue sections (2 μm) of the epididymal adipose tissue with an In Situ Cell Detection Kit, POD (Roche Applied Science, Penzberg, Germany), based on the TUNEL method [15]. Tissue sections were deparaffinized, dehydrated, and endogenous peroxidase activity was blocked by treatment with methanol containing 1% H_2O_2. After washing with distilled water and phosphate buffered saline (PBS, pH 7.4) sections were treated with 20 μg/ml proteinase K (10 mM Tris–HCl, pH 7.4; Sigma-Aldrich) for 15 min at 37 °C. Sections were then washed with PBS, overlaid with PBS containing 10% normal sheep serum, and incubated with the TUNEL reaction mixture for one h at 37 °C. Sections were then washed in PBS and incubated for 30 min at 37 °C with Converter-POD (anti-fluorescein antibody, Fab fragment from sheep, conjugated with horseradish peroxidase; all supplied in the kit). Apoptotic cells were visualized by incubating the sections with a DAB kit (Sk-4100, Vector Laboratories, Burlingame, CA, USA). Sections were counter-stained with Mayer's hematoxylin. For a negative control, sections were treated by the same procedure without Terminal deoxynucleotidyl transferase. The numbers of TUNEL positive spots and total number of adipocytes in ten×400 light-microscopic fields were counted. The percentage of TUNEL positive spots was calculated and presented as the mean±SD for each group.

2.9. Analysis for mRNA levels in white adipose tissue

Cytokine mRNA expression was assayed with semi-quantitative RT-PCR. RNA were isolated from liver using TRIzol® Reagent (Invitrogen, Carlsbad,

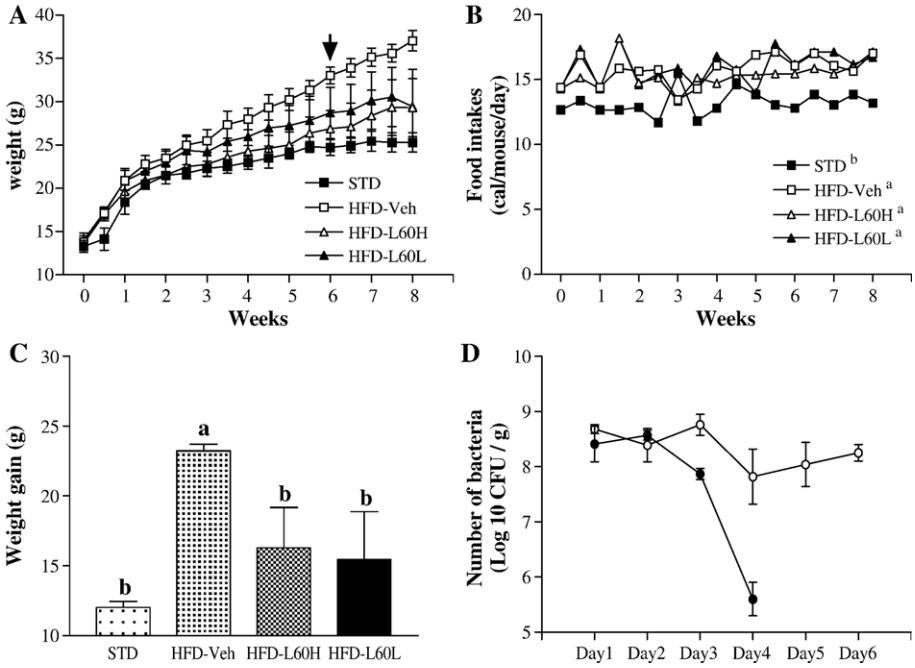

Fig. 1. The abilities of *L. rhamnosus* PL60 for reducing body weight gain and for attaching to intestines. Body weights (A), food intakes (B) and weight gain (C) during experimental periods. Arrow indicates when the HFD-PL60H fed group showed significantly lower body weight compared to HFD-Veh (A). Values of each group with same letters in each panel (B and C) were not significantly different ($P<0.05$). The panel D indicated that *L. rhamnosus* PL60 recovered from the mouse feces after oral administration of 10^9 CFU once (●) or twice (○) at intervals of 24 h. Data are means ($n=3$) and bars indicate SD.

