Journal of Agriculture and Food Research 15 (2024) 100965

Contents lists available at ScienceDirect

Journal of Agriculture and Food Research

journal homepage: www.sciencedirect.com/journal/journal-of-agriculture-and-food-research

Effects of plant-based heat killed lactic acid bacteria and its lithium chloride-extracted cellular protein on high-fat-induced obesity

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ARTICLE INFO

Keywords:
Heat killed lactic acid bacteria
S-layer proteins
Obesity
Plant-based

ABSTRACT

The anti-obesity action of postbiotics derived from plant-based probiotics remains unclear. The effects of heat killed lactic acid bacteria (*Lactobacillus plantarum*, LPHK and *Lactobacillus curvatus*, LCHK) along with bioactive their cell envelop components known as surface layer proteins (SLPs, LPSLP, and LCSLP) on obesity were investigated. LPHK, LCHK, LPSLP, and LCSLP significantly reduced lipid accumulation in 3T3-L1 adipocytes, concurrently regulating the expression of adipogenic genes. Notably, LPHK demonstrated significant improvements in high-fat (HF)-induced body weight gain, adipose tissue weight gain, liver weight gain, and reduced plasma concentrations of triglyceride, total-cholesterol, and LDL-cholesterol in C57BL/6J mice. Additionally, LPHK significantly regulated the expression of genes related to adipogenesis and anti-apoptosis. Moreover, while LPSLP significantly reduced liver weight, plasma triglyceride and LDL-cholesterol concentrations, and upregulated genes associated with hepatic fatty acid oxidation, it did not affect the body weight and adipose tissue weight gain. These results suggest that additional components other than SLP are necessary for the anti-obesity action of LPHK. In conclusion, non-viable plant-based LPHK can be used as a naturally occurring product for preventing obesity, especially in individuals with immunocompromised conditions.

1. Introduction

Obesity is a serious global health problem associated with the pathological hyperplasia or hypertrophy of adipose cells caused by excessive caloric intake, resulting in fat accumulation [1]. Naturally occurring products have been extensively explored to combat obesity, primarily due to the potential side effects of therapeutic drugs [2]. Recently, probiotics have become the most consumer oriented functional food products, offering several health beneficial properties, particularly against obesity [3–5].

Probiotics, as defined by the World Health Organization (WHO) in 2014, are "live microorganisms that when administered in adequate amounts confer a beneficial health effect on the host". More recently, as the next generation of probiotics, postbiotics have emerged. They are easy to store and prepare, ensuring safety for immunocompromised individuals while exhibiting anti-obesity efficacy [6–9]. Postbiotics comprise inanimate microorganisms and/or their components that confer health benefits on the host. Our previous studies have highlighted the anti-obesity effect of viable plant-food-derived probiotic *Lactobacillus curvatus* HY7601 (LC) and *Lactobacillus plantarum* (LP), in both animals and humans [4,10,11]. However, the anti-obesity potential of these heat killed lactic acid bacteria (LAB)s and postbiotics has yet to be studied.

S-layer proteins (SLP) are glycoproteins (25–71 kDa in MW) that make up the cell wall of microorganisms [12]. SLPs, known for their self-assembling properties, adhere to the intestinal wall of the host [13]. Previous studies have indicated that SLPs from LABs (*L. acidophilus, L. plantarum, L. rhamnosus, L. casei, Lentilactobacillus kefiri, and Clostridium difficile)* exhibited antimicrobial, anti-inflammatory, immunomodulatory, and anti-obesity properties [14–17]. These biological actions could potentially be mediated via pattern recognition receptors (PRRs) like Toll-like receptors (TLRs), which are associated with adipogenesis [18]. Several SLPs from *Lactobacillaceae* species have been characterized, including SlpA from *Lentilactobacillus kefiri*, *Levilactobacillus brevis*, and

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https://doi.org/10.1016/j.jafr.2024.100965
Received 19 November 2023; Received in revised form 18 December 2023; Accepted 1 January 2024
Available online 2 January 2024

L. acidophilus [19], AR326 Slp from *L. plantarum* [20]. In our previous study, we were the first to demonstrate the role of SLP as a key component in the anti-obesity activity of kefir-derived heat killed *L. kefiri* DH5 [17]. Thus, we hypothesized that the inhibition of adipogenesis by heat killed LAB (HLAB)s (LP and LC) may be conferred by the SLPs of HLABs.

This study aimed to investigate the anti-obesity effects of heat killed HLAB and their bioactive component, SLPs derived from LP and LC, in 3T3-L1 cells and HF-induced animal models.

2. Materials and methods

2.1. Preparation of heat-killed LABs

Lactobacillus curvatus HY7601 (LC) and *Lactobacillus plantarum* KY1032 (LP) previously derived from kimchi (Korea Yakult Co., Ltd., Gyeonggi, Korea), were cultured in Man-Rogosa-Sharpe (Difco Laboratories, Inc., Detroit, MI) broth under anaerobic conditions at 30 °C for 48 h. After culturing, the washed cells were adjusted to a concentration of 10^8 CFU/mL in a 0.85 % NaCl solution. The cell suspension was then heated at 70 °C for 30 min and freeze-dried before use.

2.2. Extraction of SLPs and SDS-PAGE

Bacterial cell pellets were obtained from the culture by centrifuging at 1100×*g* for 15 min at 4 °C. After washing twice with phosphate-buffered saline (PBS), the pellets were suspended in 5 M LiCl (Sigma-Aldrich, St. Louis, MO) and incubated in a shaking incubator for 1 h at 200 rpm following the method of Lortal et al. (1992). Suspension was centrifuged at 16,000×*g* for 30 min at 4 °C, and the resulting supernatant was filtered through a 0.45 μm pore size membrane. The filtered solution was dialyzed in PBS containing 0.05 % Tween 20 (Sigma-Aldrich) using a cellulose membrane (Spectra/Por Dialysis membrane MWCO 6000–8000 kDa, Spectrum Inc.). The amount of protein in the dialyzed SLP extract was quantified by a Bradford assay (Sigma-Aldrich) and stored in a −80 °C deep freezer before use.

