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Full Paper

Heat-killed *Lactobacillus plantarum* L-137 attenuates obesity and associated metabolic abnormalities in C57BL/6 J mice on a high-fat diet

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Heat-killed *Lactobacillus plantarum* L-137 (HK L-137) has anti-allergic, antitumor, and antiviral effects in mice, as well as an anti-inflammatory effect in rats with metabolic syndrome through regulation of immunity. To evaluate the influence of HK L-137 on chronic inflammation in mice with diet-induced obesity, C57BL/6 J mice were fed a normal diet (16% of energy as fat) or a high-fat diet (62% of energy as fat) with or without 0.002% HK L-137 for 4 to 20 weeks. It was found that HK L-137 supplementation alleviated weight gain and elevation of plasma glucose, cholesterol, alanine aminotransferase, and aspartate transaminase levels in mice with diet-induced obesity. Expression of several inflammation-related genes, including F4/80, CD11c, and IL-1β, in the epididymal adipose tissue of these mice was significantly downregulated by HK L-137. In addition, plasma levels of lipopolysaccharide-binding protein, a marker of endotoxemia, tended to be decreased by administration of HK L-137. These findings suggest that HK L-137 supplementation ameliorates obesity-induced metabolic abnormalities and adipose tissue inflammation, possibly through improvement of intestinal permeability.

Key words: *Lactobacillus*, inflammation, obesity, cholesterol, adipose tissue, macrophage

INTRODUCTION

Overweight and obesity are defined as abnormal or excessive fat accumulation that presents a risk to health. The excess of macronutrients in adipose tissue stimulates it to release inflammatory mediators. Adipose tissue inflammation leads to excessive infiltration of inflammatory cells into the tissue, induces systemic inflammation, and causes dysfunction in peripheral tissues [1]. Obesity and associated disorders are linked to chronic inflammation. As a risk factor, chronic inflammation is an embedded mechanism of developing cardiovascular diseases, atherosclerosis, metabolic syndrome, insulin resistance, and diabetes mellitus [1, 2].

Migration of pro-inflammatory cells, such as T helper (Th) 1 cells, neutrophils, and classically activated macrophages (M1 macrophages), into adipose tissue is promoted by monocyte chemoattractant protein (MCP) 1 and CCL5 in the obese state [3, 4]. The infiltrating macrophages secrete various pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, which induce insulin resistance by affecting phosphorylation of insulin receptor substrate-1 and inhibit the development of preadipocytes into mature adipocytes [5]. In mice with diet-induced obesity (DIO), homozygous knockout of MCP1 leads to marked improvement of insulin resistance, hepatic steatosis, and macrophage accumulation in adipose tissue [3]. In patients with type 2 diabetes, treatment with an IL-1 receptor antagonist has a beneficial effect on glycemic control and β-cell function, along with reduction of the circulating levels of pro-inflammatory markers [6]. These findings suggest that suppression of chronic inflammation can contribute to improvement of insulin resistance and type 2 diabetes. Thus, the adipose tissue immune system is considered to have a key role in causing deterioration or improvement of chronic inflammation and lipid metabolism.

Lactic acid bacteria (LAB) are widely used as probiotics with health-promoting effects related to diverse gastrointestinal disorders, cancer, metabolic syndrome, and immunomodulation [7]. Intake of live LAB such as *Lactobacillus curvatus* HY7601 and *Lactobacillus plantarum* KY1032 has been reported to decrease adipose tissue inflammation and weight gain by modulating the gut microbiota [8]. On the other hand, heat-killed *L. plantarum* OLL2712 has been also shown to ameliorate metabolic disorders through regulation of inflammatory cytokines

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[9]. Therefore, not only live but also heat-killed LAB could improve obesity or obesity-associated disorders.

L. plantarum L-137 was originally isolated from a fermented Southeast Asian dish made from fish and rice [10]. Heat-killed *L. plantarum* L-137 (HK L-137) has immunostimulating properties and shows anti-allergic, anti-tumor, and antiviral effects in mouse models [11–13]. It has also been demonstrated that daily intake of HK L-137 improves health-related quality of life and reduces the incidence of upper respiratory tract infection in healthy subjects or those under high levels of stress [14, 15]. Immunomodulatory effects of HK L-137 have also been reported. For example, administration of HK L-137 facilitated recovery of mice from dextran sulfate sodium-induced colitis and attenuated chronic inflammation in DahlS.Z-*Lepr*$^{fa/Lepr^{fa}}$ rats, a model of metabolic syndrome [16, 17]. In the latter study, HK L-137 showed prominent anti-inflammatory effects in already obese DahlS.Z-*Lepr*$^{fa/Lepr^{fa}}$ rats with metabolic syndrome, but it was unclear whether administration of HK L-137 has an effect on the pathogenesis of obesity, metabolic syndrome, and associated disorders. Accordingly, we examined the effect of HK L-137 on chronic inflammation and metabolic disorders in mice with DIO.