Table 4
The amount of linoleic acid and CLA isomers in sera[a]

	Linoleic acid	c9,t11-CLA	t10,c12-CLA
Control	850.3	9.706	–[b]
L. rhamnosus PL60	1066.8	13.006	1.906
L. rhamnosus GG	995.8	11.200	–[b]

[a] Fourteen mice were divided to 3 groups and fed 45% high fat diet. Five mice daily received 1×10^9 CFU of *L. rhamnosus* PL60 in PBS and another 5 mice received *L. rhamnosus* GG for 3 weeks. Control group (4 mice) received 0.5 ml of PBS for same periods. At third week, the sera obtained from each group were pooled and measure each CLA isomers by GC.
[b] Not detected.

CA, USA) and stored at −80 °C until use. cDNA was reverse transcribed from one μg of total RNA using a GeneAmp RNA PCR Core kit (Applied Biosystems, Foster City, CA, USA). PCR was performed with i-Taq DNA polymerase (iNtRON, Sungnam, Kyungki-Do, Korea) using cytokine-specific primer sets. Primer sets for proliferator-activated receptor-γ (PPAR-γ), uncoupling protein-2 (UCP-2), fatty acid synthase (FAS), tumor necrosis factor-α (TNF-α), and β-actin are shown in Table 2. PCR was initiated with denaturation for three min at 95 °C followed by 30 cycles of denaturation (one min, 95 °C), annealing (one min, 50–60 °C), chain extension (two min, 72 °C), and then seven min of final extension at 72 °C after amplification. PCR products were detected by electrophoresis using a 1.5% (w/v) agarose gel followed by staining with ethidium bromide. PCR products were visualized under ultraviolet light and band densities were measured with an analyzing program (Kodak Digital Science 1D, NEN Life Science Products, Boston, MA, USA), normalized by the density of *β*-actin, and evaluated statistically.

2.10. Statistics

Significant differences among groups were determined using Duncan's Multiple Range Test (SAS ver. 8.1, SAS Institute Inc., Cary, NC, U.S.A.). Values of $P<0.05$ were considered as significant.

3. Results

3.1. Isolation and identification of bacteria

The LAB selected in this study was identified as *L. para paracasei* (*L. rhamnosus*) with API50CH. Its 16S rRNA sequence showed 99% identity (842/844) with *L. rhamnosus* (GenBank Accession No. AY 675254), and was named as *L. rhamnosus* PL60. *L. rhamnosus* PL60 was submitted to the Korea Agriculture Culture Collection (KACC) under the number KACC-91105.

3.2. Assay of c9, t11-CLA and t10, c12-CLA produced by washed cells with gas chromatography

Gas chromatography demonstrating the amounts of c9,t11- and t10,c12-CLA produced by each strain are as shown in Table 3. The amounts of c9,t11- and t10,c12-CLA produced by washed cells of *L. rhamnosus* PL 60 were 2836.5±260.92 μg/mg and 1602.50±391.03 μg/mg, respectively. And as reported before, *L. pentosus* IFO 12011 produced both CLA isomers and *L. paracasei* IFO 12004 produced c9,t11-CLA but not t10, c12-CLA.

3.3. Colonisation of mice with PL60

We investigated the colonisation of mice with *L. rhamnosus* PL60 by detecting *L. rhamnosus* PL60 in feces after oral administration in C57BL/6J mice. *L. rhamnosus* PL60 was recovered from feces for up to 96 h in mice singly inoculated, at levels greater than 1×10^8 CFU/g for the first 2 days and then lower thereafter (Fig. 1D). The counts of *L. rhamnosus* PL60 in the feces of mice inoculated with two doses were similar to those obtained after only one administration (between 8 log10 and 9 log10 CFU/g) and were detected at the same level even 4 days after termination of feeding (Fig. 1D).

3.4. The amount of CLA isomers and linoleic acid in sera

When the 5 mice daily received 1×10^9 CFU of *L. rhamnosus* PL60 for 3 weeks, 1.906 μg/ml of t10,c12–CLA was detected in the sera, but this isomer was not detected in those of mice fed with *L. rhamnosus* GG or PBS (Table 4).

3.5. Food intake, body weight, organ weights, and fat weight

The use of a high fat diet promoted a significant increase in animal body weight over the eight-week period (Fig. 1A). Differences in body weight among each group became noticeable at the third week of the diet and became significant after the sixth week. HFD-Veh-fed animals were clearly obese after about 5 weeks on the diet with the weights of these animals 20% above the controls (Fig. 1A). In a HFD-PL60 fed group, significant different occurred between six and 8 weeks of age. At the end of the experiment, gain in body weight was higher in the HFD-Veh group than in the HFD-PL60 fed group.