The extracted SLP was weighed and suspended in sterile water and treated with sonication on ice (5 min, 5 s on, 30 s off, AMPL 30 %). The supernatant was obtained by centrifugation at 9000×*g* at room temperature. The concentration of the extracted SLP solution was quantified by a Bradford assay (Sigma-Aldrich). SDS-PAGE was used to analyze the SLP extract on a 4–20 % polyacrylamide gel, and Coomassie brilliant blue staining was used to visualize the protein bands.

2.3. 3T3-L1 adipocytes culture and differentiation

5×10^4 cells/mL of mouse 3T3-L1 preadipocytes purchased from Korea Cell Line Bank (Seoul, Korea) were grown in a growth medium (high-glucose DMEM, 10 % BS, and 1 % P/S) in a 12-well plate. After two days, the growth medium was replaced and was allowed to become confluent. Post-confluence, designated as day 0, the medium was changed to a differentiation medium (DMEM, 10 % FBS, 1 % P/S, MDI cocktail: 0.5 mM IBMX, 1 μm DEX, and 10 μg/mL insulin solution). After two days, a fresh medium was introduced, and after four days, the differentiated preadipocytes were grown in a maturation medium (high-glucose DMEM, 10 % FBS, 1 % P/S, and 10 μg/mL insulin solution). LPHK and LCHK were added from day 0 to day 6 at a concentration of 1×10^8 CFU/ml, while LPCHK was added at a 1:1 ratio of LPHK to LCHK. LPSLP (7.5 μg/mL), LCSLP (7.5 μg/mL), and LPCSLP (1:1 mixture of LPSLP and LCSLP) were added from day 0 to day 6.

Once removed from the culture medium, the cells were treated with 10 % formaldehyde solution for 1 h at room temperature. The solution was removed and washed twice with PBS. Next, the ORO stock solution, prepared at a concentration of 3 mg/mL, was dissolved in 100 % 2-propanol and stirred for 24 h. The ORO stock solution was diluted with sterile water at a ratio of 6:4 and filtered to prepare a 60 % ORO solution. The resulting solution was added to the cells and left for 10 min. The stained cells were observed under an optical microscope, and the stained oil red O dye was dissolved in 100 μL of isopropyl alcohol solution per well on a 96-well plate. The content of intracellular fat accumulation was determined by measuring the absorbance at 480 nm, and the percentage of intracellular lipid accumulation was determined as $(OD_{sample}/OD_{control}) \times 100$.

To evaluate the viability of cells against LAB, an MTT assay was performed as previously described, which was slightly modified [21]. Cells were placed in a 96-well plate at 5.5×10^4 cell/mL and cultured for 24 h (D_0). Samples were treated for 48 h (D2). It was prepared by dissolving MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich, USA) in PBS at a concentration of 5 mg/mL. The prepared MTT reagent was treated with the cells and incubated for 4 h. The formed formazan crystals were dissolved in 150 μL of dimethyl sulfoxide (DMSO, Daejung, Chemical Co., Seoul, Korea) and incubated at room temperature for 15 min. The optical density (OD) was obtained by measuring the absorbance of wells at 570 nm using plate readers (BioTek, USA).

2.4. Animals and diets

C57BL/6J mice (male, 4 weeks old) supplied by Samtako Bio Korea (Osan, Korea) were housed under the following conditions: room temperature of 21–25 °C, humidity of 50 %, and 12 h light/dark cycles. The study was conducted according to the institutional and national guidelines for animal care and approved by the Animal Experiment Ethics Committee (#19031ET1). After a week of acclimation to a chow diet (LabDiet #5001, PMI International, Redwood, CA), the mice ($n = 10$/group) were randomly divided into the following six groups: HF diet (HFD)-saline group (Saline), HFD-LPHK group (120 mg of heat-killed LP/kg body weight, LPHK), HFD-LCHK group (120 mg of heat-killed LC/kg body weight, SDH5), and HFD-LPSLP group (120 mg of LP SLP/kg body weight, LPSLP), and HFD-LCSLP group (120 mg of LC SLP/kg body weight, LCSLP), and Garcinia extract (ES Ingredients, Gyeonggi, Korea, 400 mg/kg body weight, positive control group) subjected to treatment for 8 weeks. The HLABs were prepared from LC and LP after mass culture at 37 °C for 48 h. LABs were heat killed at 70 °C for 30 min. 1×10^{10} CFU/mL culture of LP and LC were lyophilized. Before administration to the animals, the HLAB were rehydrated with sterilized distilled water at room temperature for 0.5–1 h. The HLABs were orally administered at 10 mL/kg body weight. The saline groups were administered by gavage at 10 mL of saline/kg body weight. The composition of macronutrients and total dietary fiber was balanced across the diets (37 %, 17 %, and 46 % calories derived from carbohydrates, protein, and fat, respectively). Microcrystalline cellulose, demonstrating little effect on sterol metabolism, was applied to balance total dietary fiber content (5 %) across the diets. Food intake and body weight were recorded twice a week.