MATERIALS AND METHODS

Animals and diets

Specific pathogen-free male C57BL/6 J mice (6 weeks old) were purchased from Charles River Laboratories Japan Inc. (Kanagawa, Japan) and fed a standard rodent diet (CE-2, Clea Japan, Tokyo). After 1 week of acclimatization, mice were randomly divided into 3 groups by body weight (n=30–32 per group) and were fed a normal diet (ND; 16% of energy as fat, 20% as protein, and 64% as carbohydrates; AIN-93G, Oriental Yeast Co., Ltd., Tokyo, Japan) or a high-fat diet (HFD; 62% of energy as fat, 18% as protein, and 20% as carbohydrates) with (HFD(+)) or without (HFD(−)) 0.002% HK L-137 (Table 1). The animal room was maintained at 23 ± 1°C with 55 ± 5% humidity on a 12-hr light/dark cycle. During the feeding period, mice were housed in individual cages and given free access to the experimental diet and water. Food consumption was calculated by subtracting the amount of residual diet from the amount dispensed. The sample size was determined from the results of a previous study of the effect of Orlistat on C57BL/6 J mice fed a high-fat diet [18]. The estimated sample size of 5 mice was based on expected serum alanine aminotransferase (ALT) levels of 35 (SD 2) U/L in DIO mice, a targeted 11% restoration of serum ALT levels, a statistical power of 80%, and Type I error of 5%. We allocated 5 mice to each group. Every 4 weeks, 5 mice were randomly selected from each group based on body weight and were anesthetized by inhalation of diethyl ether to allow collection of blood from the inferior vena cava. Blood samples were collected into ice-chilled tubes containing 2 IU of heparin sodium (AY Pharmaceutical Co., Ltd., Tokyo, Japan) and were centrifuged at 2,000 × g for 15 min at 4°C, after which the supernatant was stored at −80°C until analysis. After collection of blood, epididymal white adipose tissue (eWAT) was removed from each mouse and frozen in liquid nitrogen for storage at −80°C until analysis. This study was approved by the University of The Ryukyus Animal Experiment Committee (approval number A2018108), and all experiments were conducted according to the Animal Experiment Guideline.

Table 1. Composition of the diets

Ingredient	g/kg diet		
	ND[1]	HFD(−)	HFD(+)
soybean oil[2]	70	20	20
lard[2]		330	330
casein[2]	200	256	256
maltodextrin[2]	0	60	59.9
a-cornstarch[2]	132	160	160
cornstarch[2]	397.486		
calcium carbonate[3]		1.8	1.8
choline bitartrate[3]	2.5	2.5	2.5
L-cystine[3]	3	3.6	3.6
AIN-93 Vitamin mix[2,4]	10	10	10
AIN-93G Mineral mix[2,4]	35	35	35
sucrose[2]	100	55	55
cellulose[2]	50	66.1	66.1
t-butylhydroquinone[2]	0.014		
LP20			0.1

[1] The ND was based on the AIN-93G diet, which was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan).
[2] Purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan).
[3] Purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan).
[4] Composition described by Reeves [43].
ND: normal diet; HFD(−): high-fat diet without 0.002% HK L-137; HFD(+): high-fat diet with 0.002% HK L-137.

Preparation of heat-killed L. plantarum *L-137*

LP20 (House Wellness Foods Corp., Hyogo, Japan) containing 20% HK L-137 and 80% dextrin was used in this study. HK L-137 was prepared by the method described previously [16].

Blood chemistry tests

Plasma levels of glucose, total cholesterol, triglycerides, nonesterified fatty acids (NEFA), aspartate transaminase (AST), and alanine aminotransferase (ALT) were measured by using a Glucose C-II Test Wako, Cholesterol E-Test Wako, Triglyceride E-Test Wako, NEFA C-Test Wako, and Transaminase C-II Test Wako, respectively (all from Fujifilm Wako Pure Chemical Corporation, Osaka, Japan). Plasma leptin and insulin levels were measured with a Mouse/Rat Leptin ELISA Kit and an Ultra Sensitive Mouse Insulin ELISA Kit, respectively (both from Morinaga Institute of Biological Science, Kanagawa, Japan). Plasma lipopolysaccharide-binding protein (LBP) levels were measured with a Mouse LBP ELISA Kit (Hycult Biotech, Uden, Netherlands).

