Nutrition 128 (2024) 112565

Contents lists available at ScienceDirect

Nutrition

journal homepage: www.nutritionjrnl.com

Applied nutritional investigation

Lactobacillus plantarum NCHBL-004 modulates high-fat diet–induced weight gain and enhances GLP-1 production for blood glucose regulation

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ARTICLE INFO

Article History:
Received 1 May 2024
Received in revised form 27 June 2024
Accepted 16 August 2024

Keywords:
Lactobacillus plantarum NCHBL-004
Obesity
Short-chain fatty acids
GLP-1
Liver lipid metabolism

ABSTRACT

Objectives: This study investigated the therapeutic potential of *Lactobacillus plantarum* NCHBL-004 (NCHBL-004) in the treatment of obesity and associated metabolic disorders.
Methods: Mice were fed either a normal diet (ND) or a high-fat diet (HFD) with oral administration of NCHBL-004. After euthanasia, blood, liver and adipose tissue were collected. Furthermore, the microbiome and short-chain fatty acids (SCFAs) were analyzed from feces.
Results: Oral administration of live NCHBL-004 to mice fed a HFD resulted in notable reductions in weight gain, improvements in glucose metabolism, and maintenance of balanced lipid levels. A comparative analysis with other *Lactobacillus* strains highlighted the superior efficacy of NCHBL-004. Moreover, heat-killed NCHBL-004 demonstrated beneficial effects similar to those of live NCHBL-004. Additionally, administration of live NCHBL-004 induced glucagon-like peptide 1 (GLP-1) production and increased the levels of short-chain fatty acids (SCFAs), including acetate and propionate, in feces, positively influencing liver lipid metabolism and mitigating inflammation. Consistent with this, analysis of the gut microbiome following NCHBL-004 administration showed increases in SCFA-producing microbes with increased proportions of *Lactobacillus* spp. and a significant increase in the proportion of microbes capable of promoting GLP-1 secretion.
Conclusions: These findings underscore the potential of both live and inactivated NCHBL-004 as potential therapeutic approaches to managing obesity and metabolic disorders, suggesting avenues for further investigation and clinical applications.
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Abbreviations: ACE, abundance-based coverage estimator; ALT, alanine aminotransferase; AMPK, AMP-activated protein kinase; ANOVA, one-way analysis of variance; AST, aspartate aminotransferase; BA, butyric acid; DE, diethyl ether; EI, electron impact; ELISA, enzyme-linked immunosorbent assay; eWAT, epididymal white adipose tissue; GLP-1, glucagon-like peptide 1; HDL, high-density lipoprotein; HFD, high-fat diet; LDL, low-density lipoprotein; NAFLD, non-alcoholic fatty liver disease; NCHBL-003, *Lactobacillus kunkeei* NCHBL-003; NCHBL-004, *Lactobacillus plantarum* NCHBL-004; ND, normal chow diet; OGTT, oral glucose tolerance test; PCoA, principal coordinate analysis; QIME2, quantitative insights into microbial ecology; SAF, steatosis, activity, and fibrosis; SAT, subcutaneous WAT; SCFAs, short-chain fatty acids; SFN, sulforaphane; SIM, selected ion monitoring; TC, total cholesterol; TG, triglyceride; VAT, visceral WAT

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https://doi.org/10.1016/j.nut.2024.112565
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Introduction

Probiotics are emerging as a promising approach for treating obesity by regulating metabolic pathways, gut microbiota, and various physiological mechanisms [1]. In particular, specific probiotic strains, such as *Lactobacillus plantarum*, have the potential to reduce body weight, improve lipid metabolism, and enhance glucose tolerance in high-fat diet (HFD)–fed mice [2]. Furthermore, probiotics alter gut microbiota composition, favoring beneficial bacteria while decreasing harmful ones, suggesting their role in restoring microbial balance and mitigating obesity-related complications [3]. Probiotics have the potential to modulate various physiological mechanisms, including the regulation of hormones such as glucagon-like peptide-1 (GLP-1), leading to improved insulin sensitivity and influencing pathways related to appetite control and energy metabolism [4]. These findings collectively implicate probiotics as a novel approach to tackling obesity and the associated metabolic dysregulation.

The efficacy of probiotics in obesity treatment extends to specific mechanistic actions involving butyrate, AMP-activated protein kinase (AMPK), and GLP-1. Short-chain fatty acids (SCFAs), including acetate, propionate, and butyrate—which are byproducts of probiotic metabolism—play vital roles in regulating metabolism and improving glucose tolerance [5]. Additionally, probiotics induce the transcriptional activation of genes related to fatty acid β-oxidation, inhibit fatty acid synthesis, activate AMPK, and stimulate GLP-1 production, contributing to weight loss and regulation of lipid metabolism [6]. These specific mechanistic actions underscore the potential of probiotics to address obesity and its metabolic consequences through the modulation of key regulatory pathways.

Studies have also indicated that probiotics, including *Lactobacillus* spp., positively affect liver health and alleviate complications associated with chronic liver disease. For instance, *L. plantarum* improves liver function and reduces damage in mice with nonalcoholic fatty liver disease (NAFLD) [7–9], whereas *L. rhamnosus* GG mitigates liver inflammation and enhances liver function in mice with alcoholic liver disease [10]. Furthermore, probiotics can regulate lipid metabolism and reduce fat accumulation in the liver and adipose tissue. Specific strains, such as *Bifidobacterium bifidum* and *L. acidophilus*, show promise in reducing insulin resistance and preventing fat accumulation, suggesting their potential for improving liver health [11].

Moreover, probiotics exert a positive influence on blood glucose regulation through various mechanisms, including enhanced insulin sensitivity, modulation of gut microbiota composition, anti-inflammatory properties, and effects on gut peptide GLP-1 secretion [12]. Specific probiotic strains have the potential to limit lipid accumulation and promote lipolysis, indicating anti-adipogenic activities that contribute to improved glucose control [13–15]. Studies administering *L. acidophilus* to diabetic rats and supplementing *B. lactis* B94 to overweight and obese adults have demonstrated improved glucose tolerance and insulin sensitivity, highlighting the potential role of probiotics in managing metabolic disorders such as diabetes [16,17].

The use of heat-killed bacteria, specifically heat-killed *L. plantarum* LRCC5314, has shown promise in promoting butyric acid (BA)-producing bacteria, including *Akkermansia* spp., in the mouse gut microbiome [18]. This is associated with protective effects against stress-related type 2 diabetes, suggesting that postbiotics may directly impact lipid metabolism and overall metabolic health. Additionally, inactivated *L. brevis* YM1301 has demonstrated significant efficacy as a postbiotic in combating obesity and regulating blood glucose levels. *L. brevis* YM1301 exhibits hypoglycemic effects and improves insulin sensitivity in mice with type 2 diabetes mellitus, positioning it as a safer and more stable alternative to live bacteria [19]. However, further in-depth research on these mechanisms is crucial for developing obesity treatments or blood glucose regulators using postbiotics, including inactivated bacteria.

In this study, we demonstrated the significant potential of live *L. plantarum* NCHBL-004 (NCHBL-004) to mitigate obesity-related effects. The administration of NCHBL-004 led to reduced weight gain, improved adipose tissue characteristics, and favorable regulation of glucose, triglycerides (TGs), cholesterol, and liver function, surpassing the efficacy of other *Lactobacillus* strains. Both live and heat-killed NCHBL-004 (HK NCHBL-004) showed anti-obesity effects, suggesting promising interventions for managing metabolic disorders. Significant increases in the composition of the GLP-1–promoting metabolite SCFAs and secondary bile acid-producing gut microbiota further emphasize the potential benefits of NCHBL-004. This study's findings position NCHBL-004 and potentially other probiotics and postbiotics as valuable candidates for innovative strategies to address obesity and promote metabolic health.

Materials and methods

Bacterial preparation

To isolated NCHBL-004 (KCTC 14909BP) and *L. kunkeei* NCHBL-003 (NCHBL-003, KCTC 14908BP), honeybees were collected in Gwangju, Republic of Korea, and their midguts were homogenized in PBS (Corning, NY, USA). The homogenates were spread on MRS agar plates and incubated under anaerobic conditions at 37°C for 3 days. The isolated bacteria were identified by 16s rRNA sequencing using universal bacterial primer (27F: 5'-GAGTTTGATCCTGGCTCAG-3' and 1492R: 5'-GGTTACCTTGTTACGACTT—3'). For species identification, the homology of the 16s rRNA gene sequences was compared using the BLAST database on NCBI. Finally, the strains were designated as NCHBL-004 and NCHBL-003. Cell pellets of NCHBL-004 or NCHBL-003 were collected, washed, and suspended in PBS (Corning, NY, USA) for the treatment of mice. To prepare heat-killed NCHBL-004, the cell suspension was boiled in a water bath at 100°C for 30 min, then lyophilized, diluted in PBS to the desired concentration, and administered to mice. Live and heat-killed NCHBL-004 were provided by NodCure, Inc. (Gwangju, Korea).

