Probiotics and Antimicrobial Proteins (2021) 13:1054–1067
https://doi.org/10.1007/s12602-021-09752-0

Antiobesity Effect of Novel Probiotic Strains in a Mouse Model of High-Fat Diet–Induced Obesity

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Accepted: 29 January 2021 / Published online: 10 February 2021

Abstract

Obesity is one of the major causes of the development of metabolic diseases, particularly cardiovascular diseases and type-2 diabetes mellitus. Increased lipid accumulation and abnormal adipocyte growth, which is an increase in cell numbers and differentiation, have been documented as major pathological characteristics of obesity. Thus, the inhibition of adipogenic differentiation prevents and suppresses obesity. Recently, specific probiotic strains have been known to regulate lipid metabolism in vitro and/or in vivo. Previously, we demonstrated that *Lactobacillus johnsonni* 3121 and *Lactobacillus rhamnosus* 86 could act as novel probiotic strains and reduce cholesterol levels. Moreover, both strains significantly reduced lipid accumulation and inhibited adipocyte differentiation by downregulating the adipogenic transcription factor in 3T3-L1 adipocytes. Therefore, *L. johnsonni* 3121 and *L. rhamnosus* 86 were selected for in vivo evaluation of their anti-obesity effects using a high-fat diet-induced obese mouse model. Daily oral administration of *L. johnsonni* 3121 and *L. rhamnosus* 86 for 12 weeks significantly improved serum lipid profile and downregulated the expression of genes related to adipogenesis and lipogenesis in epididymal white adipose tissue of high-fat diet fed obese mice ($p < 0.05$). Fecal analysis also suggested that the two probiotic strains could normalize the altered obesity–related gut microbiota in high-fat diet–fed obese mice. These results collectively demonstrate that oral administration of *L. johnsonni* 3121 and *L. rhamnosus* 86 could prevent obesity, thereby improving metabolic health.

Keywords Obesity · Adipogenesis · Gut microbiota · Probiotics · Lactic acid bacteria

Introduction

Obesity is a multifactorial disorder, resulting from a long-term imbalance between energy intake and expenditure and is influenced by genetic and environmental factors. Therefore, obesity is a major risk factor for morbidity and mortality in many societies. Recently, it has been claimed by Ballini et al. that estimated overweight or obese people were more than 2 billion worldwide [1]. Moreover, studies have demonstrated that obesity is associated with an impaired quality of life and the incidence of obesity is related to an increased risk for cardiovascular disease, diabetes, and cancer [1, 2]. Obesity is also characterized by chronic inflammation and increased adipose tissue mass, which results from an increase in the number (hyperplasia) and size (hypertrophy) of fat cells (adipocytes) [3]. Adipose tissue, which functions as an endocrine organ, regulates metabolism in other tissues by secreting hormones and cytokines and plays key roles in regulating the overall energy balance. Therefore, an understanding of the functions of adipocytes involved in molecular and cellular biology will be important to determine the causes of obesity and develop therapies for this disease [4].

Several studies have demonstrated that gut dysbiosis, which is induced by a high-fat and high-calorie diet, is an important factor affecting the development of obesity [5, 6]. Changes in intestinal microbial ecology result in an increase in the number of the distal gut microbiota, promoting host adiposity [7, 8]. Colonization with intestinal microbes from

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obese mice led to more substantial body weight gain and fat accumulation in germ-free mice than when microbes from lean mice were transferred [8, 9]. Further, transfer of gut microbes from lean donors improves insulin resistance in individuals with metabolic syndrome [10]. Taken together, these results of previous studies support the concept that targeting high-fat diet–induced disturbance of gut microbiota is an effective approach to the treatment of obesity.

Probiotics are defined as live microorganisms that provide beneficial health effects to the host when administered in adequate amounts [11]. Oral treatment with probiotic bacteria appears to be a promising strategy to reverse the metabolic alterations relevant to dysbiosis in obesity and related disorders, for example as nomalizing the increased ratio of Firmicutes/Bacteroidetes [12, 13]. Several studies have reported that probiotics have health-promoting effects, including the amelioration of hypertension [14], hypercholesterolemia [15], cancer prevention [16], and immunomodulation [17]. Other studies demonstrated that administration of probiotics to obese rats led to reductions in body weight and adipose tissue weight [18]. Moreover, Adams et al. [19] demonstrated that live cells, dead cells, and even cell components of probiotics could significantly exert biological effects outside the digestive tract. Nevertheless, probiotics are considered an important part of the dietary strategy for maintaining health. The anti-obesity effect of some probiotics seems to be strain or dose dependent; however, the underlying molecular mechanism of action of probiotics remains largely unknown.

Therefore, this study was conducted to investigate the effects of three individual probiotic strains on the gut microbiota and adipose tissue metabolism in a mouse model of high-fat diet (HFD)-induced obesity. *Lactobacillus johnsonni* 3121 (isolated from porcine gut) and *Lactobacillus rhamnosus* 86 (isolated from Korean infant feces) were chosen based on our previous study that showed anti-obesity activity via inhibition of adipogenesis and lipid accumulation in 3T3-L1 adipocytes (under publication). Moreover, *Pediococcus pentosaceus* KID7 was also selected because a previous in vivo study demonstrated that this strain improves hypercholesterolemia in an atherogenic diet-fed mouse model [20].

Materials and Methods

Preparation of Bacterial Strains

L. johnsonni 3121, *L. rhamnosus* 86, and *P. pentosaceus* KID7 were used for oral gavaging. The three strains were individually grown in Man Rogosa Sharpe (MRS) broth (BD Co., Franklin Lakes, NJ, USA) at 37 °C for 18 h. The strains were then sub-cultured thrice for activation. Cultured cells were harvested by centrifugation (10,000×*g*, 4 °C, 5 min) and washed three times with phosphate-buffered saline (PBS). These cell pellets were lyophilized and stored at −20 °C until use.

Experimental Animals

A total of thirty 10-week-old male C57BL/6 J mice (Samtako, Seoul, South Korea) were obtained. The animals were housed for 1 week and acclimatized in a room with controlled temperature (23 ± 2 °C) and a cycle of 12/12 h light/dark cycle. The feed and water were provided ad libitum. The initial body weight of mice did not differ among the study groups.

