Probiotic *Bifidobacterium Lactis* V9 attenuates hepatic steatosis and inflammation in rats with non-alcoholic fatty liver disease

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Abstract

Both steatosis and inflammation are key pathological events in the progression of non-alcoholic fatty liver disease (NAFLD). Probiotics are beneficial in the prevention and treatment of NAFLD. *Bifidobacterium animalis* subsp.*lactis* V9 (V9) is a newly isolated strain with favorable probiotic properties. The study aims to evaluate the effects and mechanisms of V9 on the hepatic steatosis and inflammatory responses in a rat model of NAFLD induced by high-fat diets (HFD). Our results showed that administration with V9 significantly attenuated HFD-induced increases in the levels of alanine transaminase (ALT) and aspartate aminotransferase (AST), accompanied by alleviated hepatic steatosis. V9 supplementation decreased the accumulation of hepatic triglyceride (TG) and free fatty acid (FFA), while increasing the levels of glycogen. The levels of serum glucose were also decreased in HFD rats administrated with V9. Meanwhile, the transcription of SREBP-1c and FAS was reduced and the hepatic expression of phosphorylated-AMPK and PPAR-α was restored by V9 administration. V9 suppressed the production of inflammatory cytokines (e.g. IL-6, IL-1β, and TNF-α) in HFD-fed rats. The anti-inflammatory effect of V9 was found to be associated with the inhibition of hepatic expression of TLR4, TLR9, NLRP3, and ASC mRNA. Furthermore, the activation of ERK, JNK, AKT and NF-κB was suppressed by V9 treatment. These results indicated that *Bifidobacterium Lactis* V9 improved NAFLD by regulating *de novo* lipid synthesis and suppressing inflammation through AMPK and TLR-NF-κB pathways, respectively.

Key Points

V9 supplementation alleviates HFD-induced metabolic disorder.

V9 inhibited HFD-induced expression of FAS, SREBP1c and activated AMPK.

V9 suppressed the TLR-NF-κB signaling pathway in HFD-fed rats.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the chronic liver pathology which occurs when fat is deposited (steatosis) in the liver with no history of excessive alcohol consumption. NAFLD patients may progress to nonalcoholic steatohepatitis (NASH) combined with the fat deposition, oxidative stress and a series of inflammation in the liver. Some NAFLD patients may progress to cirrhosis and even hepatocellular carcinoma (Hashimoto et al. 2011). The prevalence of NAFLD has been rapidly increasing due to lifestyle changes such as increased consumption of high-fat diets and lack of exercise (Williams et al. 2011). NAFLD is now recognized as a worldwide health concern since it is closely associated with diabetes mellitus (type II) and cardiovascular diseases (Ratziu et al. 2010; Chacko et al. 2016). Besides lifestyle modifications (Malaguarnera et al. 2012) and bariatric procedures (Shouhed et al. 2017), there is no effective pharmacological treatment for NAFLD.

The pathogenesis of NAFLD is largely unknown. The widely accepted “two-hit” theory holds that the first “hit” of dysregulated lipid metabolism and insulin resistance lead to hepatic “second-hit” including

inflammatory cytokine production, oxidative stress-mediated lipotoxity and other mechanisms (Musso et al. 2010). Growing evidence indicates that the gut microbiota participates in NAFLD pathogenesis and lipotoxicity (Bibbò et al. 2018; Borrelli et al. 2018), through the metabolism of nutrients and a number of secretory factors that eventually target the liver (Szabo et al. 2011; Lu et al. 2016). Both experimental and clinical studies have shown that probiotics are beneficial in the prevention and treatment of NAFLD (Mattace et al. 2014, Nobili et al. 2018). The molecular mechanisms of the beneficial effects of probiotics are not entirely understood.

Bifidobacterium animalis subsp.lactis V9 (V9) is a novel strain isolated from healthy Mongolian children, possessing favorable probiotic properties such as aciduricity and bile resistance (Sun et al. 2010). The strain has been used in the industrial fermentation of dairy starter cultures by Inner Mongolia Yili Industrial Group Co. Ltd, China. The results of a recent research have demonstrated that the fermentation of whole oat flour with V9 and Lactobacillus plantarum TK9 yield a synbiotic food rich in lactic acid bacteria and prebiotics (Wang et al. 2015). A study has shown that Bifidobacterium and dietary blueberry supplementation could attenuate hepatic lipid accumulation in NAFLD rats (Ren et al. 2014). Administration of Bifidobacterium longum and fructo-oligosaccharides reduces the production of pro-inflammatory cytokines, HOM-IR, steatosis and NASH in patients with nonalcoholic steatohepatitis (Malaguarnera et al. 2012). We are wondering if the novel probiotic strain of V9 has beneficial effects against NAFLD. In this study, we established an HFD-induced rat model of NAFLD which was demonstrated by fatty liver, hyperglycemia, as well as systemic inflammation. We then explored the potential effects and underlying mechanisms of V9 on the improvement of metabolic dysfunction and inflammation in HFD-fed rats by examining various physiological parameters, the molecular changes of metabolism-related genes and the inflammatory signaling pathways. Our results indicate that Bifidobacterium Lactis V9 is a potential candidate for the treatment of HFD-induced metabolic syndrome.

Materials And Methods

Bacteria strain and growth conditions

The strain of Bifidobacterium animalis subsp. lactis V9 CGMCC5470 (V9), deposited at the China General Microbiological Culture Collection Center (CGMCC), was originally isolated from healthy Mongolian children and identified as described previously (Sun et al. 2010). The strain was cultured in MRS broth for 72 h under anaerobic environment. The cell pellets of V9 were harvested, washed twice with PBS and then lyophilized. The lyophilized powder of V9 was suspended in physiological saline and adjusted to 1×10^9 CFU/ml for treatment of the rats.