Animal experiments

Seven-week-old male C57BL/6 mice were obtained from Koatech (Pyeongtaek, South Korea). Mice were maintained on a normal chow diet (ND, 5L79; Orient Bio, Inc., Seongnam, Korea) and had ad libitum access to water. They were housed in an animal facility under controlled conditions, including a temperature of 21 ± 1° C, 40-60% relative humidity, and a 12-h light-dark cycle. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Kangwon National University (Approval No. KW-231212-1) and the IACUC of Chonnam National University (Approval No. CNU-IACUC-YB-2021-89). Following a 1-week acclimatization period, the mice were weighed and randomly divided into four groups to evaluate the anti-obesity effect of NCHBL-004, and six groups were established for a comparison study between *Lactobacillus* spp., specifically, NCHBL-003 and NCHBL-004—isolated from honeybees. Oral administration of the experimental materials commenced simultaneously with either a HFD (60% kcal from fat) or a ND (10% kcal from fat). The vehicle (PBS) and *Lactobacillus* spp. were administered by oral gavage five times per week for 12 and 16 weeks, respectively. The sulforaphane (SFN) or fenofibrate (Feno) used as positive control [20,21]. The group assignments for the two experiments were as follows: Experimental 1: 1) ND (n = 7), 2) HFD (n = 7), 3) HFD + SFN (30 mg/kg; n = 7), and 4) HFD + NCHBL-004 (1×10^9 CFU/mouse; n = 7). Experiment 2: 1) ND (n = 10), 2) HFD (n = 10), 3) HFD + NCHBL-003 (1×10^7 CFU/mouse; n = 10), and 4) HFD + NCHBL-004 (1×10^7 CFU/mouse; n = 10). To confirm the anti-obesity effect of heat-killed NCHBL-004, 4-week-old male ICR mice were randomly divided into five groups after 1 week of acclimation: 1) ND (n = 7), 2) HFD (n = 7), 3) HFD + Feno (2 mg/kg; n = 7), 4) HFD + HK NCHBL-004L (1×10^8 CFU/mouse; n = 7), and 5) HFD + HK NCHBL-004H (1×10^9 CFU/mouse; n = 7).

Weight of liver and adipose tissue

Mice were fasted overnight, anesthetized with isoflurane inhalation, and euthanized by cervical dislocation. The liver, epididymal white adipose tissue (eWAT), visceral WAT (VAT), and subcutaneous WAT (SAT) were dissected and weighed. Half of each tissue was fixed in 10% neutral buffered formalin for

histological analysis, while the remaining tissue was stored at −80°C for real-time PCR analysis.

Histological analysis

The liver and eWAT were fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin for histological analysis. The liver histology score was evaluated according to the steatosis, activity, and fibrosis (SAF) scoring system, which includes the assessment of the number and size of liver lipid droplets, infiltration of inflammatory cells, and extent of hepatocyte ballooning [22].

Measurement of liver TG content

Liver tissue was homogenized in 1 ml of a 5% NP-40 solution. Subsequently, the lysed tissue was heated to 80−100°C in a water bath for 5 min and then cooled down to room temperature. The lysed tissue was centrifuged to obtain lipid extracts. Hepatic TG levels were measured using a commercial TG assay kit (Abcam, Cambridge, UK).

Oral glucose tolerance test and the insulin tolerance test

Blood samples were collected from the tail vein of the mice, and blood glucose levels were measured using Accu-Chek (Roche Diagnostic, Mannheim, Germany). For the oral glucose tolerance test (OGTT), mice were fasted overnight, and then D-glucose (2 g/kg; Sigma-Aldrich, St. Louis, MO, USA) was administered orally. Blood glucose levels were measured at 0, 15, 30, 60, 90, 120, and 180 min after glucose administration. For the insulin tolerance test, mice were fasted for 6 h, and then insulin (0.75 U/kg, Sigma-Aldrich) was injected intraperitoneally. Blood glucose levels were measured 0, 15, 30, 60, 90, 120, and 150 min after insulin injection. Glucose and insulin tolerance were determined by measuring the area under the curve.

Serum analysis

Serum levels of TG, total cholesterol (TC), low-density lipoprotein (LDL), high-density lipoprotein (HDL), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured using a Fujifilm Dri-Chem 3500i (Fujifilm, Kanagawa, Japan). Serum adiponectin levels were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Microbiome analysis

The fecal samples collected were preserved at −80°C until DNA extraction. Genomic DNA (gDNA) samples were extracted from fecal samples using a commercial DNA isolation kit, QIAamp Fast DNA Stool Mini Kit (QIAGEN, Germany). The V4 region of the bacterial 16S rRNA gene of the gDNA samples was amplified using barcoded forward primers (515F: GTGCCAGCMGCCGCGGTAA) and a reverse primer (806R: GGACTACHVGGGTWTCTAAT). The V4 hypervariable region of the 16S rRNA gene was amplified for the microbial community analysis. Quality control and analysis of microbiome sequencing data were performed using the Quantitative Insights into Microbial Ecology (QIIME2) pipeline. The ASVs were identified using the SILVA database (version 138). The α-diversity (Shannon, Simpson, and abundance-based coverage estimator [ACE]) and β-diversity (unweighted UniFrac) were analyzed using QIIME. The R software package was used to construct microbial composition graphs. α-Diversity plots were generated using GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA), and principal coordinate analysis (PCoA) plots were developed using the complimentary Plotly Make Chart program (Montreal, Canada). Sequencing reads were deposited in the NCBI short read archive under the accession number PRJNA1083183.

SCFA analysis

The mice were divided into two groups: the control group (n = 11) and the NCHBL-004-treated group (n = 11). Mice in the control group were orally administered PBS, whereas mice in the NCHBL-004-treated group were orally administered a mixture of heat-killed NCHBL-004 at a concentration of 1×10^9 CFU per mouse daily for 3 consecutive days. Fecal samples (30 mg) were mixed with 300 μL of water and homogenized for 5 min at 40 oscillations per second in a TissueLyser (TissueLyser LT, Qiagen, Hilden, Germany) using beads. After centrifugation for 10 min at 13,000 × g and 4°C, 200 μL of supernatant (fecal homogenate) was transferred to a new microtube containing 2 μL of 1 M hydrochloric acid to adjust the pH of the fecal solution to 2. To extract the SCFAs from the acidified fecal homogenate, 200 μL of diethyl ether (DE) was added, vortexed, and centrifuged for 5 min at 10,000 × g at 4°C. The supernatant (90 μL) was transferred into a polypropylene vial, and 9 μL of BSTFA+TMCS and 10 μL of internal standard (100 μg/mL menthol in DE) were added. The mixture was placed in a vial, capped tightly, and incubated at 70°C for 30 min. The samples were then left for 2 h at room temperature and loaded with GC-MS. The derivatized samples were analyzed using an Agilent GC-MS instrument (7890A-5975C) with an HB-5 ms capillary column (30 m × 250 μm × 0.25 μm film thickness). The carrier gas was helium (99.999% purity), with a flow rate of 1.0 mL/min. The injector, ion source, and quadrupole temperatures were 190°C, 230°C, and 150°C, respectively. The sample injection volume was 1 μL, and the split ratio was 10:1. The initial oven temperature was maintained at 30°C for 3 min, ramped to 120°C at a rate of 15°C/min, and held for 1 min. Then, it was ramped to 250°C at a rate of 50°C/min, with a final hold for 3 min. The total run time was 15.6 min. Ionization was performed in electron impact (EI) mode at 70 eV. The analytes were quantified in selected ion monitoring (SIM) mode, and the target ions (m/z) of acetic, propionic, butyric acid, and menthol (internal standard) were 117, 131, 145, and 123, respectively.

