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ORIGINAL CONTRIBUTION

Anti-obesity effect of *Lactobacillus rhamnosus* LS-8 and *Lactobacillus crustorum* MN047 on high-fat and high-fructose diet mice base on inflammatory response alleviation and gut microbiota regulation

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Abstract

Purpose The objective of the study was to evaluate the anti-obesity effect of *Lactobacillus rhamnosus* LS-8 and *Lactobacillus crustorum* MN047, and illustrate the potential functional mechanism about the alleviation of high fat and high fructose diet (HFFD) induced obesity and related metabolic abnormalities.

Methods C57BL/6J mice were subjected to a standard or HFFD with or without supplementation of *L. rhamnosus* LS-8 and *L. crustorum* MN047 for 10 weeks. Obesity related metabolic indices including glucose tolerance, insulin resistance, serum lipid, liver function, hormones and inflammatory cytokines were assessed by standard protocols. For the monitoring of inflammatory response and lipid metabolism, transcriptional levels were profiled in liver and/or adipose tissues. Furthermore, gut microbiota composition analyses in the fecal samples were performed using 16S rRNA gene sequencing, and gut microbial metabolites, including lipopolysaccharide (LPS) and short-chain fatty acids (SCFAs), were also tested for the assessment of the relationship between gut microbiota variation and inflammatory response.

Results Administration with *L. rhamnosus* LS-8 and *L. crustorum* MN047 significantly mitigated body weight gain and insulin resistance, and inflammatory response (TNF-α, IL-1β and IL-6 levels in serum and corresponding mRNA levels in adipose tissues) was significantly inhibited in these two strains-treated mice. Moreover, *L. rhamnosus* LS-8 and *L. crustorum* MN047 could partially normalized mRNA expression levels involved in lipid metabolism including *Pparγ*, *Srebp-1c*, *CD36*, *Fabp2* and *FAS*. In addition, these two strains manipulated gut microbiota by decreasing the abundance of *Bacteroides* and *Desulfovibrio* and increasing that of *Lactobacillus* and *Bifidobacterium*, which in turn raised the levels of feces SCFAs and lowered the levels of circulating LPS.

Conclusion These results indicated that *L. rhamnosus* LS-8 and *L. crustorum* MN047 supplementation possessed the anti-obesity effect on the HFFD fed mice by alleviating inflammatory response and regulating gut microbiota, which further suggested that these two probiotics can be considered as an alternative dietary supplement in combination with the preventive and therapeutic strategies against obesity and related complications.

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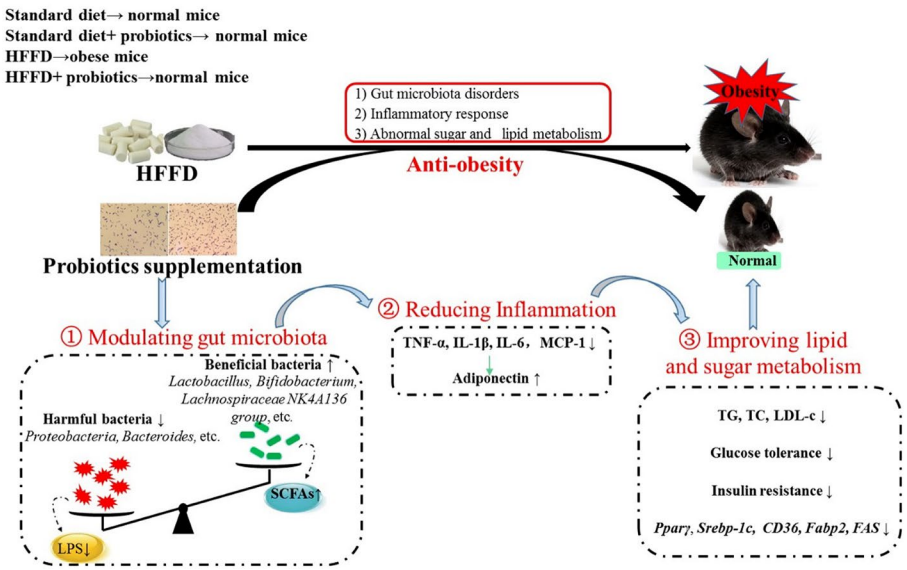
Graphic abstract



Keywords Diet-induced obesity · Lipid metabolism · Inflammation · *Lactobacillus rhamnosus* LS-8 · *Lactobacillus crustorum* MN047 · Gut microbiota

Introduction

The worldwide explosion of obesity and obesity-related metabolic syndrome, such as insulin resistance [1] and type 2 diabetes mellitus [2], has become a serious public health concern in the whole world. Over several decades, there has been a global shift in modern diets toward a high-energy diet that is rich in fats and/or sugars, especially in the western diet, which has been associated with high prevalence of overweight and metabolic syndrome [3]. At present, several diet pills are available commercially, such as orlistat and lorcaserin. However, adverse effects and little prospect of long-term benefit by using these drugs have greatly limited the clinical applications [4, 5]. Therefore, it is necessary to exploit more effective and better anti-obesity dietary supplement substance from natural biological sources for the application alone or combination with the preventive and therapeutic strategies to prevent or treat obesity and obesity-related disease.

Obesity is characterized by an excess accumulation of adipose tissues, which causes the increase of chronic low-grade inflammatory response [6, 7]. However, numerous literatures also reported that inflammation may play a causative role in the generation of insulin resistance and diabetes [6, 8]. Previous studies also suggested that adipose tissues macrophage infiltration can increase the secretion of some pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6, which interfere with the insulin-signaling pathway in peripheral tissues and facilitate the development of insulin resistance [9–11]. In addition, the overproduction of chemokine monocyte chemotactic protein-1 (MCP-1) was also proven to impair insulin sensitivity and led to the development of metabolic syndrome [12, 13]. Hence, the inflammatory response might be one of the key point for the development of obesity, therefore, which can be seen as the target for the screening of anti-obesity supplementations.

Compelling evidences support that high fat diet (HFD) or high fat and high fructose diet (HFFD) can profoundly, rapidly and sustainably alter intestinal microbiota communities in as little as 24–28 h in mice or humans [14, 15], which may lead to the occurrence of obesity-related metabolic diseases through alteration of energy metabolism, immune and chronic low-grade inflammatory responses [16]. Metabolic products from gut microbiota have both positive and negative effects on host health. For example, short-chain fatty acids (SCFAs), a major metabolite in the colon, are an important energy source for the host and helpful in regulating lipid metabolism, immunity and adipocyte development [17, 18]. But lipopolysaccharide (LPS), a major constituent of the Gram-negative bacterial outer membrane, has been proved an inducement to trigger insulin resistance [19]. Therefore, in recent years, growing attention has been paid to the possible role of the gut microbiota as a novel potential contributor in reducing or increasing the prevalence of obesity and obesity related complications [4, 20].

As an important part of gut microbiota, probiotics are gradually being proven to be beneficial in preventing and/or treating obesity. For example, intervention of *Lactobacillus gasseri* SBT2055 in obese adults has been shown to reduce abdominal adiposity and improve metabolic disorder [21]. In addition, administration with *Lactobacillus curvatus* HY7601 and *Lactobacillus plantarum* KY1032 in HFD-induced obese mice was able to alleviate weight gain and fat accumulation [22]. More recently, supplementation of *Lactobacillus rhamnosus* GG displayed an obvious inhibitory effect on dyslipidemia in HFD-induced obese mice [23]. It is deduced that the anti-obesity effects of probiotics mainly consists of three parts: (1) modulation of gut microbiota by influencing pathogen colonization through competing with pathogens for attachment sites and nutrients, and producing antimicrobial substances; (2) increasing the integrity of epithelial barrier by modulating mucus properties and influencing the turnover of epithelial cells; (3) inhibition of the inflammatory response by producing SCFAs. Despite these findings, there are still limitations in using *Lactobacillus* as preventive and therapeutic agents for obesity and metabolic syndrome, because the microorganisms exist species-specific effects that may further lead to different mechanistic actions. Therefore, it is necessary to pay more attention to the screening of *Lactobacillus* with anti-obesity effect and the functional mechanism of this activity.

Lactobacillus rhamnosus LS-8 and *Lactobacillus crustorum* MN047 are two novel probiotics screened from traditional fermented foods, and both of them had been proved in our previous studies to possess potential health benefits by producing novel antimicrobial substance, especially for *L. crustorum* MN047 which can secrete multiple novel bacteriocins [24, 25]. Since different bacteriocins possess different antimicrobial spectrum and action mechanism, it was hypothesized that supplementation of these two probiotics could reshape a new balance of gut microbiota. In addition, to our knowledge no previous study has focused on examining the anti-obesity ability of *L. crustorum*. To investigate the anti-obesity effects of the proposed two strains, as well as the modulation function of the glucose and lipid metabolism, inflammatory response and gut microbiota, the present study was conducted.

Thus, in the present study, the anti-obesity effects of *L. rhamnosus* LS-8 and *L. crustorum* MN047 were investigated, in addition, the modulations of serum lipid, hormones, liver function, systemic insulin resistance, hepatic lipid accumulation and epididymal fat expansion were also determined. To illustrate the mechanism of anti-obesity, the influences of these two strains on the inflammatory response, gut microbiota and its metabolites of HFFD-induced mice were systematically analyzed, as well as the relationship of the parameters. The results of the present study will provide new knowledge about the action mode of anti-obesity effects of *L. rhamnosus* LS-8 and *L. crustorum* on HFFD-induced mice, which also promotes the development of the proposed strains as function food for the prevention or therapy of obesity and obesity-related complications in the future.

Materials and methods

Preparation of *L. rhamnosus* LS-8 and *L. crustorum* MN047

Lactobacillus rhamnosus LS-8 and *L. crustorum* MN047 were isolated from fermented milk and homemade koumiss of Xinjiang Autonomous Region, China, respectively [24, 25]. Both of them were cultured in MRS medium at 37 °C for 16 h. Cells were harvested by centrifugation at 6500*g* for 5 min at 4 °C, and then the collected cells were washed twice and adjusted to 5×10^9 CFU/mL with sterile saline and used for gavage feeding.

Animals and diets

Eight-week-old C57BL/6J specific pathogen-free male mice were purchased from Xi'an Jiaotong University Health Science Center (Xi'an, China) and housed in standard polycarbonate cages (four mice per cage) under a controlled environment (temperature 22 ± 2 °C and humidity 55 ± 5% with 12 h light–dark cycle). All animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals: Eighth Edition, ISBN-10: 0-309-15396-4, and experimental procedures were approved by the Animal Ethics Committee of Xi'an Jiaotong University.

