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Lactobacillus plantarum NA136 improves the non-alcoholic fatty liver disease by modulating the AMPK/Nrf2 pathway

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Abstract
Hepatic lipid metabolic disorders and oxidative stress are involved in the development of non-alcoholic fatty liver disease (NAFLD). This study is to determine the protective effects of *Lactobacillus plantarum* NA136 on high-fat diet and fructose (HFD/F)-induced NAFLD and to elucidate its underlying molecular mechanisms. Male C57BL/6J mice had been fed with normal diet (ND), HFD/F, or HFD/F supplemented with *L. plantarum* NA136 for 16 weeks. Treatment with *L. plantarum* NA136 significantly lowered the body weight gain and decreased the mass of fat tissues, lipids, AST, and ALT levels of HFD/F-treated mice. Our results showed that *L. plantarum* NA136 activated AMPK pathway to phosphorylate ACC and to suppress the SREBP-1/FAS signaling to inhibit the de novo lipogenesis and increase the fatty acid oxidation. Furthermore, with treatment of *L. plantarum* NA136, the nuclear translocation of NF-E2-related factor 2 (Nrf2) was also increased which could activate antioxidant pathway. These findings suggested that *L. plantarum* NA136 improved NAFLD by regulating the fatty acid metabolism and defending against oxidative stress through AMPK and Nrf2 pathways, respectively.

Keywords *Lactobacillus plantarum* · NAFLD · AMPK · Nrf2

Introduction

Non-alcoholic fatty liver disease (NAFLD) is considered to be the most common liver disease, which ranges from benign steatosis to severe liver injury, affecting the health of both adults and children around the world (Younossi et al. 2016). NAFLD is a chronic metabolic disorder disease associated with insulin resistance (IR), oxidative stress, and dyslipidemia (Michelotti et al. 2013). Hepatic lipid accumulation during the development of NAFLD occurs due to the increased uptake of cholesterol and TG, which are regulated by raising of circulating lipids, exporting very low-density lipoproteins, increasing de novo lipogenesis, and enhancing fatty acid oxidation (Ipsen et al. 2018). In addition, oxidative stress as the main role in the development of NAFLD has been well-established (Sumida et al. 2013). It has been discussed in association with the alterations in lipid metabolic and the expression of proinflammatory transcription factors (Browning and Horton 2004). Therefore, therapeutic options that alleviate hepatic lipid metabolism and oxidative stress are crucial in managing NAFLD. Due to the vital role of gut microbiota in obesity-associated fatty liver, recent researches in food science have focused on gut microbiota in order to expand the treatment options for NAFLD (Compare et al. 2012; Leung et al. 2016; Ma et al. 2017).

It is well established that probiotics play a key role in modulating the gut microbiota composition, enhancing the intestinal barrier function, and regulating the host immune responses (Marchesi et al. 2016). Probiotics are a collection of active microorganisms beneficial to the host and can have definite health effects on improving the micro-ecological balance of the host. Selected strains of probiotic bacteria, especially *Bifidobacteria*, *Streptococcus*, and *Lactobacilli*, show such health benefits as bowel improvement, immune potentiation, antiinflammatory, diabetes delaying, and even cancer prevention (Fuller 1992). In addition, probiotics have potential beneficial effects on NAFLD. Recent evidence suggests that overgrowth of small intestinal bacterial induced liver injury by gut-derived lipopolysaccharides (LPS) and

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expression of TNF-α that linked to NAFLD (Loguercio et al. 2002). Similar study showed that *Lactobacillus rhamnosus* GG protects NAFLD by suppressing immune cell infiltration and pro-inflammatory cytokine secretion (Kim et al. 2016). Furthermore, probiotics may improve hepatic enzymes activity and decrease the serumal TC and LDL levels that are useful to the treatment of NAFLD (Nabavi et al. 2014).

Our research group initially isolated *Lactobacillus plantarum* NA136 from Chinese traditional pickle in Yanbian Korean Autonomous Prefecture, China. Twenty kinds of probiotics were selected for preliminary screening to alleviate NAFLD. Surprisingly, we found that *L. plantarum* NA136 showed excellent performance in reducing blood lipids and enhancing antioxidative status. Thus, we hypothesized that *L. plantarum* NA136 can be effective in improving NAFLD. In the present study, we observed that *L. plantarum* NA136 treatment attenuated NAFLD through activating AMPK/Nrf2 pathway to improve lipid metabolic disorders and to ameliorate oxidative stress response in the liver.