Chemical and reagents

Acetic acid and hydrochloric acid were purchased from Junsei Chemicals (Tokyo, Japan). Menthol, used as an internal standard, and propionic acid were purchased from TCI (Tokyo, Japan). Butyric acid (BA) and N, O-bis (trimethylsilyl) trifluoroacetamide with trimethylchlorosilane (containing 1% TMCS and 99% BSTFA) was purchased from Sigma-Aldrich Co. DE was purchased from Daejung (Seoul, South Korea). High-performance−grade water as a replacement for Omni-Solv pure water was purchased from J. T. Baker (Avantor Performance Materials Inc., Center Valley, PA, USA).

Plasma GLP-1 analysis

Sitagliptin (10 mg/kg; MedChemExpress, NJ, USA) was orally administered to fasted mice 1 h before oral glucose (2 g/kg) treatment [23]. Blood samples were collected from the retro-orbital plexus at 0 (n = 3), 5 (n = 5), and 10 (n = 3) min after glucose administration for GLP-1 analysis. Blood samples were immediately transferred to EDTA tubes supplemented with 5 μL of 100 mM sitagliptin [24], then centrifuged at 4000 rpm for 10 min. The plasma was stored at −20°C. Plasma active GLP-1 levels were measured using an active GLP-1 ELISA kit (ALPCO, NH, USA). The assays were performed according to the instructions in the active GLP-1 ELISA kit manual.

Crypt isolation and intestinal organoid culture

The mouse intestine was cut into 1−2 cm segments, which were then opened longitudinally. Villi and mucus were removed using curved forceps by scratching. The cut intestinal segments were washed multiple times with ice-cold PBS and then incubated in Cell Recovery Solution (Corning) at 4°C for 30 min. The crypts were scraped off using curved forceps and centrifuged at 800 rpm for 5 min. The crypts were washed three times with Advanced DMEM/F12 (Gibco, Grand Island, NY, USA), pipetted using a 200 μL tip, and then centrifuged at 1250 rpm for 5 min. The centrifuged crypts were resuspended in Matrigel (Corning) and seeded in prewarmed 24-well plates. After 30 min of incubation at 37°C, 500 μL of organoid culture medium (Advanced DMEM/F12 contained with P/S (Invitrogen), Glutamax (Invitrogen), and HEPES (Invitrogen) with 50% L-WRN Media (Advanced DMEM/F12 contained with 20% FBS (Gibco) and P/S) supplemented with B27 (Invitrogen), N2 (Invitrogen), N-acetylcysteine (Sigma-Aldrich), mEGF (Invitrogen), Y-27632 (Abmole, Houston, TX, USA)) was added. Organoids were cultured at 37°C by changing the culture medium every 2 days.

Intestinal organoid treatment

To assess the GLP-1−inducing effects of NCHBL-004, intestinal organoids were co-cultured with NCHBL-004. Intestinal organoids were treated with live NCHBL-004 at concentrations of 1×10^8 CFU/well and 1×10^9 CFU/well in the medium. After 48 h, the intestinal organoids were incubated in the cell recovery solution for 30 min, followed by real-time PCR analysis.

Real-time PCR analysis

Total mRNA was extracted from liver or intestinal organoids using TRIzol (Ambion, Austin, TX, USA). Total mRNA (1 μg of total mRNA was synthesized using ReverTra Ace qPCR RT Master Mix (TOYOBO Bio-Technology, Osaka, Japan). Real-time PCR was performed to detect the gene expression of C/ebpα, Pparγ, Srebp1, Fabp4, Acc, Il-1β, Tnf-α, Il-6, and Mcp-1 using the CFX Connect Real-Time PCR System (Bio-Rad, Hercules, CA, USA) with a two-step procedure (40 cycles at 95°C for 10 s and then at 60°C for 30 s). The primers used for qPCR were as follows: mPparγ forward: 5′- CAAGCCCTTTACCACAGTTGA-3′, reverse: 5′- CAGGTTCTACTTTGATCGCACTT -3′; mSrebp1 forward: 5′- AACCAGAAGCTCAAGCAGGA-3′, reverse: 5′- TTTCATGCCCTCCATAGACA -3′; mC/ebpα forward: 5′- AAACAACGCAACGTGGAGA -3′, reverse: 5′- GCGGTCATTGTCACTGGTC -3′; mFabp4 forward: 5′- AATCACCGCAGACGACAG -3′, reverse: 5′- ACGCCTTTCATAACACATTCC -3′; mAcc forward: 5′- GGTCTTCGAGTGGATTGGCA -3′, reverse: 5′- ATCCCTTTCCCTCCTCCTCC -3′; mIl-1β

forward: 5′- CAACCAACAAGTGATATTCTCCATG -3′, reverse: 5′- ATCCACACTCTC-CAGCTGCA -3′; mTnf-α forward: 5′- CACCACCATCAAGGACTCAA -3′, reverse: 5′-AGGCAACCTGACCACTCTCC -3′; mIl-6 forward: 5′- GCTACCAAACTGGATATAAT-CAGGA -3′, reverse: 5′- CCAGGTAGCTATGGTACTCCAGAA -3′; mMcp-1 forward: 5′- AAGCTTCCCGTTCTCAG -3′, reverse: 5′- ACCTGAGGTTCTAAGAGCAGAGAGTC -3′; mGcg forward: 5′-: AAGAGGAACCGGAACAACATTG -3′, reverse: 5′-GCCCTCCAAGTAAGAACTCACA -3′; mβ-actin forward: 5′-: AGGCCCAGAGCAAGA-GAG -3′, reverse: 5′- TCAACATGATC TGGGTCATC -3′; mGapdh forward: 5′-: TGTGTCCGTCGTGGATCTGA -3′, reverse: 5′- CCTGCTTCACCACCTTCTTGAT-3′

Statistical analysis

Data are presented as mean ± SD and were analyzed using the two-tailed Student's t-test. To compare multiple groups, we performed one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. Statistical analyses were performed using GraphPad Prism 8. Results with $p < 0.05$ were considered statistically significant.

Results

Oral administration of live NCHBL-004 attenuates weight gain in mice subjected to a high-fat diet

We investigated the influence of live NCHBL-004 on body weight gain in mice exposed to a 12-week HFD. Eight-week-old wild-type C57BL/6 male mice received daily oral doses of live NCHBL-004 starting from the onset of HFD feeding. SFN, a naturally occurring isothiocyanate found in cruciferous vegetables such as broccoli, Brussels sprouts, and cabbage, was employed as a positive control, known for its potential in treating obesity, improvement of glucose metabolism, and insulin sensitivity [25]. Weekly body weight monitoring revealed reduced weight gain in mice treated with live NCHBL-004 compared to the vehicle-treated control group during the 12-week HFD period (Fig. 1A and B) and ultimately reduced VAT (Fig. 1C). Live NCHBL-004 treatment markedly reduced hepatic lipid accumulation in HFD-induced obese mice (Fig. 1D and E). Likewise, liver weight and hepatic TG levels, which were elevated by HFD, significantly decreased with NCHBL-004 administration (Fig. 1C and F). Analysis of eWAT demonstrated positive effects on adipocyte size, indicating potential improvements in adipose tissue characteristics (Fig. 1G and H). Thus, live NCHBL-004 mitigated weight gain, reduced liver weight, regulated hepatic TG levels, and positively affected adipocyte size in mice fed a HFD.

Glucose tolerance tests were performed to assess glucose regulation, and various biochemical markers associated with lipid metabolism and liver function, including total TG, TC, LDL, HDL, ALT, and AST, were measured. HFD feeding led to increased levels of TG, TC, LDL, HDL, ALT, and AST, along with induction of glucose intolerance (Fig. 1I–P). The administration of live NCHBL-004 regulated glucose levels, leading to a significant reduction in TG, TC, AST, and ALT levels that were elevated by the HFD. However, no significant impact was observed on the LDL and HDL levels that were increased by HFD. Additionally, the decreased serum adiponectin levels induced by the HFD were restored by NCHBL-004 administration (Fig. 1Q). These findings suggest a potential mitigating influence of live NCHBL-004 on the adverse effects induced by HFD consumption.

To investigate the dose-dependent anti-obesity effects of NCHBL-004, mice were orally administered two different doses (10^8 or 10^9 CFU/mouse) for 8 wk. During the experimental period, the increased body weight and eWAT and SAT weights induced by HFD decreased in a dose-dependent manner with NCHBL-004 administration (Supplementary Fig. S1A-C).