Table 5
Weights of body, fat, spleen, kidney, liver, and brain in each experimental group

Diet	STD	HFD-Veh	HFD-PL60H	HFD-PL60L
Body weight (g)	25.30±1.10^{c}	37.05±1.18^{a}	29.92±3.35^{b}	29.44±4.28^{b}
Epididymal WAT (g)	0.35±0.13^{d}	2.01±0.23^{a}	1.39±0.37^{bc}	1.29±0.39^{c}
Perirenal WAT (g)	0.06±0.04^{d}	0.903±0.12^{a}	0.65±0.23^{bc}	0.59±0.24^{c}
Inguinal WAT (g)	0.12±0.06^{b}	1.40±0.51^{a}	1.29±0.41^{a}	1.11±0.48^{a}
Mesenteric WAT (g)	0.20±0.11^{b}	0.60±0.17^{a}	0.45±0.18^{a}	0.46±0.07^{a}
Spleen (g)	0.05±0.004^{b}	0.07±0.003^{a}	0.07±0.012^{a}	0.08±0.019^{a}
Kidney (g)	0.31±0.02^{a}	0.35±0.04^{a}	0.33±0.02^{a}	0.32±0.04^{a}
Liver (g)	1.01±0.10^{ab}	1.15±0.14^{a}	0.99±0.11^{ab}	0.93±0.21^{b}
Brain (g)	0.44±0.01^{a}	0.43±0.01^{a}	0.43±0.02^{a}	0.44±0.02^{a}

Values were expressed as mean±SD. Values of each group with same letters for each serological factor were not significantly different ($P<0.05$).

Table 6
Serum levels of glucose, leptin, and lipids

Diet	STD	HFD-Veh	HFD-PL60H	HFD-PL60L
Glucose (mg/dl)	64.0±14.98[b]	159.3±32.45[a]	89.75±22.03[b]	67±14.65[b]
Leptin (pg/ml)	262.5±104.12[d]	3855.0±961.38[a]	1855.0±515.64[c]	2436.25±966.66[bc]
HDL-C (mg/dl)	76.32±8.99[c]	96.06±5.36[a]	83.06±8.86[ab]	92.82±4.59[ab]
LDL-C (mg/dl)	6.76±3.34[c]	17.98±5.08[ab]	14.02±2.90[b]	22.12±4.38[a]
TC (mg/dl)	128.94±15.24[b]	180.24±25.54[a]	162.20±21.45[a]	193.44±16.96[a]

The values expressed as mean±SD. Values of each group with same letters for each serological factor were not significantly different ($P<0.05$).

The average energy intake of animals fed HFD was higher than that of STD-fed animals (Fig. 1B), but did not differ between HFD-Veh and HFD-PL60 fed mice. Mean body weight increased by 12.0±0.43 g in STD, 23.2±0.48 g in HFD-Veh, 16.3±2.88 g in HFD-PL60H, and 15.5±3.41 g in HFD-PL60L fed mice. The weight gain (Fig. 1C) at the end of the experiment in HFD animals was two times higher than STD-fed animals. This is a clear sign that the high fat diet induced a profound accumulation of energy in the form of body fat, including epididymal, mesenteric, linguinal, and perirenal fat masses (Table 5). In addition, spleen, kidney, liver, and brain were weighed, but no significant difference was observed.

3.6. Plasma leptin, glucose, and cholesterol measurements

Plasma leptin, glucose, HDL-C, LDL-C, and TC in the various groups of mice used in this study are summarized in Table 6. Control animals presented the normal range for plasma leptin [16]. HFD-Veh animals showed a 15-fold increase in plasma leptin compared with control animals (Table 6). After treatment with PL60, circulating leptin levels were slightly (two folds), but significantly, reduced compared with HDF-Veh treated mice. Plasma HDL-C, LDL-C, and TC levels increased in HDF-Veh fed mice as expected, but were not altered in HLF-PL60H fed mice. PL60H-fed animals showed a remarkable reduction in plasma glucose level, normalized to the levels of HFD-fed animals (Table 6).

