J Appl Biol Chem (2023) 66:45, 328–337
https://doi.org/10.3839/jabc.2023.045

Online ISSN 2234-7941
Print ISSN 1976-0442

Article: Food Science

Improvement of blood lipid metabolism and obesity through the administration of mixed lactic acid bacteria including *Lactobacillus plantarum* K-1 in mice fed a high-fat diet

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Received: 4 July 2023 / Accepted: 31 July 2023 / Published Online: 9 August 2023

Abstract We investigated the effects of single and combined administrations of *Lactobacillus* species (*L. plantarum*, LP; *L. gasseri*, LG; *L. casei*, LC) on blood lipid metabolism and obesity in mice fed a high-fat diet (HFD). The mice were continuously supplemented with LP, LP/LG, or LP/LG/LC, along with HFD, for 12 weeks. The consumption of HFD led to significant increases in body weight, total cholesterol, and triglyceride levels compared to the normal control group. However, administration of LP, LP/LG, or LP/LG/LC to HFD-fed mice reduced body weight gain and showed a tendency to suppress the levels of total cholesterol, triglycerides, and LDL-cholesterol, while increasing HDL-cholesterol levels. The HFD group exhibited increased abdominal fat weight and larger adipocytes in the epididymal adipose tissue compared to the NC group. However, the administered probiotics led to a significant reduction in adipocyte size with decreasing tendency in abdominal fat weight compared with the HFD group. Additionally, the deposition of giant vesicular fat cells in the liver of the HFD group considerably decreased in the probiotic-administered group. Microbiome analysis revealed an imbalance in intestinal microbes in the HFD group, characterized by lower *Bacteroidetes* and higher *Proteobacteria* ratios. However, probiotic administration tended to restore the microbial distribution by controlling the abundance of *Bacteroidetes* and *Proteobacteria*, resulting in decreased *Firmicutes*/*Bacteroidetes* and *Proteobacteria*/*Bacteroidetes* ratios. These results suggest that single and combined administration of LP and other probiotics holds enormous potential in reducing obesity in HFD-fed mice as they regulate lipid metabolism, reduce adipocyte size, and restore the balance of intestinal microbes.

Keywords Anti-obesity · Body fat · Hyperlipidemia · Lactic acid bacteria · Microbiome

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Introduction

Obesity refers to the accumulation of excess energy and typically occurs due to an imbalance between energy intake and expenditure, leading to body fat accumulation. It is not just a simple increase in body weight but rather a state in which excessive proliferation and differentiation of fat cells occur [1]. Currently, obesity in humans is mainly attributed to westernized dietary habits characterized by high-fat and high-calorie intakes. This continuous increase in lipid content within the body leads to abnormal lipid metabolism, such as elevated blood cholesterol and triglyceride levels, which are considered the major causes of cardiovascular and metabolic disorders, such as atherosclerosis, heart disease, diabetes, stroke, and hyperlipidemia [2]. Currently available pharmacological treatments for obesity mainly focus on appetite suppression, inhibition of carbohydrate or fat absorption, and promotion of energy expenditure. However, several forms of these medications have been withdrawn from the market, and their approval has been canceled owing to various severe side effects [3-5]. Therefore, recently, probiotics, which are considered safe natural microorganisms that can improve lipid metabolism and be used in obesity treatment without drug-induced side effects, have gained attention as health supplements [6,7].

Probiotics are living microorganisms that are added as supplements to improve the balance of the gut microbiota in the host gastrointestinal tract and benefit host's health [8]. As the

imbalance and reduced diversity of the gut microbiota have been identified as significant factors in the development of metabolic disorders such as obesity, there has been growing interest in the overall gut microbiota [9]. Lactic acid bacteria, which are commonly used as probiotics, have been shown to inhibit the growth of harmful bacteria, stabilize the balance of the microbiota, and improve the immune system of individuals with compromised immunity, thus playing a beneficial role in humans and animals [10-14]. In particular, the administration of probiotics from the *Lactobacillus* genus has been reported to improve lipid metabolism by reducing total cholesterol, LDL cholesterol, and triglyceride levels and increasing HDL cholesterol levels in the plasma of obese mice [2,7,15,16]. *Lactobacillus* probiotics have also been found to have anti-obesity activity by suppressing the growth of fat cells and alleviating hypercholesterolemia [15,16].

In this study, we aimed to analyze the impact of probiotics on lipid metabolism and evaluate their anti-obesity activity by administering a mixture of probiotics containing *Lactobacillus plantarum* K-1, *Lactobacillus gasseri*, and *Lactobacillus casei* to mice with high-fat diet (HFD)-induced obesity.

Materials and Methods

Reagents, animal management and dietary administration

L. plantarum K-1 (LP), *L. gasseri* (LG), and *L. casei* (LC) were obtained in powder form from BioRhythm Co., Ltd. (Cheongju, South Korea) and dissolved in sterilized distilled water. Four-week-old C57BL/6 mice were purchased from DBL (Eumseong, South Korea). The animals were acclimatized for one week in an animal facility at a temperature of 18±2 ºC, relative humidity of 50±10%, ventilation rate of 10-20 times/h, and a light-dark cycle of 12 h (lights on at 08:00, lights off at 20:00). During the acclimatization period, mice were fed solid rodent chow (RodFeed, DBL). After the acclimatization period, mice with an average body weight of 20-23 g were divided into five groups with seven mice per group. The care, housing, and experimental procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Jungwon University (JWU-IACUC-2022-4) to ensure compliance with ethical standards.

The normal control (NC) group was fed regular solid rodent chow as a normal low-fat diet ad libitum. The HFD groups were provided a 45 kcal % fat diet (D12451; Research Diets Inc., New Brunswick, NJ, USA) ad libitum. Among the four groups receiving the high-fat diet for 12 weeks, one group was classified as the HFD group and continued to receive only the HFD. The other three groups were administered LP, LG, or LC probiotics along with a high-fat diet. The probiotic groups were classified as follows: LP (only LP), LP/LG (combination of LP and LG), and LP/LG/LC (combination of LP, LG and LC). Body weight and food intake were measured at a fixed time once a week. The food efficiency ratio (FER) was calculated by dividing the weight gain during the experimental period by the amount of food consumed during the same period.