Isolation of RNA and real-time PCR

Total RNA was isolated from adipose tissue samples by using an RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany), and the RNA concentration was estimated by measuring the UV absorbance at 260 nm with a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription and real-time PCR were performed with One Step SYBR PrimeScript RT-PCR Kit II (Takara Bio, Shiga, Japan) and Thermal Cycler Dice® Real Time System II (Takara Bio) according to the manufacturer's protocol. The primer sequences for the target genes (F4/80, CD11c, MCP1, TNF-α, and IL-1β) and the endogenous control (GAPDH) are listed in Table 2. Expression of the target genes was normalized for GAPDH

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Table 2. Sequences of the PCR primers used in this study

Gene		Sequence
MCP1	Forward primer	5′- GACCCCAAGAAGGAATGGGT -3′
	Reverse primer	5′- ACCTTAGGGCAGATGCAGTT -3′
F4/80	Forward primer	5′- TGACTCACCTTGTGGTCCTAA -3′
	Reverse primer	5′- CTTCCCAGAATCCAGTCTTTCC -3′
CD11c	Forward primer	5′-ACACAGTGTGCTCCAGTATGA-3′
	Reverse primer	5′- GCCCAGGGATATGTTCACAGC -3′
TNF-α	Forward primer	5′- CCTGTAGCCCACGTCGTAG -3′
	Reverse primer	5′- GGGAGTAGACAAGGTACAACCC -3′
IL-1β	Forward primer	5′- TCTTTGAAGTTGACGGACCC -3′
	Reverse primer	5′- TGAGTGATACTGCCTGCCTG -3′
GAPDH	Forward primer	5′- AATGTGTCCGTCGTGGATCTGA -3′
	Reverse primer	5′- AGTGTAGCCCAAGATGCCCTTC -3′

expression by the 2−ΔΔCt method.

Statistical analysis

Results are expressed as the mean ± standard deviation (SD). Two-way analysis of variance (ANOVA), followed by the two-tailed Student's t-test, was used to compare mean values between the HFD(−) and HFD(+) group. Analyses were performed using the Statcel2 software (OMS Publishing, Inc., Saitama, Japan).

RESULTS

HK L-137 decreases weight gain without affecting food intake in DIO mice

First, we measured the body weight of mice fed the ND, HFD(−), or HFD(+) for 20 weeks. We found that obesity was induced by the HFD, while weight gain was significantly lower or tended to be lower in the HFD(+) group compared with the HFD(−) group in weeks 8, 11, 12, 14, and 16 (Fig. 1A, p=0.04, 0.07, 0.06, 0.06, and 0.08, respectively), even though food intake did not differ between the two groups (Fig. 1B). No adverse events were observed in any experimental groups during the experiment. There was no significant difference in eWAT weight between the two groups, but liver weight was lower in the HFD(+) group than in the HFD(−) group (Table 3, p=0.09).

HK L-137 improves metabolic dysfunction in DIO mice

Next, we measured various biochemical markers to examine whether administration of HK L-137 improved lipid metabolism (Table 4). After 4 and 8 weeks on the respective diets, the plasma cholesterol level was significantly lower in the HFD(+) group compared with the HFD(−) group ($p<0.01$). The plasma glucose level of the HFD(+) group was significantly lower in week 8 compared with the HFD(−) group, and the plasma insulin level tended to be lower in week 20 ($p<0.01$ and $p=0.06$ respectively). Plasma AST and ALT levels were also significantly lower in the HFD(+) group than in the HFD(−) group during the study ($p=0.02$). In contrast, the plasma levels of triglycerides, NEFA, and leptin were similar in both groups.

HK L-137 decreases pro-inflammatory gene expression in epididymal adipose tissue

To investigate the impact of HK L-137 on infiltration and accumulation of macrophages in the eWAT of DIO mice, the gene