After acclimatization for one week, a total of seventy-two male mice were randomly divided into six groups ($n = 12$/group) as follows: C group fed with standard diet (AIN-93M); CLS group fed with standard diet and administration with *L. rhamnosus* LS-8; CMN group fed with standard diet and administration with *L. crustorum* MN047; M group fed with high-fat diet (45% kcal from fat, TP23100, purchased from Trophic Animal Feed High-Tech Co., Ltd. Nantong, China) and 10% w/v fructose (99%, F108331, purchased from Aladdin Biochemical Technology Co., Ltd. Shanghai, China) in drinking water (HFFD); MLS group fed with HFFD and administration with *L. rhamnosus* LS-8; MMN group fed with HFFD and administration with *L. crustorum* MN047. The compositions of experimental diets were shown in Table S1. All the probiotics administration mice (mice in CLS group, CMN group, MLS group and MMN group) were gavaged with 200 µL prepared bacterial suspension (1×10^9 CFU/day per mouse), and other mice (mice in C group and M group) were gavaged with the same volume of saline during the same period. Body weight, food and water intake of the mice were monitored weekly throughout the duration

of the study. At the end of the 10-week experimental, the 12 h-fasted mice were euthanized with 10% chloral hydrate (300 mg/kg body weight, intraperitoneal injection). Blood samples were collected into 2 mL of centrifuge tubes by eyeball picking method, and centrifuged at 3000*g* for 10 min at 4 °C after standing for 30 min at room temperature, and then the serum was taken out slowly and stored in sterile EP tubes at − 80 °C for further analysis. Liver, kidney, spleen, epididymal and inguinal fat tissues were collected, washed with ice-cold saline, and weighed after drying the surface water by filter paper, and then stored at − 80 °C until use.

Oral glucose tolerance test and insulin tolerance test

Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) were performed as described in Balakumar et al. [26] with slight modifications. Briefly, for OGTT analysis, mice were fasted overnight and then orally administered glucose solution (2 g/kg body weight) at 8th week. Blood samples were collected from the tail vein and glucose levels were measured using a glucometer (Sannuo Biological Transmission Co., Ltd. Changsha, China) prior to glucose intake, and at 15, 30, 60, 90 and 120 min after glucose administration. For ITT, the mice were fasted 6 h and then were intraperitoneally injected with insulin (0.5 U/kg body weight, novolin R, Novo Nordisk) at 9th week. Glucose levels were determined at 0, 15, 30, 60, 90 and 120 min after insulin injection. The total glucose areas under the curve ($AUC_{glucose}$) represented the magnitude of the glucose response and were calculated using the trapezoidal rule.

Blood serum analysis

The serum insulin, leptin, adiponectin, LPS, TNF-α, IL-1β and IL-6 levels were tested by ELISA (Xin le Biotechnology Co., Ltd. Shanghai, China). Serum total alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl transferase (γ-GT), blood urea nitrogen (BUN), creatinine (CRE), C-reactive protein (CRP), total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were analyzed using a biochemical automatic analyzer (Hitachi High Technologies Corporation, Tokyo, Japan). The insulin resistance index (HOMA-IR) was calculated using the following formula: HOMA-IR = fasting insulin levels × fasting blood glucose/22.5 [27].

Histological analysis

After euthanizing of mice, the liver and epididymal fat tissues were subsequently fixed in 4% paraformaldehyde overnight at 4 °C, and then embedded in paraffin for hematoxylin and eosin (H&E) staining analyses. The stained area was viewed and photographed with an Olympus microscope (Olympus Corporation, Tokyo, Japan) under the objective of 20×. Histological sections of liver and epididymal fat tissues were evaluated and analyzed by a pathologist, and the sizes of adipocytes were calculated using float morphology in the Image J software (developed by Wayne Rasband from NIH, USA).

Short-chain fatty acid analysis

The concentrations of SCFAs (acetic acid, propionic acid, isobutyric, butyric acid, isovaleric and valeric acid) analyses were performed as described by Zhao et al. [28] with minor modifications. Briefly, approximately 200 mg of fecal samples were homogenized with 2 mL of distilled water and centrifuged at 10,000*g* for 10 min at 4 °C. The supernatant was filtered through a 0.22 μm nylon filter (EMD Millipore). Taken 1 mL of filtrate and acidified by adding 200 μL 50% H_2SO_4 (v/v). After vortexing and standing for 5 min, 1 mL of diethyl ether was added, and the mixed solution was incubated for 30 min at 4 °C. After centrifuging at 10,000*g* for 10 min at 4 °C, the organic phase was filtered through a 0.22 μm nylon filter and collected for gas chromatography analysis. SCFAs were determined with gas chromatography (GC-2014C, Shimadzu Corporation, Japan), which equipped with a RTX-wax column (30 m × 0.25 μm × 0.25 μm) and a flame ionization detector. The initial temperature was 50 °C, which was maintained for 1 min and raised to 120 °C at 15 °C/min, and then increased to 170 °C at 5 °C/min, finally, increased to 240 °C at 15 °C/min and held at this temperature for 3 min. The injector and the detector temperature were 250 °C and 270 °C, respectively.

Quantitative PCR analysis

Total RNA was extracted from liver and epididymal fat tissues using the RNAiso Plus reagent (TaKaRa, Dalian, China) according to the manufacturer's instruction. The total RNA quality was determined using a Micro-Spectrophotometer Nano-200 (Hangzhou Allsheng Instruments, Korea) with the ratios of 260 nm/280 nm (1.9–2.1) and 260 nm/230 nm (2.0–2.5), and 2 μL of total RNA (0.5–0.6 mg/mL) was used to synthesize cDNA (20 μL of total reaction volume) using the FastKing RT Kit (With gDNase) (TIANGEN, Beijing, China). Two μL of the diluted cDNA (1:10) was performed quantitative PCR (qPCR) by using SYBR Green BioEasy Master mix (BIOER, Hangzhou, China) and a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Cycle conditions: 1 min at 95 °C, followed by 39 cycles of incubation at 95 °C for 15 s, 60 °C for 15 s, and then 72 °C for 30 s. The sequences of the primers used for RT-qPCR are shown in Table 1. Relative mRNA expression level of

Table 1 Sequences of the primers used for quantitative real-time PCR

Target gene	Forward primer (5′→3′)	Revere primer (3′→5′)	Product size (bp)	Accession number
TNF-α	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG	61	NM_013693.3
IL-1β	CCTGCAGCTGGAGAGTGTGGAT	TGCTCTGCTTGTGAGGTGCTG	150	NM_008361.4
IL-6	GGCCTTCCCTACTTCACAAG	ATTTCCACGATTTCCCAGAG	126	NM_001314054.1
FAS	GGAGGTGGTGATAGCCGGTAT	TGGGTAATCCATAGAGCCCAG	140	NM_007988.3
Fabp2	TTCTCAGAGCCTGGAGCAAC	GATGACGAATGAGCCTGGCA	86	NM_007980.3
CD36	CAGATGACGTGGCAAAGAAC	TGGCTCCATTGGGCTGTA	144	NM_001159558.1
PPARγ	CGCTGATGCACTGCCTATG	ATGCGAGTGGTCTTCCATCA	124	NM_011146.3
MCP-1	CAGGTCCCTGTCATGCTTCT	CCCATTCCTTCTTGGGGTCA	121	NM_011333.3
Srebp-1c	GATCAAAGAGGAGCCAGTGC	TAGATGGTGGCTGCTGAGTG	191	NM_001358315.1
β-actin	GGACTGTTACTGAGCTGCGTT	CGCCTTCACCGTTCCAGTT	209	NM_007393.5

target genes were normalized using the mRNA level of the housekeeping gene *β-actin* in each sample and the data were analyzed according to the $2^{-\Delta\Delta Ct}$ method.

Fecal microbiota analysis

Total bacterial DNA of each feces sample was extracted using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories) according to the manufacturer's protocol. The V3–V4 regions of the bacterial 16S rRNA gene were amplified with the common primers (forward primer, 5′-ACTCCTACG GGAGGCAGCA-3′; reverse primer, 5′-GGACTACHVGGG TWTCTAAT-3′) combined with adapter sequences and barcode sequences after evaluation of DNA quality and quantity by the ratios of 260 nm/280 nm (1.8–2.0) and 260 nm/230 nm (2.0–2.5). Two steps of PCR amplification were performed, and the first round PCR was operated using High-Fidelity DNA Polymerase (Q5, NEB) under the following thermal cycling conditions: denaturation at 95 °C for 5 min, followed by 15 cycles at 95 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, with a final extension at 72 °C for 7 min. The PCR products from the first step were purified through VAHTSTM DNA Clean Beads. And the second round PCR was then performed under the following thermal cycling conditions: an initial denaturation at 98 °C for 30 s, followed by 10 cycles at 98 °C for 10 s, 65 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 5 min. Finally, all PCR products were quantified by Quant-iT™ dsDNA HS Reagent and pooled together. High-throughput sequencing analysis of bacterial rRNA genes was performed on the purified, pooled sample using the Illumina Hiseq 2500 platform (2 × 250 paired ends) at Biomarker Technologies Corporation, Beijing, China.

After sequencing, raw sequence reads were filtered with the QIIME pipeline to obtain high-quality clean sequences. Paired-end reads were first merged using FLASH (version 1.2.7) with default parameters [29]. Chimeric sequences were then identified and removed by UCHIME (version 4.2) software. Remaining sequences with similarity > 97% were classified into an operational taxonomic unit (OTU) using USEARCH software (version 10.0) and finally classified as species and genus level using Ribosomal Database Project (RDP) Classifier (version 2.2) [30]. Alpha-diversity indices (ACE, Chao 1, Simpson and Shannon) were calculated with the Mothur package [31]. The weighted and unweighted principal coordinate analyses (PCoA) and unweighted pair group method with arithmetic mean (UGMA) clustering were performed with the QIIME software. Linear discriminant analysis (LDA) effect size (LEfSe) was used to detect significant changes in relative abundance of microbial taxa among different groups. Briefly, LEfSe first identifies features that are significantly different among biological classes using the non-parametric factorial Kruskal–Wallis ran-sum test, and then LEfSe utilizes linear discriminant analysis (LDA) to estimate the effect of each differentially abundant feature.

Statistical analysis

All experimental data sets were tested for normality using Anderson–Darling test (Minitab statistical software, v. 16.2.3), and the *p* value of all data sets was greater than 0.05, indicating that all data sets were normally distributed. Data were presented as the mean ± standard deviation (SD) of at least three independent experiments. Significant differences between mean values were determined by Duncan's multiple range test with SPSS V20 software (IBM, Armonk, NY, USA). A value of $p < 0.05$ was regarded as statistically significant.

Results

Effects of *L. rhamnosus* LS-8 and *L. crustorum* MN047 on body weight and tissues weight in HFFD-fed mice

During the experimental period, the body weight, food and energy intake of mice in six groups were recorded. Although HFFD-fed groups (M group, MLS group and MMN group) led to lower food intake compared to standard diet fed groups (C group, CLS group and CMN group), the total energy intake had no difference among all groups (Table 2). At 0th week, the body weight showed no significant difference in all groups, but body weight and weight gain in the M group were significantly increased compared to the C group at 10th week, while these alterations were obviously ameliorated by the administration with *L. rhamnosus* LS-8 and *L. crustorum* MN047 (Table 2). In contrast, supplementation of *L. rhamnosus* LS-8 and *L. crustorum* MN047 in standard diet fed mice did not affect the body weight and weight gain at the end of feeding period compared to the C group. Therefore, the food efficiency ratio in the M group was higher than the C group, while it could be significantly alleviated by supplementation of *L. rhamnosus* LS-8 and *L. crustorum* MN047 (Table 2). Moreover, compared to the C group, oral *L. rhamnosus* LS-8 and *L. crustorum* MN047 in standard diet fed mice did not influence the average weight of epididymal and inguinal fat, liver and kidney tissues (Table 2); however, which significantly increased in the M group and dramatically decreased after *L. rhamnosus* LS-8 and *L. crustorum* MN047 administration (Table 2).