Blood biochemical analysis

To perform blood biochemical assays, mice that completed 12 weeks of oral administration were anesthetized. And blood was then collected from the carotid artery, and the samples were centrifuged at 13,000 rpm for 10 min to obtain blood serum, which was stored at −80 ºC until use. The levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the blood were measured using the International Federation of Clinical Chemistry method. Total cholesterol (T-CHO) was measured using the cholesterol oxidase-HMMPS (N-(3-sulfopropyl)-3-methoxy-5-methylaniline) method. Triglycerides (TG) were measured using the glycerol-3-phosphate oxidase-HMMPS method. LDL-cholesterol (LDL-C) and HDL-cholesterol (HDL-C) levels were measured using the selective elimination method (direct method) on a biochemical analyzer (HITACHI 7080, Japan). The Atherogenic index (AI) was calculated using Hablund's formula: AI=([T-CHO]-[HDL-C])/[HDL-C] [17]. Cardiovascular risk factors (CRF) were calculated using Rosenfeld's formula by dividing the amount of total cholesterol by the amount of HDL cholesterol [18].

Analysis of blood hormone concentration

The concentration of leptin in the blood was quantified using a mouse leptin ELISA kit (CSB-E04650m; Cusabio, Houston, TX, USA). Adiponectin concentration was measured using a mouse adiponectin/Acrp30 ELISA kit (MRP300; R&D Systems, Minneapolis, MN, USA). Insulin concentration was determined using a mouse insulin ELISA kit (AKRIN-011T; Shibayagi, Japan) [19].

Gut microbiome analysis

Fecal samples were collected from mice immediately before sacrifice for gut microbiome analysis. Total genomic DNA was extracted using a DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). The V3-V4 region of the 16S rRNA gene was amplified for analysis, and sequence information was obtained using the Illumina platform (Illumina, San Diego, CA, USA). Subsequently, DADA2 software was used to remove PCR and low-quality sequence reads. The resulting sequences were used for amplicon sequence variant analysis, which classified microbial species diversity based on the sequenced amplicons (Macrogen, Daejeon, South Korea) [20].

Histological staining

For histological analysis, experimental animals that had been orally administered the probiotics for 12 weeks were anesthetized and the liver and epididymal adipose tissues were extracted and fixed in 10% neutral-buffered formalin solution. Paraffin blocks

were prepared, and sections with a thickness of 0.4 μm were obtained. The sections were stained with hematoxylin and eosin (H&E) to analyze adipocyte size and morphology using an optical microscope. To measure the adipocyte size in the epididymal adipose tissue, the average diameter of adipose cells in each experimental group was calculated using ImageJ 1.53k software (National Institutes of Health, Bethesda, MD, USA).

Statistics

All experimental results are expressed as mean ± standard error (SE). Data analysis was performed using the GraphPad Prism software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA). Statistical comparisons between groups were conducted using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test to assess the significance of differences between the control and experimental groups. The significance level was set at p <0.05.

Results and Discussion

Weight gain, food intake and food efficiency ratio (FER)

The results of body weight, food intake, and FER for the NC and the experimental groups (HFD, LP, LP/LG, and LP/LG/LC) are presented in Table 1. The HFD group, which received only the HFD, showed a significant increase in body weight compared with the NC group, confirming the successful induction of obesity. The probiotic groups LP, LP/LG, and LP/LG/LC were administered each probiotic at a dose of 2×10^8 CFU daily for 12 weeks. Regarding the weight gain, the probiotic groups (LP group: 15.93±0.71 g, LP/LG group: 15.71±0.92 g, LP/LG/LC group: 16.29±0.67 g) showed a significant decrease in body weight compared to the HFD group (20.11±0.74 g). Food intake showed a decreasing trend in all HFD groups compared to that in the control group (NC group). This trend may be attributed to the lower carbohydrate content of the HFD than that in the normal diet, resulting in differences in preference among experimental animals [21,22]. However, among the experimental groups provided with the HFD, there was no significant difference in food intake. The FER in the HFD group (0.111±0.004) was approximately 3.8 times higher than that in the NC group (0.029±0.003), indicating greater weight gain for the same amount of food consumed. In contrast, FER in the LP, LP/LG, and LP/LG/LC groups showed a general tendency to decrease significantly compared with that in the HFD group. A lower FER indicates less weight gain for the same amount of food intake, suggesting that daily administration of a certain amount of probiotics can control HFD-induced weight gain and regulate obesity [23]. Therefore, when administered in combination with the HFD, LP, LP/LC, and LP/LG/LC clearly reduced FER, thereby inhibiting weight gain in HFD-fed mice (Table 1). However, no significant differences were observed between the single and combined probiotic administration.

Measurement of organ weights

After providing a HFD to the mice and daily oral administration of LP, LP/LG, or LP/LG/LC for 12 weeks, the relative weights of the visceral adipose tissue, epididymal adipose tissue, and liver were compared (Table 2). The weight of visceral adipose tissue per body weight in the HFD group significantly increased by approximately 2.4 times (5.02±0.18%) compared to the NC group (2.08±0.25%). However, when comparing the weight of visceral adipose tissue per body weight between the probiotic administration groups (LP, LP/LG, and LP/LG/LC) and the HFD group, there was a numerical tendency toward a decreased proportion, but no significant differences were observed. In addition, the relative epididymal adipose tissue weight was significantly increased by

Table 1 Body weight, food intake and food efficiency ratio of high-fat diet-fed mice

