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ARTICLE

Effect of *Lactobacillus plantarum* FH185 on the Reduction of Adipocyte Size and Gut Microbial Changes in Mice with Diet-induced Obesity

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Abstract

This study aimed to investigate the effects of *Lactobacillus plantarum* FH185 on the reduction of adipocyte size and gut microbial changes in mice with diet-induced obesity. The strain was found to have a lipase inhibitory activity of 70.09±2.04% and inhibited adipocyte differentiation of 3T3-L1 cells (18.63±0.98%) at a concentration of 100 μg/mL. To examine the effect of the strain supplementation on gut microbial changes in mice with diet-induced obesity, male C57BL/6J mice were fed on four different diets (i.e., A, normal diet (ND); B, high-fat diet (HFD); C, HFD with ABT-3 (10^9 CFU/day); and D, HFD with *L. plantarum* FH185 (10^9 CFU/day)) for 6 wk. According to the results of fecal pyrosequencing, the ratio of *Firmicutes* to *Bacteroidetes* in groups C and D was lower than in the control groups at the phylum level. At the family level, *Lactobacillaceae* in groups C and D was observed to dominate, while *Lachnospiraceae* in groups A and B was observed to dominate. At the genus level, *Lactobacillus* in groups C and D was comparatively higher than in groups A and B. To examine the effects of strain supplementation on the reduction of adipocyte size, the left and right epididymal fat pads were quickly isolated after the animals were sacrificed, and the adipocyte sizes were measured. In groups A, C and D, the percentage of 2,000 m^2 of adipocyte was higher than in the other size of adipocyte, while the percentage of over 5,000 m^2 of adipocyte was highest in group B. The mean adipocyte size of group D was significantly larger than that of group A, but smaller than that of group B.

Key words: *Lactobacillus plantarum*, anti-lipase activity, anti-adipogenetic activity, gut microbiota, adipocyte size

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Introduction

Obesity, a condition in which an abnormally large amount of fat is stored in the body's adipose tissue, resulting in an increase in body weight, is one of a number of critical pandemics and health problems that it is rapidly becoming a worldwide epidemic (An *et al*., 2011). Obesity has been linked to a variety of chronic diseases such as comorbidities of cardiovascular diseases (CVD), type-2 diabetes, certain cancers, and sleep apnea/sleep-disordered breathing, and is thought to be responsible for almost 300,000 deaths each year (Haslam and James, 2005; Stein and Colditz, 2004). It is generally known that a high-fat diet (HFD) is one of various factors that cause obesity, and that the long-term intake of such a diet induces a significant increase in abdominal fat weight. Therefore, many food science researchers have concentrated on finding a food or beverage with a function that prevents HFD-induced obesity (Iwashita *et al*., 2002; Tanida *et al*., 2008).

Recently, there has been an increase in studies of the differences in the composition of gut microbiota between obese and lean individuals, and these have demonstrated that obesity is associated with an imbalance in the normal gut microbiota (Arora *et al*., 2012; Turnbaugh *et al*., 2009). In healthy adults, 80% of the identified fecal microbiota can be classified into three dominant phyla: *Bacteroidetes*, *Firmicutes* and *Actinobacteril* (Lay *et al*. 2005). In general, the *Firmicutes* to *Bacteroidete*s ratio is thought to be particularly relevant to the composition of human microbiota. The ratio differs in obese and lean humans, and this proportion decreases with weight loss on a low-calorie diet (Ley *et al.* 2006).

Probiotics, consisting of individual or multiple live bacteria species such as *Lactobacillus* and bifidobacteria, confer a health benefit on the host when administered in adequate amounts. Probiotics directly alter the gut microbiota (Delzenne *et al.*, 2011; FAO and WHO, 2001). Several studies have reported the health-promoting effects of probiotics, including the maintenance of intestinal mucosal resistance to pathogenic microorganisms (Mennigen and Bruewer, 2009), the prevention of diarrhea (Guanda-

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lini, 2008), immunomodulation (Perdigón *et al*., 2001), reduced serum cholesterol levels (Nguyen *et al*., 2007), and the prevention of certain allergies and cancers (Isolauri and Salminen, 2008; Kumar *et al*., 2010). Other studies also demonstrate that some probiotic can be used to control bodyweight and metabolic disorders (Bhathena *et al*., 2009; Lee *et al*., 2007).