Effects of *L. rhamnosus* LS-8 and *L. crustorum* MN047 on metabolic parameters in HFFD-fed mice

For the purpose to investigate the effect of *L. rhamnosus* LS-8 and *L. crustorum* MN047 on amelioration of systemic insulin resistance induced by HFFD feeding, glucose tolerance and insulin tolerance tests were performed. As shown in Fig. 1a–c, it was clearly that an impaired glucose tolerance in the M group compared with the C group was observed, which was clearly verified by a higher area under the curve (Fig. 1c), while *L. rhamnosus* LS-8 and *L. crustorum* MN047 supplementation significantly reversed this abnormity (Fig. 1a–c). In addition, insulin tolerance test was also conducted to determine the sensitivity of insulin receptors by measuring blood glucose levels before and after insulin injection. It was obviously found that the mice in the M group exhibited distinct insulin tolerance compared to the C group (Fig. 1d–f), while insulin tolerance in HFFD-fed mice was significantly ameliorated by supplementation of *L. rhamnosus* LS-8 and *L. crustorum* MN047. Furthermore, glucose levels after a 12-h fast were 53.67% higher in the M group compared with the C group (Fig. 1g). However, it was interesting to find that the fasting blood glucose levels

Table 2 Effects of *Lactobacillus rhamnosus* LS-8 and *Lactobacillus crustorum* MN047 on diet intake, body weight and tissue wet weights in HFFD-induced mice

Parameters	Dietary group[i]					
	C	CLS	CMN	M	MLS	MMN
Initial body weight (g)	21.64 ± 0.66^{aiii}	21.10 ± 1.13^{a}	21.10 ± 2.05^{a}	20.83 ± 0.60^{a}	21.21 ± 1.08^{a}	20.43 ± 1.41^{a}
Final body weight (g)	25.42 ± 0.76^{a}	24.73 ± 0.62^{a}	24.51 ± 2.80^{a}	28.18 ± 1.50^{b}	25.48 ± 1.61^{a}	25.60 ± 2.40^{a}
Body weight gain (g)	3.78 ± 0.47^{a}	3.63 ± 0.93^{a}	3.41 ± 1.30^{a}	7.34 ± 0.91^{c}	4.28 ± 0.66^{ab}	5.17 ± 1.58^{b}
Food intake (g/day per mouse)	2.80 ± 0.13^{b}	2.79 ± 0.11^{b}	2.84 ± 0.16^{b}	2.09 ± 0.08^{a}	2.05 ± 0.12^{a}	1.98 ± 0.05^{a}
Water intake (mL/day per mouse)	3.89 ± 0.42^{a}	3.77 ± 0.46^{a}	3.86 ± 0.50^{a}	5.84 ± 0.87^{b}	5.42 ± 0.61^{b}	5.23 ± 0.43^{b}
Energy intake (kcal/day per mouse)	10.06 ± 0.46^{a}	10.04 ± 0.41^{a}	10.22 ± 0.58^{a}	10.09 ± 0.49^{a}	9.88 ± 0.43^{a}	9.58 ± 0.60^{a}
Food efficiency ratio[iii]	0.38 ± 0.05^{a}	0.36 ± 0.09^{a}	0.33 ± 0.13^{a}	0.73 ± 0.09^{c}	0.43 ± 0.07^{ab}	0.54 ± 0.16^{b}
Epididymal fat (g)	0.427 ± 0.042^{a}	0.443 ± 0.016^{a}	0.440 ± 0.027^{a}	0.681 ± 0.020^{c}	0.509 ± 0.111^{b}	0.499 ± 0.078^{ab}
Inguinal fact (g)	0.246 ± 0.016^{a}	0.222 ± 0.020^{a}	0.243 ± 0.020^{a}	0.422 ± 0.021^{c}	0.340 ± 0.017^{b}	0.327 ± 0.024^{b}
Liver (g)	0.853 ± 0.005^{ab}	0.835 ± 0.022^{a}	0.894 ± 0.045^{bc}	1.068 ± 0.023^{d}	0.929 ± 0.015^{c}	0.835 ± 0.029^{a}
Kidney (g)	0.304 ± 0.007^{a}	0.295 ± 0.007^{a}	0.306 ± 0.007^{a}	0.342 ± 0.007^{b}	0.300 ± 0.007^{a}	0.296 ± 0.007^{a}
Spleen (g)	0.006 ± 0.001^{a}	0.006 ± 0.001^{a}	0.006 ± 0.001^{a}	0.007 ± 0.001^{a}	0.007 ± 0.001^{a}	0.006 ± 0.001^{a}

[i] C: mice fed with standard diet, CLS: mice fed with standard diet and *Lactobacillus rhamnosus* LS-8, CMN: mice fed with standard diet and *Lactobacillus crustorum* MN047, M: mice fed with HFFD, MLS: mice fed with HFFD and *Lactobacillus rhamnosus* LS-8, MMN: mice fed with HFFD and *Lactobacillus crustorum* MN047

[ii] All data were expressed as the mean ± SD ($n = 8$ mice/group), different letters within a row indicate significant differences ($p < 0.05$) by Duncan tests

[iii] Food efficiency ratio (FER) = body weight gain (g)/energy intake (kcal/day per mouse)

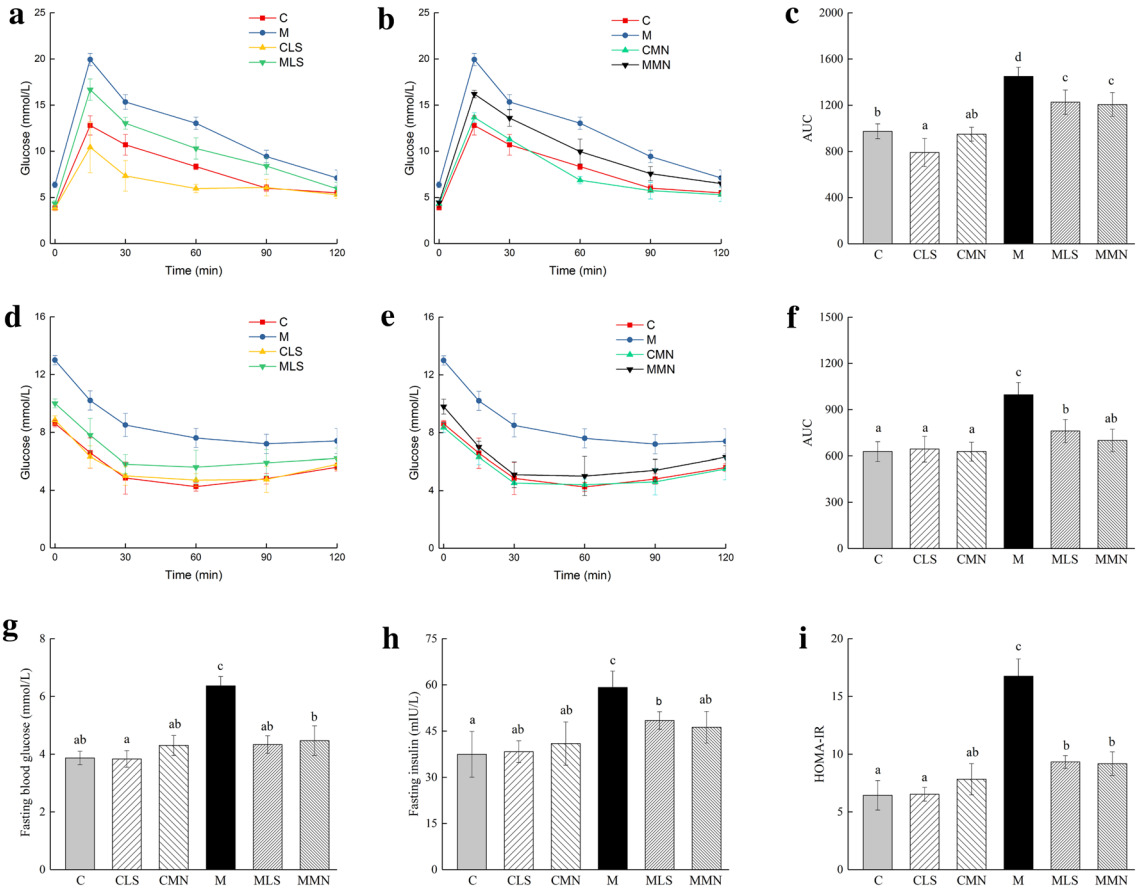


Fig. 1 Effects of *L. rhamnosus* LS-8 and *L. crustorum* MN047 administration on oral glucose tolerance test, insulin tolerance test, insulin and homeostasis model of insulin resistance (HOMA-IR) in HFFD-fed mice. Oral glucose tolerance test results of administration with **a** *L. rhamnosus* LS-8 and **b** *L. crustorum* MN047, respectively, and **c** area under curve (AUC) analyses for glucose tolerance test at week 8; Insulin tolerance test results of administration with **d** *L. rhamnosus* LS-8 and **e** *L. crustorum* MN047, respectively, and **f** AUC analyses for insulin tolerance tests at week 9; **g** Fasting glucose level, **h** fasting insulin level, and **i** HOMA-IR at week 10. Bars represent the mean ± SD ($n = 8$ mice/group), bars with different letters indicate significant differences ($p < 0.05$) by Duncan tests. C: mice fed with standard diet, CLS: mice fed with standard diet and *Lactobacillus rhamnosus* LS-8, CMN: mice fed with standard diet and *Lactobacillus crustorum* MN047, M: mice fed with HFFD, MLS: mice fed with HFFD and *Lactobacillus rhamnosus* LS-8, MMN: mice fed with HFFD and *Lactobacillus crustorum* MN047

decreased 31.94% and 29.84% after the intervention of *L. rhamnosus* LS-8 and *L. crustorum* MN047 in HFFD-fed mice compared with the M group, respectively (Fig. 1g). Moreover, overnight fasting insulin levels in the M group were 58.12% higher than the C group, while *L. rhamnosus* LS-8 and *L. crustorum* MN047 administration in HFFD-fed mice both significantly prevented these changes by an 18.17% and 21.91% reduction, respectively (Fig. 1h). The changes in fasting glucose and insulin levels were further determined by HOMA-IR index. Results showed that the value of HOMA-IR index in the M group was approximately 2.6-fold higher than the C group (Fig. 1i), while the value of HOMA-IR in the MLS group and MMN group both showed an apparent reduction compared to the M group (Fig. 1i). Meanwhile, supplementation of *L. rhamnosus* LS-8 and *L. crustorum* MN047 in standard diet fed mice did not affect the values of HOMA-IR compared to the C group (Fig. 1i).

The serum lipid levels were also tested at the end of the experimental period to analysis the improvement of lipid homeostasis caused by intervention of *L. rhamnosus* LS-8 and *L. crustorum* MN047 in HFFD-fed mice. It indicated that the serum contents of TC, TG and LDL-C in the M group were dramatically increased compared with the C group, while both of these alterations were significantly ameliorated by *L. rhamnosus* LS-8 and *L. crustorum* MN047 administration (Table 3). In addition, it also needed to mentioned that the levels of HDL-C in all HFFD-fed groups (M group, MLS group and MMN group) were significantly higher than that in standard diet fed groups (C group, CLS group and CMN group) (Table 3).