Next, we compared the anti-obesity effects of NCHBL-004 with those of other *Lactobacillus* spp. Administered alongside the HFD, live NCHBL-004 was associated with significant reductions in body weight and SAT weight (Fig. 2A–C). However, NCHBL-003 did not yield significant differences except for the glucose response (Fig. 2A–F). Furthermore, comprehensive assessments of glucose and insulin responses reinforced the favorable impact of NCHBL-004 on blood glucose levels and insulin sensitivity in the obese mouse model, highlighting its superior efficacy in mitigating obesity-related effects (Fig. 2D–F). Histological analysis of the liver and adipose tissue indicated a reduction in fat accumulation, suggesting distinctive influences on hepatic and adipose tissue metabolism associated with NCHBL-004 (Fig. 2G-I). In conclusion, NCHBL-004 demonstrated robust potential for alleviating obesity-related effects, outperforming NCHBL-003. This encompasses reductions in body weight gain, improvements in glucose metabolism and insulin sensitivity, and distinctive influences on liver and adipose tissue characteristics within the experimental framework.

Heat-killed NCHBL-004 demonstrates anti-obesity effects, mirroring live NCHBL-004 benefits

Subsequent investigations sought to assess the efficacy of inactivated NCHBL-004 in addressing weight gain and metabolic disorders associated with obesity, as demonstrated using live NCHBL-004. The administration of heat-killed NCHBL-004 resulted in significant mitigation of body weight gain, liver weight, and eWAT weight, accompanied by a decrease in adipocyte size (Fig. 3A–D). Moreover, it displayed the ability to maintain glucose tolerance, indicating the potential of inactivated bacteria to regulate glucose and manage body weight (Fig. 3E and F). As a positive control, fenofibrate, recognized for its cholesterol- and TG-lowering effects, was used to treat obesity and metabolic diseases associated with fatty liver under HFD conditions [26]. Overall, our findings suggest that both heat-killed and live NCHBL-004 contribute to the mitigation of weight gain in the context of a HFD. Furthermore, heat-killed NCHBL-004 contributed to the regulation of metabolic parameters, including TG, TC, and LDL, while restoring ALT and AST levels in HFD-fed mice (Fig. 3G–L). In light of these results, this study underscores the potential of heat-killed NCHBL-004 as a promising intervention for the treatment of obesity and associated metabolic disorders.

Measuring SCFAs and microbiome changes in the feces of mice treated with NCHBL-004

To determine the effect of NCHBL-004 administration on gut microbiota composition in mice, we performed a gut microbiota composition change analysis. Analysis of the gut microbiota composition in fecal samples after 3 d of oral administration of NCHBL-004 showed changes in composition at the phylum, family, and genus levels (Fig. 4A–C). When comparing the abundance of microbial composition, the Shannon's and diversity indices did not change, but the ACE index significantly increased due to NCHBL-004 administration (Fig. 4D and E). To compare the similarity between the groups, PCoA was performed using weighted UniFrac and Bray-Curtis as the distance matrices, which showed that significant clusters were formed between each group (Fig. 4F and G).

We investigated the influence of NCHBL-004 on the gut microbiota and its subsequent effects on the regulation of host metabolism. The concentration of SCFAs in fecal samples was analyzed from mice orally administrated NCHBL-004 for three consecutive days. Our results revealed a significant increase in the levels of acetic acid and propionic acid, but not butyric acid, in the feces of mice treated with NCHBL-004 compared to those in the group administered PBS (Fig. 4H–J). This suggests that NCHBL-004 administration positively affects the production of SCFA by the gut

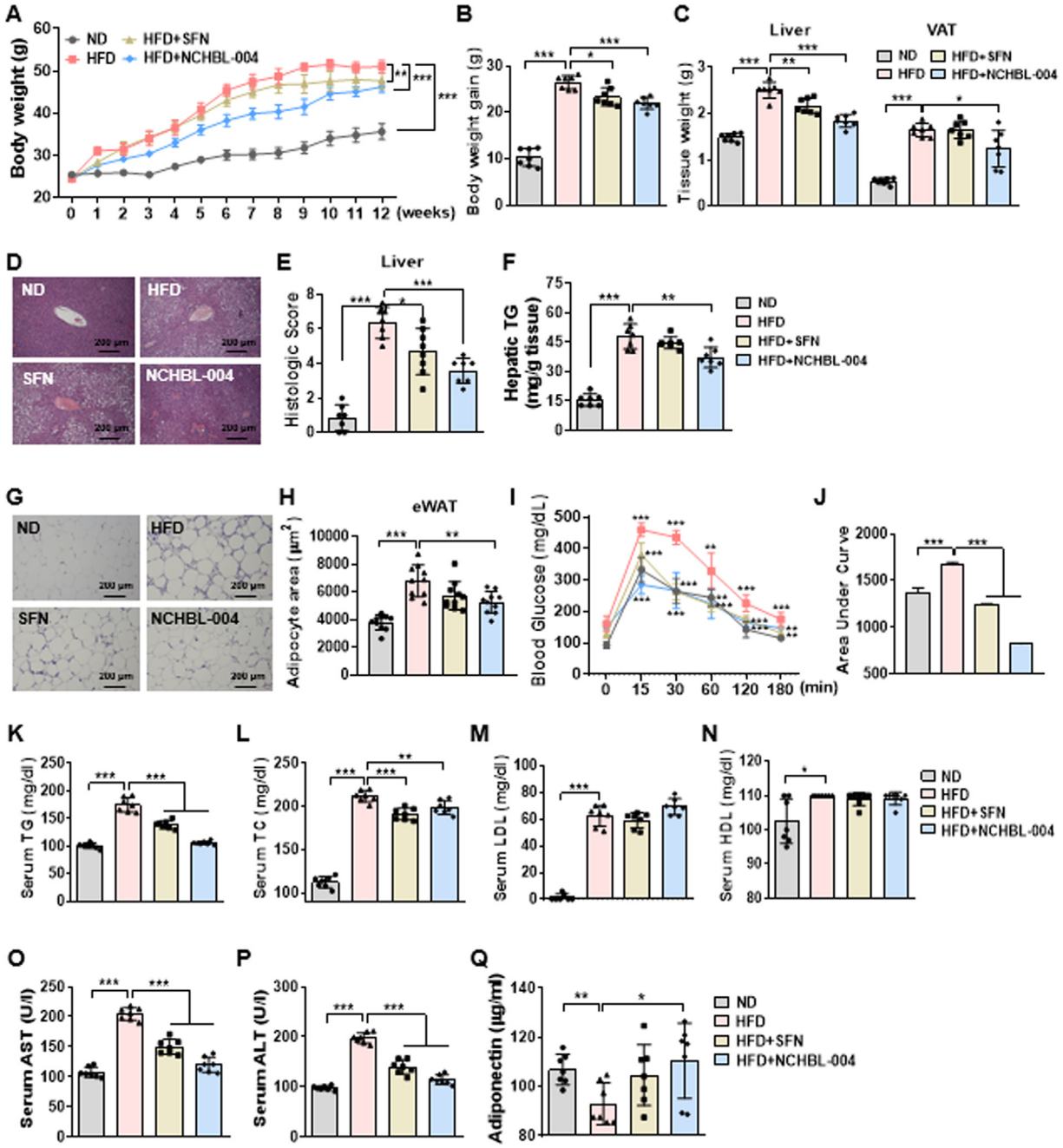


Fig. 1. *L. plantarum* NCHBL-004 supplementation improves fat accumulation, blood glucose tolerance, lipid profile, and adiponectin levels in high-fat diet–induced obese mice. (A and B) Body weight change and body weight gain of mice over 12 wk. (C) Liver and VAT weight. (D) H&E staining image of liver tissue. (E) Histological score of liver tissue. (F) Hepatic TG level. (G) H&E staining image of eWAT tissue. (H) Histological score of eWAT tissue. (I and J) Oral glucose tolerance test and area under the curve (AUC). (K–Q) Serum levels of TG, TC, LDL, HDL, AST, ALT, and adiponectin. Data are presented as mean ± standard deviation (n = 7). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. ND, normal diet group; HFD, high-fat diet group; HFD + SFN, high-fat diet treated with sulforaphane; HFD + NCHBL-004, high-fat diet treated with *L. plantarum* NCHBL-004; VAT, visceral adipose tissue; TG, triglyceride; TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

microbiota, highlighting its potential role in modulating host metabolism.