Animal experiments

Male Wistar rats (120–140 g) were purchased from Vital River Laboratories Animal Co. Ltd. (Beijing, China) and housed under controlled condition (20–22 °C, 55 ± 10% relative humidity, 12-h light/dark cycle). Experiments on rats began after one week's adaptation. During the adaption period, rats were fed a standard chow diet (10% energy from fat, Ke Ao Xie Li Diet Co. Ltd, Beijing, China) and received water

ad libitum. All protocols for animal experiments were approved by the Animal Care and Use Committee at Inner Mongolia Agricultural University. Rats were randomly divided into five groups (n = 8 in each group): control, high-fat diet (HFD), HFD + Berberine, V9 treatment (HFD/V9) and V9 control (CON/V9). With the exception of the control and CON/V9 group fed with a normal chow diet, rats in the remaining groups were given a high-fat diet (60% energy from fat, Ke Ao Xie Li Diet Co. Ltd, Beijing, China) to establish a model of non-alcoholic fatty liver disease. Six weeks later, all groups of rats were fed with a normal chow diet. Rats in the HFD/V9 and CON/V9 groups were gavaged with V9 (1×10^9 CFU) for 4 weeks. At the same time, the HFD + Berberine group of rats was orally administrated with Berberine (300 mg/kg). At the end of the experiment, all the rats were fasted overnight and then anesthetized with isoflurane. Samples of blood and liver were collected. Parts of liver tissues were fixed in 10% formalin for histological analysis and other samples were snap frozen and stored at – 80 °C until use.

Biochemical analysis

The serum samples were obtained by centrifuging clotted blood samples at 3,500 × g for 10 min, which was then stored at − 80 °C for subsequent analysis. The levels of serum ALT, AST and glucose were measured using an automatic biochemical analyzer (Olympus 2700) according to the instructions of the clinical laboratory of Inner Mongolia People's Hospital (Hohhot, China). Hepatic TG,glycogen, and free fatty acid (FFA) levels were measured by the corresponding kits according to the manufacturer's instructions (Jiancheng Institute of Biotechnology, Nanjing, China). Briefly, the liver samples (100–200 mg) were homogenized by mechanical crushing and the protein concentration was quantified using the Sangon Biotech BCA protein kit according to the manufacturer's instructions. The content of TG, glycogen, and FFA was measured by spectrophotography and calculated based on the protein content of liver homogenates.

Histological examination

The liver samples were fixed in 10% paraformaldehyde at room temperature for 24 h. After dehydration through a graded ethanol series, the samples were immersed in xylene, embedded in paraffin, and then cut into 5 μm sections for hematoxylin-eosin (HE) staining. The images of HE stain were taken under a light microscope and assessed blindly by a pathologist.

Enzyme-linked immunosorbent assay

Serum TNF-α, IL-1β, and IL-6 levels were detected using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, USA) according to the manufacturer's instructions.

Real-time PCR assay

Total mRNA from liver tissues was extracted by using a Trizol reagent (Invitrogen, Carlsbad, CA). RNA concentrations and purities (OD260/280) were measured by a NanoDrop Lite spectrophotometer. The total mRNA (1 μg) was reversed into first strand cDNA using the RT-PCR kit (Takara, Dalian, China). Real-time PCR assay was performed by using the specific primers as listed in Table S1. GAPDH was used as

an endogenous control to normalize the relative expression of target transcripts. Relative quantification of gene expression was performed by the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

The liver proteins were extracted by using the tissue protein extraction solution or the nucleoprotein extraction kit (Sangon Biotech, Shanghai, China). The protein content was measured as previously described. The same amount of total protein from each sample was electrophoresed into SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were probed using rabbit polyclonal antibodies against p-ERK/ERK, p-JNK/JNK, p-AKT/AKT, p-NF-κB/NF-κB and p-AMPK/AMPK (Cell Signaling, USA) overnight at 4 °C. After washing five times with TBST, the membranes were incubated with Goat Anti-Rabbit IRDye 800 CW secondary antibodies for 1 h. The images of specific proteins were detected and analyzed using the LICOR ODYSSEY CLx infrared imaging system.

Statistical analysis

All the data were expressed as mean ± standard deviation (SD) and analyzed using the one-way analysis of variance (ANOVA) with Duncan's multiple-range test. P-Values < 0.05 were regarded as statistically significant.

Results

V9 treatment attenuates high-fat diet- induced liver injury

In order to explore the mitigating effects of Bifidobacterium Lactis V9 on high-fat diet induced liver injury, the development of liver damage was histologically examined by H&E staining. Administration with the high-fat diet for 6 weeks caused evident damage in the liver as shown by the disturbed structure of lobules, hepatocyte hypertrophy and severe hepatic steatosis represented by the formation of numerous lipid droplets. In contrast, Bifidobacterium Lactis V9 and Berberine treatment significantly improved the structure of liver lobules and alleviated hepatic steatosis (Fig. 1a). In addition, serum alanine transaminase (ALT) and aspartate aminotransferase (AST) levels were measured in different groups. As compared with the HFD group, both V9 and Berberine treatment significantly decreased the serum ALT and AST levels (Fig. 1b and c). These results indicate that V9 alleviate high fat diet-induced hepatic injury and steatosis.

V9 treatment improves HFD-induced lipid and glucose disorder

We went on to measure the parameters of lipid and glucose metabolism in liver and serum. The hepatic TG and FFA levels were increased in the HFD group while decreased in the V9 and Berberine treatment group (Fig. 2a and b). V9 and Berberine treatment restored the HFD-induced decrease in the hepatic glycogen content (Fig. 2c). Compared with the HFD group, V9 supplementation also induced a significant

reduction in serum TC and fasting glucose levels (Fig. 2d). These results indicate that V9 supplementation improved the disorder in lipid and glucose metabolism.

V9 supplementation prevents the deregulation of genes involved in lipid and glucose metabolism

To understand the underlying mechanisms of the observed effects, we evaluated the key signaling pathways and proteins involved in lipid and glucose metabolism modulation. The mRNA expression of sterol regulatory element-binding protein 1c (SREBP-1c) and the lipogenic enzyme fatty acid synthase (FAS) was increased in HFD rats and reduced by V9 and Berberine treatment (Fig. 3a and b). Hepatic PPAR-α mRNA expression was deeply reduced by HFD challenge and restored by V9 treatment. There is no significant change in the Berberine treatment group as compared with the HFD group. Compared with the control group, an increase in the expression of PPAR-α mRNA was also found in the V9 control group (Fig. 3c). Furthermore, Western blotting analysis showed that treatment with V9 and Berberine restored the expression of phosphorylated-AMPK (Fig. 3d).