The aim of this study was to investigate the effect of *L. plantarum* FH185 on the reduction of adipocyte size and gut microbial changes in mice with diet-induced obesity.

Materials and Methods

Isolation of lactic acid bacteria

The strain FH185 was isolated from the feces of healthy adults in a modified MRS medium (Lim *et al*., 2011), and incubated in lactobacilli MRS broth (Difco, USA) as the growth medium at 37°C for 18 h.

Identification of FH185 strain

The properties of the FH185 strain were investigated by testing with Gram staining and microscopic observation after cultivation in an MRS broth for 18 h at 37°C. Bergey's Manual of Systematic Bacteriology by Rogosa (1974) was used to examine the morphological and physiological properties of the isolated strains. The FH185 strain was identified using the 16S rDNA sequencing method. The chromosomal DNA of the isolated strain was separated using a SolGent Genomic DNA prep kit (SolGent, Korea). The DNA extracts were used for the polymerase chain reaction (PCR) with universal primers [27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3')]. PCR was carried out in a programmable therma cycler (SolGent EF-Taq, Korea), according to the following steps: one cycle of denaturation at 95°C for 15 min, followed by 30 cycles of 95°C for 20 s, 50°C for 40 s, and 72°C for 90 s. The final extension was carried out at 72°C for 5 min. The purified PCR product obtained using a SolGent PCR purification kit (SolGent) was used for sequencing with an ABI 3730XL DNA analyzer (Applied Biosystems, USA).

Anti-lipase activity

A modified version of a method of lipase activity determination (by Lee *et al.*, 1993) was used. The pancreatic lipase activity was measured using porcine pancreatic lipase (Sigma chemical, USA). 0.1 mg/mL of a sample solution dissolved in water, and 0.167 mM *p*-Nitrophenylpalmitate (PNP; Sigma) solution and 0.061 M (pH 8.5) Tris-HCL buffer were mixed in the well of a plate, to which 0.3 mg/mL of the lipase solution was then added to start the enzyme reaction. After incubation at 25°C for 10 min, its absorbance was measured at 405 nm.

Anti-adipogenetic activity

Cell line and cell culture

3T3-L1 cells were obtained from the American Type Culture Collection (ATCC, USA) and cultured at 37°C in a humidified 5% CO_2 atmosphere. 3T3-L1 cells were cultured as described by Hemati *et al.* (1997). Briefly, the cells were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO Lab., USA) containing high glucose content, supplemented with 10% bovine calf serum (BCS; GIBCO) and 1% penicillin/streptomycin in six well culture plates. Two days later, confluence cells were cultured in an adipocyte differentiation cocktail medium containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma), 1 M dexamethasone (Dex; Sigma), and 5 g/mL insulin (Sigma) in DMEM supplemented with 10% fetal bovine serum (FBS; GIBCO) for 2 d. The differentiation was complete after 6 d.

Sample preparation and treatment of *L. plantarum* FH185

L. plantarum FH185 was incubated at 37°C for 18 h in MRS broth. All of the purified strains were kept at -70°C until use. After culturing *L. plantarum* FH185, all of the strains were harvested in a refrigerated centrifuge (1,500 *g* for 15 min at 4°C) and washed three times with distilled water to remove any remaining MRS broth. The washed *L. plantarum* FH185 was freeze-dried and re-suspended in distilled water at a concentration of 10 mg/mL, and then homogenized for 50 s followed by 1 min of rest (repeated 3 times) using a sonicator. The 3T3-L1 cells were treated with three different concentrations of supernatant, i.e., 0 g/mL (control), 10 g/mL and 100 g/mL.