3.7. Histopathological analysis of liver steatosis and adipocyte diameter

The livers of HDF-Veh-fed mice were engorged with microglobular and macroglobular fat (Fig. 2B). After 8 weeks of treatment with HDF-PL60H, there was nearly complete resolution of hepatic steatosis (Fig. 2C) and the grading score for steatosis was significantly lowered (Fig. 2D). However, HDF-PL60L only slightly decreased hepatic fat accumulations (Fig. 2D).

In addition, within the epididymal adipose tissue, the adipocyte size of HDF-Veh-fed mice was measured in ×400 microscopic fields using thin sectioned adipose tissues stained with hematoxylin. The epididymal adipocytes were enlarged, compared to those of STD-fed mice however, 8 weeks of

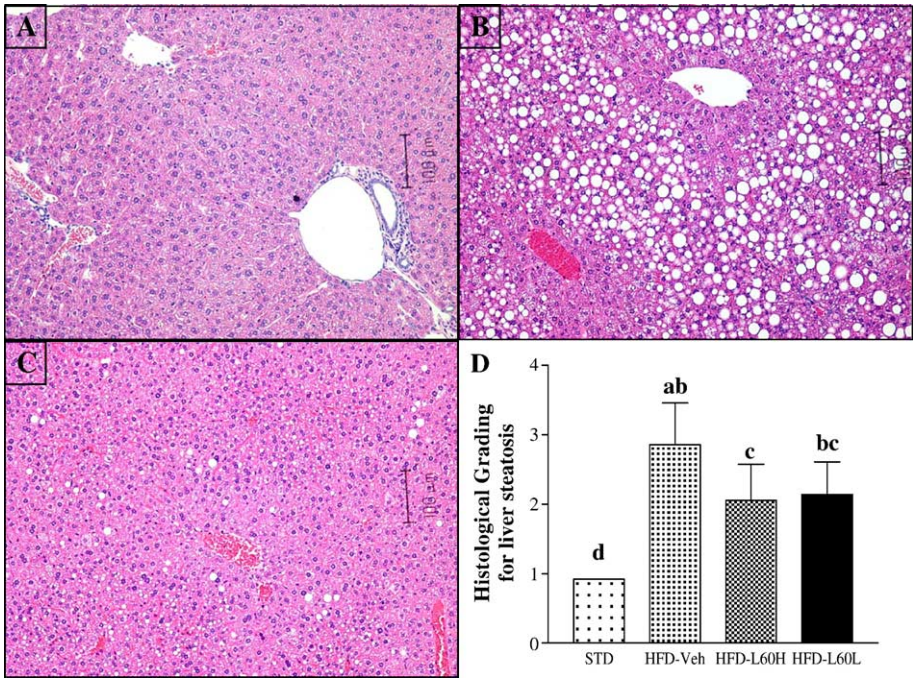

Fig. 2. Histopathological analysis for liver and epididymal adipose tissues. Panels A–C show that PL60H elevated liver steatosis. Samples were H&E stained, examined in×100 light microscopy field. Panels A, B, and C represent STD, HFD-Veh, and HFD-PL60H, respectively. The HFD-PL60H fed group showed significantly lower liver steatosis scoring compared to HFD-Veh (Panel B). Values of each group with same letters in panel D were not significantly different ($P<0.05$).

treatment with either HDF-PL60H or HDF-PL60L did not modulate the amount of fat (data were not shown).

3.8. In situ detection of apoptotic cells in adipose tissue; TUNEL assay

In the epididymal adipose tissue, scattered apoptotic adipocytes were seen in HDF-PL60-fed mice (Fig. 3B, D). Nuclear pyknosis and karyorrhexis were observed in tunnel-positive adipocytes. However, lipolysis and other severe morphological changes did not occur in all experimental groups. Few TUNEL-positive signals were observed in the epididymal white adipose tissues of STD and HFD-Veh-fed mice (Fig. 3E).