Experimental Design

The experimental flow is graphically illustrated in Fig. 1. After 1 week of acclimatization, mice were randomly divided into five groups (six mice per group): normal diet-fed mice

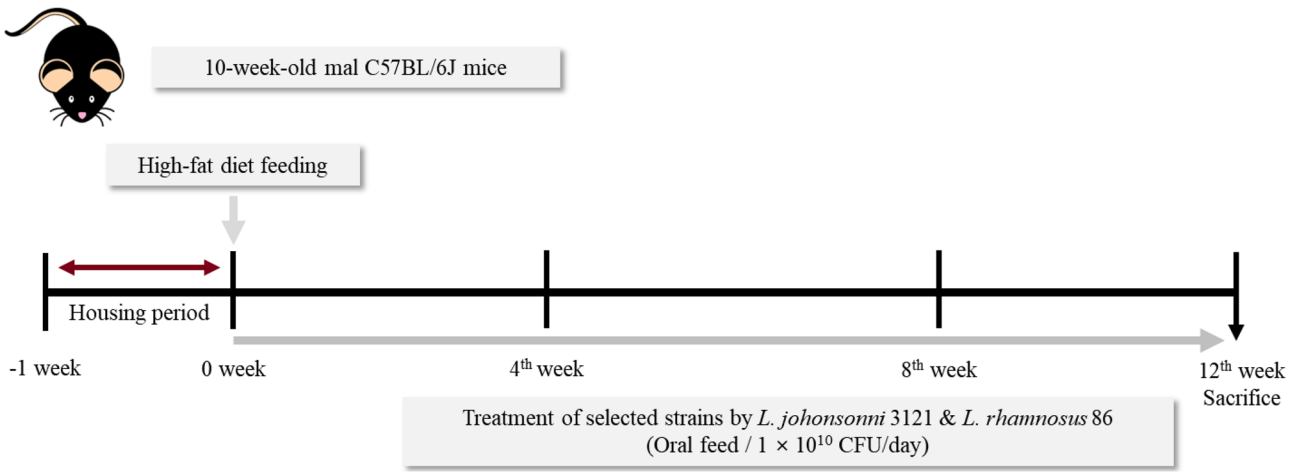

Fig. 1 Experimental design of the study

(ND), high-fat diet fed mice with 45% fat of the calories and 1.25% cholesterol (HFD), HFD-fed mice treated with *L. johnsonii* 3121 (3121), HFD-fed mice treated with *L. rhamnosus* 86 (86), and HFD-fed mice treated with *P. pentosaceus* KID7 (KID7). Each of the three strains was suspended in PBS and administered to the mice by oral feeding needle at a dose of 10^{10} CFU/day for 12 weeks. ND and HFD mice received the same amount of PBS. Three strains were used in this experiment because *L. johnsonii* 3121 and *L. rhamnosus* 86 were shown to have anti-adipogenic effects in vitro. In a previous study, *P. pentosaceus* KID7 was verified to show cholesterol-lowering activity in an atherogenic diet–fed mouse model [20] and therefore used as a positive control group. The composition of the high-fat diet (D08062402, Research Diets, New Brunswick, NJ, USA) and normal diet (D12450B, Research Diets, New Brunswick, NJ, USA) are given in Table S1–3. Body weight and feed intake were measured weekly. At the end of the experimental period, mice underwent fasting for 12 h and were anesthetized using a CO_2 chamber prior to organ and blood collection. Blood samples were collected via cardiac puncture and transferred to SST Plastic Venous Blood Collection Serum Tubes (Vacuette, 191 Kremsmünster, Austria). The serum was separated by centrifugation (1000×*g*, 24 °C, 15 min). Brown adipose tissue (BAT) was collected, immediately frozen in liquid nitrogen, and stored at − 80 °C until further use. Liver and white adipose tissue (WAT) from two different parts (epididymal and inguinal) were carefully dissected and weighed. The whole liver and WAT samples were divided for histology (fixed in 10% buffered formalin) and qRT-PCR analysis (frozen in liquid nitrogen prior to storage at − 80 °C). Fecal samples were also collected and frozen in liquid nitrogen prior to storage at − 80 °C.

Blood and Liver Lipid Analyses

Serum triglyceride and total cholesterol concentrations were measured using a triglyceride quantification colorimetric kit and total cholesterol colorimetric assay kit (Biovision, Milpitas, CA, USA), respectively. High-density lipoprotein cholesterol (HDL-cholesterol) and low-density lipoprotein/very low–density lipoprotein cholesterol (LDL/VLDL cholesterol) concentrations were determined using an HDL and LDL/VLDL cholesterol assay kit (Abcam, Cambridge, MA, USA). HDL cholesterol fraction was separated from serum by precipitation of LDL/VLDL cholesterol fraction, and then the concentration of each fraction was measured. For quantification of hepatic triglyceride and total cholesterol, 100 mg of liver tissue was homogenized in 5% Tween 20 (Promega, Madison, WA, USA) solution and chloroform/isopropanol/Tween 20 (7:11:0.1) solution, respectively. Extracted hepatic lipids were analyzed using the same kit as used in the serum analysis.

Oil Red O Staining Analysis

Samples of epididymal white adipose tissue (eWAT) and inguinal white adipose tissue (iWAT) fixed in 10% buffered formalin were dehydrated in ethanol, embedded in paraffin,

Table 1 Primer sequences used in qRT-PCR analysis

Gene	Sequence	Ta, °C	Reference
PPARγ	F: 5′-GGA AGA CCA CTC GCA TTC CTT-3′	61.0	[21]
	R: 5′-GTA ATC AGC AAC CAT TGG GTC A-3′		
C/EBPα	F: 5′-GCG GGA ACG CAA CAA CAT C-3′	61.0	[22]
	R: 5′-GTC ACT GGT CAA CTC CAG CAC-3′		
aP-2	F: 5′-AAG GTG AAG AGC ATC ATA ACC CT-3′	58.5	[23]
	R: 5′-TCA CGC CTT TCA TAA CAC ATT CC-3′		
CD36	F: 5′-GCC AAG CTA TTG CGA CAT GA-3′	59.0	[24]
	R: 5′-ATC TCA ATG TCC GAG ACT TTT CAA-3′		
LPL	F: 5′-TCT GTA CGG CAC AGT GG-3′	58.8	[25]
	R: 5′-CCT CTC GAT GAC GAA GC-3′		
ACC	F: 5′-AAT GAA CGT GCA ATC CGA TTT G-3′	62.7	[26]
	R: 5′-ACT CCA CAT TTG CGT AAT TGT TG-3′		
FASN	F: 5′-TTC CAA GAC GAA AAT GAT GC-3′	57.0	[27]
	R: 5′-AAT TGT GGG ATC AGG AGA GC-3′		
UCP-1	F: 5′-CAA AAA CAG AAG GAT TGC CGA AA-3′	64.3	[26]
	R: 5′-TCT TGG ACT GAG TCG TAG AGG-3′		
UCP-2	F: 5′-ATG GTT GGT TTC AAG GCC ACA-3′	59.9	[26]
	R: 5′-TTG GCG GTA TCC AGA GGG AA-3′		
GAPDH	F: 5′-GTA TGA CTC CAC TCA CGG CAA A-3′	58.5	[28]
	R: 5′-GGT CTC GCT CCT GGA AGA TG-3′		