This is consistent with microbiome analysis showing an increase in SCFA-producing microbes. Predictably, the analysis of the gut microbiome of mice treated with NCHBL-004 showed a significant increase in the proportion of *Lactobacillus* spp. (Fig. 4K). In addition, further analysis was conducted on the composition of microbes known to produce SCFAs, including *Akkermansia, Bacteroides, Bifidobacterium, Eubacterium, Ruminococcus, Roseburia,* and *Lactobacillus* spp., as well as the composition of secondary BA-producing microbes, including *Bacteroides, Bifidobacterium, Clostridium,* and *Lactobacillus* spp [27,28]. The administration of NCHBL-004 significantly increased the composition of SCFAs and secondary BA-producing microbes (Fig. 4L–N). These results were also confirmed in the HFD model. The same analysis of the gut microbiota composition in the model where high concentrations of NCHBL-004 were administered along with the HFD revealed changes in composition at the phylum, class, and genus levels

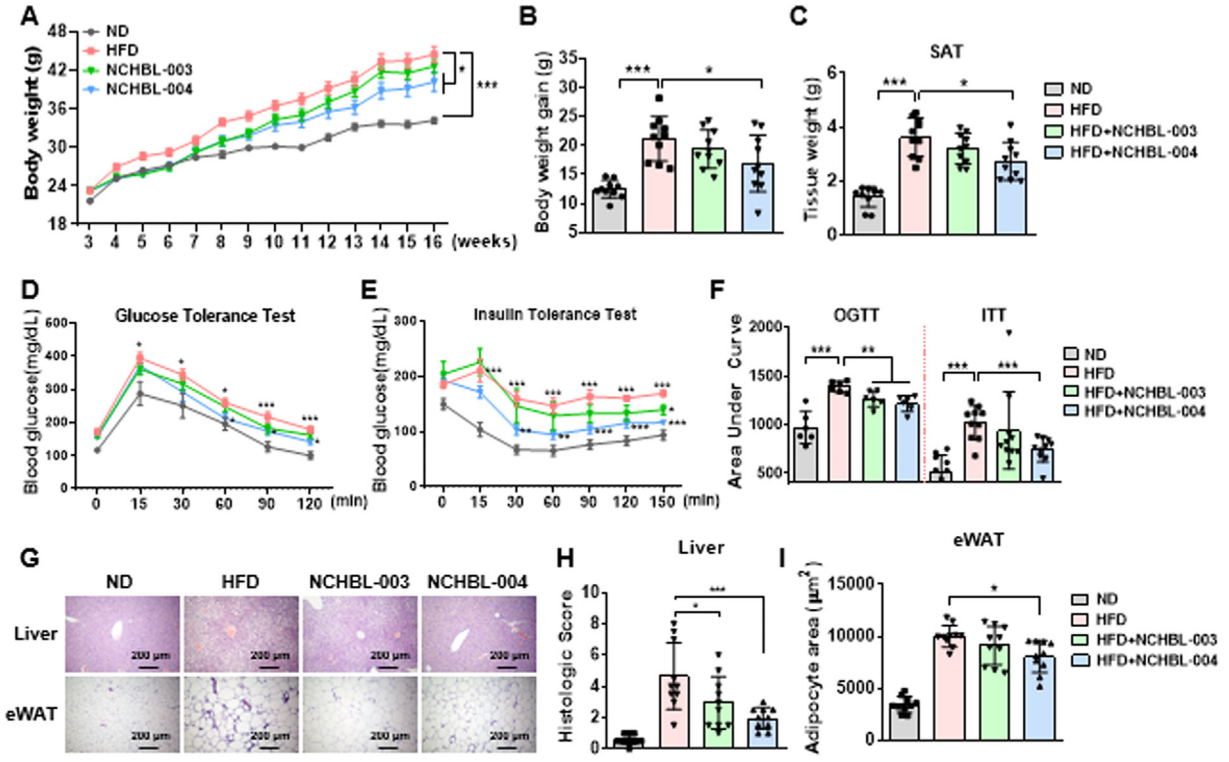


Fig. 2. The anti-obesity effect of *L. kunkeei* NCHBL-003 or *L. plantarum* NCHBL-004 in high-fat diet–induced obese mice. (A and B) Body weight change and body weight gain of mice over 16 wk. (C) SAT weight. (D–F) Oral glucose tolerance test, insulin tolerance test, and area under the curve (AUC). (G) H&E staining images of liver tissue and eWAT tissue. (H) Histological score of liver. (I) Adipocyte size. Data are presented as mean ± standard deviation (n = 10). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. ND, normal diet group; HFD, high-fat diet group; HFD + NCHBL-003, high-fat diet treated with *L. kunkeei* NCHBL-003 (1×10^7 CFU/mouse) group; HFD + NCHBL-004, high-fat diet treated with *L. plantarum* NCHBL-004 (1×10^7 CFU/mouse) group; SAT, subcutaneous adipose tissue; eWAT, epididymal white adipose tissue.

(Fig. 5A–C). When comparing the abundance of the microbial composition, the Shannon and ACE indices were significantly reduced by the HFD compared to the RD, and in this case, NCHBL-004 administration did not restore it (Fig. 5D and E). However, when PCoA was performed using weighted UniFrac and Bray-Curtis as distance matrices independent of abundance, we found that the NCHBL-004 treatment group became relatively closer to the RD group (Fig. 5F and G). In line with the same prediction, we found that HFD caused a decrease in SCFA-producing strains or secondary bile acid-producing strains, including *Lactobacillus* spp., but NCHBL-004 administration significantly improved this effect (Fig. 5H–K). This further suggests that the administration of NCHBL-004 may also increase SCFAs and secondary BAs in the HFD model.

Regulation of hepatic lipid metabolism and anti-obesity effects through NCHBL-004–induced GLP-1 activation

GLP-1 is an incretin hormone that enhances insulin secretion for blood glucose control, suppresses appetite, aids in weight management, and is regarded as a valuable tool for obesity treatment and blood glucose regulation in type 2 diabetes [12]. Recent studies have revealed that gut microbiota metabolites stimulate GLP-1 secretion from enteroendocrine L-cells through mediators Gcg), a GLP such as SCFAs and secondary BAs [29]. To evaluate the direct effect of NCHBL-004 on GLP-1 secretion, normal mice were orally administered live NCHBL-004 at a concentration of 1×10^9 CFU/mouse for three consecutive days. After an overnight fast, mice were administered oral glucose, and blood samples were collected at different time points for plasma-active GLP-1 analysis. The results showed a significant increase in active GLP-1 levels in the NCHBL-004-treated group compared to the control group administered PBS, particularly 5 min after oral glucose administration (Fig. 6A). Additionally, we assessed the mRNA transcription level of proglucagon (-1 encoding gene, and observed that it was significantly increased in heat-killed NCHBL-004-treated intestinal organoids compared with vehicle-treated organoids (Fig. 6B).

Next, we measured the parameters of lipid metabolism and inflammation in the liver. In HFD-fed obese mice, there was an increase in the transcription levels of lipogenesis-associated genes (Pparγ, Srebp1, C/ebpα, Fabp4, and Acc) in the liver (Fig. 7A–E), but C/ebpα, Fabp4, and Acc genes were downregulated by live NCHBL-004 treatment (Fig 7C–E). Additionally, the administration of NCHBL-004 mitigated the elevated levels of pro-inflammatory cytokine genes (Il-1β, Tnf-α, and Il-6) as well as the chemokine gene Mcp-1 in HFD-fed mice (Fig. 7F–I). These observations highlight the potential of *L. plantarum* NCHBL-004 in modulating hepatic gene expression and attenuating inflammation, further supporting its beneficial effects on hepatic lipid metabolism.