2.5. Sample collection and plasma lipid analysis

Mice were fasted for 12 h and then anesthetized using a vaporizer (VetEquip, Livermore, CA) containing 4 % isoflurane (Phoenix Pharmaceutical, St. Joseph, MO). Blood samples were collected using a cardiac puncture method with a potassium EDTA solution (15 % w/v). Tissues were obtained and frozen in liquid nitrogen for further analysis. Adipose and liver tissues were fixed in 10 % formalin, followed by staining with hematoxylin and eosin. The plasma was obtained and centrifuged at 3000×*g* for 15 min. Plasma total cholesterol (T-CHO), triglycerides (TG), HDL cholesterol (HDL), and LDL cholesterol (LDL) concentrations were determined using a biochemical automatic analyzer (HITACHI3100, Tokyo, Japan).

Table 1
PCR primer sequence used in gene expression analysis.

Genes		Sequences	
36B4	forward	TCTAGGACCCGAGAAGACCTC	
	reverse	GTTGTCAAACACCTGCTGGAT	
Cpt-1	forward	AGCACACCAGGCAGTAGCTT	(Wang et al., 2013)
	reverse	AGGATGCCATTCTTGATTCG	
Fabp4	forward	AAGAAGTGGGAGTGGGCTTTG	(Weisberg et al., 2006)
	reverse	CTGTCGTCTGCGGTGATTTC	
Fasn	forward	AGCACTGCCTTCGGTTCAGTC	(Nie et al., 2017)
	reverse	AAGAGCTGTGGAGGCCACTTG	
Bcl-2	forward	TCTGGTTGGGATTCCTACGG	(Li et al., 2020)
	reverse	AGGAGGGTTTCCAGATTGGG	
Ppar γ	forward	AACTCTGGGAGATTCTCCTGTTGA	(Zhang et al., 2016)
	reverse	GAAGTGCTCATAGGCAGTGCAT	
Ppar α	forward	ATGGTGGACACGGAAAGCC	(Ju et al., 2019)
	reverse	CGATGGATTGCGAAATCTCTTGG	

Bcl-2, B-cell lymphoma 2; *Cpt-1,* Carnitine palmitoyltransferase 1*; Fabp4*, Fatty Acid Binding Protein 4;*Fasn*, ; *Ppar γ*, Peroxisome Proliferator-Activated Receptor gamma; *Ppar α*, Peroxisome Proliferator-Activated Receptor alpha.

2.6. Real-time quantitative (RT)-PCR analysis

RNA was extracted using the TRIzol Plus RNA Purification Kit (Invitrogen, Life Technologies, Carlsbad, CA, USA). The concentration and purity of the extracted RNA were measured using a ND-1000 spectrophotometer (NanoDrop, Wilmington, USA). The RNA was then mixed with the PrimeScript RT Reagent Kit (Takara Biotechnology, Japan) to synthesize the cDNA. Next, the synthesized cDNA was diluted tenfold and mixed with SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). The analysis was performed using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories). The amplification conditions were 95 °C for 30 s, followed by 40 cycles of incubation at 94 °C for 15 s, and 55–60 °C for 1 min. Target gene expression related to fatty acid metabolism and anti-apoptosis was normalized using a housekeeping gene, 36B4, and the gene expression level was calculated using the $^{\Delta\Delta}$CT method. The target gene primers used in the experiment are shown in Table 1.