Table 2 Bacterial primer sequences used in fecal bacterial analysis

Gene	Sequence	Ta, °C	Reference
A. muciniphila	F: 5′-CAG CAC GTG AAG GTG GGG AC-3′ R: 5′-CCT TGC GGT TGG CTT CAG AT-3′	62.7	[29]
Bacteriodetes	F: 5′-GAA GGT CCC CCA CAT TG-3′ R: 5′-CTT TGA GTT TCA CCG TTG CCG G-3′	61.4	[30]
Firmecutes	F: 5′-CTG ATG GAG CAA CGC CGC GT-3′ R: 5′-ACA CYT AGY ACT CAT CGT TT-3′	50.0	[30]
F. prausnizii	F: 5′-GAT GGC CTC GCG TCC GAT TAG-3′ R: 5′-CCG AAG ACC TTC TTC CTC C-3′	62.2	[30]
Roseburia spp.	F: 5′-AAG CGA CGA TCA GTA GCC GA-3′ R: 5′-TTC TTC TTC CCT GCT GAT AGA G-3′	59.9	[29]
Total bacteria	F: 5′-TCC TAC GGG AGG CAG CAG T-3′ R: 5′-GGA CTA CCA GGG TAT CTA ATC CTG TT-3′	60.9	[31]

and then sectioned at 4 μm. Sections were stained with hematoxylin solution (Sigma Aldrich, St. Louis, MO, USA) and eosin Y solution (Daejung, Gyeonggi-do, South Korea) to quantify the mean adipocyte size. The slides were examined using an Olympus CH30 microscope. The mean surface area of the adipocytes in WAT was calculated using ImageJ software (NIH, Bethesda, MD, USA). For oil red O staining, frozen liver samples were sectioned at 8 μm, and cryosections on glass slides were stained with 0.1% (w/v) oil red O in 75% (v/v) isopropanol.

qRT-PCR Analysis

Total RNA from eWAT, BAT, and colon was isolated using the GeneJET RNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. Final mRNA concentration and quality were determined by ultraviolet absorbance using a Nanodrop spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Forster City, CA, USA). RNA expression levels were quantified by qRT-PCR using SYBR® Green (Sigma Aldrich) and a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The GAPDH gene was analyzed simultaneously as a housekeeping gene, and each qRT-PCR reaction was performed in triplicate in the same run. Relative gene expression levels of the targeted genes were calculated using Bid-Rad CFX Manager software (Bio-Rad). The primer sequences of the targeted mouse genes are listed in Table 1 [21–28].

Fecal Microbiota Analysis

At the end of the experiment, fecal samples were taken out and immediately stored at − 80 °C. To investigate the intestinal microbial community composition, genomic DNA (gDNA) was extracted from the fecal samples of all mice using the QIAamp DNA stool kit (Qiagen, Hilden Germany) according to the manufacturer's instructions. qRT-PCR was performed to measure the relative amount of bacteria using SYBR® Green (Sigma Aldrich) and a CFX96 Touch™ Real-Time

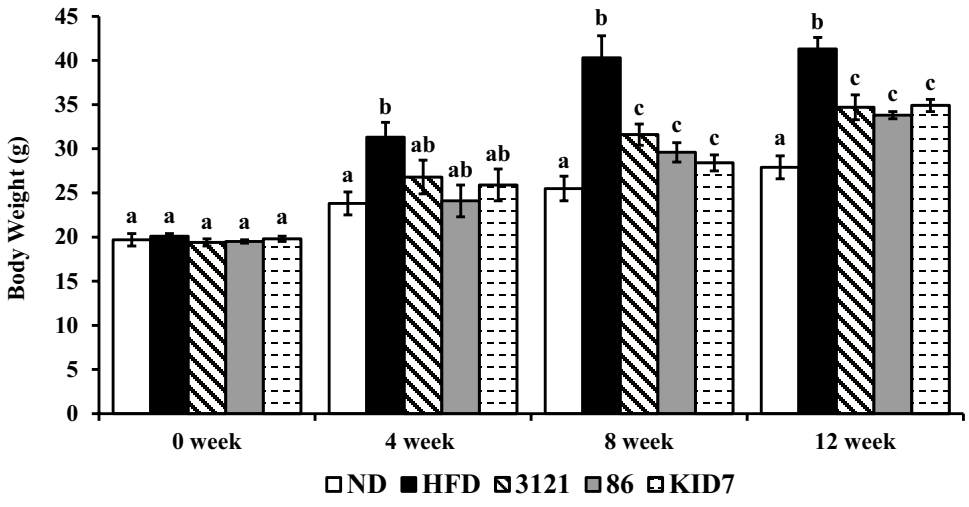

Fig. 2 Effect of probiotic strains on the weekly body weight difference in HFD-induced obese mice. Results are expressed as mean ± SE ($n = 6$). [abc]Means in the same series with different lowercase superscript letters are significantly different ($p < 0.05$)

Table 3 Effect of probiotic strains on the growth performance

	ND	HFD	3121	86	KID7
Initial body weight (g)	19.7 ± 0.7^{a}	20.1 ± 0.3^{a}	19.4 ± 0.4^{a}	19.5 ± 0.2^{a}	19.8 ± 0.3^{a}
Final body weight (g)	27.9 ± 1.3^{a}	41.3 ± 1.3^{c}	34.7 ± 1.4^{b}	33.8 ± 0.4^{b}	34.9 ± 0.7^{b}
Body Mass Index (kg/m^2)	2.95 ± 0.22^{a}	4.10 ± 0.24^{c}	3.28 ± 0.30^{ab}	3.32 ± 0.14^{ab}	3.40 ± 0.14^{b}
Feed Intake (g/day)	2.71 ± 0.10^{a}	2.74 ± 0.07^{a}	2.67 ± 0.07^{a}	2.58 ± 0.04^{a}	2.63 ± 0.04^{a}
Calorie intake (g/day)	10.44 ± 0.37^{a}	12.82 ± 0.34^{b}	12.47 ± 0.35^{b}	12.04 ± 0.21^{b}	12.30 ± 0.19^{b}

Results are expressed as mean ± SE ($n = 6$). Means in the same column with different lowercase letters are significantly different ($p < 0.05$)

PCR Detection System (Bio-Rad). The relative abundance of bacterial populations was determined using Bid-Rad CFX Manager software (Bio-Rad). The primer sequences of the targeted bacterial genes are listed in Table 2 [29–31].

Statistical Analysis

Data were analyzed using IBM SPSS statistics software version 25.0 (IBM Corp, New York, USA). One-way analysis of variance was used to compare sample means. Multiple comparisons of means were performed using Tukey's post hoc test. $p < 0.05$ was considered significant.