3.9. Semi-quantitative RT-PCR

As *L. rhamnosus* PL60 greatly decreased white adipose mass especially epididymal and perirenal deposits (Table 5), the effect of *L. rhamnosus* PL60 for epididymal adipose tissue was studied by analyzing the mRNA levels of several proteins that are involved in the regulation of energy metabolism (UCP-2), adipogenesis (PPAR-γ, FAS), and adipolysis (TNF-α) in the white adipose tissues that were assayed. The mRNA levels estimated by quantitative RT-PCR were corrected by comparison to those of a house-keeping gene (β-actin), and expressed as percentages with the value from mice fed the HDF-Veh as 100%. *L. rhamnosus* PL60 did not affect the gene expression of β-actin in white adipose tissues in the C57BL/6J mice (99.98–130.4% in all groups relative to STD-fed groups). *L. rhamnosus* PL60 significantly decreased TNF-α and FAS mRNA levels in epididymal white adipose tissues, compared to HFD-Veh (Fig. 4). In particular, *L. rhamnosus* PL60 greatly increased UCP-2 expression, approximately two fold higher than in STD and HFD fed mice. However, *L. rhamnosus* PL60 did not affect PPAR-γ mRNA levels in epididymal adipose tissue.

4. Discussion

4.1. Colonisation of mice with L. rhamnosus PL60

L. rhamnosus PL60 was recovered from feces for up to 96 h in mice singly inoculated, at levels greater than 1×10^8 CFU/g

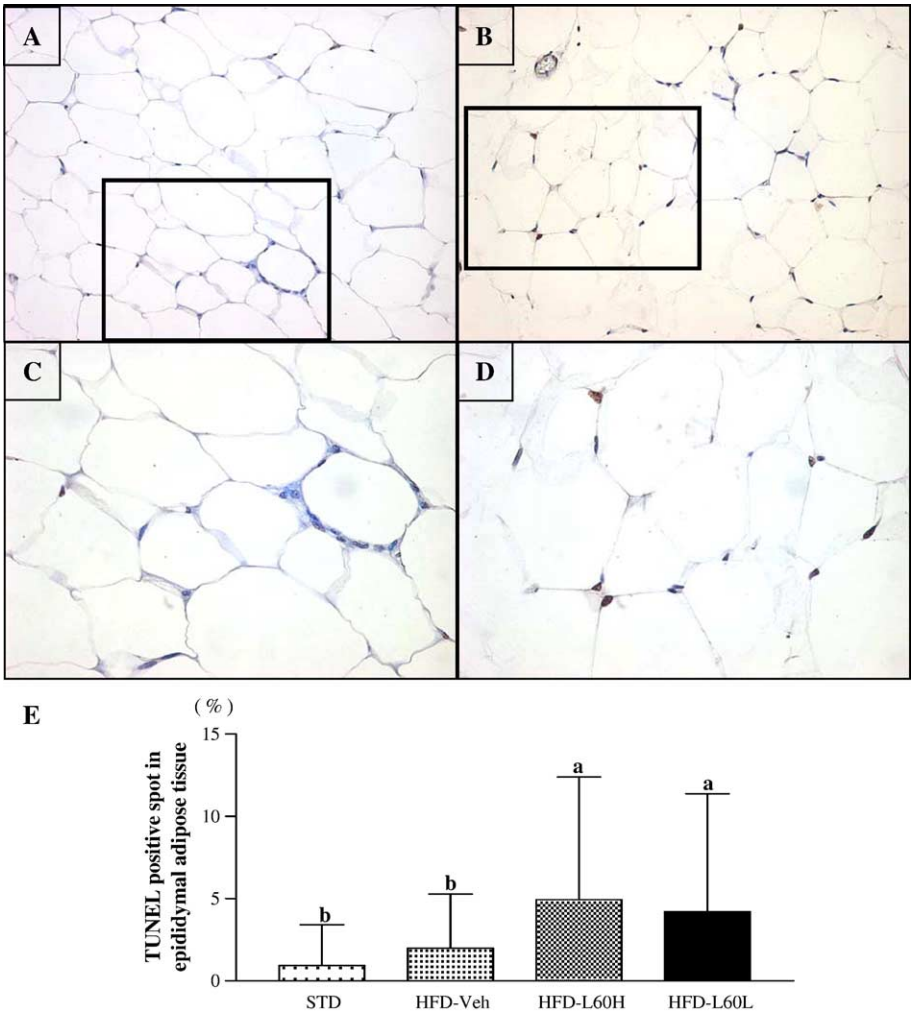

Fig. 3. TUNEL positive signals in white adipose tissue. The left panel (A and C) shows the epididymal adipose tissue of the HFD-Veh group. The right panel (B and D) shows that of the HFD-PL60H group. The percentage of TUNEL positive cells in twenty ×400 light microscopy fields was calculated (E). Light microscopy fields: A and B = ×200, C and D = ×400. Values of each group with same letters in panel E were not significantly different ($P < 0.01$).