	Groups[1)]				
	NC	HFD	LP	LP/LG	LP/LG/LC
Weight (g)					
0 week	22.50±0.15	22.21±0.24	21.57±0.40	22.71±0.42	21.86±0.28
1st week	22.07±0.47	22.36±0.51	22.07±0.41	23.50±0.52	23.14±0.36
12th week	29.07±0.58	42.47±0.76[a]	38.00±0.89[a,c]	39.21±0.87[a,d]	39.43±0.58[a]
Weight gain (g)[1)]	7.00±0.68	20.11±0.74[a]	15.93±0.71[a,c]	15.71±0.92[a,c]	16.29±0.67[a,c]
Intake					
Food intake (g/day)	2.89±0.07	2.15±0.06[a]	2.11±0.04[a]	2.22±0.05[a]	2.22±0.05[a]
FER[2)]	0.029±0.003	0.111±0.004[a]	0.090±0.004[a,c]	0.084±0.005[a,b]	0.087±0.004[a,c]

[1)]NC, normal control mice; HFD, high-fat diet-fed mice; LP, high-fat diet-fed mice treated orally with *Lactobacillus plantarum* (2×10^8 CFU); LP/LG, high-fat diet-fed mice treated orally with the combination (2×10^8 CFU) of *Lactobacillus plantarum* and *Lactobacillus gasseri*; LP/LG/LC, high-fat diet-fed mice treated orally with the combination (2×10^8 CFU) of *Lactobacillus plantarum*, *Lactobacillus gasseri* and *Lactobacillus casei*.
[1)] Weight gain (g) = body weight at 12th week – body weight at 1st week
[2)] Food efficiency ratio = [weight gain (g/day)] / [food intake (g/day)]
Values are mean ± SE of seven mice per group
[a]p <0.001 compared to the NC group; [b]p <0.001 compared to the HFD group; [c]p <0.01 compared to the HFD group; [d]p <0.05 compared to the HFD group

Table 2 Relative organ weight of high-fat diet-fed mice (%)

Relative organ weight[2)]	Groups[1)]				
	NC	HFD	LP	LP/LG	LP/LG/LC
Epididymal fat	1.39±0.14	2.46±0.16[a]	1.90±0.12[b,c]	1.91±0.10[b,c]	1.87±0.08[c]
Abdominal fat	2.08±0.25	5.02±0.18[a]	4.88±0.11[a]	4.67±0.21[a]	4.78±0.15[a]
Liver	4.45±0.10	3.30±0.10[a]	3.12±0.08[a]	3.42±0.15[a]	3.18±0.11[a]

[1)] NC, normal control mice; HFD, high-fat diet-fed mice; LP, high-fat diet-fed mice treated orally with *Lactobacillus plantarum* (2×10^8 CFU); LP/LG, high-fat diet-fed mice treated orally with the combination (2×10^8 CFU) of *Lactobacillus plantarum* and *Lactobacillus gasseri*; LP/LG/LC, high-fat diet-fed mice treated orally with the combination (2×10^8 CFU) of *Lactobacillus plantarum*, *Lactobacillus gasseri* and *Lactobacillus casei*.
[2)] Relative organ weight (%) = [Organ gain (g) / Body weight (g)] × 100
Values are mean ± SE of seven mice per group
[a]p <0.001 compared to the NC group; [b]p <0.05 compared to the NC group; [c]p <0.05 compared to the HFD group

Table 3 Effect of lactic acid bacteria on the blood biochemical index and atherogenic index of high-fat diet-fed mice

Index[2)]	Groups[1)]				
	NC	HFD	LP	LP/LG	LP/LG/LC
AST (U/L)	94.94±17.13	130.9±13.93	131.7±14.11	139.7±8.69	131.6±14.62
ALT (U/L)	27.83±1.95	92.20±10.25[a]	89.71±6.13[a]	90.34±7.71[a]	80.66±6.01[a]
T-CHO (mg/dL)	106.3±1.60	232.0±14.24[a]	184.6±5.29[a, d]	209.7±5.88[a]	196.0±8.99[a, e]
TG (mg/dL)	20.29±2.45	27.71±0.92[a]	21.14±1.50[f]	22.57±0.72	22.29±1.11
HDL-C (mg/dL)	53.69±0.77	79.00±3.48[a]	81.83±2.95[a]	86.97±3.35[a]	86.37±1.86[a]
LDL-C (mg/dL)	4.74±0.33	16.40±1.58[a]	11.31±0.91[b, f]	13.26±0.83[a]	13.46±1.28[a]
AI	0.98±0.04	1.94±0.15[a]	1.26±0.05[e]	1.39±0.05[c, e]	1.27±0.07[d]
CRF	1.98±0.04	2.94±0.15[a]	2.26±0.05[e]	2.39±0.05[c, e]	2.27±0.07[c, e]

[1)] NC, normal control mice; HFD, high-fat diet-fed mice; LP, high-fat diet-fed mice treated orally with *Lactobacillus plantarum* (2×10^8 CFU); LP/LG, high-fat diet-fed mice treated orally with the combination (2×10^8 CFU) of *Lactobacillus plantarum* and *Lactobacillus gasseri*; LP/LG/LC, high-fat diet-fed mice treated orally with the combination (2×10^8 CFU) of *Lactobacillus plantarum*, *Lactobacillus gasseri* and *Lactobacillus casei*.
[2)]AST, aspartate aminotransferase; ALT, alanine aminotransferase; T-CHO, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; AI, Atherogenic index = (Total cholesterol – HDL cholesterol)/HDL cholesterol; CRF, Cardiac risk factor = Total cholesterol/HDL cholesterol. Values are mean ± SE of seven mice per group
[a]p <0.001 compared to the NC group; [b]p <0.01 compared to the NC group; [c]p <0.05 compared to the NC group; [d]p <0.001 compared to the HFD group; [e]p <0.01 compared to the HFD group; [f]p <0.05 compared to the HFD group

approximately 1.8 times in the HFD group (2.46±0.16%) compared to the NC group (1.39±0.14%). However, in all probiotic administration groups, there was a significant decrease in the amount of epididymal adipose tissue per body weight compared to the HFD group, with the LP/LG/LC probiotic group showing a decrease of approximately 24% compared to the HFD group. These results indicate that the continuous long-term consumption of probiotics leads to a reduced accumulation of body fat caused by an HFD. The relative liver weight showed a decreasing trend in both the HFD and probiotic administration groups compared to that in the NC group, but there was no statistically significant difference between the HFD and probiotic administration groups.