V9 supplementation reduces HFD-induced pro-inflammatory responses

To investigate the anti-inflammatory effects of probiotic V9, hepatic mRNA levels and serum concentrations of TNF-α, IL-1β, and IL-6 were measured. Compared with the control group, HFD challenge resulted in significantly increased levels of serum TNF-α, IL-1β and IL-6 as well as corresponding hepatic mRNA expression. V9 supplementation markedly inhibited HFD-induced expression of TNF-α, IL-1β, and IL-6 both in mRNA and protein levels. A similar trend was found in the Berberine treatment group (Fig. 4a-f). These results indicated that both V9 and Berberine can reduce HFD-induced pro-inflammatory cytokine production.

V9 suppresses the hepatic expression of TLRs and NLRP3

As shown in Fig. 5a and b, the hepatic TLR4 and TLR9 mRNA expression increased significantly in the HFD group in comparison with the control group. However, V9 and Berberine treatment significantly reduced the expression of TLR4 and TLR9 mRNA in HFD rats ($P < 0.05$). We found a similar tendency in the TLR4 and TLR9 protein expression after the administration of V9 and Berberine (Figure S1). A significant decline in NLRP3 and ASC mRNA expression was also found in V9 and Berberine treatment group (Fig. 5c and d).

V9 inhibits the activation of MAPK, AKT and NF-κB

In order to further study the mechanisms of the protective effects of V9 against HFD-induced NAFLD, we went on evaluating the changes of the TLR-mediated downstream signaling pathways. As shown in Fig. 6a, HFD challenge resulted in elevated phosphorylation of JNK, ERK and AKT. However, V9 and

Berberine supplementation evidently reduced the activation of JNK, ERK and AKT. V9 treatment also inhibited HFD-induced phosphorylation of NF-κB (Fig. 6b). These results indicate that modulation the activation of MAPK, AKT and NF-κB contribute to the anti-inflammatory effects of V9.

Discussion

The chronic liver disease of NAFLD has emerged as a growing worldwide health burden without effective treatment. Probiotics confer a health benefit on the host with NAFLD by regulating glycolipid metabolism and steatosis-induced inflammatory responses (Kim et al. 2017; Ritze et al. 2014). However, the beneficial effects of probiotics are evidently related to specific strains and dosage (Xu et al. 2012; Ferolla et al. 2015). In this study, our results revealed that the novel strain of probiotic V9 exhibits beneficial effects against HFD-induced NAFLD through its hypolipidemic and anti-inflammatory potential.

In our study, a rat model of NAFLD was successfully established as evidenced by elevated serum glucose, increased hepatic TG and FFA. In addition, histopathological observations revealed severe steatosis in the model group. Studied have demonstrated that the levels of ALT and AST are increased in patients of NAFLD with insulin resistance (Sheng et al. 2018). Similar to this result, our study also showed that ALT and AST levels are significantly increased in HFD rats. All these results indicate that there is a metabolic disorder, hepatic steatosis, and hepatotoxicity in the HFD rats.

Evidence from the clinical trial demonstrated that administration with multi-strain probiotics reduces the fatty liver index, pro-inflammatory cytokines and ALT levels of NAFLD patients (Kobyliak et al. 2018). Animal studies have shown that co-administration with cholesterol-lowering probiotics (two strains) and anthraquinone from Cassia obtusifolia L. has potential treatment effects for NAFLD (Mei et al. 2015). A similar study also indicates that supplementation with Bifidobacterium longum and Lactobacillus acidophilus attenuate liver fat accumulation in NAFLD rats, and the superior effect of Bifidobacterium longum was found (Xu et al. 2012). Our results are consistent with the above studies by showing that V9 improved hepatic steatosis and HFD-induced lipid and glucose disorder. There was markedly reduced serum ALT, AST, TG and glucose accompanied by reduced hepatic lipid accumulation (TG and FFA) and increased content of hepatic glycogen in V9 treatment group. These results suggest the single Bifidobacterium strain of V9 has beneficial effects on the progression of NAFLD. Whether the V9 strain is superior in terms of alleviating liver fat accumulation than other identified Lactobacillus strains needs to be clarified in the future.

In order to elucidate the underlying mechanisms of the hypolipidemic effect of V9, the expression of lipogenic sterol regulatory element binding protein 1c (SREBP1c) and its target gene fatty acid synthase (FAS) was analyzed. The results of decreased expression of SREBP1c and FAS in HFD/V9 group indicate that lipid de novo synthesis was suppressed by V9 treatment. Peroxisome proliferative-activated receptors (PPARs) are transcription factors belonging to the nuclear receptor superfamily, which are involved in lipid metabolism, energy homeostasis and inflammation (Derosa et al. 2017). PPARα is highly expressed in the liver, and is the master regulator of hepatic lipid flux by modulating fatty acid transport

and β-oxidation. In addition, PPARα activation counteracts the expression of the inflammatory genes induced by NF-κB (Tanaka et al. 2017, Alves et al. 2017, Staels et al. 1998). Experimental evidence shows that PPARα deficiency leads to susceptibility to NAFLD, NASH and hepatic inflammatory responses (Ip et al 2003). And thus, PPARα is an ideal therapeutic target for hyperlipidemia. A research has shown that selective activation of PPARa by K877 improves lipid metabolism in mice (Takei et al. 2017). The results of our study showed that HFD-induced decreased expression of hepatic PPAR-α mRNA was restored in the V9 treatment group while not in the Berberine group, suggesting different underlying mechanisms as to the function of V9 and Berberine. Considering the role of PPAR-α in lipid metabolism, we conclude that PPAR-α is one of the underlying target genes that are induced in the V9-mediated attenuation of NAFLD.