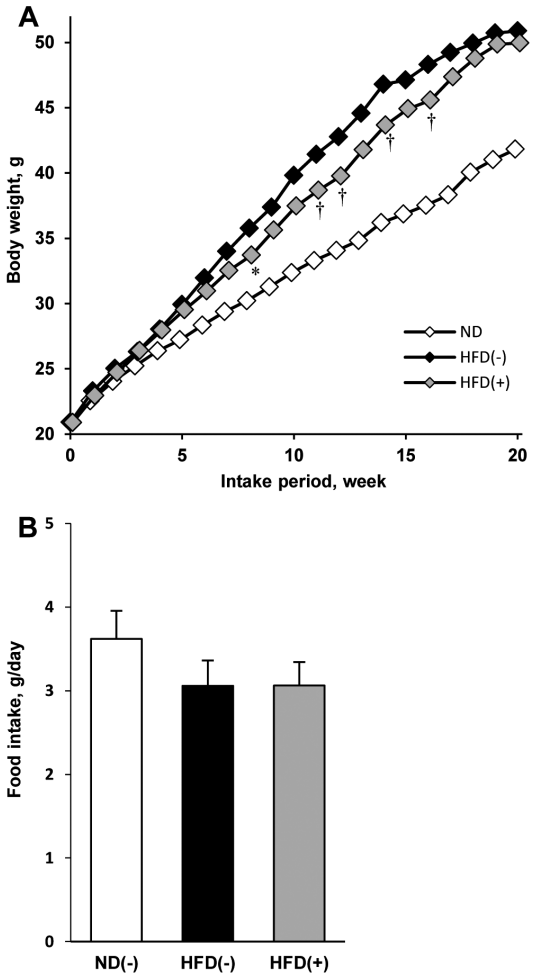

Fig. 1. Effect of HK L-137 on body weight and food intake in C57BL/6 J mice fed the HFD. Mice were fed a normal diet (ND, white bars) or a high-fat diet without (HFD(−), black bar) or with (HFD(+), gray bar) 0.002% HK L-137 for 20 weeks. Body weight changes (A) and total food intake (B) are shown. Values are means, with their SDs represented by vertical bars (n=32 at 0–4 weeks; n=27 at 5–8 weeks; n=22 at 9–12 weeks; n=17 at 13–16 weeks; n=12 at 17–20 weeks). Significant differences between the HFD(−) and HFD(+) groups were evaluated by Student's t-test. Differences from the HFD(−) group are indicated as follows: *$p<0.05$; †$p<0.1$. Data from the ND group are shown for reference.

Table 3. Effect of HK L-137 on tissue weight in C57BL/6 J mice fed the HFD

							ANOVA (p value)		
			8 weeks	12 weeks	16 weeks	20 weeks	Intervention	Time point	Interaction
Tissue weight (g)	eWAT	ND	1.02 ± 0.3	1.50 ± 0.7	1.99 ± 0.6	2.20 ± 0.3			
		HFD(−)	1.98 ± 0.6	3.03 ± 1.0	2.25 ± 0.4	1.94 ± 0.6	0.55	<0.01	0.11
		HFD(+)	1.60 ± 0.3	2.31 ± 0.6	2.70 ± 0.1	2.15 ± 0.5			
	Liver	ND		1.50 ± 0.3	1.46 ± 0.3	1.89 ± 0.5			
		HFD(−)		1.68 ± 0.3	2.40 ± 0.9	3.27 ± 1.2	0.09	<0.01	0.78
		HFD(+)		1.47 ± 0.2	1.80 ± 0.4	2.62 ± 0.9			
Relative tissue weight (mg/g)	eWAT	ND	33.3 ± 8.2	41.9 ± 13	51.2 ± 10	52.3 ± 4.5			
		HFD(−)	54.9 ± 11	68.8 ± 17	47.7 ± 13	38.6 ± 16	0.9	<0.01	0.18
		HFD(+)	47.6 ± 7.1	57.9 ± 14	59.4 ± 6.7	43.2 ± 13			
	Liver	ND		43.0 ± 3.4	38.0 ± 2.9	44.3 ± 5.0			
		HFD(−)		38.9 ± 2.7	48.7 ± 14	62.2 ± 19	0.09	<0.01	0.66
		HFD(+)		36.8 ± 2.6	38.9 ± 5.2	51.1 ± 15			

Values are expressed as the mean ± SD (n=5 mice per group).
Significant differences between the HFD(−) and HFD(+) groups over the time periods were evaluated by two-way ANOVA.
Data from the ND group are shown for reference (n=5).
eWAT: epididymal white adipose tissue; ND: normal diet; HFD(−): high-fat diet without 0.002% HK L-137; HFD(+): high-fat diet with 0.002% HK L-137.