Results

Effect of Probiotic Strains on the Growth Performance of HFD-Induced Obese Mice

The weekly body weight gain of the experimental groups from week 0 to week 12 is shown in Fig. 2. The body weight of HFD-fed mice gradually increased each week, and after 12 weeks of treatment, the HFD group showed a significantly higher final body weight compared with the ND group ($p < 0.05$). Notably, all three strains, *L. johnsonni* 3121, *L. rhamnosus* 86, and *P. pentosaceus* KID7, could significantly decrease the final body weight of obese mice ($p < 0.05$). Additional growth performance data are represented in Table 3. Moreover, the body mass index (BMI) was also significantly increased in the HFD group; however, all three probiotic supplements were able to normalize the increased BMI to values similar to those of the ND group ($p < 0.05$). Additionally, the HFD group showed a significant increase in daily calorie intake ($p < 0.05$). However, no significant difference was observed between the probiotic-fed groups. Moreover, no significant difference was detected in the average daily feed intake (ADFI) among all experimental groups.

Effect of Probiotic Strains on Hypertrophy of WAT in HFD-Induced Obese Mice

The WAT of epididymal and inguinal regions were measured to investigate the reason for the decreased body weight gain through probiotic strains (Table 4). The weight of both WATs in the epididymal and inguinal areas was significantly higher in the HFD group than in the ND group ($p < 0.05$). Therefore, the total WAT weight was also significantly higher in the HFD group than in the ND group ($p < 0.05$). Notably, all three strains were capable of significantly decreasing the adipose tissue of HFD-fed mice in both epididymal and inguinal regions, which also resulted in a significant lowering of the total WAT ($p < 0.05$). Mild hepatomegaly was also observed to significantly increase the liver weight in the HFD group, which was also normalized by the three probiotic strains, providing results similar to that observed in the ND group ($p < 0.05$). No significant differences were observed in heart weight. In order to compare the mean size of the adipocytes, hematoxylin and eosin staining analysis

Table 4 Effect of probiotic strains on the weight of white adipose tissue (WAT), liver, and heart

	ND	HFD	3121	86	KID7
Epididymal white adipose tissue (g)	0.95 ± 0.12^{a}	2.08 ± 0.06^{c}	1.65 ± 0.09^{b}	1.77 ± 0.07^{bc}	1.67 ± 0.07^{b}
Inguinal white adipose tissue (g)	0.92 ± 0.11^{a}	2.88 ± 0.18^{c}	2.12 ± 0.10^{b}	1.92 ± 0.08^{b}	1.94 ± 0.18^{b}
Total white adipose tissue (g)	1.87 ± 0.22^{a}	4.96 ± 0.20^{b}	3.77 ± 0.18^{b}	3.69 ± 0.09^{b}	3.61 ± 0.22^{b}
Liver (g)	1.07 ± 0.13^{a}	2.64 ± 0.23^{b}	1.39 ± 0.10^{a}	1.23 ± 0.04^{a}	1.34 ± 0.07^{a}
Heart (g)	0.13 ± 0.06^{a}	0.14 ± 0.02^{a}	0.13 ± 0.07^{a}	0.14 ± 0.04^{a}	0.14 ± 0.02^{a}

Results are expressed as mean ± SE ($n = 6$). Means in the same column with different lowercase letters are significantly different ($p < 0.05$)

was performed (Fig. 3a–c). The size of adipocytes in WAT was significantly larger in the HFdD group than in the ND group, whereas all three probiotic treatments normalized this parameter in both eWAT and iWAT ($p < 0.05$).

Effect of Probiotic Strains on Serum and Hepatic Biochemical Markers in HFD-Induced Obese Mice

The effects of the three probiotic strains on lipid metabolism biomarkers were evaluated in the serum and liver of HFD-fed obese mice (Table 5). Total and LDL/VLDL cholesterol levels in the serum were significantly higher in the HFD group compared with the ND group ($p < 0.05$). However, this increase in serum total and LDL/VLDL cholesterol levels was significantly decreased by the three probiotic strains ($p < 0.05$). No significant differences were observed in serum triglyceride and HDL cholesterol levels. To measure hepatic lipid accumulation in the liver, triglyceride and total cholesterol levels were evaluated. Both hepatic triglyceride and total cholesterol levels were significantly higher in the HFD group than in the ND group ($p < 0.05$). Similar to the serum levels, total cholesterol level in the liver was significantly decreased in all three probiotic-supplemented groups ($p < 0.05$). However, hepatic triglyceride level was significantly decreased only by *L. johnsonni* 3121 ($p < 0.05$). Furthermore, the staining of the liver with the oil red O method revealed that HFD-stimulated lipid droplet formation in the liver was more prominent in the HFD group than in the ND group (Fig. 4). However, consistent with the effect on liver weight, treatment with *L. johnsonni* 3121, *L. rhamnosus* 86, and *P. pentosaceus* KID7 clearly decreased lipid droplet formation.

Effect of Probiotic Strains on mRNA Expression Levels of Obesity-Related Markers in HFD-Induced Obese Mice

To further understand the gene expression pathway related to the anti-adipogenic effects of *L. johnsonni* 3121, *L. rhamnosus* 86, and *P. pentosaceus* KID7, mRNA expression levels of genes downstream of peroxisome proliferator-activated receptor γ (PPARγ) and genes correlated to adipogenesis were measured in eWAT (Fig. 5a–c). The mRNA expression level of PPARγ was significantly higher in the HFD group compared to that in the ND group ($p < 0.05$). Moreover, HFD treatment significantly increased the expression levels of CD36 and lipoprotein lipase (LPL), which are target genes of PPARγ and associated with fatty acid uptake, in the eWAT ($p < 0.05$). Furthermore, the mRNA expression levels of CCAAT/enhancer-binding protein α (C/EBPα) and adipocyte protein 2 (aP2), which are downstream of PPARγ, were also significantly increased by HFD ($p < 0.05$), indicating the stimulation of adipogenesis in obese mice. Notably, treating the HFD-fed mice with the three probiotic strains drastically downregulated the mRNA expression of most of these genes in eWAT. The gene expression levels of PPARγ, C/EBPα, LPL, and CD36 were significantly decreased by *L. johnsonni* 3121 and *L. rhamnosus* 86 supplementations ($p < 0.05$). Moreover, only *L. johnsonni* 3121 could significantly downregulate the expression of the aP2 gene in obese mice ($p < 0.05$). For further exploration, gene expression levels of lipid metabolism regulators, fatty acid synthase (FASN), and acetyl-CoA carboxylase (ACC) were measured. Both FASN and ACC were significantly upregulated in all HFD-fed groups ($p < 0.05$). However, only *L. johnsonni* 3121 significantly decreased the increased gene expression levels of FASN and ACC in eWAT ($p < 0.05$). Uncoupling protein (UCP), also known as thermogenin, is a gene involved in thermogenesis and energy expenditure usually found in BATs. No significant difference in UCP-1 and UCP-2 was observed between the ND and HFD groups. Of note, only *the P. pentosaceus* KID7-supplemented group showed significantly increased expression levels of both UCP-1 and UCP-2 genes ($p < 0.05$). Therefore, the decreased body weight gain in the *P. pentosaceus* KID7-supplemented group might be due to increased thermogenesis and energy expenditure.