Table 3 Effects of *Lactobacillus rhamnosus* LS-8 and *Lactobacillus crustorum* MN047 on serum lipids, liver function and kidney function in HFFD-induced mice

Serum parameters	Dietary group[i]					
	C	CLS	CMN	M	MLS	MMN
TC (mmol/L)	2.75 ± 0.06^{aiii}	2.57 ± 0.29^{ab}	2.97 ± 0.31^{ab}	5.22 ± 0.43^{d}	3.51 ± 0.37^{bc}	3.84 ± 0.79^{c}
TG (mmol/L)	0.70 ± 0.07^{a}	0.79 ± 0.05^{a}	0.74 ± 0.06^{a}	0.92 ± 0.08^{b}	0.72 ± 0.03^{a}	0.72 ± 0.06^{a}
HDL-C (mmol/L)	2.12 ± 0.07^{a}	2.18 ± 0.15^{a}	2.42 ± 0.30^{a}	3.52 ± 0.21^{c}	3.00 ± 0.20^{b}	3.36 ± 0.30^{bc}
LDL-C (mmol/L)	0.18 ± 0.03^{a}	0.23 ± 0.05^{ab}	0.18 ± 0.03^{a}	0.41 ± 0.06^{c}	0.26 ± 0.01^{b}	0.27 ± 0.05^{b}
ALT (U/L)	22.90 ± 3.13^{a}	24.37 ± 1.18^{ab}	27.63 ± 2.61^{b}	41.07 ± 2.55^{d}	26.17 ± 2.06^{ab}	34.80 ± 1.37^{c}
AST (U/L)	105.43 ± 6.76^{a}	104.73 ± 9.66^{a}	113.97 ± 6.42^{a}	143.37 ± 5.01^{b}	104.83 ± 10.59^{a}	113.07 ± 9.35^{a}
γ-GT (U/L)	0.30 ± 0.05^{c}	0.13 ± 0.03^{ab}	0.11 ± 0.02^{a}	0.47 ± 0.06^{d}	0.20 ± 0.05^{b}	0.10 ± 0.01^{a}
BUN (mmol/L)	9.67 ± 0.43^{b}	9.63 ± 0.67^{b}	8.60 ± 1.76^{ab}	8.97 ± 0.52^{ab}	8.10 ± 0.24^{ab}	7.69 ± 0.71^{a}
CRE (μmol/L)	17.53 ± 2.10^{a}	17.97 ± 1.69^{a}	14.93 ± 1.72^{a}	22.47 ± 0.96^{b}	16.57 ± 2.74^{a}	18.07 ± 0.96^{a}
CRP (mg/L)	0.87 ± 0.07^{a}	0.69 ± 0.18^{a}	0.78 ± 0.13^{a}	1.12 ± 0.04^{b}	0.84 ± 0.07^{a}	0.81 ± 0.10^{a}

[i]C: mice fed with standard diet, CLS: mice fed with standard diet and *Lactobacillus rhamnosus* LS-8, CMN: mice fed with standard diet and *Lactobacillus crustorum* MN047, M: mice fed with HFFD, MLS: mice fed with HFFD and *Lactobacillus rhamnosus* LS-8, MMN: mice fed with HFFD and *Lactobacillus crustorum* MN047

[ii]All data were expressed as the mean ± SD ($n = 8$ mice/group), different letters within a row indicate significant differences ($p < 0.05$) by Duncan tests

The levels of γ-GT, AST and ALT were tested in serum to evaluate the prevention of HFFD-induced liver function injury caused by the supplementation of *L. rhamnosus* LS-8 and *L. crustorum* MN047. Table 3 revealed that γ-GT, AST and ALT were significantly elevated in the M group, while these alterations were obviously reversed by treatment with *L. rhamnosus* LS-8 and *L. crustorum* MN047. Interestingly, the levels of γ-GT in the MLS group and MMN group were even significantly lower than that in the C group (Table 3). In addition, the levels of serum CRE and BUN were also investigated to verify the amelioration of HFFD-induced kidney function injury after intervention of the proposed strains. It demonstrated that serum CRE levels were obviously raised in HFFD-induced obesity mice (Table 3), and which showed a significant reduction after treatment with *L. rhamnosus* LS-8 and *L. crustorum* MN047 (Table 3). For the levels of serum BUN, except for MMN group had an obvious decrease compared to the M group, there was no significant difference between the other groups (C group, M group, MLS group, CLS group and CMN group) (Table 3). In addition, the concentration of serum CRP was also determined and it was markedly elevated compared to the C group, while both *L. rhamnosus* LS-8 and *L. crustorum* MN047 administration could ameliorate these changes (Table 3).

The analysis of lipid-associated cytokines (such as serum adiponectin and leptin) indicated that HFFD feeding led to the decreasing of serum adiponectin level and increasing of serum leptin level, and which were both inhibited or reversed by the supplementation of *L. rhamnosus* LS-8 and *L. crustorum* MN047 as shown in Fig. 2a, b. It was also found that the intervention of *L. rhamnosus* LS-8 and *L. crustorum* MN047 could relieve HFFD-induced inflammatory response. In detail, the serum pro-inflammatory factors (TNF-α, IL-1β and IL-6) in the M group were markedly increased when compared to the C group (Fig. 2c–e). On the contrary, the increasing trend of these pro-inflammatory factors in HFFD-fed mice were apparently down-regulated by the administration with *L. rhamnosus* LS-8 and *L. crustorum* MN047 (Fig. 2c–e). Interestingly, the levels of IL-1β in the CMN group and MMN group were both extremely lower than the C group (Fig. 2d), and the ability of *L. crustorum* MN047 in preventing the elevation of IL-6 was better than *L. rhamnosus* LS-8 (Fig. 2e). In addition, the serum LPS levels, which had been proved to be an inducement to trigger insulin resistance by stimulating systemic inflammatory response [21], significantly higher in the M group than that in the C group, while it were distinctly decreased after administration with *L. rhamnosus* LS-8 and *L. crustorum* MN047 (Fig. 2f).

Effect of *L. rhamnosus* LS-8 and *L. crustorum* MN047 on liver and epididymal fat histology in HFFD-fed mice

At the end of the experimental period, the effects of *L. rhamnosus* LS-8 and *L. crustorum* MN047 intervention on liver and epididymal fat histology were evaluated. Compared to the C group, HFFD feeding induced prominent diffuse macro-vesicular steatosis in the liver tissue (Fig. 3a). Interestingly, the supplementation of *L. rhamnosus* LS-8 and *L. crustorum* MN047 significantly reversed the formation of hepatic steatosis (Fig. 3a). In addition, epididymal fat histology analysis also indicated that the average adipocyte size

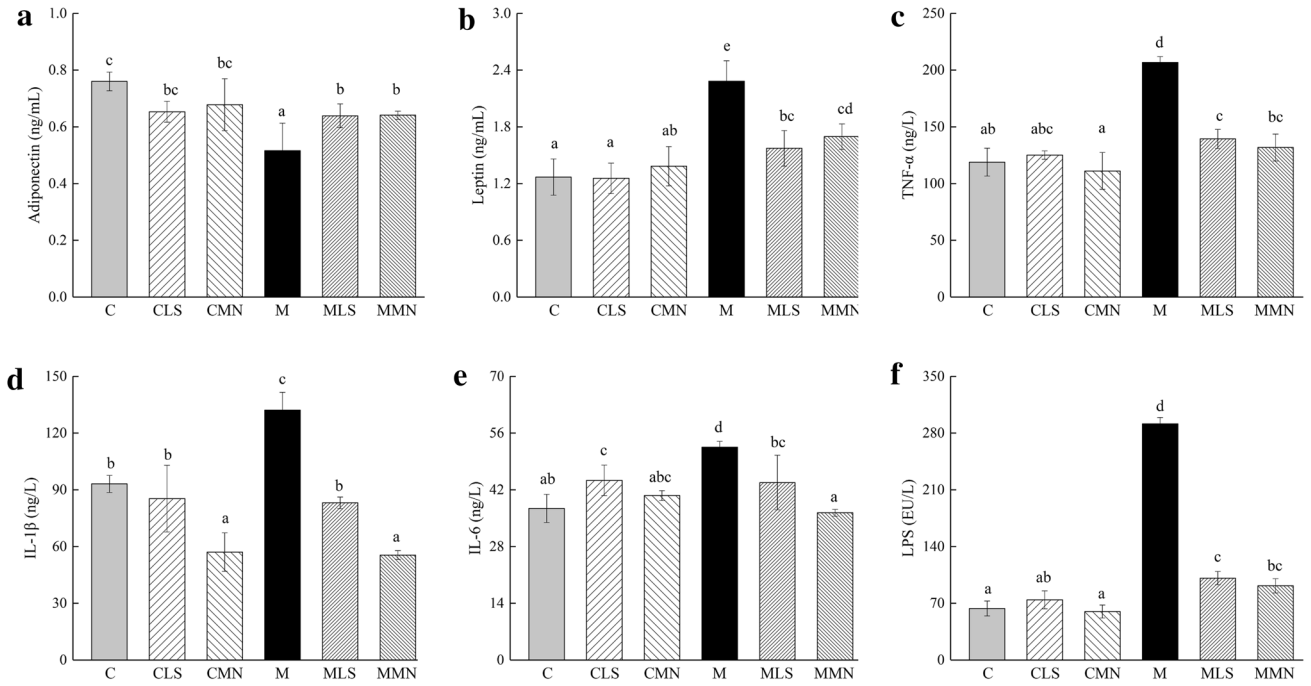


Fig. 2 Effects of *L. rhamnosus* LS-8 and *L. crustorum* MN047 administration on serum cytokines in HFFD-fed mice. **a** Adiponectin; **b** leptin; **c** TNF-α; **d** IL-1β; **e** IL-6; and **f** LPS. Bars represent the mean ± SD ($n = 8$ mice/group), bars with different letters indicate significant differences ($p < 0.05$) by Duncan tests. C: mice fed with standard diet, CLS: mice fed with standard diet and *Lactobacillus rhamnosus* LS-8, CMN: mice fed with standard diet and *Lactobacillus crustorum* MN047, M: mice fed with HFFD, MLS: mice fed with HFFD and *Lactobacillus rhamnosus* LS-8, MMN: mice fed with HFFD and *Lactobacillus crustorum* MN047

in the M group was distinctly increased compared to the C group, while the mean value of adipocyte cell size was markedly reduced by treatment with *L. rhamnosus* LS-8 and *L. crustorum* MN047 (Fig. 3b, c).