2.7. Statistical analysis

SPSS Version 25.0 was used to perform the statistical analysis (SPSS

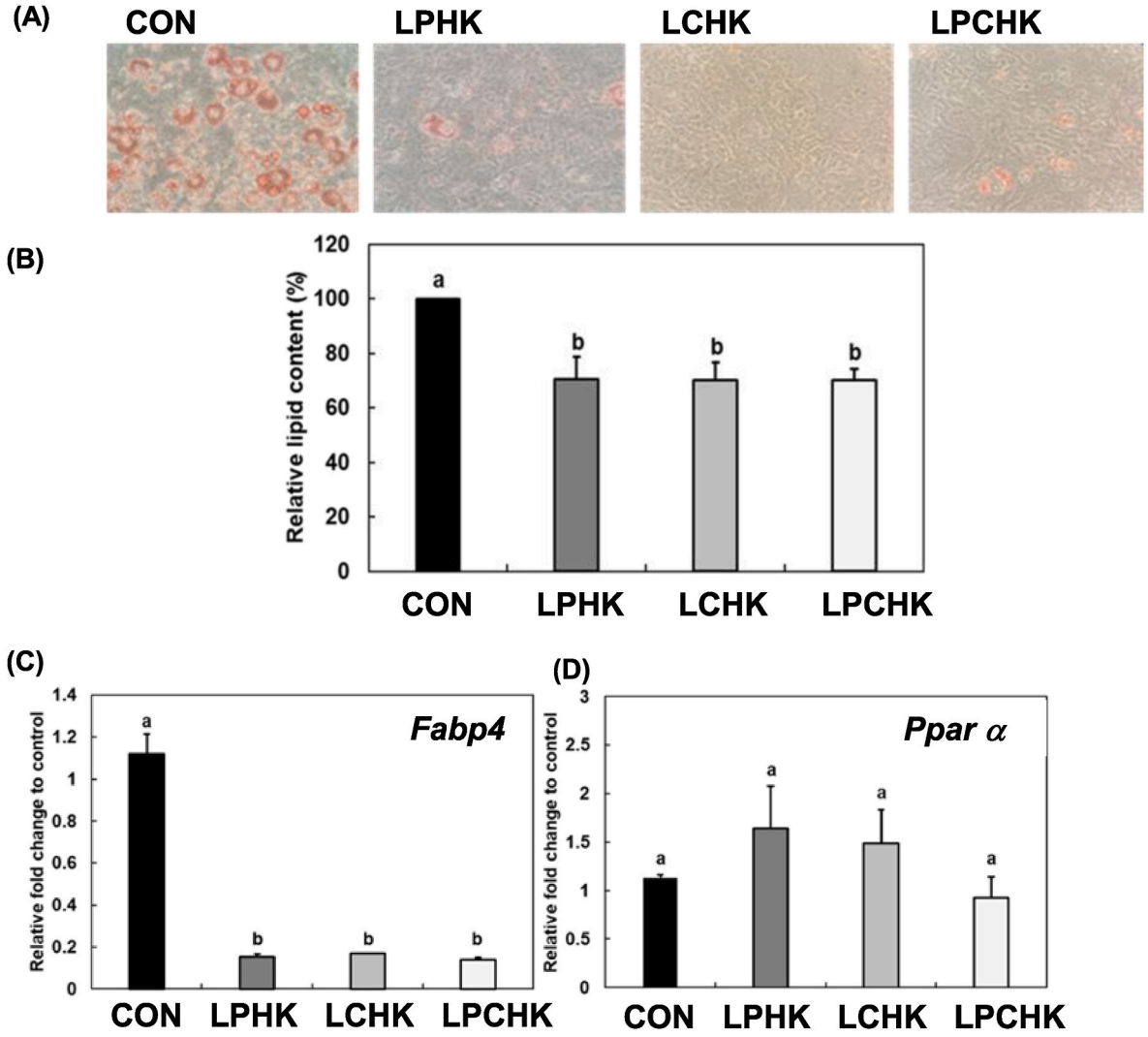

Fig. 1. Inhibition effects of non-viable heat-killed LABs (LPHK, *Lactobacillus plantarum*, LPHK, and *Lactobacillus curvatus*, LCHK, and LPCHK) on relative lipid content in 3T3-L1 adipocytes after differentiation (day 6). Oil red O staining was observed (A) microscopically and (B) colorimetrically (Abs at 540 nm). Effect of non-viable heat-killed LABs (LPHK, LCHK, and LPCHK) on gene expression levels related to adipogenic differentiation (*Fabp4*) (C) and oxidation of fatty acid (*Ppar*-α) (D). Values are presented as percentages relative to the values of the untreated control (CON). Data are expressed as mean ± SEM ($n = 6$). Mean values with different letters are significantly different at $p < 0.05$.

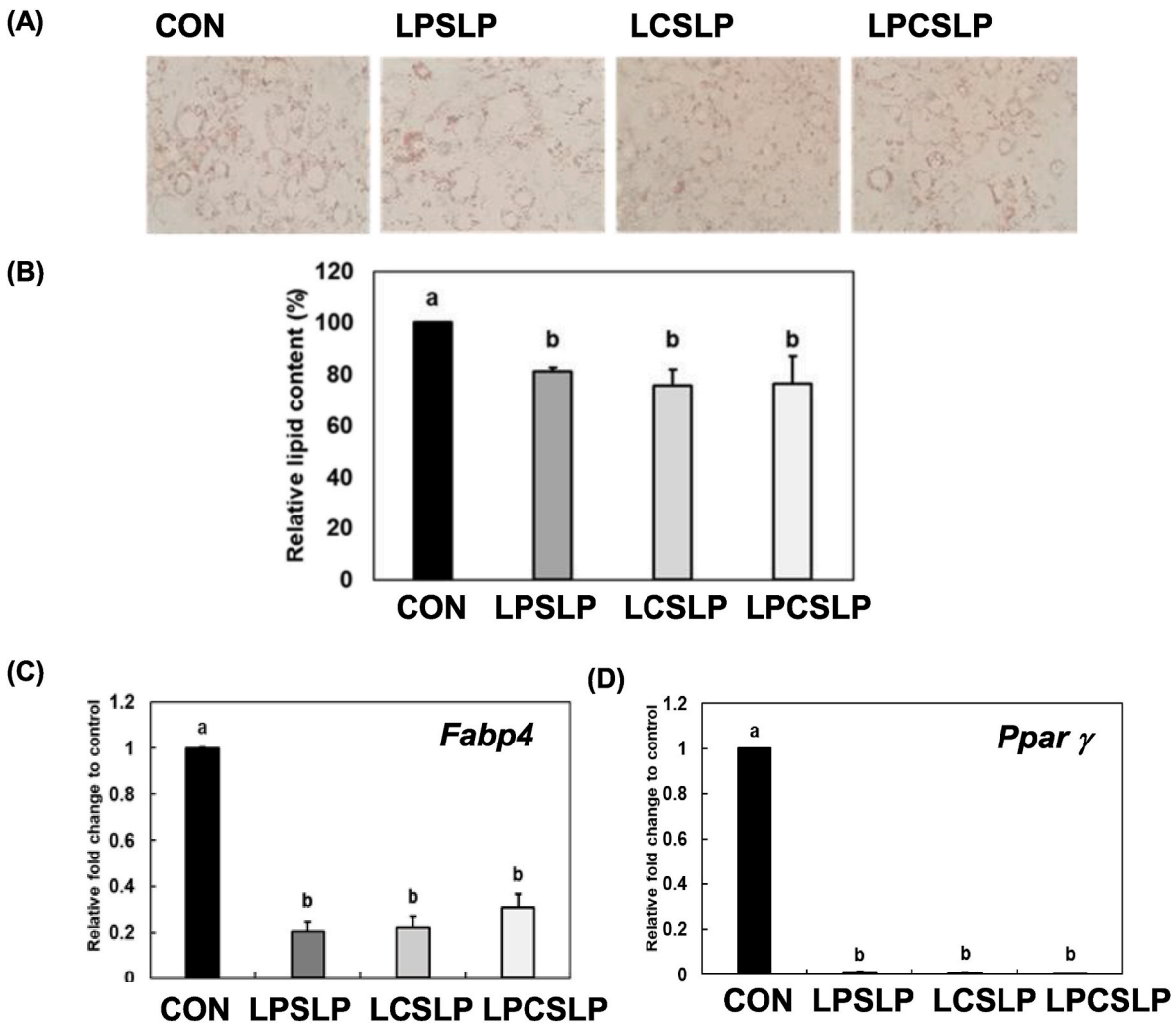

Fig. 2. Inhibition effects of cellular surface layer proteins (SLP)s derived from LP and LC in 3T3-L1 adipocytes after differentiation (day 6). Oil Red O staining was investigated (A) microscopically and (B) colorimetrically (Abs at 540 nm). Levels of gene expression related to adipogenic differentiation (*Fabp4* (C) and *Ppar* γ (D)) are shown. Values are presented as percentages relative to the values of the untreated CON. Data are expressed as mean ± SEM ($n = 6$). Mean values with different letters are significantly different at $p < 0.05$.