Table 4. Effect of HK L-137 on blood parameters in C57BL/6 J mice fed the HFD

							ANOVA (p value)		
		4 weeks	8 weeks	12 weeks	16 weeks	20 weeks	Intervention	Time point	Interaction
Glucose (mg/dL)	ND	196 ± 14	206 ± 21	195 ± 12	222 ± 15	212 ± 30			
	HFD(−)	221 ± 24	262 ± 15	238 ± 12	233 ± 37	227 ± 30	0.4	0.17	0.06
	HFD(+)	252 ± 60	218 ± 19**	207 ± 41	232 ± 26	212 ± 23			
Total cholesterol (mg/dL)	ND	103 ± 11	112 ± 13	134 ± 18	120 ± 12	126 ± 16			
	HFD(−)	139 ± 7.3	148 ± 10	172 ± 29	180 ± 29	205 ± 39	0.01	<0.01	0.93
	HFD(+)	115 ± 14**	127 ± 10**	149 ± 24	172 ± 27	177 ± 42			
TG (mg/dL)	ND	83.2 ± 10	81.2 ± 12	69.1 ± 7.6	67.0 ± 17	71.1 ± 13			
	HFD(−)	98.0 ± 53	72.6 ± 24	57.2 ± 17	71.1 ± 23	75.3 ± 17	0.14	0.09	0.52
	HFD(+)	69.8 ± 10	54.6 ± 18	57.7 ± 5.0	64.9 ± 6	78.1 ± 15			
NEFA (μEq/L)	ND	559 ± 582	326 ± 129	285 ± 58	263 ± 69	307 ± 67			
	HFD(−)	337 ± 103	377 ± 133	258 ± 46	331 ± 101	343 ± 38	0.66	0.84	0.17
	HFD(+)	351 ± 128	277 ± 47	354 ± 93	285 ± 76	323 ± 58			
AST (IU/L)	ND	9.10 ± 0.7	11.4 ± 2.2	14.2 ± 6.1	11.3 ± 2.7	18.2 ± 3.7			
	HFD(−)	10.0 ± 0.5	10.6 ± 1.2	19.9 ± 8.1	27.5 ± 17	52.8 ± 26	0.02	<0.01	0.23
	HFD(+)	9.28 ± 1.0	9.7 ± 0.8	14.9 ± 3.9	14.1 ± 5.0	31.9 ± 16			
ALT (IU/L)	ND	3.53 ± 1.8	3.17 ± 1.1	3.51 ± 2.4	3.42 ± 1.3	6.91 ± 5.4			
	HFD(−)	3.24 ± 0.3	3.54 ± 0.7	7.73 ± 5.1	18.4 ± 13	31.3 ± 16	0.02	<0.01	0.3
	HFD(+)	2.89 ± 0.3	3.22 ± 0.3	4.37 ± 2.1	8.14 ± 4.9	18.8 ± 13			
Leptin (ng/mL)	ND								
	HFD(−)	21.2 ± 7.3	60.2 ± 37	104 ± 46	112 ± 35	112 ± 25	0.26	<0.01	0.49
	HFD(+)	11.1 ± 10	39.2 ± 17	73.6 ± 35	114 ± 28	124 ± 22			
Insulin (mg/mL)	ND								
	HFD(−)	1.90 ± 1.4	2.30 ± 1.1	3.85 ± 2.0	3.10 ± 1.5	6.93 ± 2.8	0.12	<0.01	0.21
	HFD(+)	1.63 ± 1.4	1.91 ± 0.5	3.84 ± 1.4	3.05 ± 2.4	3.77 ± 1.5†			

Values are expressed as the mean ± SD (n=5 mice per group).
Significant differences between the HFD(−) and HFD(+) groups over the time periods were evaluated by two-way ANOVA, followed by comparison at each time point by Student's t-test.
Differences from the HFD(−) group are indicated as follows: **p<0.01; †p<0.1.
Data from the ND group are shown for reference (n=5).
TG: triglycerides; NEFA: nonesterified fatty acids; AST: aspartate transaminase; ALT: alanine aminotransferase; ND: normal diet; HFD(−): high-fat diet without 0.002% HK L-137; HFD(+): high-fat diet with 0.002% HK L-137.

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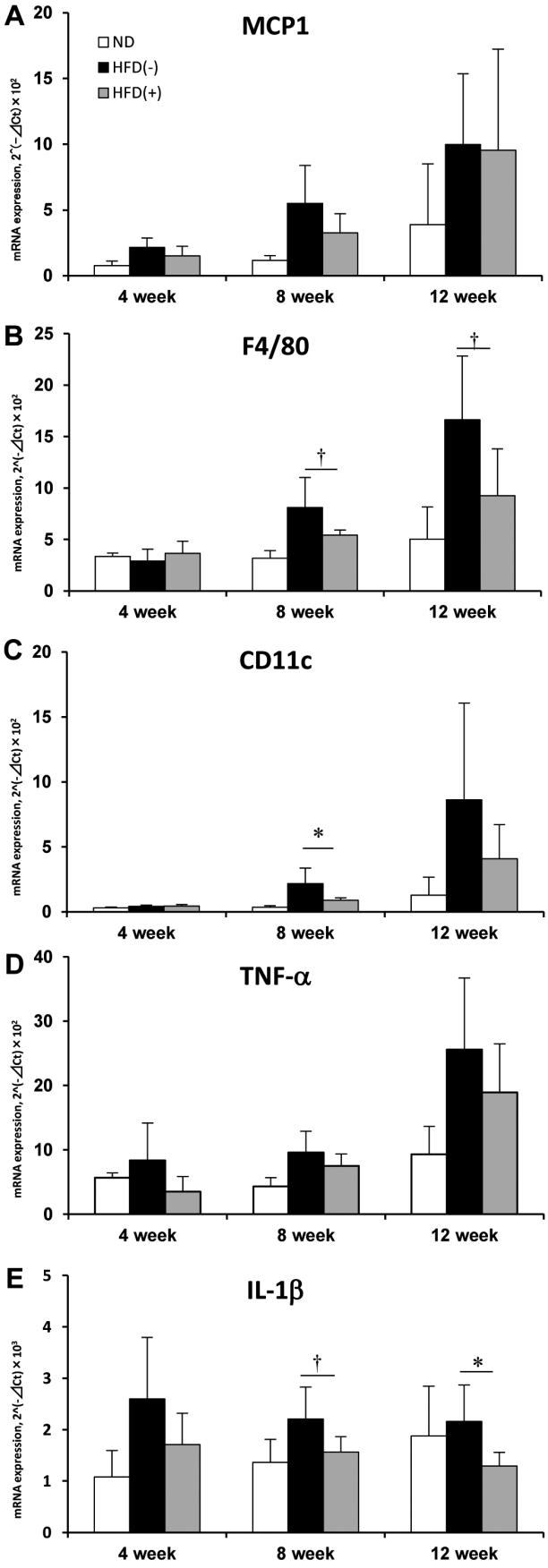