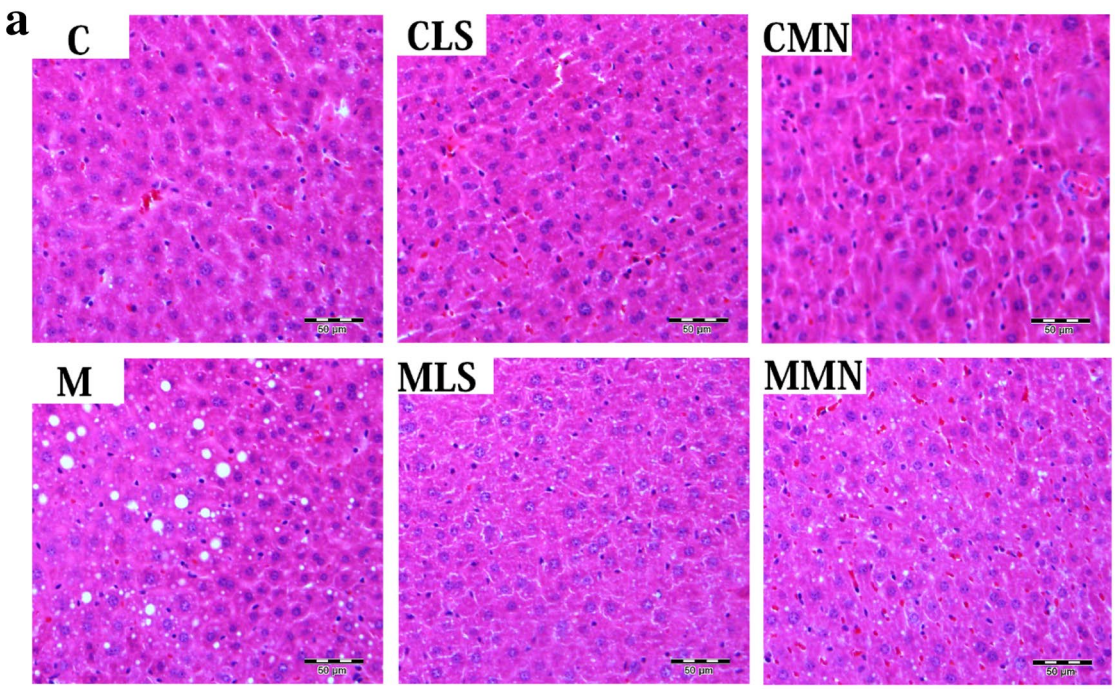
Effect of *L. rhamnosus* LS-8 and *L. crustorum* MN047 on liver and epididymal fat gene expressions

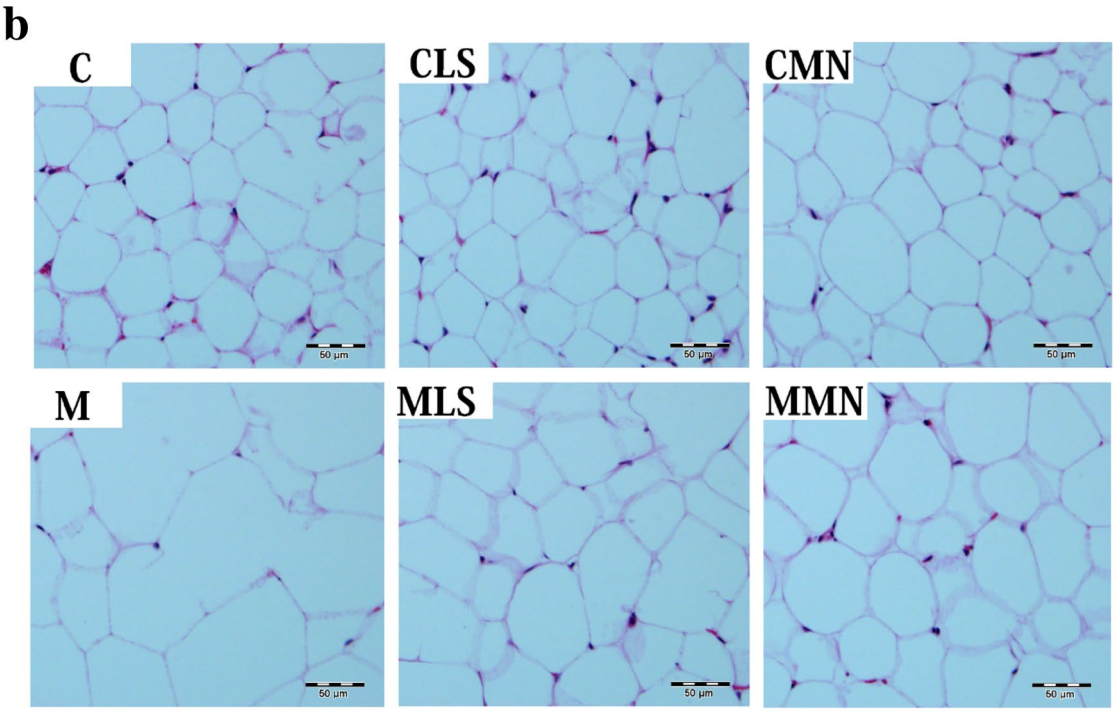
To further understand the underlying molecular mechanisms about the inhibitory effect of *L. rhamnosus* LS-8 and *L. crustorum* MN047 on hepatic lipid accumulation, the mRNA levels related with lipid metabolism were assessed, including peroxisome proliferator-activated receptor gamma (*Pparγ*), sterol regulatory element-binding protein 1 (*Srebp-1c*), platelet glycoprotein 4 (*CD36*) and fatty acid-binding protein (*Fabp2*). Results indicated that the mRNA expressions of hepatic lipogenic genes (*Pparγ* and *Srebp-1c*) were higher in the HFFD-fed mice than that in the C group mice (Fig. 4a), while the expression of these genes were significantly reduced via supplementation of *L. rhamnosus* LS-8 and *L. crustorum* MN047 (Fig. 4a). Moreover, the expressions of fatty acid metabolic genes, including *CD36* and *Fabp2*, were also dramatically increased in the M group compared to the C group and showed a down-regulated expression in *L. rhamnosus* LS-8 and *L. crustorum* MN047 treatment group (Fig. 4a).

The prevention effects of supplementation of *L. rhamnosus* LS-8 and *L. crustorum* MN047 on the change of genes expression (related to lipid metabolism and inflammatory response) in white fat tissue were investigated by the total RNA analysis in epididymal fat. Compared to the C group, the expressions of lipid metabolism-related genes, including fatty acid synthase (*FAS*), *Pparγ*, *CD36* and *Fabp2*, were significantly increased in HFFD-fed mice, while these alterations were obviously ameliorated by the treatment of *L. rhamnosus* LS-8 and *L. crustorum* MN047 (Fig. 4b). Moreover, the expression of some pro-inflammatory genes, including *TNF-α*, *IL-1β*, *IL-6* and *MCP-1* also showed an increase trend in HFFD-fed mice, which were also significantly reduced by supplementation of *L. rhamnosus* LS-8 and *L. crustorum* MN047 (Fig. 4c).

Effect of *L. rhamnosus* LS-8 and *L. crustorum* MN047 on the SCFAs levels in mice feces

It was reported that SCFAs produced in colon, such as acetic acid, propionic acid, isobutyric, butyric acid, isovaleric and valeric acid, showed multiple beneficial effects on metabolic syndrome, therefore, the effects of *L. rhamnosus* LS-8 and


a
C
CLS
CMN
M
MLS
MMN
50 μm


b
C
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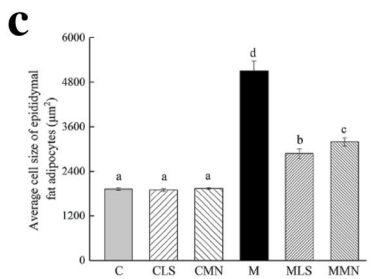

c
Average cell size of epididymal fat adipocytes (μm²)
6000
4800
3600
2400
1200
0
a
a
a
d
b
c
C
CLS
CMN
M
MLS
MMN

◂**Fig. 3** Effects of *L. rhamnosus* LS-8 and *L. crustorum* MN047 administration on liver and epididymal fat tissues histological in HFFD-fed mice under the objective of ×20. **a** Representative H&E staining of liver tissue (scale bar 50 μm); **b** representative H&E staining of epididymal fat tissue (scale bar 50 μm); **c** average cell size of epididymal fat. Bars represent the mean ± SD, bars with different letters indicate significant differences ($p < 0.05$) by Duncan tests. C: mice fed with standard diet, CLS: mice fed with standard diet and *Lactobacillus rhamnosus* LS-8, CMN: mice fed with standard diet and *Lactobacillus crustorum* MN047, M: mice fed with HFFD, MLS: mice fed with HFFD and *Lactobacillus rhamnosus* LS-8, MMN: mice fed with HFFD and *Lactobacillus crustorum* MN047

L. crustorum MN047 on SCFAs levels in HFFD-fed mice were analyzed. From Fig. 5a–f, it was observed that acetic acid, propionic acid and butyric acid were the main colonic SCFAs metabolites in all groups, and the concentration of acetic acid in each group was the highest among the six detected SCFAs (Fig. 5a–f). Furthermore, it was interesting to find that except for the CLS group, the concentration of acetic acid in all other groups were significantly decreased compared to the C group (Fig. 5a). For the concentration of propionic acid, the M group was lower than the C group (Fig. 5b), and interestingly, no matter whether with HFFD feeding or not, the treatment with *L. crustorum* MN047 dramatically increased the concentration of propionic acid, and it was even significantly higher than that in the C group (Fig. 5b). Similarly, the level of butyric acid was significantly decreased in HFFD-fed mice, and which was significantly reversed by administration with *L. rhamnosus* LS-8 and *L. crustorum* MN047 (Fig. 5c). Although the concentration of isobutyric, isovaleric and valeric acid were extremely lower than the SFCAs mentioned above, a distinct decreasing in HFFD-fed mice compared to the C group and obviously elevated by supplementation of *L. rhamnosus* LS-8 and *L. crustorum* MN047 were also found (Fig. 5d–f).

Effect of *L. rhamnosus* LS-8 and *L. crustorum* MN047 on gut microbiota

16S rRNA gene sequencing analysis were conducted for the investigation of effects of *L. rhamnosus* LS-8 and *L. crustorum* MN047 administration on gut microbiota. A total of 1,909,884 clean sequences were obtained after the sequences were quality-filtered with the QIIME pipeline and an average of 63,663 clean sequences per sample were used for the downstream analysis. For alpha diversity analysis, Simpson, Shannon, Ace and Chao indexes were used to assess community diversity and richness. Except for the Simpson indexes were significantly elevated in HFFD-fed mice, another three indexes (Shannon, Ace and Chao) were markedly decreased as compared to the standard diet fed mice, which suggested that HFFD feeding induced a decreasing in the alpha diversity of gut microbiota (Fig. 6a). Although both supplementation of *L. rhamnosus* LS-8 and *L. crustorum* MN047 in HFFD-fed mice had no significant difference in the alpha diversity compared to the M group, it was also found that these alterations were improved by *L. rhamnosus* LS-8 but were aggravated by *L. crustorum* MN047 (Fig. 6a). Correspondingly, the alpha diversity of the MLS group was significantly better than that of the MMN group (Fig. 6a). In addition, analysis by UPGMA clustering (Fig. 6e), as well as the unweighted and weighted UniFrac distance based PCoA (Fig. 6f, g) showed a distinctive microbiota profiles between the standard diet fed groups (C, CLS and CMN group) and the HFFD-fed groups (M, MLS and MMN group), which indicates these two probiotics supplementation almost did not lead to any significant recovery of beta diversity in the gut microbiota of HFFD-fed mice.