Inc., Chicago, IL, USA). The data were analyzed using a one-way analysis of variance (ANOVA) followed by Duncan's multiple range test and expressed as the mean standard error of the mean (SEM). A *p*-value of <0.05 was considered statistically significant.

3. Results

3.1. Effect of heat killed LABs on lipid accumulation in 3T3-L1 cells

To investigate the effect of HLABs against obesity, the inhibitory activities of heat-killed LPHK and LCHK on lipid accumulation in 3T3-L1 cells were determined using oil red O staining. The viability of 3T3-L1 cells treated with LPHK and LCHK at 1×10^8 CFU/mL was found to be greater than 90 % compared with that of the CON group (data not shown). LPHK (1×10^8 CFU/mL), LCHK (1×10^8 CFU/mL), and LPCHK (a combination of LPHK:LCHK, 1:1 ratio) showed significant inhibition of lipid accumulation of 30 % compared to the CON (Fig. 1B). No significant synergistic effect was observed in the LPCHK group when a 1:1 mixture of LPHK and LCHK was treated (Fig. 1B).

LPHK, LCHK, and LPCHK significantly reduced the level of fatty acid binding protein 4 (*Fabp4*) gene by 80 % compared with the CON group (Fig. 1C). The expression of peroxisome proliferator-activated receptor α (*Ppar α*) was not significantly affected by the heat killed LABs (Fig. 1D).

3.2. Effect of HLAB-derived cellular components, SLPs, on lipid accumulation in 3T3-L1 cells

LPSLP and LCSLP, prepared from LP and LC, revealed the presence of a major band of approximately 37 kDa (Supporting Information Fig. 1), consistent with the previous finding in *L. plantarum* [20]. To explore the bioactive component in HLAB against lipid accumulation in 3T3-L1 cells, the inhibitory activities of SLPs isolated from LP KY1032 and LC HY7601 on lipid accumulation were investigated in 3T3-L1 cells. The viability of 3T3-L1 cells treated with LPSLPs and LCSLPs was greater than 80 % at concentrations of 7.5 μg/mL (Data not shown).

LPSLP (7.5 μg/mL), LCSLP (7.5 μg/mL), and LPCSLP (1:1 mixture of LPSLP and LCSLP) significantly inhibited lipid accumulation by 19 %, 24 %, and 24 %, respectively, compared to the CON group (Fig. 2B). No significant synergistic effect with a 1:1 mixture of LPSLP and LCSLP (Fig. 2B) was observed.

LPSLP and LCSLP significantly downregulated the levels of *Fabp4* and *Ppar γ* gene expression in 3T3-L1 adipocytes after differentiation (day 6) (Fig. 2C and D). There was no significant difference in the expression level of *Ppar α* (data not shown).