Discussion

Obesity is a significant global public health concern associated with various health problems, including type 2 diabetes, cardiovascular disease, and certain cancers [30]. Effective strategies for obesity management are crucial for improving public health outcomes. In this study, we focused on the regulation of body weight gain and glucose levels using NCHBL-004 in HFD-driven obesity. Notably, oral administration of heat-killed NCHBL-004 and live NCHBL-004 reduced weight gain and improved blood glucose

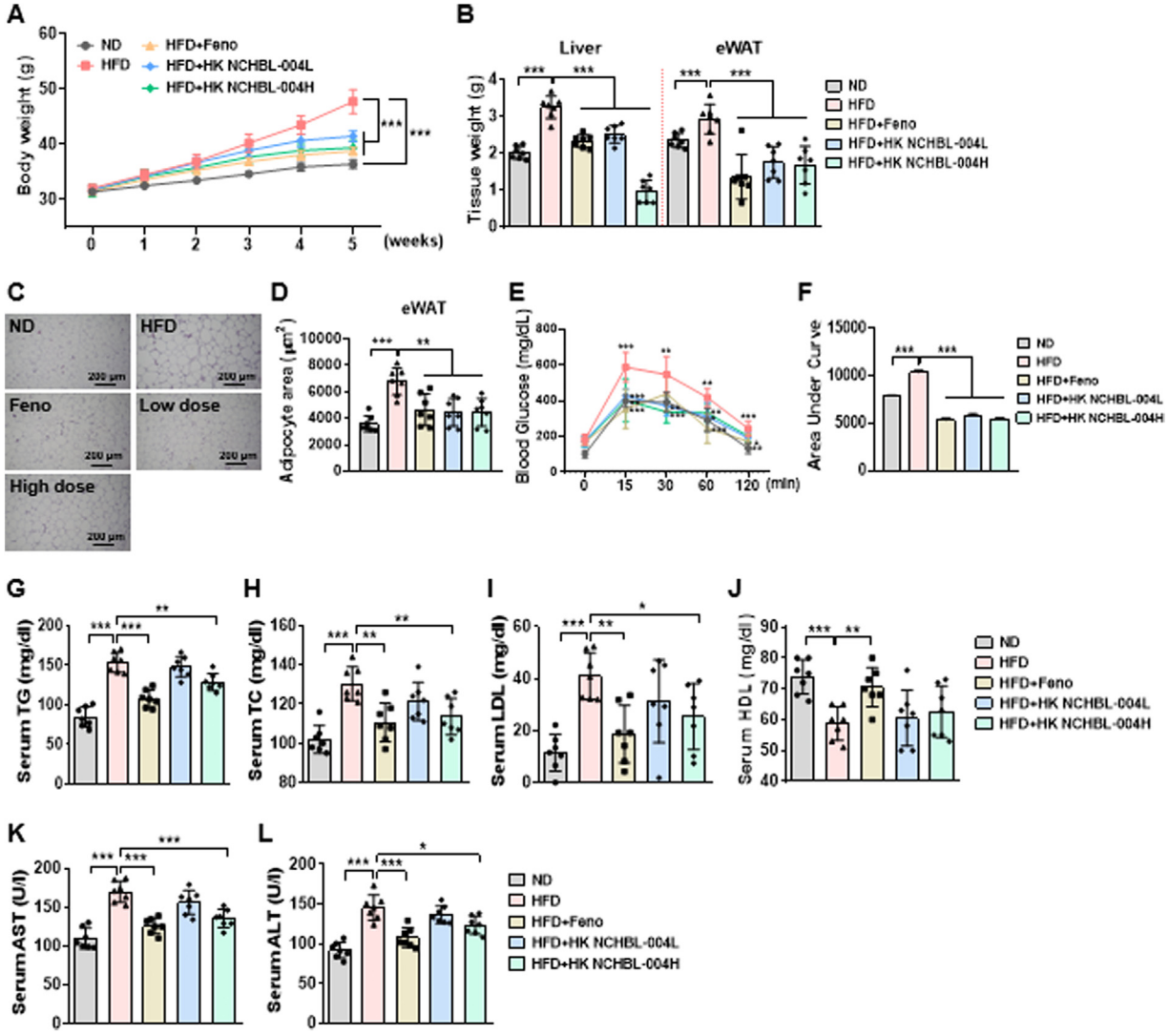


Fig. 3. Anti-obesity effect of heat-killed *L. plantarum* NCHBL-004 in high-fat diet–induced obese mice. (A) Body weight change in mice over 5 wk. (B) Liver and eWAT weights. (C) H&E staining image of eWAT tissue. (D) Adipocyte size. (E and F) Oral glucose tolerance test and area under the curve (AUC). (G–L) Serum levels of TG, TC, LDL, HDL, AST, and ALT. Data are presented as mean ± standard deviation (n = 7). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. ND, normal diet group; HFD, high-fat diet group; HFD + Feno, high-fat diet treated with fenofibrate; HFD + HK NCHBL-004 10^8 or 10^9, high-fat diet treated with 1×10^8 CFU/mouse or 1×10^9 CFU/mouse of heat-killed *L. plantarum* NCHBL-004; eWAT, epididymal white adipose tissue; TG, triglyceride; TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

regulation. Intriguingly, mice subjected to daily oral administration of live NCHBL-004 for 3 days exhibited a significant increase in GLP-1 production when glucose was orally administered after an overnight fast. This suggests that NCHBL-004 may have a direct stimulatory effect of GLP-1 production. Additionally, it is possible that NCHBL-004 influences the gut microbiome composition, which could enhance GLP-1 production. Gut microbiome analysis revealed a significant increase in *Lactobacillus* spp. in mice treated with NCHBL-004, which is unsurprising. However, there was a corresponding increase in the levels of other bacterial species that are known to produce SCFAs. Meanwhile, some gut microbiota metabolites promote GLP-1 production, most notably by SCFAs and secondary BAs [12]. SCFAs promote GLP-1 production by binding to the GPR43 receptor on L cells in the gut, while secondary BAs promote GLP-1 production by binding to TGR55 [12]. Therefore, we analyzed microbes known to produce SCFAs and secondary BAs [27,28]. The results showed that the percentage of bacteria known to contain SCFAs and secondary BAs was significantly increased by NCHBL-004 administration. These results support our hypothesis that changes in microbiome composition may promote the production of GLP-1.

GLP-1 plays a crucial role in regulating glucose metabolism and has various beneficial effects, including reducing hepatic fat accumulation, improving hepatic lipid metabolism, and stimulating glucose-dependent insulin production and secretion [31]. GLP-1 has a direct anti-lipogenic effect on the liver, leading to reduced hepatic fat accumulation and improved hepatic lipid metabolism by modulating the expression of lipogenic enzymes and promoting fatty acid oxidation [32]. Additionally, GLP-1 stimulates glucose-dependent insulin production and secretion via specific receptors expressed on islet β-cells, which is known as the incretin effect [31]. GLP-1 analogs are widely used as efficient anti-diabetic drugs because of their ability to increase insulin secretion and improve glucose metabolism. In addition, many studies have demonstrated that probiotics show an anti-diabetic effect in *db/db* mice by increasing SCFA-producing bacteria, improving intestinal barrier function, and