AMP-activated kinase (AMPK), a key regulator in energy metabolic homeostasis, which has been identified as a crucial target for drugs (Hardie et al. 2012). Activated AMPK phosphorylates SREBP and attenuates hepatic steatosis and atherosclerosis in mice with insulin resistance (Li et al. 2011). Treatment with V9 and Berberine induced the phosphorylation of AMPK, indicating that both of them alleviated hepatic steatosis through promoting the activation of AMPK. A recent study demonstrated that Lactobacillus plantarum NA136 could activate AMPK pathway to suppress the SREBP-1/FAS signaling to inhibit the de novo lipogenesis in mice with NAFLD (Zhao Z et al. 2019). Our study is consistent with the study of Lactobacillus plantarum NA136. And thus, we conclude that V9 supplementation alleviates HFD-induced metabolic disorder through upregulating the AMPK and PPARα signaling pathways.

It has been demonstrated that TLR4 plays a key role in the transition from simple steatosis to non-alcoholic steatohepatitis (NASH) in mice with obesity-induced NAFLD (Ye et al. 2012). The expression of TLR9 is increased in NASH models and activation of TLR9 signaling leads to inflammatory recruitment and cell survival (Mridha et al. 2017). Our results coincided with the previous reports by showing that the expression of TLR4 and TLR9 was increased in the model group of NAFLD. However, V9 supplementation inhibited the expression of TLR4 and TLR9 (Fig. 5). The results were correlated with reduced production of pro-inflammatory cytokines in V9 treatment group. It suggests that the beneficial effects of V9 on NAFLD are associated with the downregulation of TLR4 and TLR9-induced inflammatory responses.

The role of NLRP3 in the transition from steatosis to NASH has also been verified recently (Blasetti et al. 2017). Blocking NLRP3 by MCC950 mitigate hepatic inflammation and fibrosis as shown in two mouse models of NASH (Mridha et al. 2017). In addition, the study of Tipoe G et al (Xiao et al. 2016) showed that bee's honey attenuates the progression of NASH partly through suppressing the thioredoxin-interacting protein-NLRP3 pathway. In our study, increased transcription of NLRP3 and its adaptor protein ASC was found in the NAFLD model group. Treatment with V9 and Berberine significantly decreased the expression of NLRP3 and ASC, suggesting that regulation on the expression of NLRP3 inflammasome is one of the underlying mechanisms of the anti-inflammatory effect of V9.

To further support the anti-inflammatory effect of V9 on HFD-induced inflammatory responses, we studied the TLR4 downstream signaling changes. Our results showed that V9 and Berberine treatment reduced the phosphorylation of JNK, ERK, AKT, and NF-κB (Fig. 6). As activation of NF-κB leads to the

priming of NLRP3 inflammasome, it is rational to deduce that reduced activation of NF-κB contributes to the reduced expression of NLRP3, which was verified in our study. In summary, the anti-inflammatory effect of V9 against NAFLD is associated with the down-regulation on TLR-NF-κB-NLRP3 signaling.

In summary, our results demonstrated that administration of probiotic V9 protects against HFD-induced liver damage. The beneficial effect is mediated by a reduction in hepatic fat accumulation (through increasing AMPK-mediated fatty acid β-oxidation and decreasing de novo lipid synthesis) and in inflammation (through downregulation of TLR-NF-κB signaling pathways). Probiotic V9 has the potential to be used as an alternative treatment for NAFLD.

Abbreviations

NAFLD
Non-alcoholic fatty liver disease
V9
Bifidobacterium animalis subsp.lactis V9
HFD
High-fat diets
ALT
Alanine transaminase ALT
AST
Aspartate aminotransferase
TG
Triglyceride
FFA
free fatty acid
NASH
Nonalcoholic steatohepatitis
CGMCC
China General Microbiological Culture Collection Center
HE
Hematoxylin-eosin
ELISA
Enzyme-linked immunosorbent assay
SREBP1c
Sterol regulatory element binding protein 1c
FAS
Fatty acid synthase
PPARs
Peroxisome proliferative-activated receptors
AMPK

AMP-activated kinase

Declarations

Compliance with ethical standards

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

Ethical Approval This article does not contain any studies with human participants performed by any of the authors. All experiments were approved by the Animal Care and Use Committee of Inner Mongolia Agricultural University (China) according to the Chinese Council on Animal Care guidelines.

Consent for publication: All authors listed have read the complete manuscript and have approved submission of the paper.

Author Contribution

YW and GZ conceived the study, analyzed the data, and revised the manuscript; YY and CL executed most of the experiments, collected the data and prepared the manuscript; SZ and XW performed the Real-time PCR and ELISA assay; JW did the HE staining and analyzed the photos; HZ help to prepare with the manuscript. All authors reviewed and approved the final manuscript.