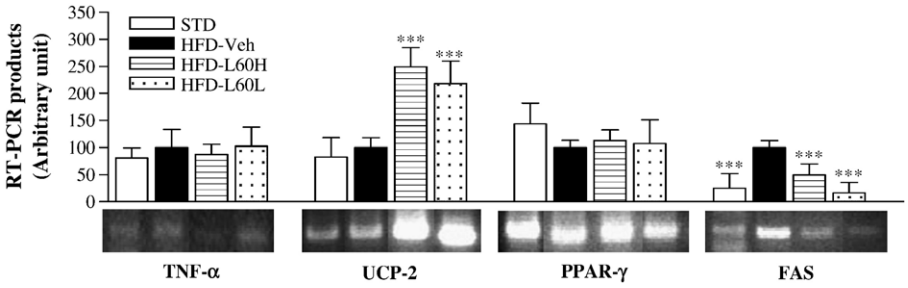

Fig. 4. RT-PCR analysis of gene expression in the epididymal adipose tissue of HFD-Veh and PL60-fed mice. Results are expressed as the relative abundance of RT-PCR product in STD-fed and HFD-fed mice as compared to HFD-Veh fed mice (=100) after normalization to the levels of b-actin mRNA. Each reaction was performed in triplicate. Data are means ($n=12$) and t-bars indicate SD, *$P<0.05$; ***$P<0.001$.

for the first 2 days and then lower thereafter (Fig. 1D). This result for the colonisation substantiates other previous studies performed with *L. rhamnosus* [17,18]. The ingested *L. rhamnosus* PL60 produced t10,c12–CLA as detected in sera (Table 4). *L. rhamnosus* PL60 is expected to continuously produce CLA after it colonises the gut, thereby continuously exerting beneficial effects from CLA.

4.2. Weight gain and energy intakes

In this study, decreased weight gain was observed in mice fed *L. rhamnosus* PL60, and the effect was particularly marked in epididymal white adipose tissues, while *L. rhamnosus* PL60 showed no effect on energy intake. Although many studies have demonstrated that dietary supplementation of CLA reduces weight gain, an issue that has remained controversial is the effect of CLA on food intake. Some studies have shown that CLA supplementation reduces energy intake [19–22], while others did not show any effect [4,23,24]. It was also reported that diet supplementation with CLA induced a significant decrease in body fat deposition, but a marginal reduction in energy intake [19]. Several studies have shown a large decrease in body fat mass after supplementation with CLA, even when there was no alteration in energy intake [4,25,26]. Data from these experiments strongly suggest that other mechanisms are involved in the observed CLA-induced decline of adipose deposition, while reduction in food intake may account in part for the reduction of weight gain and fat mass in animals.

Although *L. rhamnosus* PL60 reduce body weight gain, there was no dose response relationship between high and low dose of PL60 (Fig. 1A, Table 5, Fig. 4). As shown in Fig. 1A, after 7 weeks, the gap of body weight between PL60H and PL60L become narrower. It appears that the dose of PL60 used is sufficient to exert the maximal effect.

4.3. Mechanisms through which CLA affects weight gain and body composition in animals

In the present study, *L. rhamnosus* PL60 did not reduce the cell size in epididymal adipose tissue (data were not shown). Thus, we hypothesized that the decrease in the weight of white adipose tissues was due to reduction in cell number rather than cell size. To clarify the mechanisms of *L. rhamnosus* PL60 for the reduction of weight gain, in situ detection of apoptotic cells and analysis of the mRNA levels of several proteins that are involved in energy metabolism regulation, adipogenesis and adipolysis in white adipose tissues were performed. In the present study, the TUNEL-positive spots and mRNA levels of UCP-2 increased, while the mRNA levels of FAS decreased in the epididymal white adipose tissues of mice fed *L. rhamnosus* PL60. The overexpression of UCP-2 mRNA was also noted.