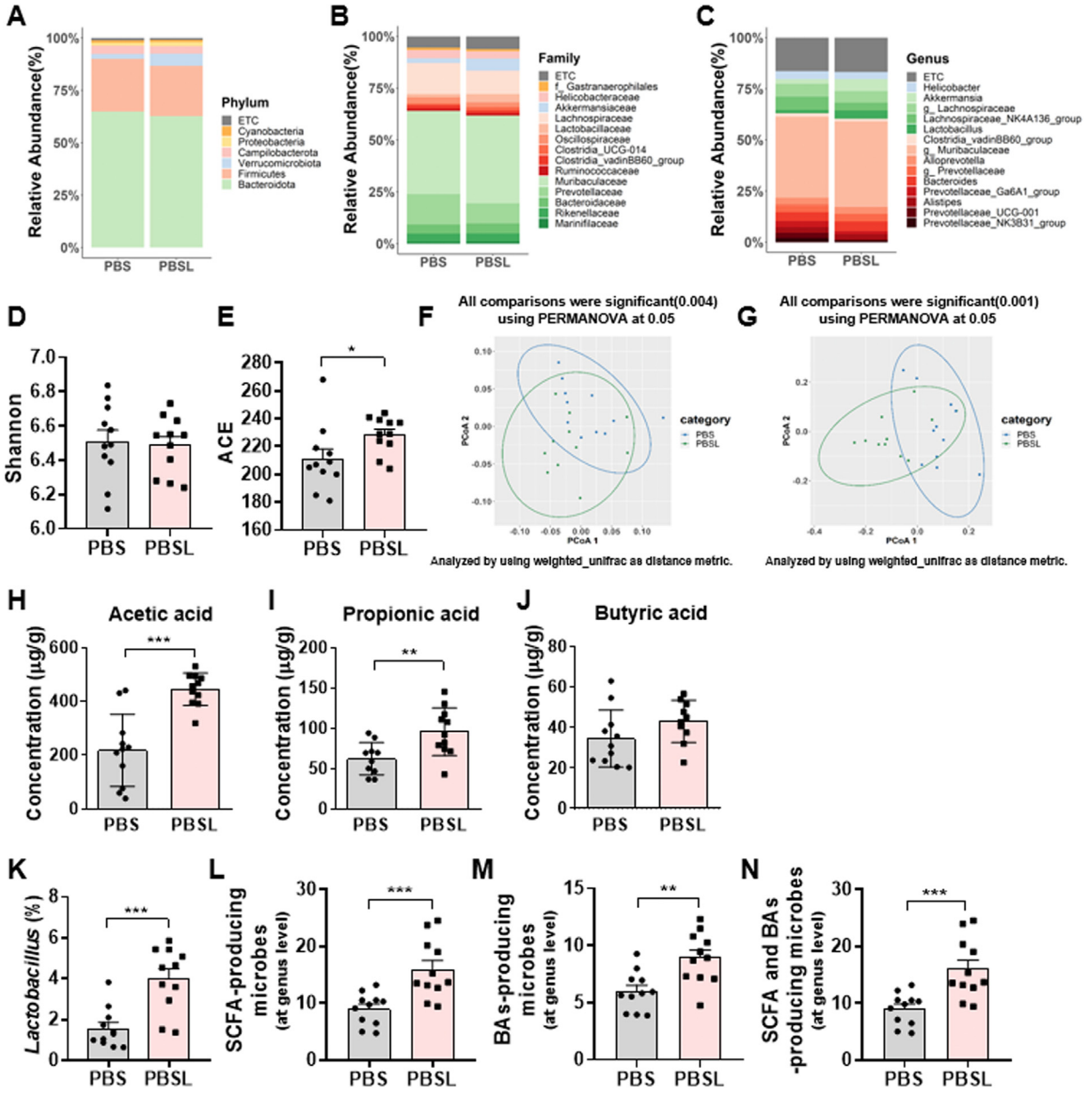


Fig. 4. *L. plantarum* NCHBL-004 modulates fecal microbiota and enhances SCFA production in mouse feces. (A–C) The relative abundances of gut microbiome at the phylum, family, and genus levels were analyzed through 16S rRNA amplicon sequencing. (D and E) Alpha diversity was analyzed based on Shannon and ACE indices (F and G), PCoA based on weighted UniFrac, and Bray-Curtis metrics. (H-J) Fecal SCFA (acetate, propionate, and butyric acid) concentrations on day 3 in mice administered PBS and NCHBL-004 (1×10^9 CFU/mouse). (K) Graphs depicting the proportion of *Lactobacillus* at the genus level. (L) The proportion of bacteria producing SCFAs. (M) The proportion of bacteria producing secondary BAs. (N) The proportion of bacteria producing both SCFAs and BAs. Data are presented as mean ± standard error of the mean (n = 11). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. PBSL, PBS treated with *L. plantarum* NCHBL-004; SCFA, short-chain fatty acid; BA, bile acids.

upregulation GLP-1 production, suggesting their potential for diabetes prevention and treatment [12,33]. Although there is evidence suggesting that probiotics can influence GLP-1 production and thus contribute to metabolic regulation, the exact mechanisms are not fully understood. Therefore, further studies are needed to clarify mode of action. Recently, postbiotics have attracted significant interest owing to their distinctive technical merits. Notably, the safety and stability of postbiotics, particularly in the form of inactivated bacteria, make them resilient during storage and transportation [34]. Therefore, this study was conducted to find out whether postbiotics (heat-killed bacteria) exhibit similar efficacy to probiotics (live bacteria) and could potentially replace them. The results indicated that heat-killed NCHBL-004 demonstrated anti-obesity effects, although direct comparison with live NCHBL-004 is challenging, necessitating further studies. Several species of heat-killed bacteria, including *L. plantarum*, have anti-obesity effects, suggesting that they can be used as functional food ingredients to alleviate metabolic diseases, including obesity [14,15,35]. The controllability of postbiotics allows precision in designing drugs targeted at specific metabolic pathways or functionalities. This safety profile positions postbiotics as advantageous in experimental investigations and regulatory scrutiny.

Their versatility across pharmaceuticals, health supplements, and food fortification underscores their adaptability to various

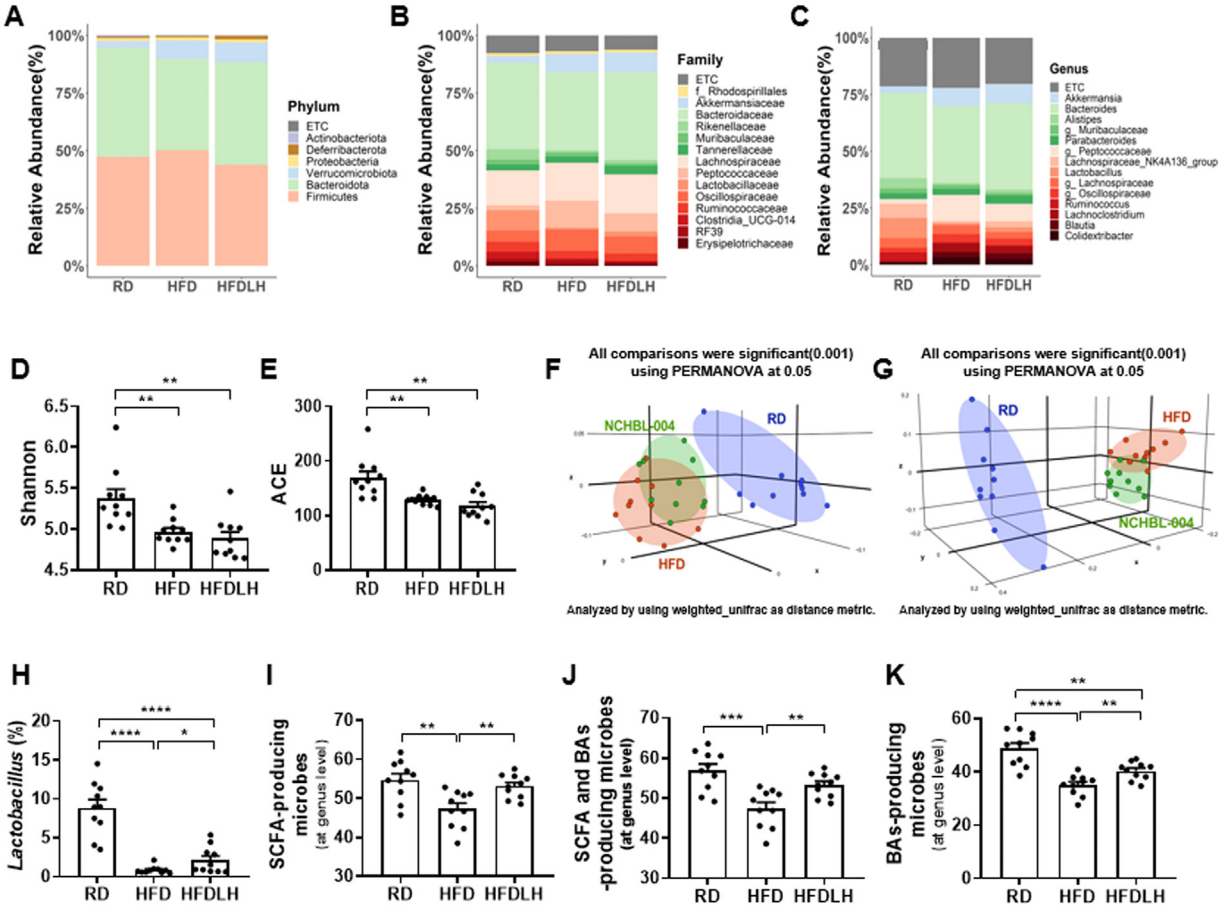


Fig. 5. *L. plantarum* NCHBL-004 modulates fecal microbiota and enhances SCFA production in the feces of high-fat diet–induced obese mice. (A–C) The relative abundances of gut microbiome at the phylum, family, and genus levels were analyzed through 16S rRNA amplicon sequencing. (D and E) Alpha diversity was analyzed based on Shannon and ACE indices. (F and G) Beta diversity was analyzed based on weighted UniFrac and Bray-Curtis metrics. (H) Graphs depicting the proportion of *Lactobacillus* at the genus level. (I) The proportion of bacteria producing SCFAs. (J) The proportion of bacteria producing BAs. (K) The proportion of bacteria producing both SCFAs and BAs. Data are presented as mean ± standard error of the mean (n = 10). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. RD, regular diet group; HFD, high-fat diet group; HFDLH, high-fat diet treated with *L. plantarum* NCHBL-004 (1×10^9 CFU/mouse); SCFA, short-chain fatty acid; BAs, bile acids.

scientific domains, emphasizing their growing significance in shaping future scientific advancements.