Materials and methods

Culture of *L. plantarum NA136*

L. plantarum NA136 is collected in China Center for Type Culture Collection (CCTCC), and the number is M2018112. *L. plantarum* NA136 was cultured in MRS broth at 37 °C for 18 h. The bacterial cells were harvested after centrifugation at 6000*g* for 10.0 min at 4.0 °C, washed twice with phosphate-buffered solution, resuspended in physiological saline, and adjusted to 1.0×10^9 CFU/day/mice for oral supplement.

Animals and grouping

Male C57BL/6J (8-week-old) mice were offered from Yisi Laboratory Animal Technology Co. Ltd. (Changchun, China) and had been fed with normal diet and water for a week to stabilize all metabolic conditions. Mice were housed at 22±1 °C and a 12-h light/dark cycle. The mice were randomly divided into three groups (n=10): (1) the normal diet group (ND) fed with a standard normal diet (10% energy from fat; Yisi, Changchun, China) and water; (2) the high-fat diet and fructose group (HFD/F) fed a high-fat diet (65% energy from fat; Yisi, Changchun, China) and 30% fructose solution (volume %); and (3) the *L. plantarum* NA136 group (HFD/F+NA136) fed a high-fat diet and fructose concomitantly supplemented with a daily dose of NA136. Animals were raised and experimented according to the guidance of the Animal Care Committee of Jilin Academy of Agricultural Sciences. After 16 weeks, the blood samples were collected and serum was extracted by centrifugation at 4000 rpm for 10 min at 4 °C. The tissues of liver were rapidly harvested, snap-frozen in liquid nitrogen, then stored at −80 °C for further analyses. Portions of liver tissue were frozen by fixing in formalin and waxing in paraffin for subsequent sectioning and mounting on microscopic slides.

Biochemical analyses

Total cholesterol (TC) and triglycerides (TG) were determined by commercial kits (Jiancheng Institute, Nanjing, China). Free fatty acid (FFA), low-density lipoprotein (LDL), high-density lipoprotein (HDL), alanine aminotransferase (ALT), aspartate aminotransferase (AST), superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) were determined by mouse ELISA kit (Jianglai, Shanghai, China).

Histological analysis

At the time of killing, small pieces of liver tissues from each group were fixed in 10% neutral formalin. After waxed in paraffin, 5-μm-thick sections of the tissues were sliced by a microtome, stained with hematoxylin and eosin (H&E), and photographed with an optical microscope (Olympus, Japan).

Western blot analysis

Sliced liver tissues were placed in RIPA cell lysis buffer (Solarbio, Beijing, China) and homogenized in homogenates. The liver lysate was centrifuged at 12000 rpm for 10 min at 4 °C. The supernatant was removed and repeatedly centrifuged. The total protein content was measured by using the BCA Protein Assay Kit (Thermo Scientific, USA). Nuclear extracts from the liver were prepared by using a Nuclear Protein Extraction Kit (Solarbio, Beijing, China). 50 μg of protein were loaded onto 7% or 10% SDS-PAGE, proteins were transferred to PVDF membranes, the membranes were blocked with 5% BSA for 1 h at room temperature, and the blocked membranes were incubated with antibodies against AMP-activated protein kinase (AMPK), phospho-AMPK (pThr172), acetyl-CoA carboxylase (ACC), phospho-ACC (pSer79), sterol-regulatory element-binding protein (SREBP-1), Nrf2, HO-1, Kelch ECH associating protein 1 (Keap-1) (Abcam, Cambridge, MA, USA), fatty acid synthase (FAS) (Bioss, Beijing, China), β-actin (Sangon Biotech, Shanghai, China), and Lambin B (Solarbio, Beijing, China). The proteins were then visualized by enhanced chemiluminescene by using horseradish peroxidase–conjugated antibody. The protein bands were visualized by image scanner (Clinx Science Instruments, Shanghai, China). Protein levels were normalized to β-actin or Lambin B.

Statistical analysis

Data were expressed as means±S.D. for each group. SPSS software version 15.0 was used for all the data analysis. Differences between the groups were carried out using one-way analysis of variance (ANOVA). $P<0.05$ was considered to be statistically significant.