Oil red O staining of 3T3-L1 adipocytes

Intracellular lipid accumulation was measured using oil red O (Sigma). Oil red O straining of 3T3-L1 cells was performed using a modified version of the method described by Ramirez-Zacarias *et al.* (1992). 3T3-L1 cells were washed with PBS twice, fixed with 10% formaldehyde/PBS at 4°C for 1 h, and stained with filtered oil red O solution (stock solution: 3.5 mg/mL in isopropanol; working solution: 60% oil red O stock solution and 40% distilled water) at room temperature for 30 min. The quanti-

fication of lipid accumulation was achieved by oil red O obtained from stained cells with isopropyl alcohol and measured spectrophotometrically at 520 nm. The material stained with oil red O was expressed on a per cell basis using the cell number determined from similar plates. The percentage of the material stained with oil red O relative to the control wells containing the cell culture medium without compounds was calculated as 520 nm (FH185)/ 520 nm (control) × 100.

Change of gut microbiota and adipocyte size

Animals and treatments

Male C57BL/6J mice aged 6 wk were purchased from DooYeol Biotech (Korea). All of the mice were individually housed at a constant temperature and humidity (20± 2ºC, 55±10%) under a 12 h light/dark cycle. After acclimatization for 1 wk on a normal chow diet, they were then randomized according to their body weights and divided into four groups of 6 mice. The mice were fed either a high-fat diet (HFD; n=18) or a normal diet (ND; n=6) for 6 wk. In calorific terms, the high-fat diet consisted of 34.3% fat, 27.3% carbohydrate, and 34.3% protein (5.1 kcal/g), whereas the normal diet contained 4.2% fat, 64.7% carbohydrate, and 18.6% protein (3.7 kcal/g). The compositions of HFD and ND are given in Table 1. The groups of mice were fed as follows: Group A was fed a normal diet and orally administrated with saline solution; Group B was fed a high-fat diet and orally administrated with saline solution; Group C was fed a high-fat diet and orally administrated with ABT-3 (at 10^9 CFU/day), a commercial probiotic start culture obtained from Chr. Hansen's Laboratory (Denmark), in saline solution; and Group D was fed a high-fat diet and orally administrated with *L. plantarum* FH185 (10^9 CFU/day) in saline solution. Food and water were freely available, and ABT-3 and *L. plantarum* FH185 in saline solution, or only saline solution, were orally administered once a day for 6 wk (2.5 g/g body weight). Probiotics were suspended in sterilized saline solution immediately before being orally administered to the mice. Before being sacrificed, the mice were fasted for 12 h and anesthetized with diethyl ether. All animal experimentation was performed after obtaining the approval of the Institutional Animal Care and Use Committee (IACUC) of Seoul National University College of Medicine.

Table 1. The formula of normal diet and high fat diet

Formula	g/kg	
	Normal diet (ND)	High fat diet (HFD)
Casein	210.0	265.0
L-Cystine	3.0	4.0
Maltodextrin	50.0	160.0
Sucrose	325.0	90.0
Lard	20.0	310.0
Soybean Oil	20.0	30.0
Cellulose	37.15	65.5
Mineral Mix, AIN-93G-MX(94046)	35.0	48.0
Calcium Phosphate, dibasic	2.0	3.4
Vitamin Mix, AIN-93-VX(94047)	15.0	21.0
Choline Bitartrate	2.75	3.0
Food Color	0.1	0.1