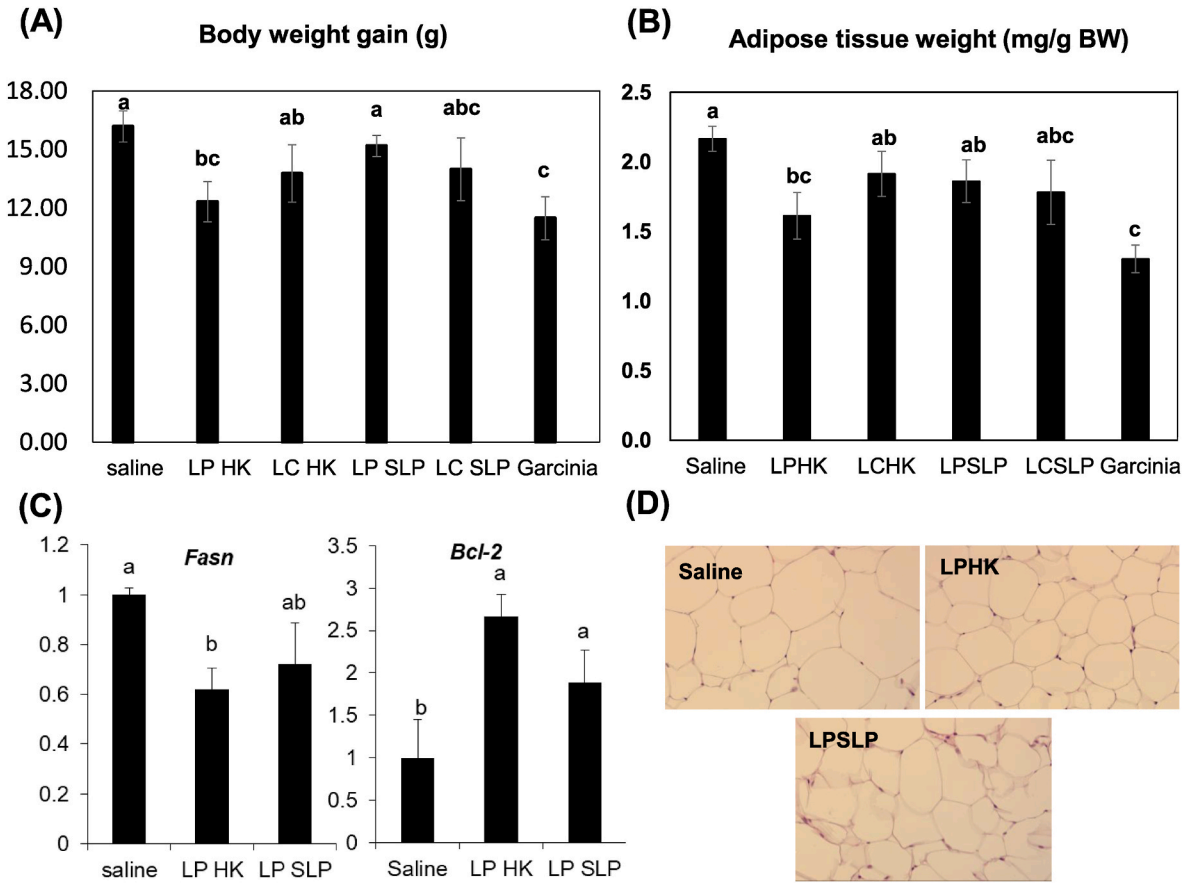

Fig. 3. Effect of non-viable heat-killed LABs and surface layer protein (SLPs) on C57BL/6J mice fed with a high-fat (HF) diet and orally administered with saline, LPHK (heat-killed *Lactobacillus plantarum*), LCHK (heat-killed *Lactobacillus curvatus*), SLPs (LPSLP and LCSLP) or Garcinia for 8 weeks. (A) Body weight gain (B) adipose tissue weight (C) expression of genes related to adipogenesis (*Fasn*) and anti-apoptosis (*Bcl-2*) (D) hematoxylin and eosin (H&E) staining of adipose tissue. Data are expressed as mean ± SEM; $n = 10$/group. Values not sharing a common letter differed significantly at $p < 0.05$.

3.3. Effect of heat killed LABs and SLP supplementation on body weight gain, organ weight, and metabolic parameters in high-fat diet (HFD)-Induced obese mice

Four-week-old male C57BL/6J mice orally administered LPHK and Garcinia cambogia for 8 weeks showed significantly lower body weight gain by 24 % and 29 %, respectively (Fig. 3A) while there was no significant difference in total energy intake among all groups (data not shown). LPHK and Garcinia significantly decreased adipose tissue weight by 27 % and 41 %, respectively, compared to the Saline group (Fig. 3B). The level of *Fasn* gene expression in adipose tissue was significantly down-regulated by 62 % and 39 in LPHK and LPSLP, respectively (Fig. 3C). Expression of *Bcl-2* gene in adipose tissue was significantly up-regulated by 267 % and 188 % in LPHK and LPSLP, respectively (Fig. 3C). Fig. 3D exhibits the difference in adipocyte size among the three groups using a hematoxylin & eosin (H & E) staining. Liver weights were significantly decreased by LPHK, LPSLP, and Garcinia, 18 %, 17 %, and 21 %, respectively, compared to the Saline group (Fig. 4A). Levels of *PPAR α and Cpt-1* gene expression in the liver were significantly up-regulated in LPHK and LPSLP (Fig. 4B). Fig. 4C shows the difference in the livers among the three groups using a H & E staining. Plasma ALT, triglyceride, LDL-C concentrations of LPHK and LPSLP were significantly reduced compared to the Saline group (Fig. 5). Plasma AST concentration was significantly lower by 68 % in LPSLP compared to that observed in the Saline group (Fig. 5A). LPHK significantly reduced Total-C concentration by 20 %, compared to the Saline group (Fig. 5E).

4. Discussion

Despite considerable attention given to research on heat killed probiotics, their mechanism and bioactive components remain unclear. Previously we demonstrated that a combination of two probiotic LABs isolated from a traditional fermented plant food, LP KY1032 and LC HY7601, induced anti-obesity effect on a diet-induced animal models [22]. In addition, the cellular membrane fraction of LP KY1032 and LC HY7601 inhibited adipogenesis in 3T3-L1 cells [23]. In the present study, we have shown that non-viable LPHK and LCHK, along with their cellular component SLPs, inhibited adipogenesis of 3T3-L1 cells. Interestingly, LPHK exhibited an ameliorative effect on HF-induced body weight, liver weight, adipose tissue weight gain, and plasma concentrations of triglyceride, total-C, and LDL-C in HF-induced obese C57BL/6J mice, which was not demonstrated by LCHK. Notable, SLP from LPHK did not affect HF-induced body weight gain but significantly reduced liver weight gain, plasma concentrations of triglyceride, ALT, AST, and LDL-C.

Consistent with present data, anti-obesity action of non-viable HLABs was partially mediated through a reduction in the expression of adipocyte genes related to fatty acid synthesis such as *Fasn, Fabp4, Ppar γ* [6]. It could be speculated that the anti-obesity action of LPHK is mediated by the TLR-related PPAR signaling pathways [24] TLR2/4 are implicated in anti-inflammation and immunomodulation in HepG2 cells [25], Caco-2 cells [26], and obese adipose tissue, and serve as pattern recognition receptors (PRRs) in response to LAB [27]. Activation of TLR2/4 by LPHK are possibly associated with the improvement of HF-induced obesity in animal models. Activation of TLR-pathway