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Figures

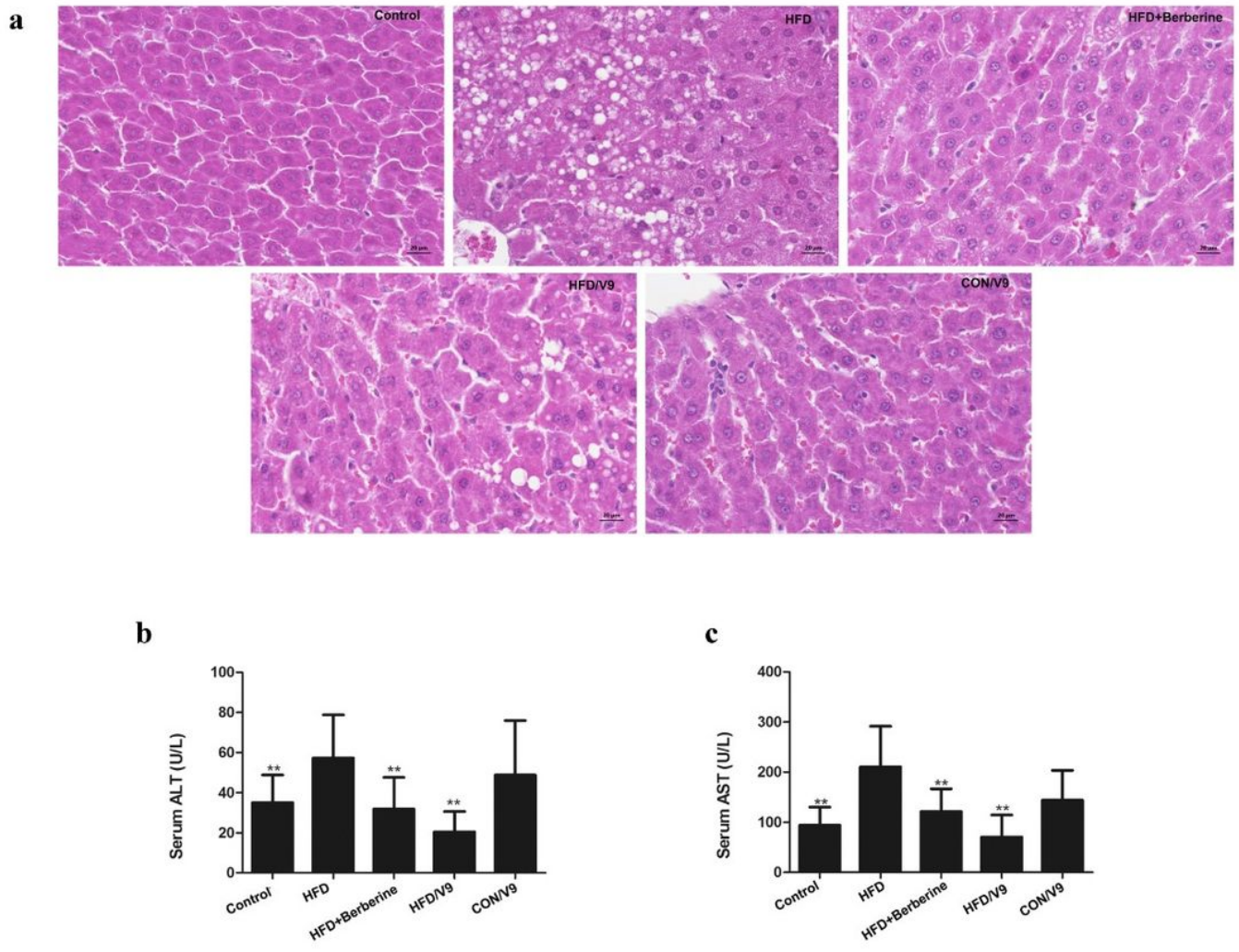

Figure 1

Probiotic V9 improves hepatic steatosis and liver damage in HFD rats. (a) H&E staining analysis of liver sections. (b) Serum ALT levels. (c) Serum AST levels. Data are expressed as means ± SD, with n=8, **P<0.01 vs HFD group.