Bacterial 16S rRNA gene amplification and barcoded pyrosequencing

Fecal samples were collected on the first day of 0 wk and on the last day of 6 wk to analyze the fecal microbiota, and the samples were stored at -80ºC prior to DNA extraction. For analysis of the microbial content, the genomic DNA was extracted from the fecal samples of all the mice using a G-spin™ Genomic DNA Extraction Kit (iNtRON Biotechnology, Seoul, Korea) according to the manufacturer's instructions. The extracted DNA was amplified using primers targeting the V1 to V3 hypervariable regions of the bacterial 16S rRNA gene (V1-9F: 5'-X-AC-GAGTTTGATCMTGGCTCAG-3' and V3-541R: 5'-X-AC-WTTACCGCGGCTGCTGG-3', where X denotes the uniquely designed barcode for each mouse followed by a common linker AC). In this study, mixtures of barcodes of varying lengths (7 to 11 base pairs) were used. To amplify the 16S rRNA genes, the polymerase chain reaction (PCR) was performed with the universal primers 8F and 338R (Liu *et al*., 1997). The PCR consisted of a 95ºC hold for 120 s, followed by 35 cycles of 1 min at 95ºC, 30 s at 58ºC, 30 s at 72ºC, followed by a final extension for 10 min at 72ºC. The amplification was performed using a PE2400 thermal cycler (Perkin-Elmer, USA). The PCR fragment was purified using a QIA Quick PCR Purification kit (QIAGEN, USA) according to the manufacturer's protocol. The DNA sequencing was performed using the standard shotgun sequencing reagents and a Genome Sequencer FLX (Roche, USA), according to the manufacturer's instructions by Macrogen Inc. (Korea). Clustering and statistical analysis of sequence data were performed using the Mothur software package (Schloss and Handelsman, 2008). The length of the fragment of the 16S rDNA pyrosequenced ranged from 118 to 526 base pairs, with an average length of 472 base pairs. The pyrosequencing data are available in the EMBL SRA

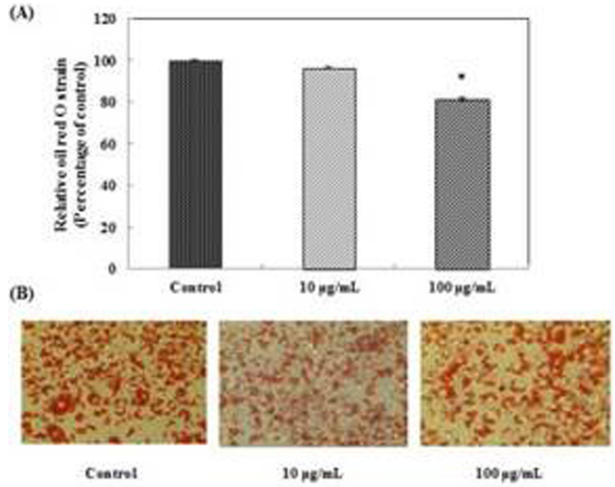

Fig. 1. The effects of *Lactobacillus plantarum* FH185 on oil red O stained in 3T3-L1 adipocyte. (A) quantification of oil red O staining * significant difference from control at $p<0.05$; (B) photograph of oil red O staining. Cells were stained with oil red O observed by using a microscope (original magnification ×200).

database under the accession number ERP000935.

Measurement of adipocyte size

The left and right epididymal fat pads were quickly isolated after the animals were sacrificed. Epididymal fat pads were fixed in 10% formaldehyde solution. According to the method of Hirsch and Gallian (1968), samples were passed through a 250-m nylon filter in order to remove fibrous tissue and intact shreds of fixed adipose tissue. The tissue shreds were flushed thoroughly with PBS. The filtrate was then passed through a 25-m nylon filter to trap fixed adipocytes, and the cells were washed thoroughly with PBS. Cell suspensions were applied to glass slides and then stained with hematoxylin and eosin solutions (H&E staining), as previously described (van Goor *et al*., 1986). The cells on the glass slides were observed with a microscope and the adipocyte size were measured using Image J Software (National Institute of Health, USA).

Statistical analysis

The statistical analysis was performed with a statistical analysis system (SAS, SAS Institute Inc., USA), and the results expressed as the mean±standard deviation (SD). The significance of the differences was analyzed by conducting a one-way analysis of variance (ANOVA) with Duncan's multiple range tests. Values of $p<0.05$ were considered statistically significant.