Effect of Probiotic Strains on Fecal Bacterial Populations in HFD-Induced Obese Mice

The interaction between the three probiotic strains and gut microbiota was evaluated by confirming the relative abundance level of fecal microbiota (Fig. 6). Bacteria belonging to two major phyla, Firmicutes and Bacteroidetes, were observed. The HFD and the three probiotic-supplemented groups significantly increased the relative abundance of the phylum Firmicutes ($p < 0.05$). However, the relative abundance of the phylum Bacteroidetes was not affected. Notably, the Firmicutes/Bacteroidetes ratio was significantly increased by HFD ($p < 0.05$), which was then normalized by all three strains—*L. johnsonni* 3121, *L. rhamnosus* 86, and *P. pentosaceus* KID7. HFD also significantly decreased the relative abundance of *Roseburia* spp., *Faecalibacterium prausnitzii*, and *Akkermansia muciniphila* to a greater extent compared with ND ($p < 0.05$). Nonetheless, each genus and the subspecies were individually affected by each probiotic strain. The decrease *in Roseburia* spp. was significantly increased only by *L. johnsonni* 3121 ($p < 0.05$). Further, the abundance of *A. muciniphila* was increased by *P. pentosaceus* KID7 and that of *F. prausnitzii* was significantly increased by *L. rhamnosus* 86 ($p < 0.05$). These results proved that *L. johnsonni* 3121, *L. rhamnosus* 86, and *P. pentosaceus* KID7 could also influence the gut microbiota composition and that changes in the microbial profiles could be strain-specific.

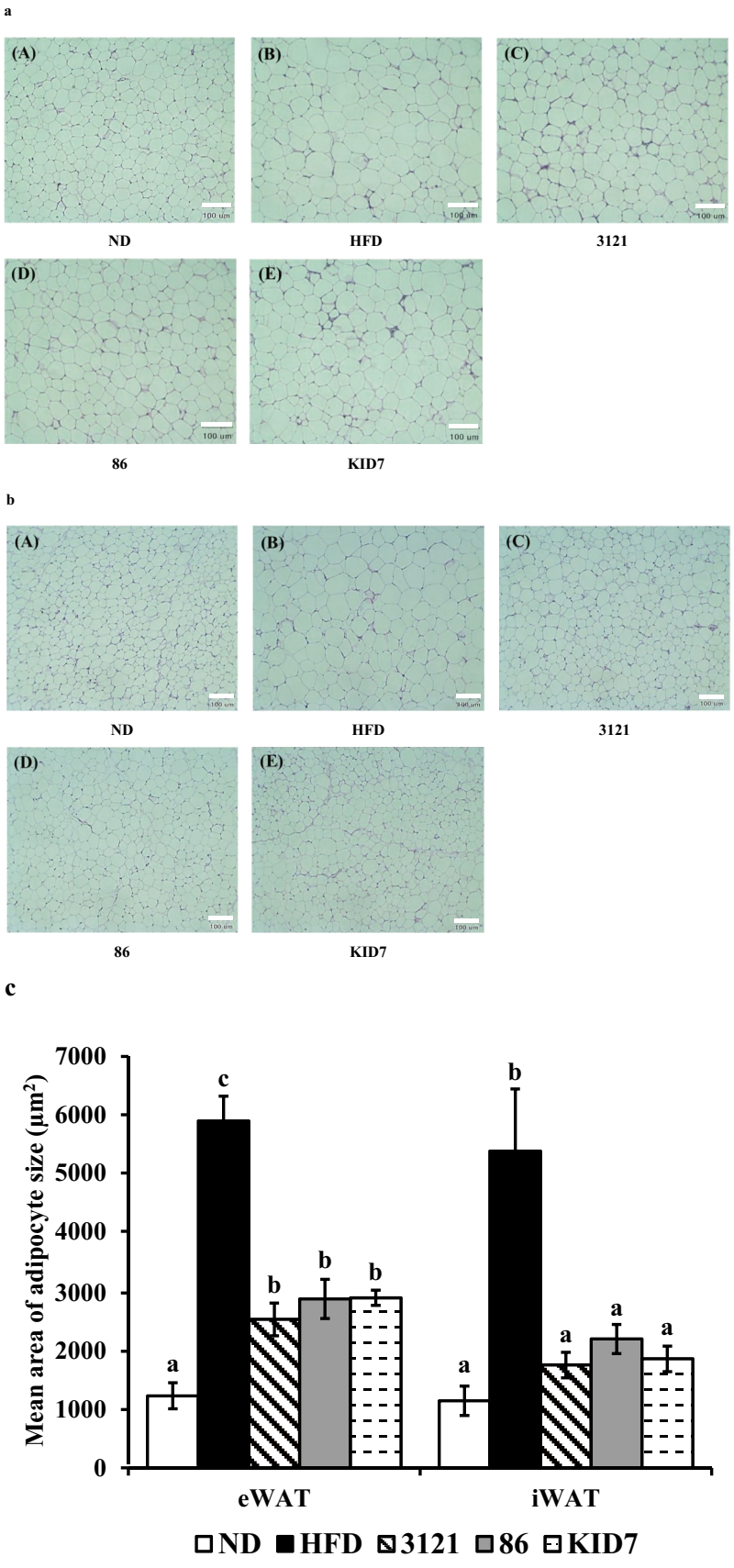

Fig. 3 Histological analysis of white adipose tissue stained with hematoxylin and eosin Y (H&E) (× 100 magnification) in HFD-induced obese mice. **a** Epididymal white adipose tissue (eWAT). **b** Inguinal white adipose tissue (iWAT). **c** Quantitative measurements of adipocyte size. [abc]Means in the same series with different lowercase superscript letters are significantly different ($p < 0.05$). Scale bar = 100 μm

Table 5 Effect of probiotic strains on the serum and liver lipid profiles

	ND	HFD	3121	86	KID7
Serum lipids					
Triglyceride (mg/dL)	44.5 ± 7.2^{a}	43.0 ± 5.7^{a}	45.2 ± 5.2^{a}	37.2 ± 4.6^{a}	40.1 ± 3.6^{a}
Total cholesterol (mg/dL)	187.5 ± 5.6^{a}	296.3 ± 12.1^{b}	222.9 ± 12.8^{a}	215.7 ± 11.2^{a}	224.2 ± 13.2^{a}
HDL-cholesterol (mg/dL)	162.3 ± 22.9^{a}	204.6 ± 16.3^{a}	199.5 ± 19.1^{a}	189.7 ± 14.5^{a}	197.6 ± 14.3^{a}
LDL/VLDL-cholesterol (mg/dL)	26.8 ± 1.9^{a}	51.9 ± 3.6^{b}	35.9 ± 2.2^{a}	30.0 ± 2.4^{a}	34.3 ± 3.6^{a}
Hepatic lipids					
Triglyceride (μg/mg)	58.2 ± 4.3^{a}	85.7 ± 2.3^{b}	65.0 ± 6.1^{ab}	79.7 ± 6.0^{ab}	75.9 ± 8.5^{ab}
Total cholesterol (μg/mg)	2.6 ± 0.1^{a}	5.5 ± 0.2^{c}	4.2 ± 0.1^{b}	4.3 ± 0.2^{b}	4.1 ± 0.3^{b}