West et al. [25] previously compared the increase of energy expenditure with the reduction of fat deposition and found that increased energy expenditure was sufficient to account for the decreased adipose deposition in CLA-treated mice. It has been hypothesized that the enhanced thermogenesis in adipose tissues is partially attributed to the altered expression of genes encoding uncoupling proteins (UCPs), a family of proteins that regulate adiposity and are expressed differently in various adipose and other tissues. While UCP-1 is expressed exclusively in brown adipose tissue, UCP-2 is expressed ubiquitously in multiple tissues, whereas UCP-3 is expressed at high levels in skeletal muscle and brown adipose tissue [27]. Several studies reported that UCP-2 plays a more important role than other UCPs in the CLA-induced alterations of energy expenditure. Increased UCP-2 mRNA levels after feeding CLA have been observed in the white adipose tissue and liver of C57BL/6J mice [22,28], ICR mice[22], ob/ob mice [29], and ZDF rats [30]. Since UCP2 is a predominant UCP in white adipose tissue, upregulation of UCP-2 is likely a primary mechanism through which CLA increases energy expenditure in the present study.

TNF-α is known as a cytokine that leads to apoptosis of adipocytes [31], inhibits the synthesis of fatty acid synthase (FAS) [32], and is positively associated with UCP-2 mRNA expression [31]. Feeding of CLA in C57BL/6J mice resulted in an increase of TNF-α mRNA in white adipose tissue [28]. However, in the present study, TNF-α mRNA level was not increased in the white adipose tissue after *L. rhamnosus* PL60 supplementation, although TUNEL-positive spots, UCP-2 mRNA level increased and FAS mRNA level decreased in adipose tissue (Fig. 4). The reason why the level of TNF-α mRNA in adipose tissue was not altered is not known, and further studies are needed to investigate this observation.

4.4. Liver steatosis

Although CLA supplementation has significantly reduced body fat and weight gain in different animal models, the

concomitant enlargements of the liver have raised safety issues [33]. Biochemical, cellular, and molecular mechanisms involved in the development of a fatty liver and spleen are not well established. It has been suggested that a fatty liver could be a consequence of the increased lipogenesis in the liver compensating for the reduced fat deposition in the adipose tissue.

Unexpectedly, oral administration of *L. rhanmosus* PL60 alleviated the liver steatosis in DIO-mice. It seems that both the doses and the isomer type of CLA have an important role for liver steatosis, because the adverse effect on the liver was observed predominantly with relatively higher doses of CLA (0.5–2%) and specific isomer-type (especially t10, c12) [28,34–37]. Moreover, it seems that the normalization of liver steatosis was due to *lactobacillus*. Previously, Li et al. showed that treatment with the mixture of viable, lyophilized *bifidobacteria, lactobacilli* and *Streptococcus thermophilus* significantly decreased hepatic steatosis [14]. Thus, the lower dose of CLA supplementation from probiotics, such as *L. rhamnosus* PL60, which were able to colonise the gut and continuously produce CLA, could be a reliable solution to control both obesity and adverse effects caused by CLA.

4.5. Serum leptin, glucose, and lipids

In the present study, correlations were also found between plasma leptin levels and white adipose tissue weights in the perirenal and epididymal tissues. Similar effects have been observed in another study using rat [38]. Results of these studies suggest that reductions in fat mass due to CLA are associated with a reduction in leptin.

Leptin is an important hormone involved in maintaining blood glucose levels by inducing insulin-mediated glucose disposal [39,40], therefore, it is reasonable to consider that a reduction of plasma leptin level by CLA affects insulin sensitivity. However, the effect of CLA on blood glucose concentration was inconsistent. In fact, in the present study, the concentration of serum glucose was rather significantly decreased. Previous studies reported that CLA significantly reduced glucose, insulinemia, and serum leptin in rats with type 2 diabetes [41,42], but it modestly increased insulin levels in non-diabetic mice [28]. These data suggested that CLA may not improve insulin sensitivity under a normoglycemic state [28]. Other studies have shown that CLA supplementation did not produce any effect on plasma glucose levels in AKR/J [21], C57BL/6J, or ICR mice [22].

4.6. Conclusion

The present study shows that an eight week oral treatment with CLA-producing bacteria, *L. rhamnosus* PL60, decreases body weight gain and liver steatosis in DIO mice, and also suggests that anti-obesity effects of *L. rhamnosus* PL60 are possibly related to both apoptosis and mRNA expression in white adipose tissues.

This is the first study where the effects of oral intake of CLA-producing bacteria on obesity have been investigated using the DIO mice model.

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