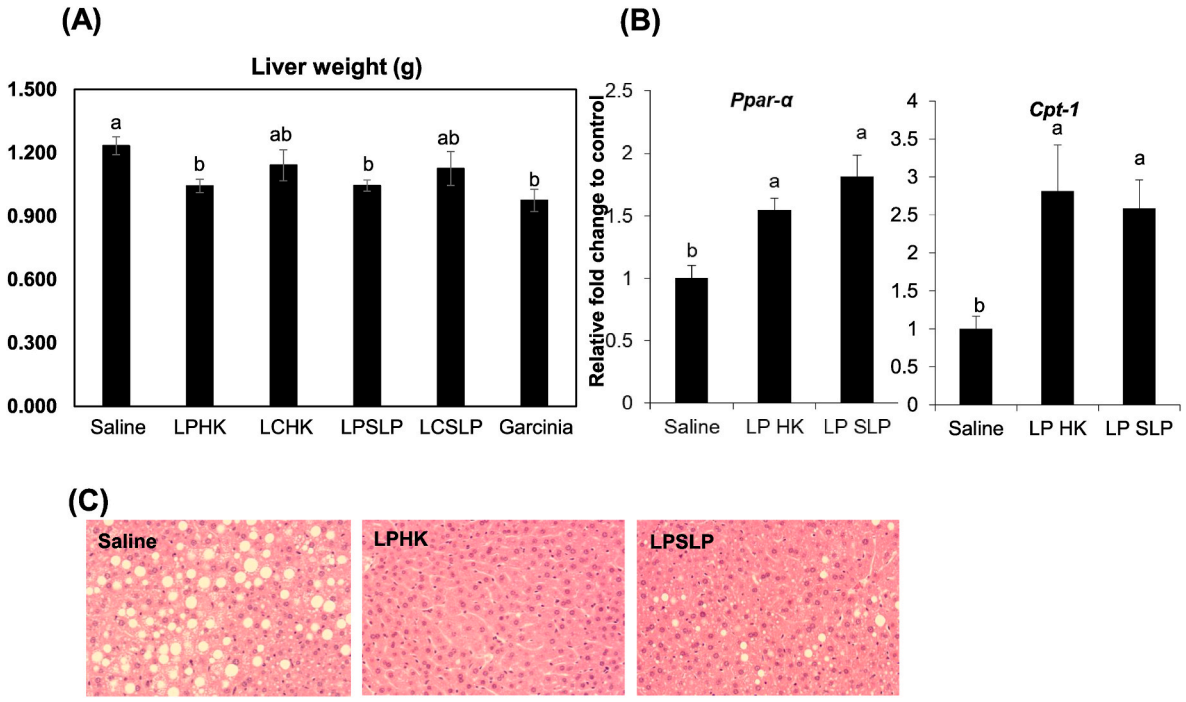

Fig. 4. Effect of non-viable heat-killed LABs and surface layer proteins (SLP)s on C57BL/6J mice fed with a high-fat (HF) diet and orally administered with saline, LPHK (heat-killed *Lactobacillus plantarum*), LCHK (heat-killed *Lactobacillus curvatus*), SLPs (LPSLP and LCSLP) or Garcinia for 8 weeks. (A) Liver weight (B) expression of genes related to fatty acid oxidation (*PPAR α*) and (*Cpt-1*) (C) hematoxylin and eosin (H&E) staining of liver tissue. Data are expressed as mean ± SEM; $n = 10$/group. Values not sharing a common letter differed significantly at $p < 0.05$.

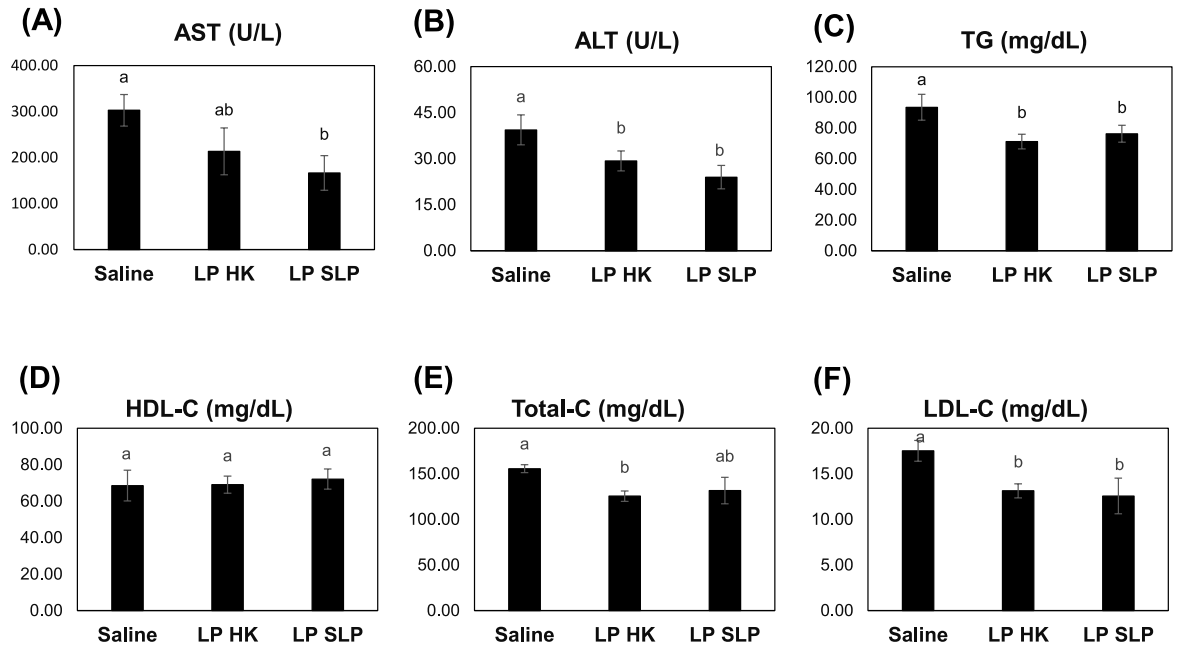

Fig. 5. Effect of non-viable heat-killed LAB and surface layer proteins (SLPs) on C57BL/6J mice fed with a high-fat (HF) diet and orally administered with saline, LPHK (heat-killed *Lactobacillus plantarum*), LPSLP for 8 weeks. Plasma concentration of (A) AST (B) ALT (C) TG (Triglyceride) (D) HDL-cholesterol (E) Total-cholesterol (F) LDL-cholesterol. Data are expressed as mean ± SEM; $n = 10$/group. Values not sharing a common letter differed significantly at $p < 0.05$.