Table 2. Physiological characteristics of *Lactobacillus plantarum* FH185

Gram reaction		+	
Cell type		rod	
Spore forming		-	
Motility		-	
Aerobic growth		+	
Anaerobic growth		+	
Catalase reaction		-	
Growth at 15		-	
Growth at 45		-	
Gas forming from glucose		-	
Ammonia production from alginin		-	
Acid production from			
Glycerol	-	Salicin	+
Erythritol	-	D-Celiobiose	+
D-Arabinose	-	D-Maltose	+
L-Arabinose	+	D-Lactose	+
D-Ribose	+	D-Melibiose	+
D-Xylose	-	D-Saccharose	+
L-Xylose	-	D-Trehalose	+
D-Adonitol	-	Inulin	-
Methyl-β-D-Xylopyranoside	-	D-Melezitose	+
D-Galactose	+	D-Raffinose	+
D-Glucose	+	Amidon(starch)	-
D-Fructose	+	Glycogen	-
D-Mannose	+	Xylitol	-
L-Sorbose	-	Gentiobiose	+
L-Rhamnose	-	D-Turanose	+
Dulcitol	-	D-Lyxose	-
Inositol	-	D-Tagatose	-
D-Mannitol	+	D-Fucose	-
D-Sorbitol	+	L-Fucose	-
Methyl-α-D-Mannopyranoside	+	D-Arabitol	-
Methyl-α-D-Glucopyranoside	-	L-Arabitol	-
N-AcetylGlucosamine	+	Potassium Gluconate	+
Amygdalin	+	Potassium 2-KetoGluconate	-
Arbutin	+	Potassium 5-KetoGluconate	-
Esculin	+		

Results and Discussion

Isolation of lactic acid bacteria

The feces samples were collected from healthy adult staff members of the Korea Food Research Institute (Korea), and 188 strains were isolated as lactic acid bacteria in a modified MRS medium. The strain was incubated in lactobacilli MRS broth as the growth medium at 37°C for 18 h.

Selection of strain of anti-obesity activity

Several approaches to the prevention and treatment of

obesity have been reported (Birari and Bhutani, 2007). Among these, both natural and synthetic pancreatic lipase inhibitors are effective in preventing obesity, which is considered likely to be due to their inhibition of intestinal lipid absorption (Hirose *et al.*, 2013). After being incubated in MRS broth at 37°C for 18 h, three kinds of strains having a lipase inhibitory activity of over 60% were selected from among the 188 strains by measuring their lipase inhibitory activity. The FH185 strain exhibited a lipase inhibitory activity of 70.09±2.04% compared with the control group. Fig. 1 shows that an 8-day treatment at various concentrations [0-100 μg/mL] of the FH 185 strain during the differentiation period significantly and dose-dependently inhibited 3T3-L1 adipogenesis in terms of lipid accumulation compared with the control cells. Among the tested concentrations of FH185, 100 μg/mL was the most effective at reducing the lipid content in differentiated cells (by 18.63±0.98% compared with the control cells). These results suggest that FH185 inhibits pancreatic lipase (PL) and the differentiation of 3T3-L1 pre-adipocytes by suppressing lipid accumulation.

Identification and DNA sequencing of the selected FH185 strain

Physiological and biochemical tests were conducted to determine the genus and species of the selected FH185 strain. The FH185 strain consisted of non-spore, rod-type, gram-positive bacteria and exhibited negative properties on catalase and motility. In addition, it cannot grow at 15°C or 45°C. As it does not produce gas and ammonia from glucose and arginine, it has been identified as a genus *Lactobacillus* (Table 2). After PCR amplification using universal primers targeting 16S rDNA and the following sequence analysis, it was identified as *L. plantarum* with a similarity of 99% (data not shown). Based on the results of previous studies, it was named *L. plantarum* FH185 (Fig. 2).