Results are expressed as mean ± SE ($n = 6$). Means in the same column with different lowercase letters are significantly different ($p < 0.05$)

Discussion

Obesity, which is characterized by excessive fat storage in tissue and increased adipose tissue mass, is a major risk factor for metabolic syndromes, such as hypercholesterolemia and hepatic steatosis [32, 33]. Therefore, as a potential approach, probiotics have been suggested as an ideal method of preventing metabolic diseases [33]. In various experimental studies, it has been suggested that specific probiotic strains have anti-obesity effects due to different efficacy and mechanisms of action [34, 35]. Furthermore, according to recent data, particular probiotics have an anorectic effect by reducing food intake and energy intake in obese mice [36]. In our study, supplementation with *L. johnsonni* 3121, *L. rhamnosus* 86, and *P. pentosaceus* KID7 reduced the final body weight of the HFD-fed mice without affecting the feed and calorie intake, which provides evidence that probiotic treatments blocked the stimulated fat accumulation in HFD-obese mice without appetite regulation. We further investigated whether the effect of probiotics on the reduction of body weight gain could be explained by a decrease in fat pad weight of white adipose tissue (WAT) in the epididymal and inguinal regions. Since the increased size of adipocytes is known to be an important factor in developing obesity [37], we also evaluated the stored adiposity of epididymal WAT representing the visceral fat and inguinal WAT representing subcutaneous fat. Moreover, each adipose tissue has individual physiological differences. Therefore, we analyzed by designating epididymal WAT and inguinal WAT, which can be expressed as representative of each adipose tissue. A clear difference in fat cell has shown that individual treatment with *L. johnsonni* 3121, *L. rhamnosus* 86, and *P. pentosaceus* KID7 can also reduce the size of fat cells in HFD-fed mice. These results suggest that *L. johnsonni* 3121, *L. rhamnosus* 86, and *P. pentosaceus* KID7 could prevent the enlargement of fat cells stimulated by HFD, thereby suppressing body weight gain.

To confirm the evidence of decreased fat mass, biomarkers related to lipid metabolism were evaluated. No significant

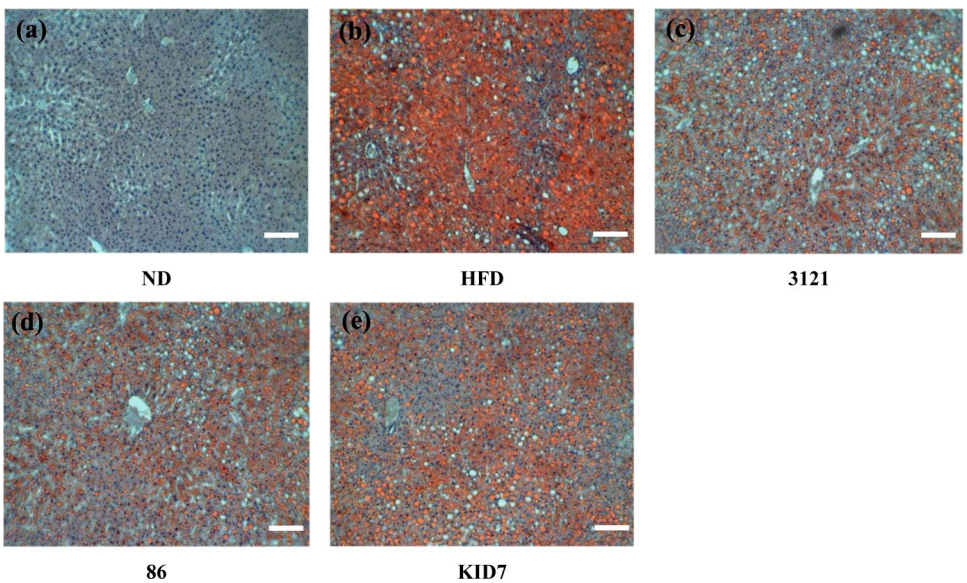

Fig. 4 Histological analysis of liver tissue stained with oil red O (× 100 magnification) in HFD-induced obese mice (red dots indicate the accumulated lipid droplets). Scale bar = 100 μm