Blood biochemical analysis

The levels of AST, ALT, T-CHO, TG, HDL-C, and LDL-C in the serum of each experimental group, as well as the AI and CRF are presented in Table 3. AST levels tended to increase in each experimental group compared to those in the NC group, but no statistically significant differences were observed. Additionally, no significant differences were found between the LP, LP/LG, and LP/LG/LC groups and the HFD group. Regarding ALT measurements, the HFD group showed a significant increase of approximately three-fold compared to the NC group. This elevation in ALT levels is likely due to the accumulation of fat in the liver tissue and subsequent hepatic cell damage resulting from long-term HFD feeding [3]. However, no significant differences in ALT levels were observed between the LP, LP/LG, and LP/LG/LC groups and the HFD group. Therefore, the absence of change in AST and ALT levels suggests that the administration of probiotics for 12 weeks did not cause additional impairment in liver function in the experimental groups, indicating the absence of liver toxicity associated with probiotics.

Changes in serum T-CHO, TG, HDL-C, and LDL-C levels were measured to analyze their potential effects on improving dyslipidemia. Of the levels of T-CHO, the NC group, which received a normal diet, were 106.3±1.60 mg/dL, while this in the HFD group were significantly higher at 232.0±14.24 mg/dL, indicating an approximately 2.2-fold increase in serum T-CHO due to the HFD. However, both the LP and LP/LG/LC groups showed statistically significant reductions of 20 and 16%,

Table 4 Plasma leptin and insulin levels of high-fat diet-fed mice

Index	Groups[1)]				
	NC	HFD	LP	LP/LG	LP/LG/LC
Leptin (ng/mL)	0.15±0.07	1.39±0.29[a]	0.70±0.18	0.68±0.21	0.58±0.13[b]
Adiponectin (mg/mL)	8.53±0.29	7.39±0.26	7.55±0.39	8.13±0.40	7.98±0.35
Insulin (ng/mL)	1.00±0.19	1.42±0.18	1.03±0.14	1.18±0.17	1.04±0.15

[1)] NC, normal control mice; HFD, high-fat diet-fed mice; LP, high-fat diet-fed mice treated orally with *Lactobacillus plantarum* (2×10^8 CFU); LP/LG, high-fat diet-fed mice treated orally with the combination (2×10^8 CFU) of *Lactobacillus plantarum* and *Lactobacillus gasseri*; LP/LG/LC, high-fat diet-fed mice treated orally with the combination (2×10^8 CFU) of *Lactobacillus plantarum*, *Lactobacillus gasseri* and *Lactobacillus casei*. Values are mean ± SE of seven mice per group

[a]p <0.001 compared to the NC group; [b]p <0.05 compared to the HFD group

respectively, in T-CHO levels compared to the HFD group. Although not statistically significant, the LP/LG group also exhibited a tendency towards decreased T-CHO levels. These results suggest that the long-term intake of probiotics may contribute to lowering serum T-CHO levels.

In the case of TG, the HFD group showed a significant increase of approximately 1.4-fold (27.71±0.92 mg/dL) compared to the NC group (20.29±2.45 mg/dL). However, the LP, LP/LG, and LP/LG/LC groups exhibited decreasing tendencies of approximately 24, 18.5, and 19.6%, respectively, compared to the HFD group, with no significant difference compared to the NC group. In particular, the LP group showed a significant decrease in TG levels compared with the HFD group. These findings suggest that long-term probiotic administration may reduce serum TG levels. In the case of HDL-C, the HFD group showed a significant increase of approximately 1.5-fold compared to the NC group, and the probiotic administration groups (LP, LP/LG, and LP/LG/LC) also exhibited a statistically significant increase of approximately 1.5 to 1.6-fold compared to the NC group. However, when comparing HDL-C levels between the HFD group and each probiotic administration group, no significant differences were observed. Nevertheless, the probiotic administration groups showed a tendency of approximately 3.5 to 9% increase in HDL-C levels compared with the HFD group. Regarding LDL-C, the HFD, LP, LP/LG, and LP/LG/LC groups showed a significant increase of approximately 2.4 to 3.5-fold compared to the NC group. However, when comparing LDL-C levels between the HFD group and each probiotic-administered group, an overall decreasing tendency was observed, with a statistically significant decrease in LDL-C levels in the LP group.

The analysis of the lipid composition in the blood indicated that prolonged consumption of HFD resulted in increased levels of T-CHO, TG, and LDL-C. However, the consumption of LP alone or LP-containing LG or LG and LC can significantly reduce their levels, indicating a positive impact on the regulation and improvement of abnormal lipid metabolism, including a small increase in HDL-C content. It has been reported that disruptions in the balance of serum lipid levels owing to factors such as obesity or genetic alterations can lead to an imbalance in the ratio of LDL-C to HDL-C, thereby increasing the risk of atherosclerosis and cardiovascular diseases [24]. In addition, when high levels of cholesterol are maintained in the blood, the incidence of atherosclerosis increases, and this is described by the AI. The plasma AI was highest in the HFD group (1.94±0.15), while the probiotic-administered groups showed significantly lower values, indicating that probiotic consumption significantly reduced AI. The CRF was also significantly lower in the probiotic-administered groups compared to the HFD group (2.94±0.15). Hyperlipidemia is one of the causes of cardiovascular diseases, such as stroke, hypertension, and atherosclerosis, where excessive lipid components accumulate in the blood vessel walls, leading to inflammation and diseases. Based on the results of this experiment, it can be inferred that the consumption of probiotics, including LP, may have a positive effect on the prevention and treatment of hyperlipidemia-related conditions, such as atherosclerosis and cardiovascular diseases, by regulating lipid levels in the blood of patients with hyperlipidemia.