Change of gut microbiota

The fecal samples collected on the first day of 0 wk and the last day of 6 wk were analyzed for their fecal microbiota. According to the results of fecal pyrosequencing, compared with 6 wk, at the phylum level, there was a greater increase in phylum *Firmicutes* than *Bacteroidetes*

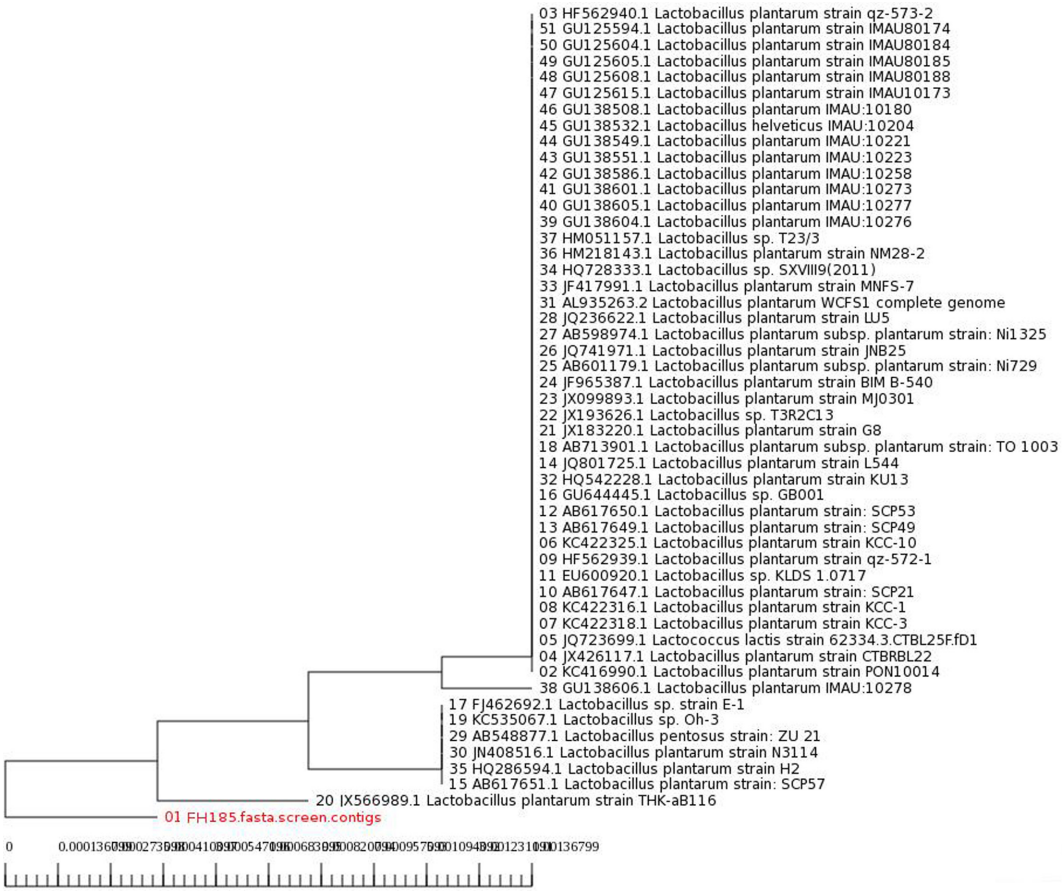

Fig. 2. Phylogenetic tree of *Lactobacillus plantarum* FH185 isolated from adult feces.

in group A, which was the control group fed with the ND. Meanwhile, in group B, the control group fed with the HFD, there was a slight increase in *Bacteroidetes*; whereas in group C, the group fed with the HFD and orally administered with ABT-3, the was a considerable increases in *Bacteroidetes*. In addition there was an increase in *Bacteroidetes* in group D, which was fed with the HFD and orally administered with *L. plantarum* FH185. Ley *et al.* (2006) have demonstrated that *ob/ob* mice had a reduction in the abundance of *Bacteroidetes* and a proportional increase in *Firmicutes*. They also found that obese people had a lower proportion of *Bacteroidetes/Firmicutes* than lean people. It means that *L. plantarum* FH185 has an anti-obesity effect (Fig. 3(a) and (b)).