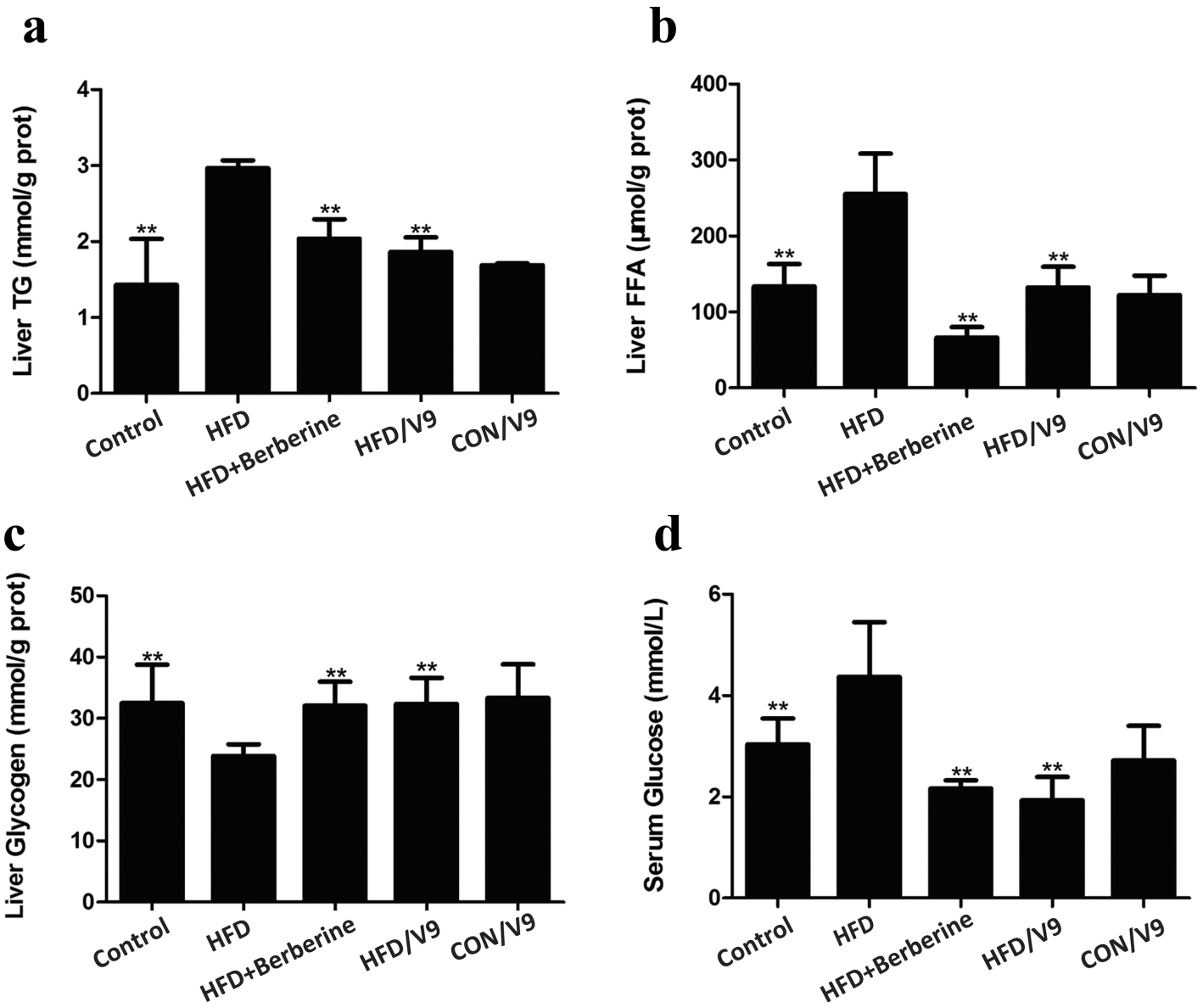

Figure 2

V9 treatment improves HFD-induced metabolic disorder. (a) Hepatic levels of TG. (b) Hepatic levels of FFA. (c) Hepatic levels of Glycogen. (d) Fasiting glucose levels in serum. Data are expressed as means ± SD with n=8, **P < 0.01 vs HFD group.

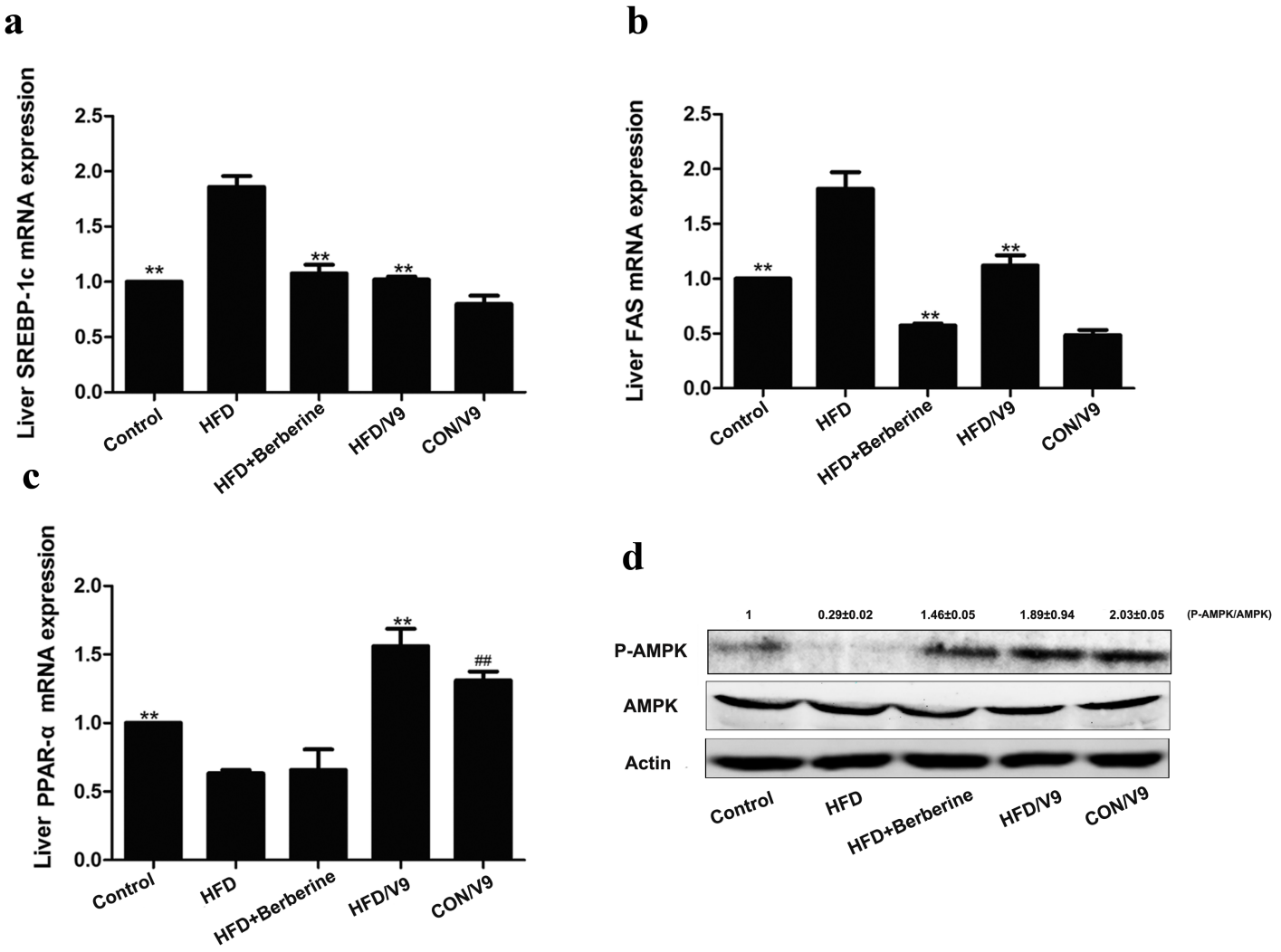

Figure 3

V9 treatment reduces HFD-induced inflammatory response. (a) Serum TNF-α production. (b) Hepatic TNF-α mRNA expression. (c) Serum IL-1β production. (d) Hepatic IL-1β mRNA expression. (e) Serum IL-6 production. (f) Hepatic IL-6 mRNA expression. Data are expressed as means ± SD, with n=8, #P < 0.05 vs control group, and **P < 0.01 vs HFD group.