Plasma adipokine and insulin concentrations

Leptin is a hormone produced and secreted by the adipose tissue. It is one of the adipokines that increase with the occurrence of obesity. Leptin stimulates the hypothalamus to regulate appetite, increase energy expenditure, and control body fat accumulation. Plasma leptin levels correlate with the size, number, and amount of the visceral adipose tissue, which decrease when the amount of adipose tissue decreases [25,26]. Adiponectin is a protein whose expression increases during adipocyte differentiation. It is highly expressed in normal mouse adipose tissue, but decreases in obese or diabetic mouse models, showing a negative correlation with obesity [27,28]. According to our study, probiotic supplementation had a greater impact on leptin expression than on adiponectin (Table 4). Plasma leptin levels were significantly increased in the HFD group compared to the NC group, whereas the probiotic-supplemented groups showed higher leptin levels compared to the NC group, but exhibited a decreasing tendency compared to the HFD group. In particular, the LP/LG/LC group showed a significant decrease in leptin levels. These results indicate that long-term oral administration of LP or LP-containing probiotic mixtures suppressed

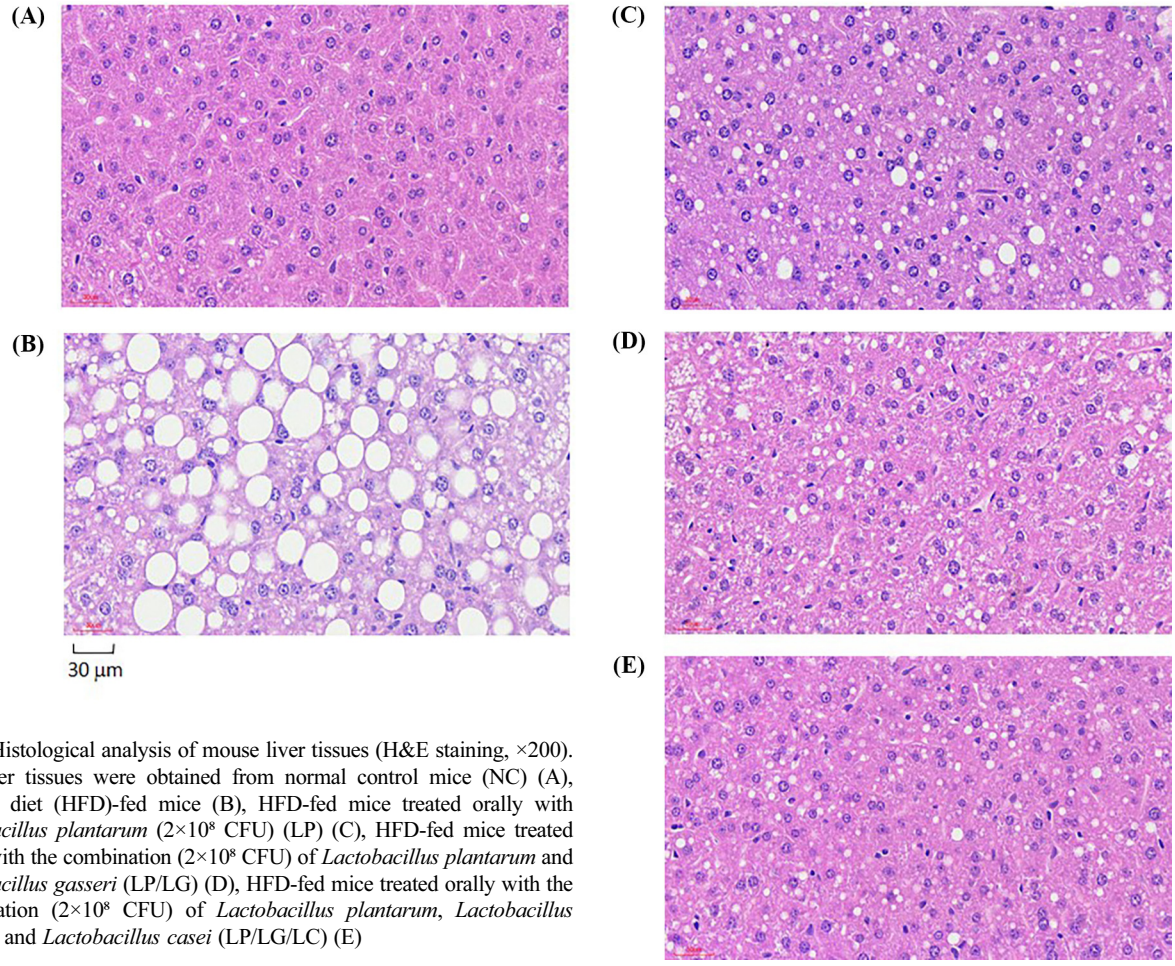

Fig. 1 Histological analysis of mouse liver tissues (H&E staining, ×200). The liver tissues were obtained from normal control mice (NC) (A), high-fat diet (HFD)-fed mice (B), HFD-fed mice treated orally with *Lactobacillus plantarum* (2×10^8 CFU) (LP) (C), HFD-fed mice treated orally with the combination (2×10^8 CFU) of *Lactobacillus plantarum* and *Lactobacillus gasseri* (LP/LG) (D), HFD-fed mice treated orally with the combination (2×10^8 CFU) of *Lactobacillus plantarum*, *Lactobacillus gasseri*, and *Lactobacillus casei* (LP/LG/LC) (E)

HFD-induced increase in the expression of leptin, resulting in a significant reduction in plasma leptin concentration. In contrast, probiotic supplementation had little effect on adiponectin levels. There was a slight decrease in the HFD group compared to the NC group, but there was no significant increase in the probiotic groups. Additionally, the plasma insulin levels increased in the HFD group and decreased in the probiotic-supplemented groups, although these changes were not statistically significant. These results suggest that probiotic supplementation, especially with LP-containing strain mixtures, improves FER, thereby inhibiting the increase in body weight and fat gain, and reducing plasma leptin levels (Tables 1, 2, 4). Therefore, it is believed that the consumption of probiotics containing LP can help regulate increased leptin levels caused by an HFD and thus inhibit fat accumulation in the body.