A closer look at the microbial community revealed the influence of probiotics supplementation at both phylum and genus levels. At phylum level, *Firmicutes*, *Bacteroidetes* and *Proteobacteria* dominated the gut microbiota in the all groups (Fig. 6b). Specifically, HFFD feeding induced a distinct elevation in the relative abundance of *Firmicutes* (from 31.9 to 47.3%) and an obvious decreasing in *Bacteroidetes* (from 53.7 to 23.3%) compared with the C group, which also resulted in the increasing of the *Firmicutes* to *Bacteroidetes* ratio (from 0.596 to 2.030) in HFFD-fed mice (Fig. 6c). However, supplementation of *L. rhamnosus* LS-8 and *L. crustorum* MN047 did not prevent these changes in HFFD-fed mice compared to the M group, and the ratio of these two phyla were even higher than that in the M group (*Firmicutes/Bacteroidetes*, from 2.030 to 5.276 and 13.267, respectively) (Fig. 6c). In contrast, the abundance of *Proteobacteria* was significantly elevated in the M group when compared with the C group (from 8.1 to 22.2%), which was strikingly ameliorated (from 22.2 to 18.5% and 15.6%, respectively) by the administration with *L. rhamnosus* LS-8 and *L. crustorum* MN047 in HFFD-fed mice (Fig. 6c). In addition, at the genus level, 30 most abundant genera were selected for the further analysis (Fig. 6d and Table S2). There were 17 genera which had significant changes (increased or decreased) in the M group compared with the C group, and there were 10 genera significantly reversed via the supplementation of *L. rhamnosus* LS-8 and/or *L. crustorum* MN047. Specifically, compared with the C group, the genera of *Lactobacillus, uncultured_bacterium_f_Erysipelotrichaceae, Olsenella* and *uncultured_bacterium_f_Bacteroidales_S24-7_group* were decreased in the M group. The results also indicated that the changes of other three genera were only prevented by *L. crustorum* MN047, except for the abundance of *Lactobacillus* increasing in both administration of *L. rhamnosus* LS-8 and *L. crustorum* MN047. Conversely, the genera *Desulfovibrio, Blautia, Catenibacterium, Bacteroides, Candidatus_Saccharimonas and Faecalibaculum* were significantly increased in the M group compared to the C group. And except for the abundance of *Blautia* only significantly

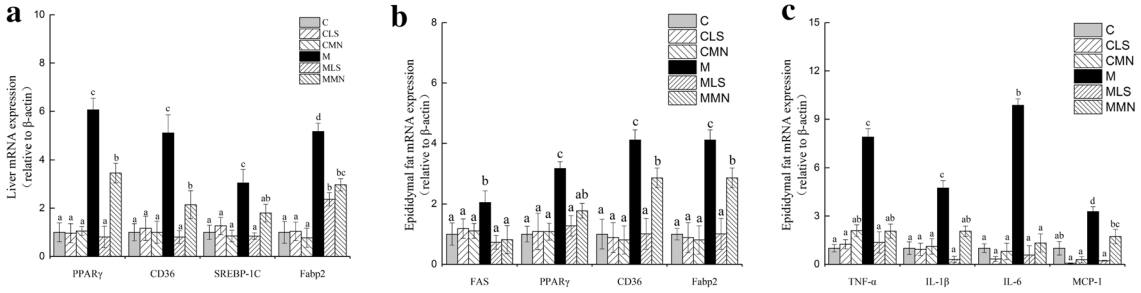


Fig. 4 Effects of *L. rhamnosus* LS-8 and *L. crustorum* MN047 administration on liver and epididymal fat tissues gene expressions in HFFD-fed mice. **a** mRNA levels of *Pparγ*, *CD36*, *Srebp-1c* and *Fabp2* in the liver tissue; **b** mRNA levels of *FAS*, *Pparγ*, *CD36* and *Fabp2* in the epididymal fat tissue; **c** mRNA levels of *TNF-α*, *IL-1β*, *IL-6* and *MCP-1* in the epididymal fat tissue. Bars represent the mean ± SD ($n = 5$ mice/group), bars with different letters indicate significant differences ($p < 0.05$) by Duncan tests. C: mice fed with standard diet, CLS: mice fed with standard diet and *Lactobacillus rhamnosus* LS-8, CMN: mice fed with standard diet and *Lactobacillus crustorum* MN047, M: mice fed with HFFD, MLS: mice fed with HFFD and *Lactobacillus rhamnosus* LS-8, MMN: mice fed with HFFD and *Lactobacillus crustorum* MN047

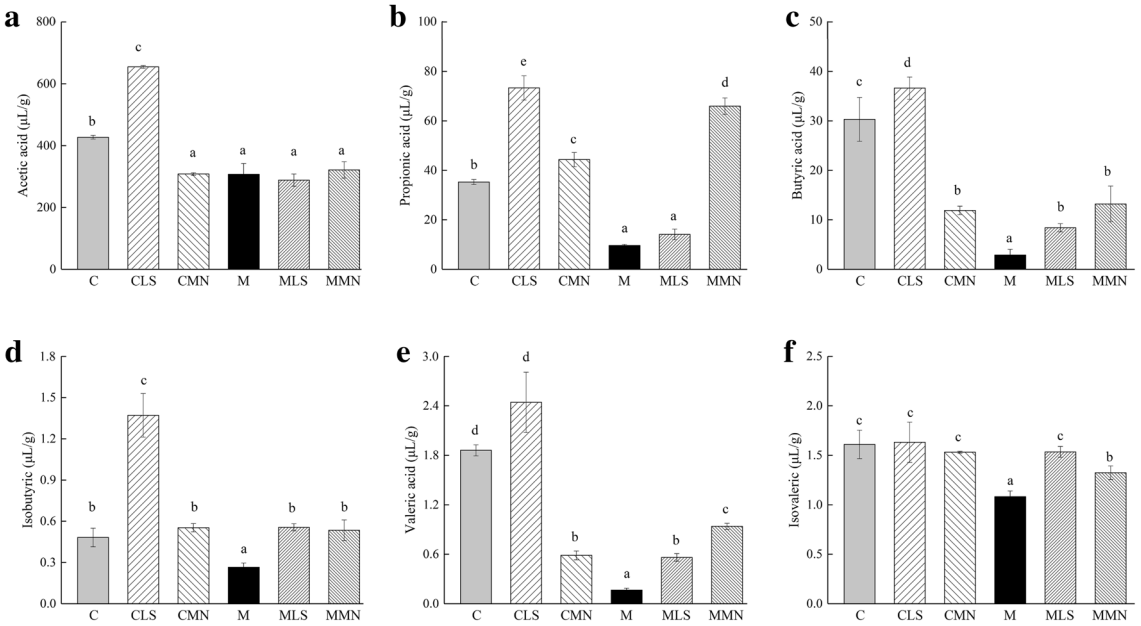


Fig. 5 Effects of *L. rhamnosus* LS-8 and *L. crustorum* MN047 administration on the concentration of SCFAs in mice feces after 10 weeks of HFFD feeding. **a** Acetic acid; **b** propionic acid; **c** butyric acid; **d** isobutyric; **e** valeric acid; and **f** isovaleric. Bars represent the mean ± SD ($n = 8$ mice/group), bars with different letters indicate significant differences ($p < 0.05$) by Duncan tests. C: mice fed with standard diet, CLS: mice fed with standard diet and *Lactobacillus rhamnosus* LS-8, CMN: mice fed with standard diet and *Lactobacillus crustorum* MN047, M: mice fed with HFFD, MLS: mice fed with HFFD and *Lactobacillus rhamnosus* LS-8, MMN: mice fed with HFFD and *Lactobacillus crustorum* MN047

decreased by *L. rhamnosus* LS-8 supplementation, other five genera were significantly reversed by both treatment with *L. rhamnosus* LS-8 and *L. crustorum* MN047. Interestingly, although some beneficial genera had no significant difference between the M group and C group, the abundance of them was apparently elevated by probiotics supplementation, such as *Bifidobacterium, Anaerotruncus, Turicibacter, Ruminiclostridium_9* and *Lachnospiraceae_NK4A136_group*, etc.

To identify the specific phylotypes that were significantly altered in response to probiotics supplementation, all effective sequences of the sample were analyzed using the LEfSe method. Taxonomy cladogram obtained from phylum to species indicated that microbiota in the *Deltaproteobacteria* class, *Desulfovibrionales* order, *Desulfovibrionaceae* family, *Desulfovibrio* genus and *uncultured_bacterium_g_Desulfovibrio* species were enriched in HFFD-fed mice, whereas

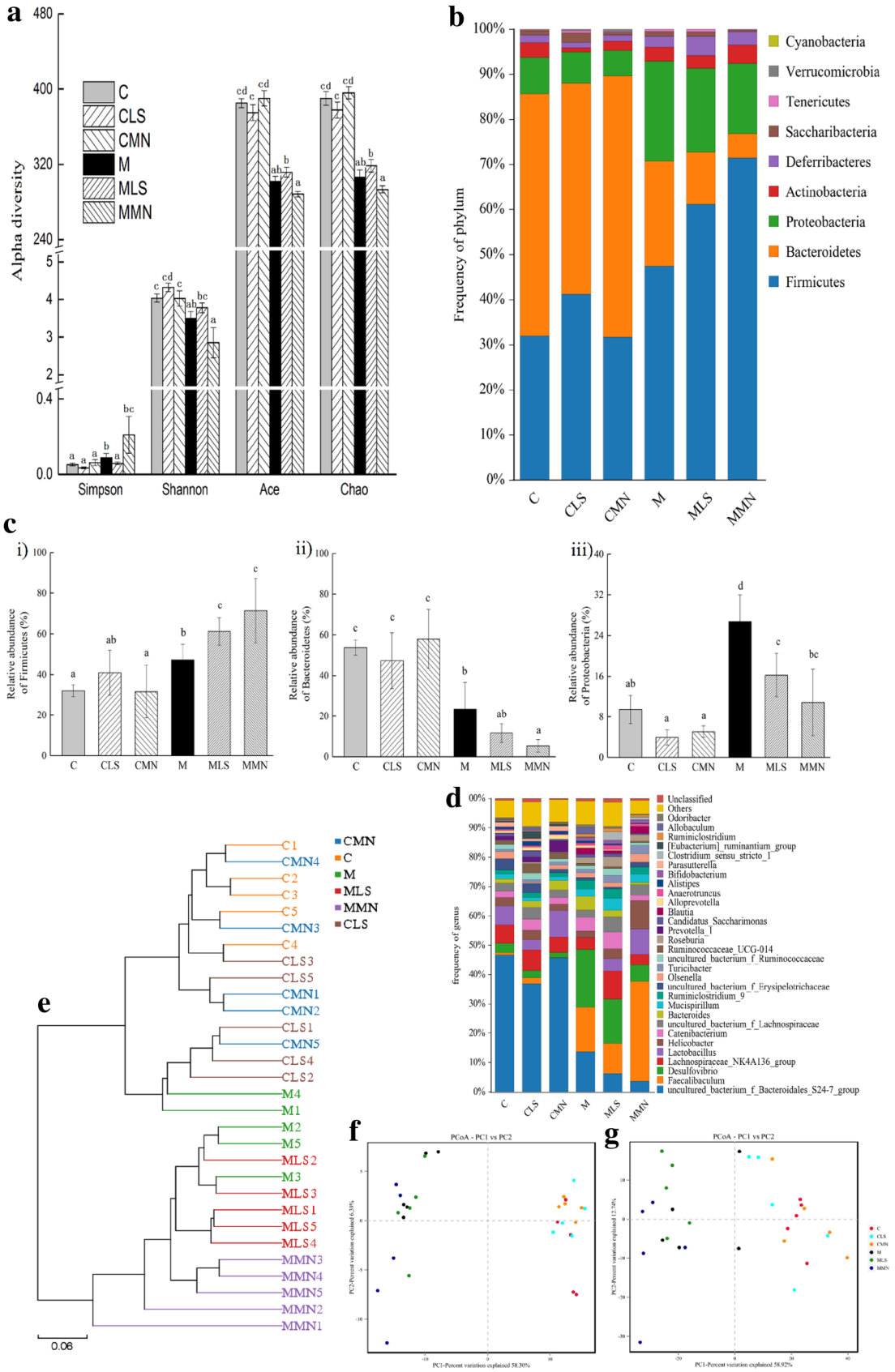
Bacteroidetes phylum, *Bacteroidia* class, *Bacteroidales* order, *Bacteroidales_S24_7_group* family, *uncultured_bacterium_f_Bacteroidales_S24_7_group* genus and *uncultured_bacterium_f_Bacteroidales_S24_7_group* species were enriched in the C group (Fig. 7a). These results were also shown in the LDA score plot (Fig. 7b). In addition, with the same method, it was found that the *Firmicutes* phylum, *Bacilli* class, *Lactobacillales* order, *Lactobacillaceae* family, *Lactobacillus* genus were enriched in both supplementation of *L. rhamnosus* LS-8 and *L. crustorum* MN047 when compared with the M group (Fig. 7c–f).