Next, we analyzed the differences in gut microbiota composition at the family level. The *Lactobacillaceae* family was observed to dominate in all groups before the start of treatment. However, the *Lachnospiraceae* family was observed to dominate in group A. In group B, *Lachnospiraceae* was also observed to dominate, but the amount of *Porphyromonadaceae* increased only slightly. In group C, *Lactobacillaceae* and *Lachnospiraceae* were observed to dominate equally. In case of group D, it displayed a similar tendency with group C, but the *Lactobacillaceae* family was observed to be slightly more prevalent than the *Lachnospiraceae* family. Also, the amount of *Bacteroidaceae* and *Porphyromonadaceae* increased slightly in groups C and D. *Lactobacillaceae* and *Lachnospiraceae* were the families of *Bacteroidetes,* while *Bacteroidaceae* and *Porphyromonadaceae* were the families of *Firmicutes* (Nava *et al*., 2011) (Fig. 3(c) and (d)).

At the genus level, an unknown spp. was observed to dominate after six weeks, while the *Lactobacillus* spp. in all groups was observed to dominate before the start of the treatment. However, the *Lactobacillus* spp. in groups C and D was comparatively higher than in groups A and B (Fig. 3(e) and (f)).

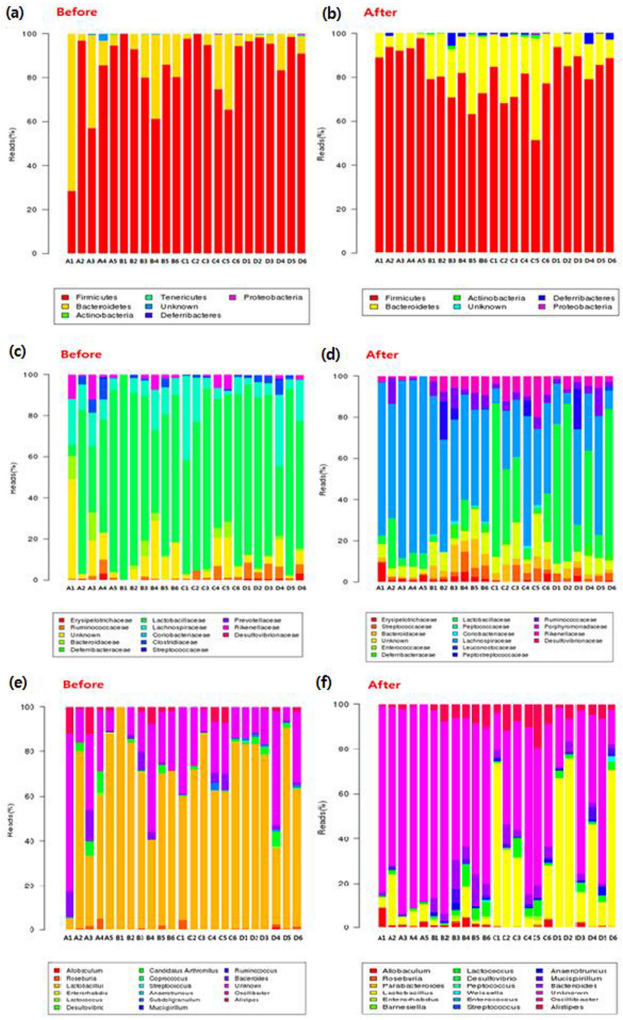

Fig. 3. Proportion or taxonomic assignment at phylum level (a, b), family level (c, d), and genus level (e, f). A, normal diet; B, high fat diet; C, high fat diet with ABT-3 (10^9 CFU/d); D, high fat diet with *L. plantarum* FH185 (10^9 CFU/d).

Adipocyte size

The epididymal fat pads of the mice were quickly isolated and the adipocyte size was observed and measured. In group A, which was the control group fed with the ND, the percentage of 2,000 m^2 of adipocyte was higher than in the other size of adipocyte. In groups C and D, i.e., those fed with the HFD and orally administrated with ABT-3 and *L. plantarum* FH185, the percentage of 2000 m^2 of adipocyte was also higher than in the other size of adipocyte. However, the percentage of over 5000 m^2 of adipocyte was highest in group B, the control group fed with the HFD. In addition, the average adipocyte size of group B was found to be higher than that of the other groups (Fig. 4). Indeed, Cani *et al*. (2007) previously reported that a high-fat diet increased the size of adipocyte cells. Moreover, in their after-study (Cani *et al.* 2008), the mean adipocyte size was reduced in high-fat antibiotic treated mice when compared with high-fat untreated mice. They concluded that the antibiotic treatment was associated with changes in the size of adipocyte cells. Thus, the results suggest that *L. plantarum* FH185 has an effect on reduction of adipocyte size.