Histopathological analysis of liver tissue

The liver tissues were harvested from HFD-fed mice, and the pattern of fat accumulation within the tissue was observed using H&E staining and optical microscopy (Fig. 1). No liver lesions or fatty deposits were observed in the NC group (Fig. 1A). However, in the HFD group, which received only the high-fat diet, widespread deposition of large foamy lipid droplets, appearing as white areas, was observed throughout the liver tissue compared with the NC group (Fig. 1B). In contrast, in the LP, LP/LG, and LP/LG/LC groups, relatively suppressed deposition and smaller foamy lipid droplets were observed, indicating a significant reduction in fatty liver lesions compared to those in the HFD group (Fig. 1C-E). These results suggest that the administration of LP probiotics, either alone or in combination with LG or LG and LC, could inhibit the development of fatty liver lesions caused by increased fat accumulation due to HFD, or enhance the breakdown of fat, thereby mitigating the risk of fatty liver. In particular, the LP/LG and LP/LG/LC groups showed more pronounced suppression of lipid droplet deposition than the other group, indicating that the intake of mixed probiotics is more effective in improving hepatic fat metabolism (Fig. 1D, E).

Analysis of epididymal adipose tissue

To examine the size of adipocytes in the experimental animals, perigonadal adipose tissue was collected, fixed, stained with H&E staining and observed using optical microscopy (Fig. 2). The

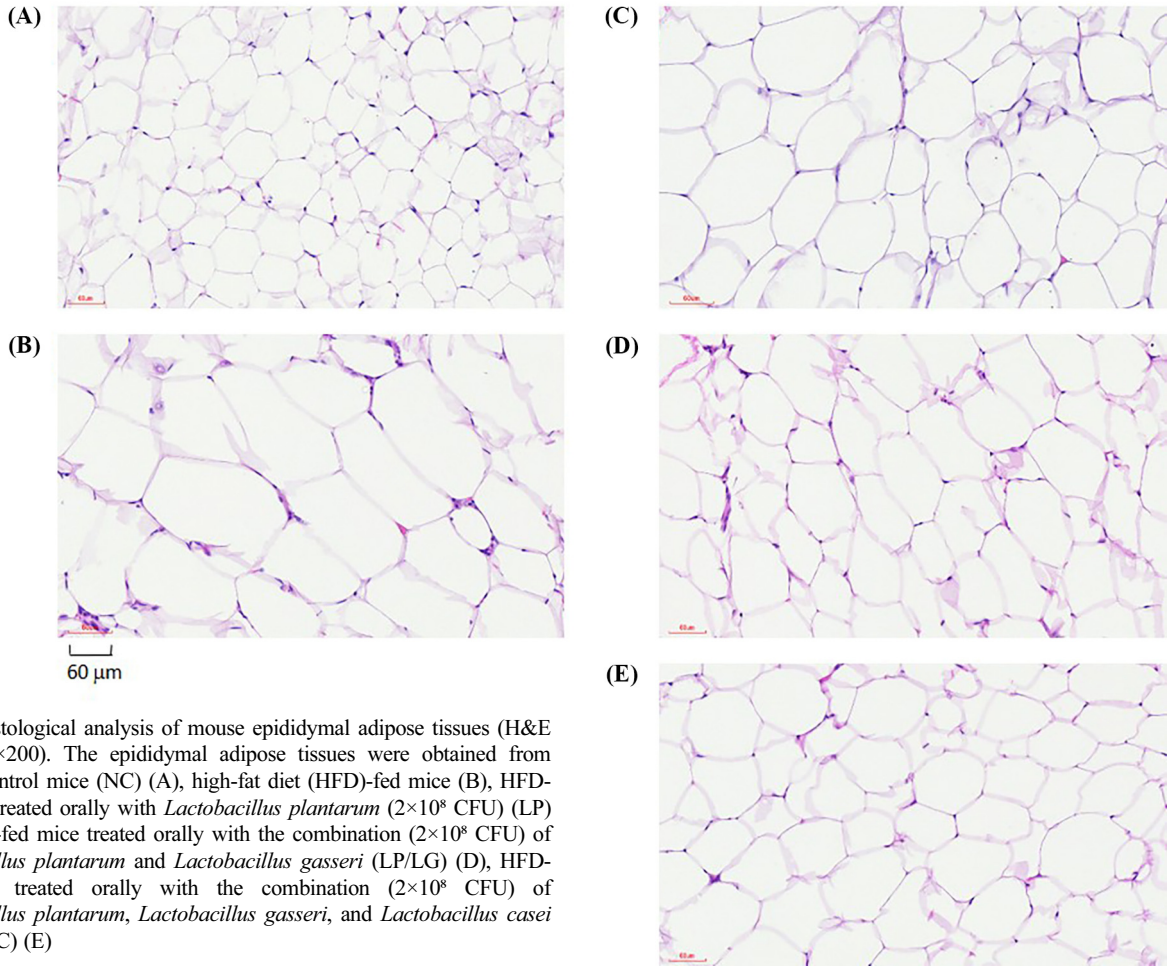

Fig. 2 Histological analysis of mouse epididymal adipose tissues (H&E staining, ×200). The epididymal adipose tissues were obtained from normal control mice (NC) (A), high-fat diet (HFD)-fed mice (B), HFD-fed mice treated orally with *Lactobacillus plantarum* (2×10^8 CFU) (LP) (C), HFD-fed mice treated orally with the combination (2×10^8 CFU) of *Lactobacillus plantarum* and *Lactobacillus gasseri* (LP/LG) (D), HFD-fed mice treated orally with the combination (2×10^8 CFU) of *Lactobacillus plantarum*, *Lactobacillus gasseri*, and *Lactobacillus casei* (LP/LG/LC) (E)