Probiotics play a crucial role in weight management by engaging various mechanisms, including promoting energy expenditure, regulating lipid metabolism, suppressing appetite, and modulating gut microbiota composition [36]. In addition, GLP-1 enhances these effects by contributing to the reduction of hepatic TG and regulating overall lipid metabolism. [32]. In the context of blood glucose regulation, probiotics demonstrate promise through mechanisms such as enhanced insulin sensitivity, increased glucose uptake, and

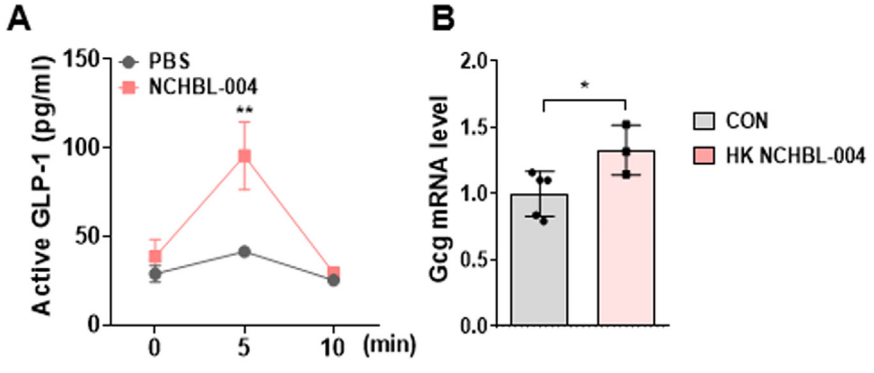


Fig. 6. Plasma active GLP-1 change after oral glucose administration in fasted mice fed with a regular diet. (A) Plasma active GLP-1 levels at 0, 5, and 10 min after oral glucose (2 g/kg) administration in the control group treated with PBS and the NCHBL-004 group treated with NCHBL-004 (1×10^9 CFU/mouse). Two-way ANOVA. $^{**}p < 0.01$. Data are presented as mean ± standard error of the mean (n = 3 to n = 5). (B) mRNA transcription level of Gcg in organoids after treatment with heat-killed NCHBL-004 (1×10^8 CFU/well) for 48 h.

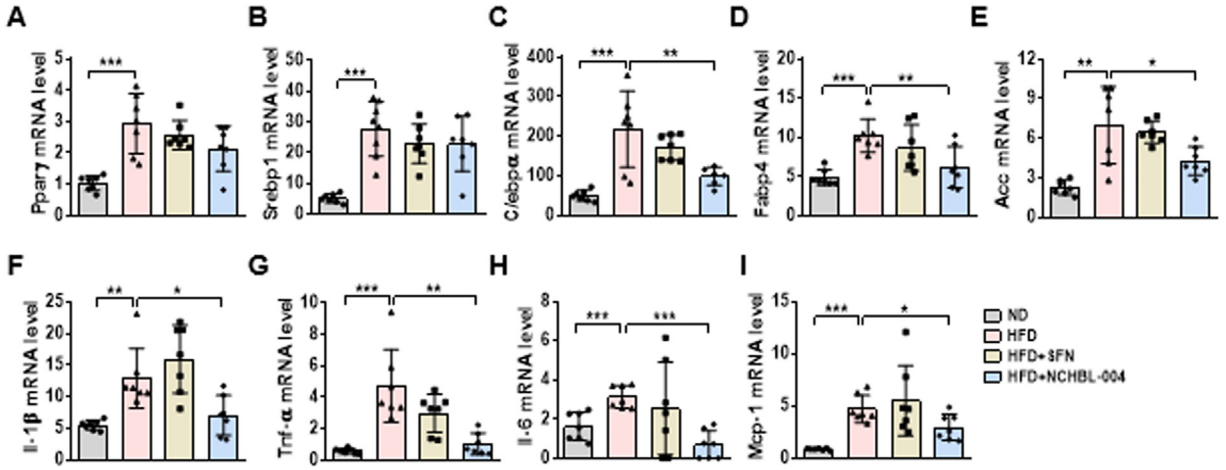


Fig. 7. Oral administration of *L. plantarum* NCHBL-004 mitigates the levels of lipogenesis-related genes and pro-inflammatory cytokine genes in liver tissue. (A–I) Hepatic mRNA expression of Pparγ, Srebp1, C/ebpα, Fabp4, Acc, Il-1β, Tnf-α, Il-6, and Mcp-1. Data are presented as mean ± standard deviation (n = 7). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. ND, normal diet group; HFD, high-fat diet group; HFD + SFN, high-fat diet treated with sulforaphane; HFD + NCHBL-004, high-fat diet treated with *L. plantarum* NCHBL-004.

modulation of the gut microbiota composition in association with glucose metabolism [37]. Additionally, probiotics contribute to the production of GLP-1, enhance insulin secretion, and facilitate glucose uptake in peripheral tissues [38]. Specific strains, such as *B. longum, B. animalis* subsp. *lactis*, and *L. gasseri* show positive effects on obesity-related metabolic functions, including improvements in blood glucose and leptin levels [1]. *L. plantarum*, for example, has proven effective in enhancing glucose tolerance and insulin sensitivity in HFD-fed mice [3]. Collectively, these findings highlight the potential of probiotics to positively influence blood glucose regulation via diverse and promising mechanisms.

Probiotics containing *Bifidobacterium* and *Lactobacillus* strains, such as VSL#3, produce the BA, which is known for its beneficial metabolic effects [4]. BA stimulates GLP-1 release from intestinal L-cells, reduces food intake, and improves glucose tolerance. Studies show that VSL#3 administration reduces food intake, protects against weight gain and insulin resistance in HFD-fed mice, and reverses obesity and diabetes in mouse models [4]. These effects involve gut flora modulation and increased GLP-1 levels, suggesting a plausible mechanism for the action of VSL#3.

The positive impacts of SCFAs, byproducts of probiotics, on liver health include reducing hepatic fat accumulation, promoting fatty acid oxidation, decreasing inflammation, and providing hepatoprotective effects. This highlights its potential use in the prevention and treatment of liver disease. Additionally, the administration of *L. plantarum* LMT1-48 to obese mice induced with obesity through a HFD resulted in reduced liver weight and TGs [3]. This treatment suppressed lipid synthesis genes (Pparγ, Hsl, Scd-1, and Fat/CD36) associated with intrahepatic lipid accumulation, accompanied by a decrease in body weight and fat volume [39]. Although these findings suggest a positive impact on liver health, further research is needed to validate the efficacy and safety of LMT1-48 in humans. In addition, *L. plantarum* SKO-001 treatment correlated with decreased body weight, reduced fat accumulation, and smaller adipocyte size, along with the downregulation of genes involved in lipid metabolism [40]. These findings emphasize the potential of *L. plantarum* to positively affect lipid metabolism, indicating its clinical relevance.

Conclusion

In conclusion, the collective evidence from these studies suggests that probiotics, especially NCHBL-004, hold promise as therapeutic strategies for liver health and blood glucose regulation. Further investigations, including human trials, are crucial to validate these potential benefits and establish the translational relevance of probiotics in clinical applications. The study on NCHBL-004 further supports the promising role of probiotics in the comprehensive management of obesity and the associated metabolic challenges.

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.

CRediT authorship contribution statement

Ah-Ra Jang: Writing – original draft, Validation, Methodology, Formal analysis. **Do-Hyeon Jung:** Methodology. **Tae-Sung Lee:** Methodology. **Jeon-Kyung Kim:** Writing – original draft, Methodology. **Yu-Bin Lee:** Visualization, Methodology. **Jae-Young Lee:** Investigation. **So-Yeon Kim:** Methodology. **Yung-Choon Yoo:** Methodology. **Jae-Hee Ahn:** Visualization, Methodology, Formal analysis. **Eun-Hye Hong:** Validation, Methodology, Formal analysis. **Chae-Won Kim:** Validation, Methodology, Formal analysis. **Su Min Kim:** Visualization, Methodology. **Hye Hyun Yoo:** Writing – original draft, Methodology. **Joo Young Huh:** Methodology. **Hyun-Jeong Ko:** Writing – original draft, Supervision, Project administration, Conceptualization. **Jong-Hwan Park:** Writing – original draft, Supervision, Project administration, Conceptualization.

Funding

This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT) (No. 2022R1A2C2012287, No. RS-2023-00243830, No. RS-2023-00217123) and it was also supported by the technology transfer and commercialization Program through INNOPOLIS Foundation funded by the Ministry of Science and ICT (No. 2023-GJ-RD-0041-01).

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.nut.2024.112565.

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