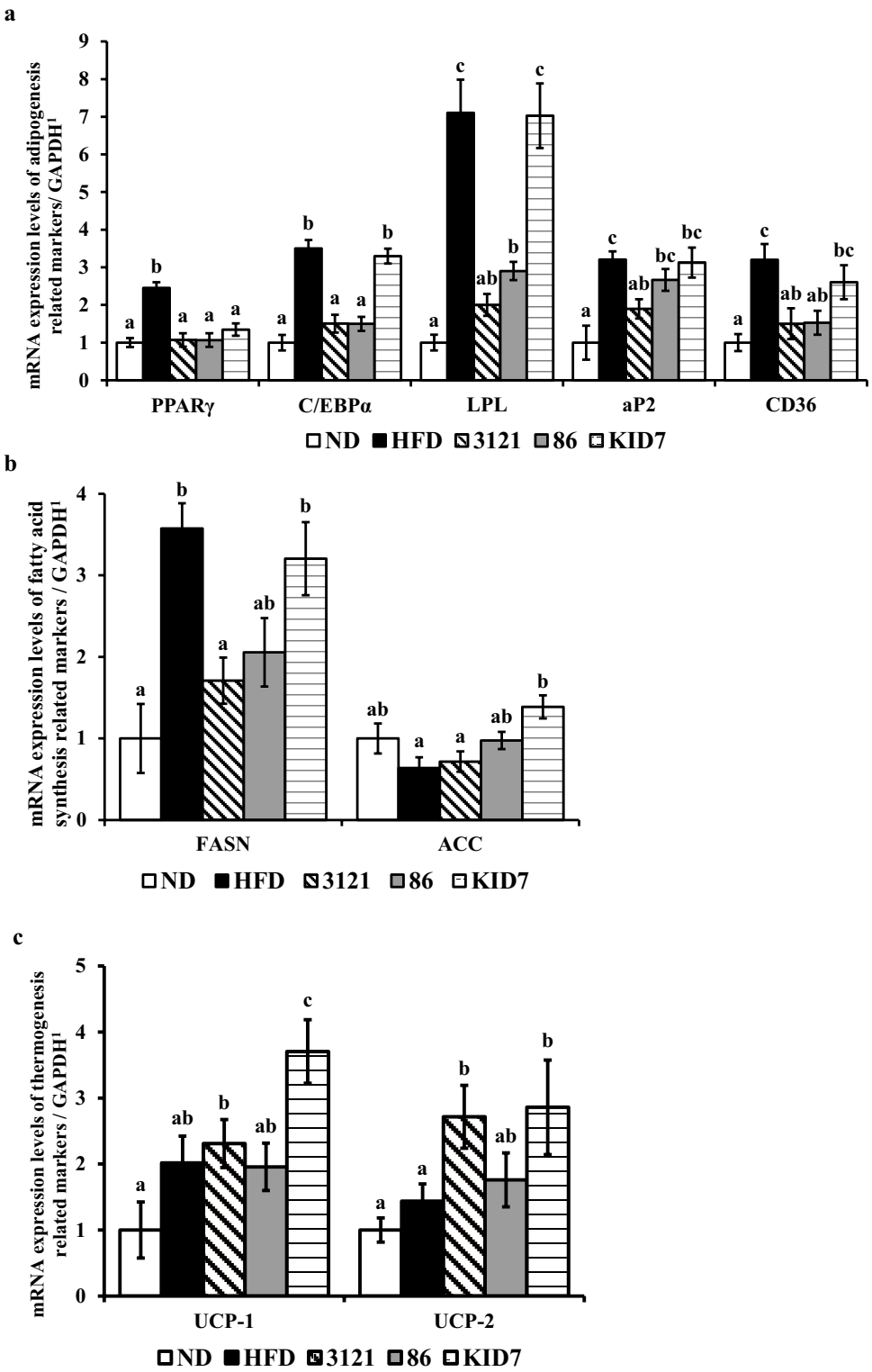

Fig. 5 Effect of probiotic strains on mRNA expression levels of body metabolism-related markers in epididymal white adipose tissue (eWAT) and brown adipose tissue (BAT) of HFD-induced obese mice. **a** Adipogenesis-related markers (Peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer-binding protein α (C/EBPα), lipoprotein lipase (LPL), adipocyte protein 2 (aP2) and CD36). **b** Fatty acid synthesis-related markers (fatty acid synthase (FASN) and acetyl-CoA carboxylase (ACC)). **c** Thermogenesis related markers (uncoupling protein (UCP) -1 and UCP-2). Results are expressed as mean ± SE ($n = 6$). [abc]Means in the same series with different lowercase superscript letters are significantly different ($p < 0.05$)

differences in serum triglyceride and HDL-cholesterol levels were observed among the groups. Interestingly, total cholesterol content, especially LDL/VLDL-cholesterol levels, in the serum and liver was significantly increased by HFD, indicating stimulated cholesterol synthesis by HFD. However, the increased total cholesterol levels in the serum and liver were normalized by the probiotic treatments. Increased levels of total cholesterol are known to be associated with an increased risk of heart failure and metabolic disorders [38]. Moreover, LDL/VLDL-cholesterol

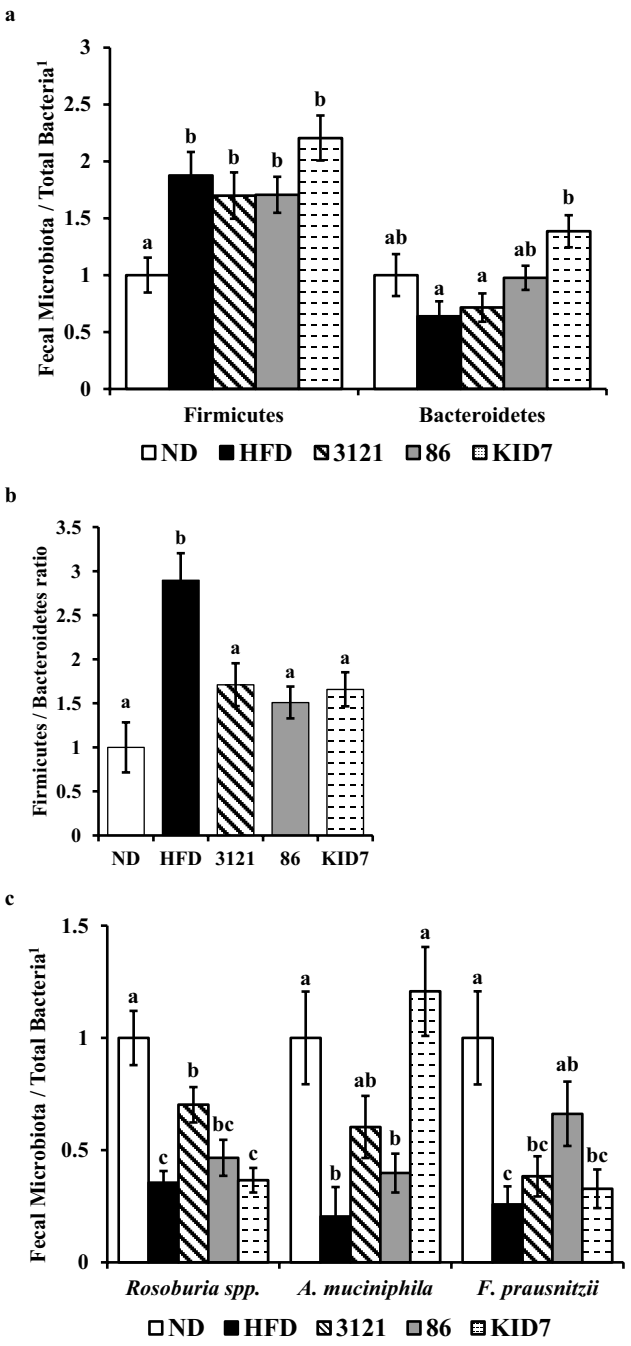

Fig. 6 Effect of probiotic strains on abundance of fecal microbiota of HFD-induced obese mice. **a** Phyla. **b** Firmicute/Bacteroidetes ratio. **c** Genus and species. Results are expressed as mean ± SE ($n = 6$). [abc]Means in the same series with different lowercase superscript letters are significantly different ($p < 0.05$)