results showed that the size of epididymal adipocytes was significantly increased in the HFD group compared to that in the NC group (Fig. 2A, B). In contrast, in the LP, LP/LG, and LP/LG/LC groups, the size of adipocytes was slightly larger than that in the NC group, but significantly smaller than that in the HFD group (Fig. 2C-E). When the diameter of adipocytes was measured using an image analyzer, the adipocyte size in the HFD group (85.76± 5.44 μm) was significantly increased by more than twofold compared to the NC group (36.03±1.89 μm) (Fig. 3). However, administration of probiotics inhibited and/or reduced adipocyte size. The adipocyte sizes in the LP, LP/LG, and LP/LG/LC groups were 59.13±2.99, 59.09±2.94, and 46.47±3.21 μm, respectively. In all groups, a significant decrease in adipocyte size was observed compared with that in the HFD group. The decrease in adipocyte size is presumed to be due to the activity of LP, LG, and LC in inhibiting the transcription and expression of genes necessary for adipocyte differentiation and adipogenesis, thereby attenuating fat accumulation [7,15,16].

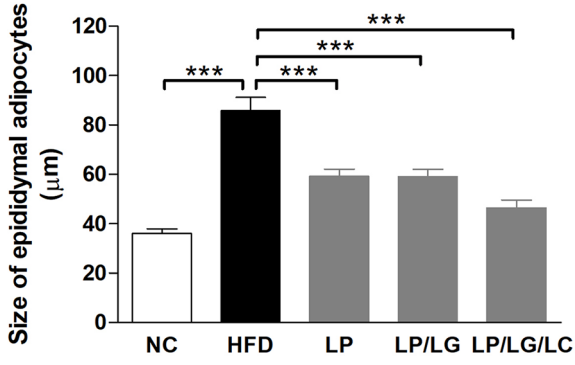

Fig. 3 Size analysis of adipocytes in epididymal adipose tissues of high fat diet (HFD)-fed mice. NC, normal control mice; HFD, high-fat diet-fed mice; LP, HFD-fed mice treated orally with *Lactobacillus plantarum* (2×10^8 CFU); LP/LG, HFD-fed mice treated orally with the combination (2×10^8 CFU) of *Lactobacillus plantarum* and *Lactobacillus gasseri*; LP/LG/LC, HFD-fed mice treated orally with the combination (2×10^8 CFU) of *Lactobacillus plantarum*, *Lactobacillus gasseri*, and *Lactobacillus casei*

Gut microbiome analysis

To investigate the effect of probiotic administration containing LP on the gut microbiome, fecal samples were collected from the experimental animals and total genomic DNA was extracted.

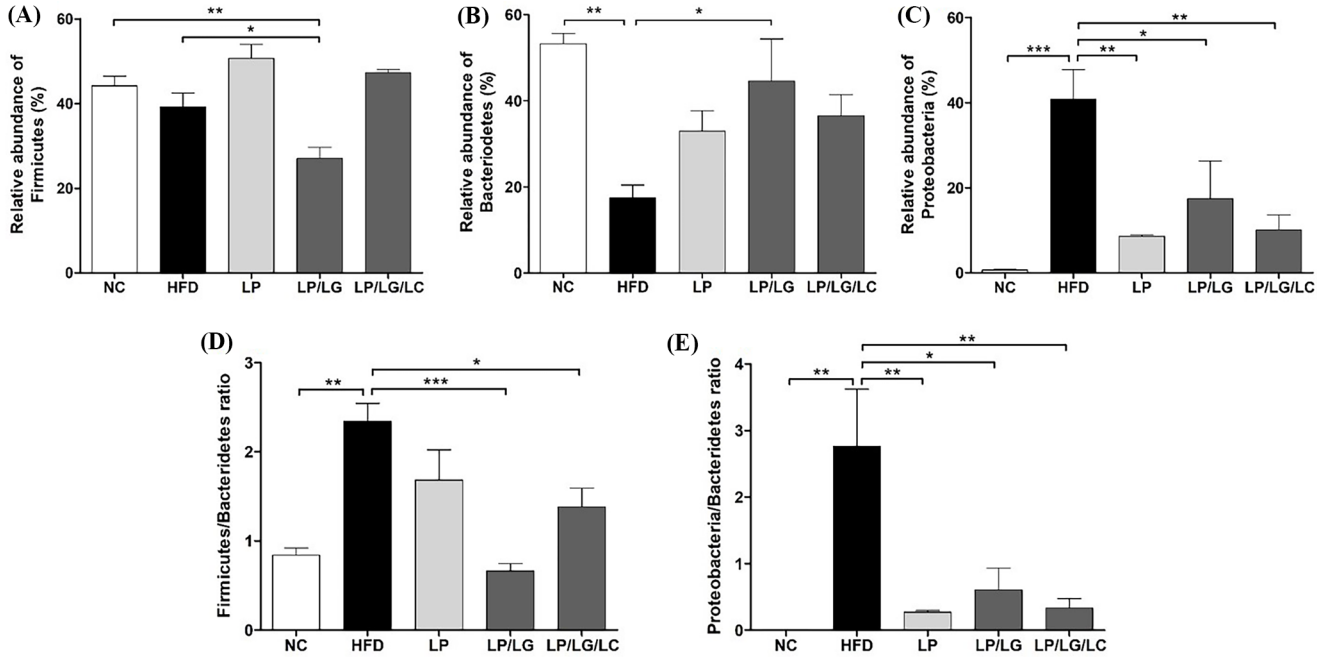

Fig. 4 Effect of supplementation of *Lactobacillus plantarum* containing probiotics on fecal microbiome composition. The relative abundance of the bacterial phylum (A, B and C), *Firmicutes* to *Bateroidetes* ratio (D) and *Proteobacteria* to *Bateroidetes* ratio (E) in the fecal samples from different groups. Values are presented as the mean ± SE (n =4 per group). **p* <0.05, ***p* <0.01, ****p* <0.001 vs high-fat diet (HFD). NC, normal control mice; HFD, high-fat diet-fed mice; LP, HFD-fed mice treated orally with *Lactobacillus plantarum* (2×10^8 CFU); LP/LG, HFD-fed mice treated orally with the combination (2×10^8 CFU) of *Lactobacillus plantarum* and *Lactobacillus gasseri*; LP/LG/LC, HFD-fed mice treated orally with the combination (2×10^8 CFU) of *Lactobacillus plantarum*, *Lactobacillus gasseri*, and *Lactobacillus casei*