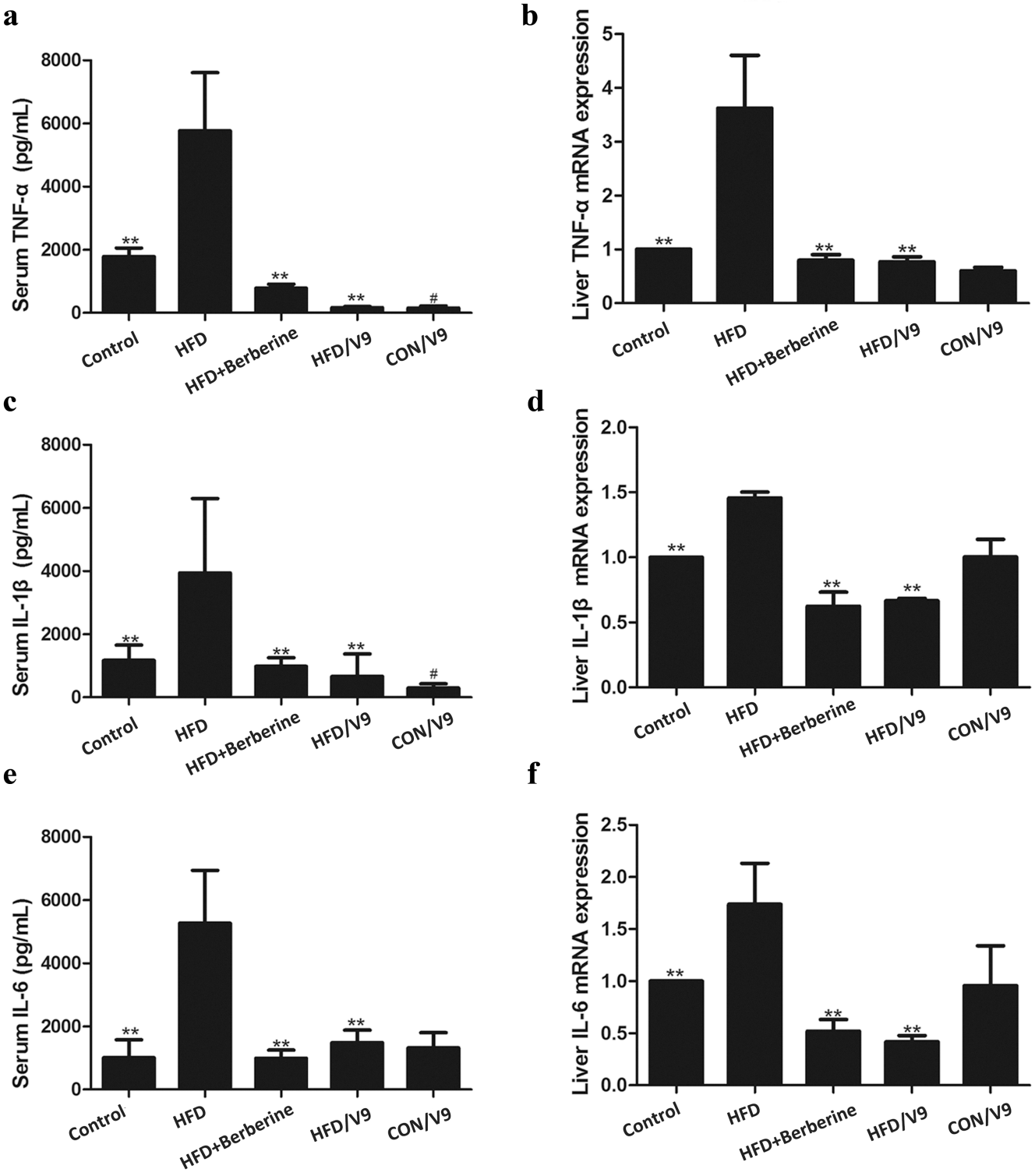

Figure 4

Probiotic V9 regulate the lipid metabolism in the liver. (a) SREAP-1c mRNA expression. (b) FAS mRNA expression. (c) PPAR-α mRNA expression. (d) Western blotting analysis of hepatic p-AMPK/AMPK. The representative picture is one from three independent experiments.