Discussion

It has been reported that excessive intake of fructose and saturated fats may cause obesity-associated metabolic syndrome and intestinal microbiota disorder [15, 32]. Although there were some evidences suggested that some *Lactobacillus* may have potentially beneficial effects on anti-obesity, the applications of specific *Lactobacillus* strains for anti-obesity were still limited as the anti-obesity mechanisms were not fully understood. Thus, the objective of this study was to evaluate the potential anti-obesity effects of two new strains (*L. rhamnosus* LS-8 and *L. crustorum* MN047) on HFFD-fed mice. Results indicated that consumption of HFFD throughout the experimental period significantly increased body weight gain and the levels of serum TG, TC and LDL-c, accelerated hepatic lipid accumulation, epididymal fat expansion and led to liver function (γ-GT, AST, ALT) injury, which were consistent with that reported by Liu et al. [33]. It was found that the levels of HDL-C in all the HFFD-fed groups (M group, MLS group and MMN group) were significantly higher than that in the standard diet fed groups (C group, CLS group and CMN group), which was in accordance with the recent reports by Lee et al. [34] and Pothuraju et al. [35], and it might be caused by the stress reaction. In addition, kidney function (serum CRE and BUN) injury was also found in the M group, which was similar to the HFD and STZ-induced diabetic rats [36]. Fortunately, by the supplementation of *L. rhamnosus* LS-8 and *L. crustorum* MN047 during the HFFD feeding period, these adverse changes were remarkably ameliorated. It was also found that although the total energy intake among all groups were not significantly different, the body weight gain was significantly suppressed by both *L. rhamnosus* LS-8 and *L. crustorum* MN047 supplementation in HFFD-fed mice, which indicated that energy intake was not the only determinant for obesity development [10, 33].

Leptin, a specific secreted protein from adipose tissues, mainly used to regulate food intake and energy expenditure by the central nervous system [37]. Leptin deficiency mice (ob/ob mice) shown hyperphagia, obesity and insulin resistance, and which was proved to be reversed by supplementation with leptin [38]. However, in the present study, the increasing of leptin levels were not accompanied with the expected anorectic responses in HFFD-induced obese mice, nevertheless, which was reversed by administration with *L. rhamnosus* LS-8 and *L. crustorum* MN047. This phenomenon was similar to the previous literature [33] and related to the occurrence of leptin resistance. Grunfeld et al. [39] found that the levels of leptin in the infected host were increased in response to pro-inflammatory stimulation, such as TNF and LPS. Therefore, it was speculate that the high levels of leptin in the M group might be mainly caused by the high secretion/expression of pro-inflammatory cytokine (TNF-α, IL-1β, IL-6, MCP-1), as well as the high production of pro-inflammatory substance (LPS) in HFFD-induced obese mice. Therefore, after the supplementation of the proposed strains, the modulation of gut microbiota and its metabolites (SCFAs) would lead to the amelioration of inflammatory response, and which then led to the decreasing of leptin levels and even obesity related reactions. For other hormones, such as adiponectin and insulin, it also played an important role in obesity. For example, adiponectin was a smaller numbers of anti-inflammatory factors secreted by adipose tissues, and the function of adiponectin was to stimulate autophagy and reduce oxidative stress, which would further improve insulin sensitivity [40]. Moreover, the secretion of adiponectin by adipocytes was inhibited by pro-inflammatory factors [41]. Consistent with these reports, in the present study, the obese mice induced by HFFD feeding had lower serum adiponectin levels, and accompanied with higher pro-inflammatory factors (TNF-α, IL-1β, IL-6 and LPS) levels and insulin resistance, and it also found that these changes were distinctly ameliorated by administration with *L. rhamnosus* LS-8 and *L. crustorum* MN047. Hence, it was clearly that the level of the inflammatory response was closely related to the secretion of leptin and adiponectin, and which were also modulated by the intervention of the proposed strains via action on the gut microbiota that had significant influence on the inflammatory response. Therefore, the results indicated that the gut microbiota can be act as an important checkpoint for the inflammatory response and further lead to the amelioration of obesity.

Chronic low-grade inflammation is closely related to obesity and metabolic disorder. It has been previously reported that chronic inflammation plays a crucial role in the development of obesity-related insulin resistance [6], and an inflammatory program is activated early in adipose expansion, and then, during chronic obesity, which would permanently lead the immune system to a pro-inflammatory phenotype [8]. Obviously, it can be seen that an increasing of some pro-inflammatory factors including TNF-α, IL-1β, IL-6 in HFFD-fed mice serum (Fig. 2c–e), and the expression of these inflammatory genes mentioned above were


a
Alpha diversity
C
CLS
CMN
M
MLS
MMN
Simpson
Shannon
Ace
Chao
b
Frequency of phylum
Cyanobacteria
Verrucomicrobia
Tenericutes
Saccharibacteria
Deferribacteres
Actinobacteria
Proteobacteria
Bacteroidetes
Firmicutes
c
i)
Relative abundance of Firmicutes (%)
ii)
Relative abundance of Bacteroidetes (%)
iii)
Relative abundance of Proteobacteria (%)
d
frequency of genus
Unclassified
Others
Odoribacter
Allobaculum
Ruminiclostridium
[Eubacterium]_ruminantium_group
Clostridium_sensu_stricto_1
Parasutterella
Bifidobacterium
Alistipes
Anaerotruncus
Alloprevotella
Blautia
Candidatus_Saccharimonas
Prevotella_1
Roseburia
Ruminococcaceae_UCG-014
uncultured_bacterium_f_Ruminococcaceae
Turicibacter
Olsenella
uncultured_bacterium_f_Erysipelotrichaceae
Ruminiclostridium_9
Mucispirillum
Bacteroides
uncultured_bacterium_f_Lachnospiraceae
Catenibacterium
Helicobacter
Lactobacillus
Lachnospiraceae_NK4A136_group
Desulfovibrio
Faecalibaculum
uncultured_bacterium_f_Bacteroidales_S24-7_group
e
CMN
C
M
MLS
MMN
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C1
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C4
CLS3
CLS5
CMN1
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MMN3
MMN4
MMN5
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MMN1
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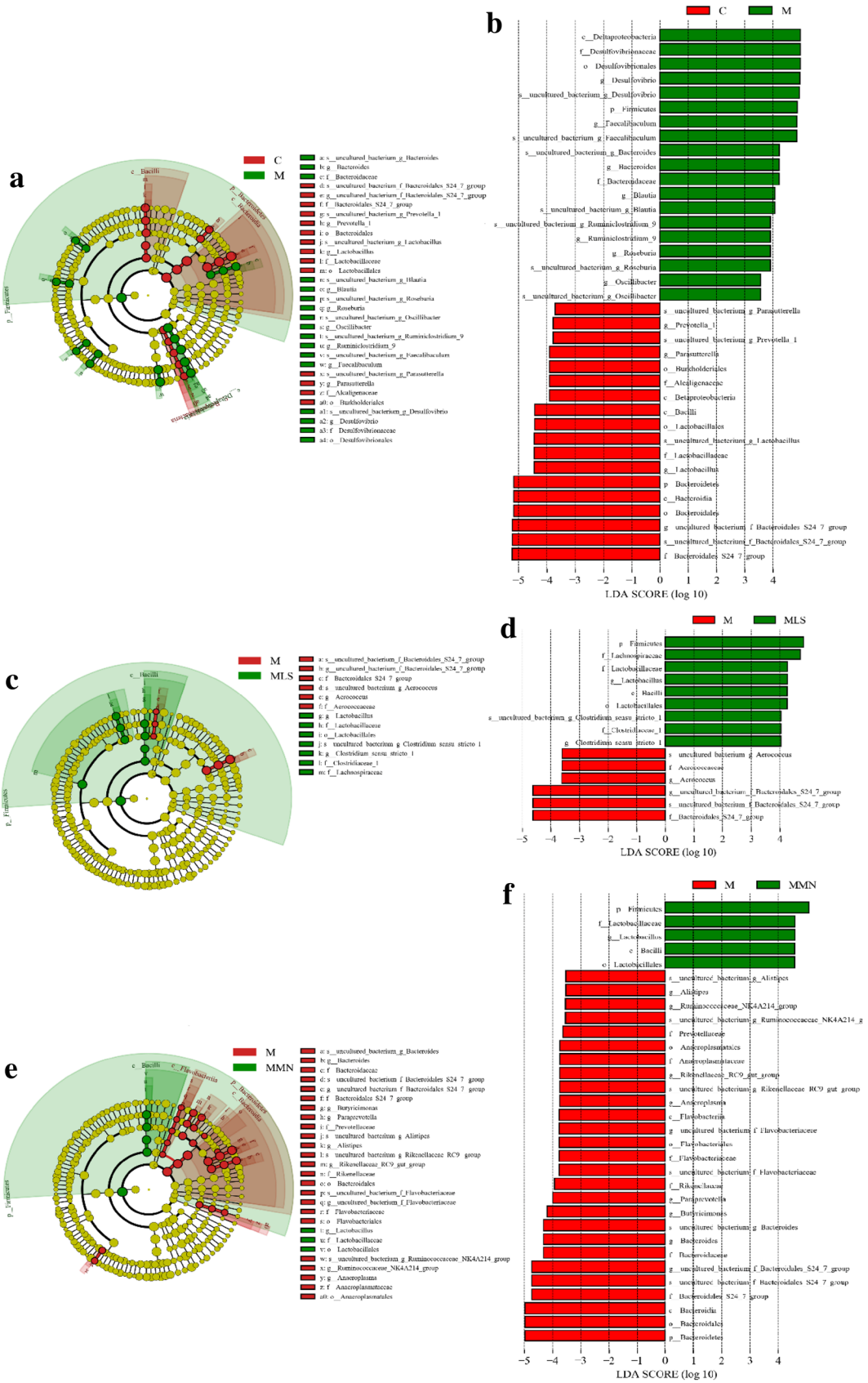
f
PCoA - PC1 vs PC2
g
PCoA - PC1 vs PC2