expression profiles of a chemokine (MCP1), two macrophage markers (F4/80 and CD11c), and two pro-inflammatory cytokines (TNF-α and IL-1β) were evaluated. As shown in Fig. 2, expression of mRNAs for these genes was strongly upregulated in the HFD(−) group compared with the ND group. In the HFD(+) group, expression of CD11c (week 8) and IL-1β (week 12) was significantly downregulated compared with that in the HFD(−) group (p=0.04 and 0.03, respectively). Expression of F4/80 (weeks 8 and 12) and IL-1β (week 8) also tended to be lower in the HFD(+) group than in the HFD(−) group (Fig. 2, p=0.08, 0.06, and 0.07, respectively). As shown in Table 5, expression of F4/80, TNF-α, and IL-1β during the study was significantly reduced or tended to be lower in the HFD(+) group (p=0.02, 0.06, and <0.01, respectively). These results indicate that HK L-137 inhibited inflammation in eWAT, possibly by reducing the accumulation of pro-inflammatory macrophages.

Effect of HK L-137 on plasma LBP levels in DIO mice

Finally, we measured the plasma levels of LBP, a marker of endotoxemia, to investigate whether HK L-137 ameliorated HFD-induced intestinal permeability of lipopolysaccharide (LPS). The plasma LBP levels of the HFD(+) group tended to be lower at 16 weeks compared with the HFD(−) group (Fig. 3, p=0.08).

DISCUSSION

The present study demonstrated that HK L-137 significantly ameliorated HFD-induced weight gain in DIO mice during the early phase of obesity induction without affecting food intake and also tended to suppress the increase of liver weight (Fig. 1A and Table 3). HK L-137 significantly decreased plasma glucose, cholesterol, ALT, and AST levels and also tended to reduce plasma insulin (Table 4). We also found significant reduction in the expression of several inflammation-related genes in eWAT, including F4/80, CD11c, and IL-1β mRNA, and a declining trend of TNF-α mRNA (Fig. 2 and Table 5). In addition, elevation of the levels of the endotoxemia marker LBP tended to be inhibited by HK L-137 (Fig. 3), and this may contribute to the improvement of glucose/lipid metabolism and inflammation in obesity.

It has been reported that small intestinal permeability is abnormal in obese persons [19], and an increase of paracellular permeability was noted after 1 week on a high-fat diet in an animal study [20]. Since the intestinal barrier normally prevents translocation of LPS derived from microbiota or the diet, its impairment resulted in chronic elevation of plasma LPS levels in obese animals/humans [21–23]. Cani *et al.* demonstrated that

Fig. 2. Effect of HK L-137 on the expression of pro-inflammatory genes in eWAT. The expression levels of MCP1 (A), F4/80 (B), CD11c (C), TNF-α (D), and IL-1β (E) are shown. Expression of the target genes was normalized for that of the endogenous control (GAPDH). Values are expressed as the mean ± SD (n=5 mice per group). Significant differences between the HFD(−) and HFD(+) groups were evaluated by Student's t-test. Differences from the HFD(−) group are indicated as follows: $^{*}p<0.05$; $^{\dagger}p<0.1$. Data from the ND group are shown for reference (n=5). MCP1: monocyte chemoattractant protein 1; CD11c: cluster of differentiation 11c; TNF-α: tumor necrosis factor-α; IL-1β: interleukin-1β; eWAT: epididymal white adipose tissue; ND: normal diet; HFD(−): high-fat diet without 0.002% HK L-137; HFD(+): high-fat diet with 0.002% HK L-137.