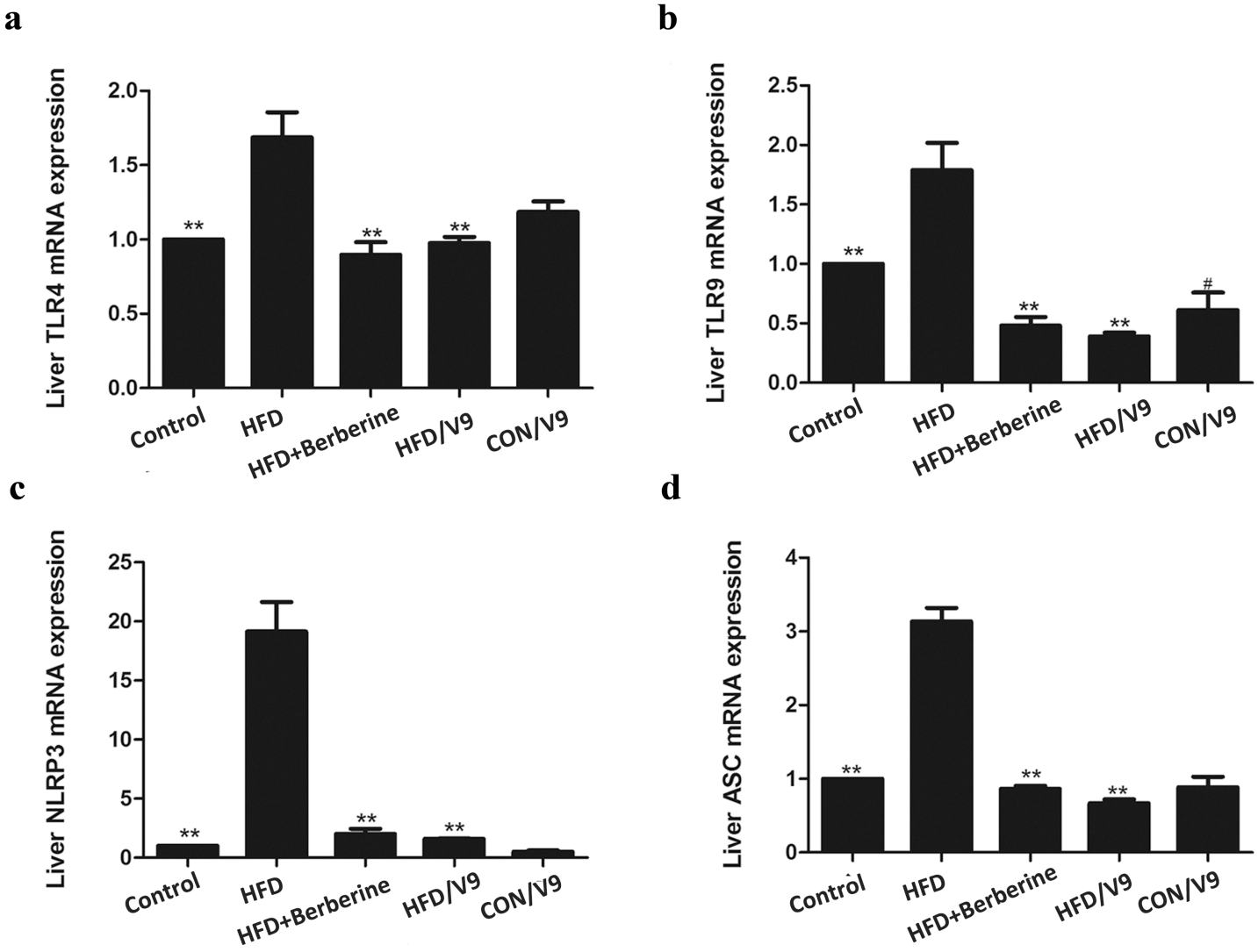

Figure 5

The effect of Probiotic V9 on hepatic TLRs, NLRP3 and ASC. (a) Hepatic mRNA levels of TLR4. (b) Hepatic mRNA levels of TLR9. (c) Hepatic mRNA levels of NLRP3. (d) Hepatic mRNA levels of ASC. Data are expressed as means ± SD with n=8, #P < 0.05 vs Control group; **P < 0.01 vs HFD group.

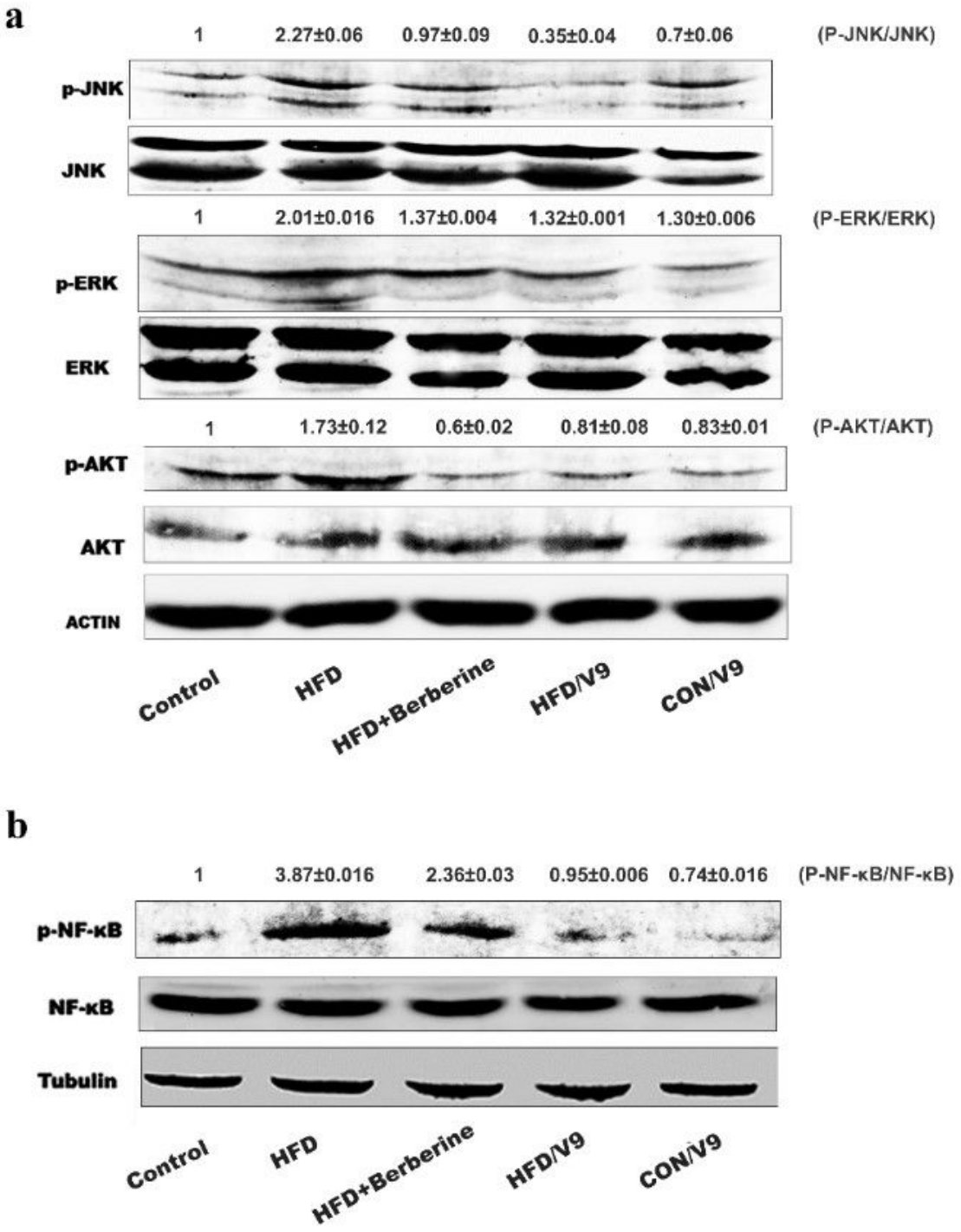

Figure 6

The effects of probiotic V9 on hepatic MAPK, AKT and NF-κB activation. (a) Western Blotting analysis of p-JNK/ JNK, p-ERK/ERK and p-AKT/ AKT. (b) Western Blotting analysis of nuclear p-NF-κB/NF-κB. The representative picture in (a) and (b) is one from three independent experiments.

Supplementary Files

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