◂**Fig. 6** Effects of *L. rhamnosus* LS-8 and *L. crustorum* MN047 administration on gut microbiota structure in HFFD-fed mice. **a** Alpha diversity of the microbial community indicated by the Simpson, Shannon, Ace and Chao indexes; **b** gut microbiota composition at phylum level; **c** The relative abundance of (i) *Firmicutes*, (ii) *Bacteroides* and (iii) *Proteobacteria*; **d** gut microbiota composition at genus level; **e** weighted UPGMA of all samples; **f**, **g** Plots of unweighted and weighted UniFrac-based PCoA, respectively. Bars represent the mean ± SD ($n = 5$ simples/group), bars with different letters indicate significant differences ($p < 0.05$) by Duncan tests. C: mice fed with standard diet, CLS: mice fed with standard diet and *Lactobacillus rhamnosus* LS-8, CMN: mice fed with standard diet and *Lactobacillus crustorum* MN047, M: mice fed with HFFD, MLS: mice fed with HFFD and *Lactobacillus rhamnosus* LS-8, MMN: mice fed with HFFD and *Lactobacillus crustorum* MN047

also significantly elevated in obese mice epididymal adipose tissues, which was consistent with the previous reports [10, 33]. What's more, these alterations were significantly reversed by the administration with *L. rhamnosus* LS-8 and *L. crustorum* MN04. Strikingly, the levels of serum IL-1β in *L. crustorum* MN04 intervention mice were significantly lower than the C group, suggesting that *L. crustorum* MN04 was more effective than *L. rhamnosus* LS-8 in inhibiting the secretion of IL-1β. Moreover, MCP-1, as a cytokine produced in adipose tissues, was an important chemokine for macrophage recruitment and responsible for the beginning of macrophages infiltration into adipose tissues [42, 43]. The overexpression of *MCP-1* in adipose tissues was finally led to the development of insulin resistance and some other obesity-related complications, such as atherosclerosis [12, 13]. In the present study, the expression of *MCP-1* was remarkably increased in HFFD-induced obese mice, which was similar to the HFD-induced obese mice [1]. While these alterations were prevented by the treatment with *L. rhamnosus* LS-8 and *L. crustorum* MN047 (Fig. 4c). In addition, LPS as a biomarker of Gram-negative bacteria has been proved as an inducement to trigger insulin resistance [19] for the effects on stimulating the release of pro-inflammatory cytokines, and disruption of the metabolism function, such as insulin function and lipid metabolism [20, 44]. Furthermore, LPS was also regarded as a biomarker of intestinal dysbiosis for the increasing of gut permeability [32, 45]. The present study found that the higher levels of LPS in HFFD-induced obese mice was significantly inhibited by supplementation of *L. rhamnosus* LS-8 and *L. crustorum* MN047 (Fig. 2f), which was similar to the results that HFD-induced obese mice had extremely higher circulatory levels of LPS than lean mice [26, 46]. Some studies also found that the abundance of LPS-producing bacteria such as *Proteobacteria*, *Bacteroides, Enterobacterium, Escherichia coli, Salmonella* sp. would be enriched in the obese individuals [47–49]. Therefore, the serum LPS levels were remarkably decreased in these two probiotics supplementation groups (MLS and MMN) was probably due to the lower abundance of LPS-producing bacteria (*Proteobacteria* and *Bacteroides*) caused by these two strains (Fig. 7 and Table S2). All these results implied that the administration with these two probiotics could ameliorate intestinal dysbiosis, and which further led to the decreasing of inflammatory response and the amelioration of obesity related metabolic abnormalities.

Meanwhile, growing literature reported that high fat and/or high fructose diet consumption led to gut microbiota dysbiosis, which further promoted an elevation in systemic LPS levels [50, 51]. However, no complete consensus reached on the composition of intestinal microbiota in obese and lean individuals up to now. Therefore, 16S rRNA gene sequencing analysis was conducted to illustrate the variation of the gut microbiota in mice during the HFFD feeding process, and the results showed that both *L. rhamnosus* LS-8 and *L. crustorum* MN047 administration had significant effects on the abundance of some specific species, although no extremely significant difference in the alpha and beta diversity. Contrary to some findings that the increasing tendency of *Firmicutes*/*Bacteroidetes* in HFFD or HFD fed mice could be inhibited by probiotics intervention [2, 52], it showed that the ratio of *Firmicutes* and *Bacteroidetes* was even higher than that in the M group after *L. rhamnosus* LS-8 and *L. crustorum* MN047 supplementation. It need to be emphasized that the ratio of *Firmicutes* and *Bacteroidetes* has not been used as a biomarker of obesity so far, and the results indicated that the change of *Firmicutes*/*Bacteroidetes* might be different in the obesity mice when administration with *Lactobacillus*. *Proteobacteria* mainly consist of Gram-negative bacteria and its amount showed a positive correlation with serum LPS levels [53]. Compared with the M group, a decreasing of fecal *Proteobacteria* in the MLS group and MMN group were observed, which was consistent with the results of serum LPS levels among the corresponding groups. In addition, at the genus level, it needed to be mentioned that some harmful genera were elevated in HFFD induce obese mice, but which were reversed by *L. rhamnosus* LS-8 and *L. crustorum* MN047 administration. For example, a higher abundance of *Desulfovibrio* and *Bacteroides* in obese mice was observed, which would inhibit the mitochondrial respiration of colonic epithelial cells and trigger inflammatory response and insulin resistance by producing hydrogen sulfide and LPS, respectively [49, 54], nevertheless, the supplementation of *L. rhamnosus* LS-8 or *L. crustorum* MN04 not only reduced the abundance of the proposed harmful genera, but also promoted the increasing of the beneficial genera, such as *Lactobacillus*, *Bifidobacterium* and *Lachnospiraceae NK4A136 group*, all of which were related to SCFAs-producing [47, 55]. As SCFAs were the important energy sources for the host and helpful in regulating lipid metabolism, immunity and adipocyte development [17, 18], the increasing of the SCFAs level in gut would be beneficial for the amelioration of inflammatory


a
C
M
b
C
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LDA SCORE (log 10)
c
M
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d
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MMN
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◂**Fig. 7** LEfSe analysis results. Only the taxa with LDA score higher than 3.5 are shown; **a**, **b** Cladogram showing the phylogenetic relationships of bacteria taxa and LDA scores between the C and the M group. **c**, **d** Cladogram showing the phylogenetic relationships of bacteria taxa and LDA scores between the M and the MLS group. **e**, **f** Cladogram showing the phylogenetic relationships of bacteria taxa and LDA scores between the M and the MMN group. Yellow dots indicate no statistical significance. C: mice fed with standard diet, CLS: mice fed with standard diet and *Lactobacillus rhamnosus* LS-8, CMN: mice fed with standard diet and *Lactobacillus crustorum* MN047, M: mice fed with HFFD, MLS: mice fed with HFFD and *Lactobacillus rhamnosus* LS-8, MMN: mice fed with HFFD and *Lactobacillus crustorum* MN047

response and other obesity related metabolic abnormalities. Therefore, similar with other related studies [4, 56, 57], the significantly increasing of the levels of SCFAs (Fig. 5a–d) showed a high consistency with the alleviation of inflammatory response, glucose tolerance and insulin resistance mentioned in each group, furthermore, which further indicated that the gut microbiota and its metabolites were the potential targets for the prevent or cure of obesity.

Meanwhile, the influence on the expression of host genes (such as *Pparγ*, *CD36*, *Fabp2*, *Srebp-1c*, *Fas*, etc.), which were closely related with lipid metabolism, was also important for the amelioration of metabolic syndrome. In the present study, it was found that the mRNA levels of these lipid metabolism-related genes, including *PPAR-γ*, *CD36*, *Fabp2*, *Srebp-1c*, *FAS*, were extremely higher in the hepatic and/or adipose tissues of mice in the M group than that in the C group, but all these up-regulations were reversed by *L. rhamnosus* LS-8 and *L. crustorum* MN047 supplementation (Fig. 4a, b), which was also corresponding to the results of recovering morphology of fatty liver and epididymal fat expansion, as well as the improving insulin sensitivity in these two probiotics administration groups. Although it was not sure whether the supplementation of the proposed probiotics directly influenced on the expression of these key genes, it would like to believe that the modulation of the gut microbiota and its metabolites can lead to the decreasing of the inflammatory response and further to ameliorate the obesity abnormalities, such as lipid accumulation, inulin resistant and even the expression of some crucial genes. In addition, there was an important point to be mentioned that *β-actin* might not be the best housekeeping gene for the analysis of adipose tissue related genes, and if the investigation has a highly requirements for the quantification and accuracy of the test, it is necessary to select more appropriate housekeeping gene as internal reference for RT-qPCR analysis by using geNorm, NormFinder or BestKeeper, etc.

The above results indicated that the *L. rhamnosus* LS-8 and *L. crustorum* MN047 supplementation possessed a good anti-obesity on HFFD fed mice by the combined action of gut microbiota modulation, inflammatory response amelioration, as well as glucose and lipid metabolism regulation. Therefore, the action mode of the proposed strains can be deduced as the following three aspects: (1) gut microbiota were manipulated firstly by these two probiotics supplementation, resulting in a decreasing of harmful bacteria abundance (such as LPS-producing bacteria, *Proteobacteria* and *Bacteroides*) and an increasing of beneficial bacteria abundance (such as SCFAs-producing bacteria, *Lactobacillus*, *Bifidobacterium* and *Lachnospiraceae NK4A136 group*), and then which in turn raised the levels of SCFAs and lowered the levels of LPS; (2) inflammatory response was then ameliorated by inhibiting the secretion/expression of pro-inflammatory factors (TNF-α, IL-1β, IL-6 and MCP-1), which mainly regulated by the related metabolites (inflammatory stimulus, LPS; and inflammatory inhibitor, SCFAs) of gut microbiota; (3) with the attenuated inflammatory response, the expression of lipid metabolism-related genes including *Pparγ*, *Srebp-1c*, *CD36*, *Fabp2* and *FAS* were further normalized, as well as the abnormal sugar and lipid metabolism (TG, TC and LDL-c, glucose tolerance and insulin resistance). Based on the proposed three action modes, the obesity caused by HFFD in mice was significantly alleviated by the intervention of *L. rhamnosus* LS-8 and *L. crustorum* MN047, which not only enriched the function of probiotics, but also provided a new insight for the prevention and therapy of obesity.

Conclusion

The present study indicated that *L. rhamnosus* LS-8 and *L. crustorum* MN047 possessed good anti-obesity effect on the HFFD-induced mice, as well as the alleviation of inflammatory response, lipid metabolic and other obesity related abnormalities. Based on the analysis of cytokines secretion, related gene expression, gut microbiota and its metabolites (LPS and SCFAs), the anti-obesity action mode of the proposed two strains was concluded as a process that the gut microbiota and its metabolites were firstly modulated by the oral intervention of these strains, and then which resulted in the attenuation of the inflammatory response, finally the lipid metabolism, expression of related genes, secretion of hormones and other obesity related abnormalities were significantly alleviated. The results of the present study provided new knowledge about the action mode of anti-obesity effects of *L. crustorum* on HFFD-induced mice, which also promotes the development of the proposed strains as function food for the prevention or therapy of obesity and obesity-related complications. In addition, the synergistic effect of these two strains on anti-obesity and the molecule mechanism of the regulation on inflammatory response of the gut microbiota or its metabolites would be further studied in the future.

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Author contributions TW and XL designed the study and wrote the manuscript; HY and YL performed the experiments; XL and XW analyzed the data; YS and YY interpreted the results of experiments; BL and YZ prepared figures. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors have declared no conflicts of interest.

Ethical standards All animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals: Eighth Edition, ISBN-10: 0-309-15396-4, and experimental procedures were approved by the Animal Ethics Committee of Xi'an Jiaotong University.

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