Conclusion

This study aimed to investigate the effects of *L. plan-*

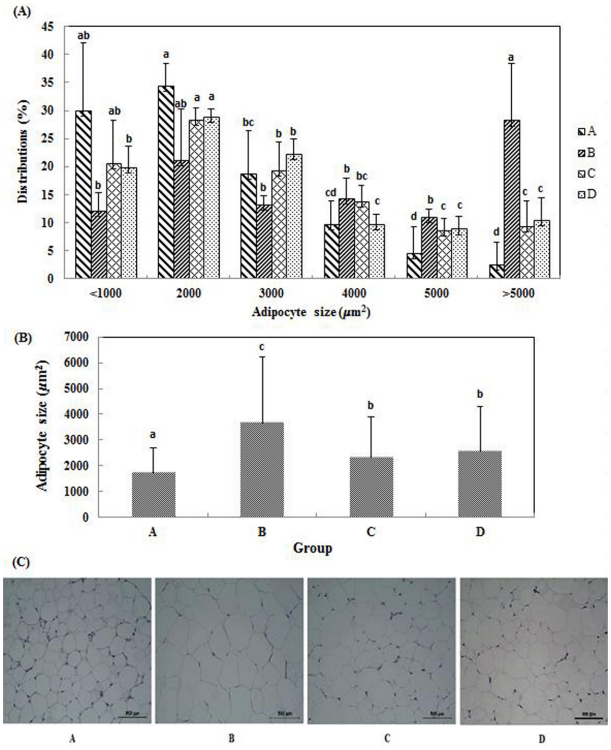

Fig. 4. Changes in adipocyte size of the epididymal fat pads in mouse. Values are mean±SD of 6 mice per group; (A) Percentage of cell number; [a-c]Means that the values with different superscripts within the same group are significantly different (p<0.05). (B) The adipocyte size, [a-c]Means that the values with different superscripts are significantly different (p<0.05). (c) Photograph of the epididymal fat pads in mouse (original magnification ×200). A, normal diet; B, high fat diet; C, high fat diet with ABT-3 (10^9 CFU/d); D, high fat diet with *L. plantarum* FH185 (10^9 CFU/d).

tarum FH185 isolated from human feces on the reduction of adipocyte size and gut microbial changes in mice with diet-induced obesity. The strain was found to have a lipase inhibitory activity of 70.09±2.04% and to inhibit the adipocyte differentiation of 3T3-L1 cells (18.63 ±0.98%) at a concentration of 100 μg/mL. The gut microbial changes in mice with diet-induced obesity were investigated. Male C57BL/6J mice were fed on different diets (A, normal diet (ND); B, high-fat diet (HFD); C, HFD with ABT-3 (10^9 CFU/day); and D, HFD with *L. plantarum* H-185 (10^9 CFU/day)) for 6 wk. According to the results of fecal pyrosequencing, the ratio of *Firmicutes* to *Bacteroidetes* in groups C and D was lower than of the control groups at the phylum level. At the family level, the *Lactobacillaceae* family was observed to dominate in groups C and D, while *Lachnospiraceae* was observed to dominate in groups A and B. At the genus level, an unknown spp. was observed to dominate after six weeks, while the *Lactobacillus* spp. in all groups was observed to dominate before the start of the treatment. However, the *Lactobacillus* spp. in groups C and D was comparatively higher than in groups A and B. In groups A, C and D, the percentage of 2,000 m^2 of adipocyte was higher than in the other size of adipocyte, while the percentage of over 5,000 m^2 of adipocyte was highest in group B. The mean adipocyte size of group D was significantly larger than that of group A, but smaller than that of group B.

According to the results, *L. plantarum* FH185 has potential as a probiotic with anti-obesity effects.

Acknowledgements

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