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infusion of LPS increased fasting glycemia and insulinemia, as well as whole-body, liver, and adipose tissue weights, to a similar extent as in DIO mice [21]. Their findings suggest that an increase in circulating LPS may increase adipose tissue weight, adipose tissue inflammation, and insulin resistance in DIO. Other researchers have indicated that increased intestinal permeability and tight junction disruption induce endotoxin translocation to the liver, which contributes to the progression of nonalcoholic steatohepatitis [24]. Recently, considerable evidence has been obtained indicating that LAB can strengthen the gut barrier in DIO mice. For example, *L. gasseri* SBT2055 was reported to improve intestinal integrity, reducing translocation of LPS from the intestine and also decreasing body weight, visceral fat mass, and inflammation [25]. In another study, Hsieh *et al.* demonstrated that both viable and heat-killed *Lactobacillus reuteri* GMNL-263 can improve gut microbiota influencing the intestinal barrier and reduce weight gain [26]. These findings indicate that non-viable bacteria, which are unable to colonize the host intestine, can still improve the gut microbiota and intestinal barrier in a similar fashion to viable bacteria. Similarly, the levels of the endotoxemia marker LBP were gradually increased after 12 weeks and tended to be suppressed in the HK L-137 group at 16 weeks of HFD intake (Fig. 3), which suggests that HK L-137 may strengthen the intestinal barrier and thus improve weight gain, glucose/lipid metabolism, and adipose tissue inflammation. This hypothesis is supported by the fact that HK L-137 induced intestinal cell growth by activating intestinal function in broiler chickens and by the fact that HK L-137 improved systemic and hepatic inflammation, possibly through restoration of the intestinal barrier in overweight human subjects [27, 28]. In this study, the effects of HK L-137 on obesity and associated metabolic markers were observed in the early phase, while a trend toward a decrease in plasma LBP levels caused by HK L-137 was seen in the late phase. We consider that the inhibitory effects of HK L-137 on increases in LPS permeability might occur in the early phase of diet-induced obesity, but we could not detect them timely by measuring plasma LBP levels because of time-lag bias between LPS translocation and LBP synthesis [29]. Further experiments are needed.

In addition to the above mechanism, non-viable LAB have been reported to improve diseases associated with obesity by (a) activation of peroxisome proliferator-activated receptor (PPAR) α/γ [30], (b) binding to bile acids [31], and (c) an anti-inflammatory effect mediated via the exosome [32]. Activation of PPARα inhibits inflammation and suppresses cholesterol absorption and synthesis [33–35]. It has been reported that the fragmented components of *Lactobacillus amylovorus* CP1563 possess potent PPARα agonist activity *in vitro* and induce an increase in plasma HDL cholesterol levels and decrease in LDL cholesterol levels *in vivo*. In addition to its anti-inflammatory effect in the present study, HK L-137 markedly reduced the plasma total cholesterol level (Table 4). It is possible that the fragmented HK L-137 generated in the intestine might be absorbed and activate PPARα in the liver and improve lipid metabolism. As another cholesterol-lowering mechanism of LAB, it was reported that heat-killed *Lactobacillus paracasei* NLB163 bind with bile acids and inhibit bile acid reabsorption [31]. Since the dietary LAB concentration was much lower in this study than in the previous study (0.002% vs. 5%), it would not have been possible for HK L-137 to reduce cholesterol by binding to bile acids. Recently, exosomes of mice fed *L. plantarum* No. 14

Table 5. Two-way analysis of variance (ANOVA) of expression of inflammation-related genes in eWAT

Gene		ANOVA (p value)		
		Intervention	Time point	Interaction
MCP1	HFD(−) HFD(+)	0.38	<0.01	0.87
F4/80	HFD(−) HFD(+)	0.02	<0.01	0.05
CD11c	HFD(−) HFD(+)	0.12	<0.01	0.29
TNF-α	HFD(−) HFD(+)	0.06	<0.01	0.71
IL-1β	HFD(−) HFD(+)	<0.01	0.39	0.91

Significant differences between the HFD(−) and HFD(+) groups at the time points were evaluated by two-way ANOVA.
eWAT: epididymal white adipose tissue; HFD(−): high-fat diet without 0.002% HK L-137; HFD(+): high-fat diet with 0.002% HK L-137.