is a well-established risk factor for atherosclerotic cardiovascular disease [39]. Therefore, regulating total cholesterol and LDL/VLDL-cholesterol levels is an ideal therapeutic method for alleviating metabolic disorders. Recently, several studies have revealed that probiotic treatments in obese animal models are capable of altering lipid metabolism in the serum and liver [40–42]. Similar to our study, Liang et al. [42] demonstrated that probiotic strains could effectively improve hyperlipidemia caused by a high-fat diet and relieve lipid accumulation in the liver. Therefore, in our study, we examined a pathway that is directly related to lipid metabolism in eWAT. Peroxisome proliferator-activated receptor γ (PPARγ) is an important transcription factor in the development and function of adipocytes [43]. Triggering the activation of PPARγ in adipocytes leads to an increased storage capacity of fatty acids in adipocytes, thereby decreasing the amount of circulating fatty acids and trapping the synthesis of triglycerides [44]. CCAAT/enhancer-binding protein α (C/EBPα) is another key transcription factor that plays an important role in promoting adipocyte differentiation [45]. Furthermore, adipocyte protein 2 (aP2), lipoprotein lipase (LPL), and CD36 have been explained as PPARγ-mediated fatty acid uptake genes [46]. Therefore, the five fatty acid uptake-stimulating genes were measured in eWAT. The expression levels of all five genes were increased in the HFD-fed obese mice; however, probiotic strains, especially *L. johnsonni* 3121 and *L. rhamnosus* 86, could normalize the increased gene expression to levels similar to those in the ND group. This provides good evidence that *L. johnsonni* 3121 and *L. rhamnosus* 86 can inhibit adipogenesis by blocking the expression levels of PPARγ-mediated fatty acid uptake genes. Further investigations were performed on the genes related to triglyceride synthesis and thermogenesis. Fatty acid synthase (FASN) and acetyl-CoA carboxylase (ACC) are transcription factors that play important roles in controlling fatty acid metabolism and adipogenesis [47]. As expected, mRNA expression levels of FASN and ACC were dramatically increased by HFD. Interestingly, *L. johnsonni* 3121 decreased the gene expression levels of the two genes, similar to that in the ND-treated group. *L. rhamnosus* 86 also downregulated the expression of FASN and ACC but was not significantly different. Since the gene expression of lipogenic genes FASN and ACC is regulated by the PPARγ pathway [34], this is good evidence that *L. johnsonni* 3121 has the potential to block fat accumulation by inhibiting the downstream pathway from PPARγ to FASN and ACC.

Obesity is the result of excessive fat accumulation due to an imbalance between intake and expenditure of energy. Uncoupling protein (UCP), also known as thermogenin, is located in the inner membrane of mitochondria and composed of a family of proton transporters, which increases thermogenesis and energy expenditure [48, 49]. UCP-1 is mostly involved in thermogenesis, while UCP-2 is involved in energy metabolism and obesity [50]. Therefore, we also measured the gene expression levels of UCP-1 and UCP-2 in BAT. Interestingly, only *P. pentosaceus* KID7 could increase the expression levels of both UCP-1 and UCP-2, but not *L. johnsonni* 3121 and *L. rhamnosus* 86. These results suggested that all three strains could ameliorate HFD-induced obesity;

however, the anti-obesity effect could be different due to the activation of individual signaling pathways. Therefore, further studies are needed to verify this claim.

Obesity and changes in diet are also correlated with the altered composition of the gut microbiota [51]. Ley et al. [6] demonstrated that obese mice showed a reduction in the abundance of *Bacteroidetes* and a proportional increase in that of *Firmicutes*. They also found that obese people had a higher *Firmicutes/Bacteroidetes* ratio than lean people. Moreover, it was revealed that gut microbiota had an effect on the host metabolism, utilization and storage of energy, and metabolic diseases [5, 52]. Several studies have shown that HFD-fed obesity murine models exhibit altered gut microbiota structure [53, 54]. A shift in the composition of the murine gut microbiota, such as a decrease in the abundance of phylum Bacteroidetes or an increase in the abundance of phylum Firmicutes, was reported to be induced by HFD [55]. Moreover, dysbiosis was reported to be associated with metabolic syndrome-related diseases such as diabetes and obesity which additionally indicates the linkage between gut microbiota and obesity [56]. Additionally, the physiological abundance of *Roseburia* spp., *F. prausnitzii*, *A. muciniphila*, and *Bacteroides/Prevotella* spp. was also reported to be decreased by HFD [53, 55, 57]. Among these bacteria, *A. muciniphila* plays an important role in controlling gut barrier function and other physiological functions homeostatically during obesity [57]. Everard et al. and the colleges have demonstrated that treating HFD-fed obese mice with *A. muciniphila* reduced the symptoms related with obesity [57]. Moreover, Shen et al. and the researchers have also shown the correlation between the increased *A. muciniphila* population in the gut bacterium and anti-obesity effect [58]. Therefore, changing the composition of gut microbiota to beneficial status with probiotics might be a good approach for treating obesity. Our results showed that *L. johnsonni* 3121, *L. rhamnosus* 86, and *P. pentosaceus* KID7 could alter several gut microbial communities at the phylum and genus levels. Firmicutes and Bacteroidetes are the most common bacterial phyla in the gut and are used as determinants of human health and disease [59]. As compared with the ND, the HFD and probiotics significantly increased the relative abundance of Firmicutes and Firmicutes/Bacteroidetes ratio, but the relative abundance of Bacteroidetes was not significantly affected. This change in ratio was counteracted by the supplementation of *L. rhamnosus* 86 and *P. pentosaceus* KID7 strains. As previous studies have shown, treatment with the HFD diet also induced a significant decrease in *Roseburia* spp., *F. prausnitzii*, and *A. muciniphila* [53, 55, 57]. Interestingly, Alard et al. have report that mixture of *Lactobacillus* and *Bifidobacterium* strains significantly decreased the phenotype obesity symptoms via increasing the gut bacterial abundance of *A. muciniphila* [60]. In our study, *L. johnsonni* 3121 treatment increased the abundance of *Roseburia* spp.; *L. rhamnosus* 86 and *P. pentosaceus* KID7 increased the abundance of *F. prausnitzii* and *A. muciniphila*, respectively, thus restoring the HFD-induced alteration of microbiota composition. Through these results, it was found that *L. johnsonni* 3121, *L. rhamnosus* 86, and *P. pentosaceus* KID7 strains could improve obesity symptoms by altering obesity-related gut microbiota.

Conclusion

In the present study, we demonstrated the anti-obesity potential of *L. johnsonii* 3121, *L. rhamnosus* 86, and *P. pentosaceus* KID7 in an HFD-induced obese murine model. All three strains were capable of significantly reducing body weight and body fat mass without affecting appetite. Moreover, they improved hypercholesterolemia and lipid accumulation in the liver. Additionally, gene expression analysis by qRT-PCR revealed that the anti-adipogenic effects were strain-specific. *L. johnsonii* 3121 and *L. rhamnosus* were capable of regulating PPARγ pathway–related genes, thereby controlling the expression levels of FASN and ACC. However, only *P. pentosaceus* KID7 was able to upregulate the expression of thermogenesis- and energy metabolism–related genes, UCP-1 and UCP2. Furthermore, treatment with the three probiotic strains could control the gut microbiota alterations in the HFD group. Taken together, these results suggest that *L. johnsonii* 3121, *L. rhamnosus* 86, and *P. pentosaceus* KID7 have the potential to be used for the treatment and prevention of obesity.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12602-021-09752-0.

Funding This work was financially supported by grants funded by the Chong Kun Dang Bio and Korea University Grant.

Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics Approval All procedures used in these experiments were approved by the Korea University Institutional Animal Care & Use Committee, South Korea (KUIACUC-2016-154).

Conflict of Interest The authors declare that they have no conflicts of interest.

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