After identifying the 16S rRNA genes, the gut microbiome was analyzed. When comparing the distribution of *Firmicutes* and *Bacteroidetes* in the gut microbiome, the HFD group showed *Firmicutes* at 39.17% and *Bacteroidetes* at 17.50%, whereas the LP group showed *Firmicutes* at 50.68% and *Bacteroidetes* at 32.98%. The LP/LG group showed *Firmicutes* at 27.05% and *Bacteroidetes* at 44.60%, whereas the LP/LG/LC group showed *Firmicutes* at 47.30% and *Bacteroidetes* at 36.46% (Fig. 4A, B). Compared with the HFD and NC groups, the LP/LG group showed a significant decrease in the proportion of *Firmicutes*. The proportion of *Firmicutes* in the LP and LP/LG/LC groups was similar to that in the NC (44.18%) and HFD groups, and there were no significant differences among the groups. The proportion of *Bacteroidetes* was significantly decreased in the HFD group compared to that in the NC group (53.25%) but showed an increasing tendency in each probiotic-administered group. In particular, the LP/LG group exhibited a significant increase in the proportion of *Bacteroidetes* compared to that in the HFD group. In all groups, *Firmicutes* accounted for approximately 27-50%, whereas *Bacteroidetes* accounted for approximately 18-53% of the total microbial population in the gut, indicating that these two phyla were dominant in the gut microbiome. The *Firmicutes*/*Bacteroidetes* (F/B) ratio, which was 2.34-fold higher in the HFD group, decreased in the LP, LP/LG, and LP/LG/LC groups with ratios of 1.68, 0.66, and 1.38, respectively (Fig. 4D). Regarding *Proteobacteria* distribution, the HFD group (40.83%) showed a significant increase compared with the NC group (0.65%). However, the LP, LP/LG, and LP/LG/LC groups exhibited statistically significant decreases in *Proteobacteria* abundance, with proportions of 8.56, 17.43, and 10.06%, respectively (Fig. 4C). The *Proteobacteria*/*Bacteroidetes* (P/B) ratio also showed a decreasing tendency in the LP, LP/LG, and LP/LG/LC groups, with ratios of 0.27, 0.61, and 0.33, respectively, in contrast to the HFD group, which had a P/B ratio of 2.76 (Fig. 4E). These results indicate that the HFD led to a significant increase in the number of bacteria belonging to *Proteobacteria* and a decrease in *Bacteroidetes*. However, the administration of LP-containing probiotics increased the abundance of *Bacteroidetes* and decreased the abundance of *Proteobacteria*, thereby restoring the F/B and P/B ratios to levels comparable to those in the NC group. In particular, the LP/LG group demonstrated superior activity compared with the other groups in reducing the ratio of *Firmicutes* to *Proteobacteria* and increasing the ratio of *Bacteroidetes*.

Changes in the distribution of gut microbiota have been reported to be associated with the obesity status of the host. Leptin-deficient ob/ob mice, which naturally develop obesity due to impaired leptin production, were analyzed for their gut bacterial communities and showed a higher abundance of *Firmicutes* and a lower abundance of *Bacteroidetes* than normal mice [29]. Similar changes have been observed in the fecal microbiota of obese

individuals. It has been reported that more than 90% of the gut microbiota in obese individuals belong to the *Firmicutes* phylum, including bacteria such as *Bacillus*, *Clostridium*, and *Streptococcus*, while the remaining 10% belong to the *Bacteroidetes* phylum, including *Bacteroides acidifaciens*, *B. distasonis*, and *B. fragilis*. However, when body weight decreases due to dietary interventions, *Firmicutes* decrease and *Bacteroidetes* increase, restoring the balance of the gut microbiome, which is closely associated with obesity [30,31]. Particularly, the gut microbial community undergoes significant changes in response to an HFD. Mice fed an HFD showed an overall decrease in *Bacteroidetes* and an increase in *Firmicutes* and *Proteobacteria*, leading to an elevated F/B and P/B ratio and substantial alterations in community structure [32]. In this study, as shown in Fig. 4, feeding an HFD (HFD group) led to a decrease in *Bacteroidetes* and an increase in *Proteobacteria*, resulting in an imbalance in the distribution of the gut microbiota, as indicated by increased F/B and P/B ratios. However, the administration of LP-only or LP-containing other probiotics tended to restore this imbalanced distribution of the gut microbiota, thus demonstrating the potential role of LP mixed with other lactic acid bacteria as an anti-obesity probiotic.

This study demonstrated that the rapid weight gain observed in the HFD group was closely related to the accumulation and preservation of body fat, as evidenced by increased fat deposition in the epididymal adipose tissue and liver. Additionally, HFD significantly increased serum T-CHO and TG levels, which indicate abnormal lipid metabolism. However, it was found that probiotics can improve abnormal lipid levels by controlling lipid metabolism. In particular, the administration of LP showed excellent anti-obesity and lipid metabolism improvement effects. The combined administration of LP/LG or LP/LG/LC also exhibited enhanced anti-obesity and lipid metabolism improvement effects. Furthermore, it was confirmed that these LP-containing probiotics can restore the imbalance in gut microbiota caused by HFD. Although it may be challenging to directly apply these findings to the treatment of obesity, based on the results obtained in this study, the combined administration of LP, LG, and LC may regulate HFD-induced abnormal lipid metabolism and control body weight, FER, and gut microbiota distribution of patients with obesity. These findings serve as a foundation for further research and improvement in obesity management.

Acknowledgments The authors sincerely thank Biorhythm Co., Ltd. for providing *lactobacillus* products. This research was supported by the "Regional Innovation Strategy (RIS)" through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (MOE) (2021RIS-001).

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