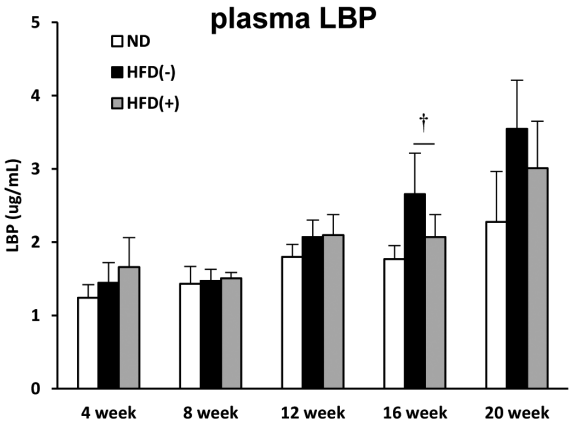

Fig. 3. Effect of HK L-137 on plasma LBP levels in C57BL/6 J mice fed the HFD. Plasma LBP levels are shown. Values are expressed as the mean ± SD (n=5 mice per group). †p<0.1 by Student's t-test. Data from the ND group are shown for reference (n=5). LBP: lipopolysaccharide-binding protein; ND: normal diet; HFD(−): high-fat diet without 0.002% HK L-137; HFD(+): high-fat diet with 0.002% HK L-137.

were reported to inhibit the production of inflammatory cytokines [32]. Exosomes are thought to be involved in regulating various physiological and pathophysiological responses by mediating cell-cell communication, including modulation of obesity and associated disorders [36]. Therefore, further studies are needed to determine the role of exosomes in the anti-inflammatory effect of HK L-137.

As shown in Fig. 1A, HK L-137 partly reduced HFD-induced weight gain but did not decrease the weight of eWAT (Table 3). It has been reported that *Lactobacillus gasseri* SBT2055 significantly lowered the weights of the body and mesenteric and perirenal/retroperitoneal adipose tissues but not that of eWAT in DIO mice [25]. Thus, HK L-137 might exert a transient anti-obesity effect through reduction of mesenteric and perirenal/retroperitoneal adipose tissues. On the other hand, no anti-obesity effects could be observed in studies of already

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overweight human subjects and in genetically obese DahlS.Z-*Lepr*^{fa}/*Lepr*^{fa} rats, despite anti-inflammatory effects being seen in both studies [17, 28]. Consequently, it is possible that HK L-137 has preventative but not therapeutic effects on obesity. After 8 weeks of HK L-137 administration, weight gain was decreased in conjunction with the downregulation of F4/80 and CD11c mRNA expression in eWAT (Figs. 1A, 2, and Table 5). Recent studies have demonstrated that immune cells, such as macrophages and natural killer cells, are involved in the regulation of lipid metabolism and obesity [37–39]. Bu *et al.* reported that growth/differentiation factor (GDF) 3, which is secreted by CD11c+ macrophages in response to low insulin levels, inhibits lipolysis of adipose tissue and accelerates obesity in the early phase of DIO [40]. They also showed that GDF3-producing CD11c+ macrophages expressed typical M2 markers, such as arginase-1 and chitinase-like 3, but not M1 markers like TNF-α and MCP1. Their findings suggest that GDF3+CD11c+M2 macrophages may be involved in promoting fat accumulation in adipose tissue. In the present study, administration of HK L-137 decreased expression of CD11c mRNA in eWAT at 8 weeks but did not cause downregulation of the M1 markers TNF-α and MCP1 (Fig. 2). Therefore, it is possible that HK L-137 temporally inhibits recruitment or differentiation of CD11c+ M2 macrophages in adipose tissue, leading to a transient anti-obesity effect by reducing GDF3 production.

In the present study, HK L-137 tended to inhibit liver weight gain and significantly reduced the plasma levels of ALT and AST, which increase in nonalcoholic steatohepatitis in the late phase of DIO (Tables 3 and 4). Several studies have demonstrated that adipose tissue inflammation stimulates lipolysis and fibrosis and enhances the release of free fatty acids, which is followed by ectopic accumulation of fat at other sites, such as the liver and skeletal muscle [41, 42]. Therefore, HK L-137 may improve liver damage by attenuating adipose tissue inflammation. The novel finding of this study was that intake of HK L-137 decreased biomarkers of hepatic inflammation. To our knowledge, this is the first report showing that lactobacilli can improve biomarkers of hepatic inflammation, such as AST and ALT, in both overweight healthy human subjects and DIO mice.

In conclusion, our findings suggest that dietary intake of HK L-137 prevents transient weight gain, adipose tissue inflammation, and liver damage, at least partly through improvement of intestinal permeability and endotoxin translocation. Further studies are needed to determine the mechanisms involved.

CONFLICT OF INTEREST

No conflict of interest was declared.

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R.Y. and Y.H. designed this research. R.Y. conducted the research. R.Y. and Y.H. analyzed the data. R.Y. wrote the paper. S.M. and G.M. participated in interpretation of the results. R.Y. has primary responsibility for the final content. All authors read and approved the final manuscript.

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