suppresses *Ppar γ* expression [28], indicating a reduction of *Fabp4* expression is related to TLR activation via LPHK. Intriguingly, our study unveiled that non-viable LPHK significantly modulated the expression of *Ppar*-associated genes (*Fabp4*, *Ppar γ*, *Bcl-2*), suggesting their possible role in fatty acid synthesis and anti-apoptosis in adipose tissues. In our previous study, SLPs with a size of 70 kDa, derived from kefir LABs, decreased the proinflammatory response in macrophage RAW 264.7 cells and ameliorated HF diet-induced obesity and its associated disorders in animal model [17]. This anti-obesity action of Kefir SLPs could be mediated via activation of TLR2/4 [17]. In the current study, the SLPs prepared from LP and LC exhibited a size range of 25–71 kDa, primarily containing a 37 kDa protein component previously identified in

L. plantarum AR326, known for its immunomodulatory effects [20,29]. These SLPs from LP and LC significantly down-regulated the expression levels of *Fabp4* (a Ppar γ target gene associated with fatty acid synthesis by the transport, lipogenesis, absorption and storage of long chain fatty acids) and *Ppar* γ genes in the 3T3-L1 cells. However, administration of the SLPs to HF-fed mice did not affect the expression of *Fasn* (a Ppar γ target gene linked to fat deposit through the de novo synthesis of long chain fatty acids from acetyl-CoA and malonyl-CoA precursors in the adipose tissue while it was significantly down-regulated by administration of LPHK in HF-fed mice. This discrepancy might arise from distinct mechanisms of action between SLPs in *in vitro* adipocyte cells and adipose tissue, possibly related to the passage through the gastrointestinal tract. Cell wall capsular exopolysaccharides (EPSs) are additional candidate cellular components of HLABs that could have a biological function in anti-obesity efficacy [17,21]. Capsular EPS of *Lactobacillus rhamnosus* GG previously showed a reduction in adipogenesis through TLR2 signaling pathways in an animal model [30]. However, in our current study, the capsular EPS from LP and LC did not affect lipid accumulation in 3T3-L1 cells treated with capsular EPS (data not shown). These findings suggest the presence of additional components, distinct from SLPs and capsular EPSs, in non-viable LPHK that ameliorate HF-induced obesity.

Numerous studies have suggested a positive relationship between adipocyte apoptosis and the proinflammatory response [31]. In this study, administration of LPHK to HF-fed mice significantly up-regulated *Bcl-2* gene associated with anti-apoptosis, suggesting a potential role of LPHK in averting adipose tissue apoptosis and inflammation. The activation of TLR2 led to the increase of antiapoptotic gene, *Bcl-2* [32]. Taken together, action of LPHK to up-regulate *Bcl-2* gene is likely to be mediated through TLR-pathways [28].

In the present study, SLPs from LPHK reduced liver weight and plasma TG content, but did not show anti-obesity effect. These SLPs up-regulated the expression of *Ppar* α and *Cpt-1* genes related to fatty acid oxidation in the liver, indicating a potential decrease in hepatic lipid content. TLR-pathway can regulate PPAR α activity and inflammatory responses [33], and lipid metabolism. This suggests that LPSLPs modulate expression of *Ppar α* and *Cpt-1* via TLR activation in the liver, resulting in a decrease in liver weight and plasma TG concentration. In addition, these SLPs significantly reduced levels of plasma AST and ALT, markers for liver disease including NAFLD [34], suggesting a possible favorable role in the liver function. Supplementation of probiotics and HLAB ameliorated hepatic steatosis by reducing liver weight and lipid accumulation in western-diet fed animals and patients with NAFLD [35–42]. Interestingly, we had previously shown that SLP derived from Kefir DH5 LAB reduced body weight and adipose tissue weight in obese mice fed a HF diet but did not affect liver weight [17]. These findings exhibited a various biological function of SLPs based on the sources. Further research is needed to establish whether this diversity consequently leads to an improvement in NAFLD.

5. Conclusion

The anti-obesity action of non-viable HK LAB is species-specific and closely related to expression of adipogenic and anti-apoptic genes in adipose tissue. This study suggests that LPHK could be a potential functional food ingredient capable of mitigating obesity and obesity-related metabolic diseases. These anti-obesity effects appear to be mediated by components within LP that extend beyond SLPs, contributing to the inhibition of inflammation and adipogenesis in an animal model. Further research is warranted to identify the bioactive components in LPHK responsible for its anti-obesity action.

CRediT authorship contribution statement

Da Hye Yoon: Writing – original draft, Methodology, Formal analysis. **Joo Yun Kim:** Data curation, Conceptualization. **Heo Keon:** Conceptualization. **Hyeon Gyu Lee:** Writing – review & editing. **Kunho Seo:** Visualization, Validation, Project administration, Data curation, Conceptualization. **Jae-Jung Shim:** Writing – original draft. **Jung-lyoul Lee:** Writing – original draft. **Hyunsook Kim:** Writing – review & editing, Writing – original draft, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Data availability

No data was used for the research described in the article.

Acknowledgment

This work was supported by the grant from the National Research Foundation of Korea (NRF), funded by the Korean government (MSIP) (grant number, 2021R1A2C2006817).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jafr.2024.100965.

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