Results

Effects of *L. plantarum* NA136 on body weight and lipid levels in serum

HFD/F-fed mice showed higher body weights than the ND group mice (Table 1). After treatment with *L. plantarum* NA136, the body weight was decreased by nearly 17% compared with that of HFD/F group. The results of various serum parameters of each group are shown in Table 1. The levels of serum TC, TG, and LDL significantly increased in the HFD/F group, but *L. plantarum* NA136 treatment could attenuate these elevations. In addition, HDL level was considerably decreased by HFD feeding, and this decrease was blocked by *L. plantarum* NA136 supplementation.

L. plantarum NA136 ameliorated fat accumulation in the liver

According to a previous study, HFD and fructose induced substantial steatosis (Ritze et al. 2014). We measured hepatic steatosis by histopathological study to investigate the effect of *L. plantarum* NA136 on hepatic lipid accumulation. Compared with that of the ND group livers, H&E staining of the HFD/F group livers revealed a diffuse macrovesicular steatosis (Fig. 1a). More importantly, HFD/F-induced hepatic steatosis and inflammation were markedly reversed by administration of *L. plantarum* NA136. Furthermore, the levels of FFA in serum and hepatic were increased significantly by HFD and fructose, and this increase was normalized by treatment with *L. plantarum* NA136 (Fig. 1b, c). These results suggested that treatment with *L. plantarum* NA136 could attenuate hepatic steatosis induced by HFD and fructose.

Effects of *L. plantarum* NA136 on liver injury

The levels of ALT and AST in the liver are routinely used as markers of liver function. The activities of ALT and AST of the HFD/F group increased significantly compared with those of the ND group, which indicated that HFD/F feeding induced a massive liver injury in the mice (Fig. 2). Nevertheless, elevated ALT and AST concentrations were almost normalized by treatment with *L. plantarum* NA136. These results suggested that treatment with *L. plantarum* NA136 could attenuate liver injury and improve the liver function.

Effects of *L. plantarum* NA136 on hepatic AMPK signaling

SREBP-1 is a major regulator for transcription of lipogenic enzymes, and it also positively regulates hepatic lipogenic proteins including FAS and ACC (Horton et al. 2002; Shimano 2001). We measured the hepatic levels of SREBP-1 to determine the effects of *L. plantarum* NA136 on HFD/F--induced lipid synthesis and clearance. As shown in Fig. 3a and c, the expression of SREBP-1 in HFD/F+NA136 group was significantly decreased and was accompanied with the decrease of FAS expression compared to the HFD/F group.

AMPK regulates the transcription factors such as SREBP-1 to improve lipid metabolism (Long and Zierath 2006). The protein levels of AMPK, ACC, and phosphorylation of AMPK and ACC were examined. The phosphorylation of AMPK was obviously decreased in the HFD/F group, and when treated with *L. plantarum* NA136, the phosphorylation of AMPK was increased in HFD/F+NA136 groups (Fig. 3b). Phosphorylation of AMPK is known to decrease the ACC activity via phosphorylation, which subsequently downregulated the expression of FAS (Yang et al. 2014). HFD/F-induced NAFLD mice demonstrated a lower level of ACC phosphorylation compared with the ND and HFD/F+NA136 groups (Fig. 3b).

L. plantarum NA136 reduced the oxidative stress in the liver

CAT could trigger Nrf2 nuclear translocation and HO-1 gene expression to activate the antioxidative action (Chang et al. 2012). The results exhibited that the level of CAT in

Table 1 Various serum parameters and body weight of mice in each group

Groups	Body weight (g)	TC (mmol/L)	TG (mmol/L)	HDL (mmol/L)	LDL (mmol/L)
ND	27.81±2.54	3.51±0.80	1.16±0.029	0.74±0.03	2.01±0.18
HFD/F	34.20±1.77$^{\#\#}$	5.06±0.26$^{\#\#}$	1.54±0.18$^{\#}$	0.23±0.053$^{\#\#}$	3.23±0.74$^{\#}$
HFD/F+NA136	28.31±1.49	3.97±0.11**	1.32±0.17*	0.63±0.03**	2.55±0.43*

Data represent the mean±SD of each group. $^{\#}P<0.05$, $^{\#\#}P<0.01$ versus ND group; $^{*}P<0.05$, $^{**}P<0.01$ versus HFD/F group

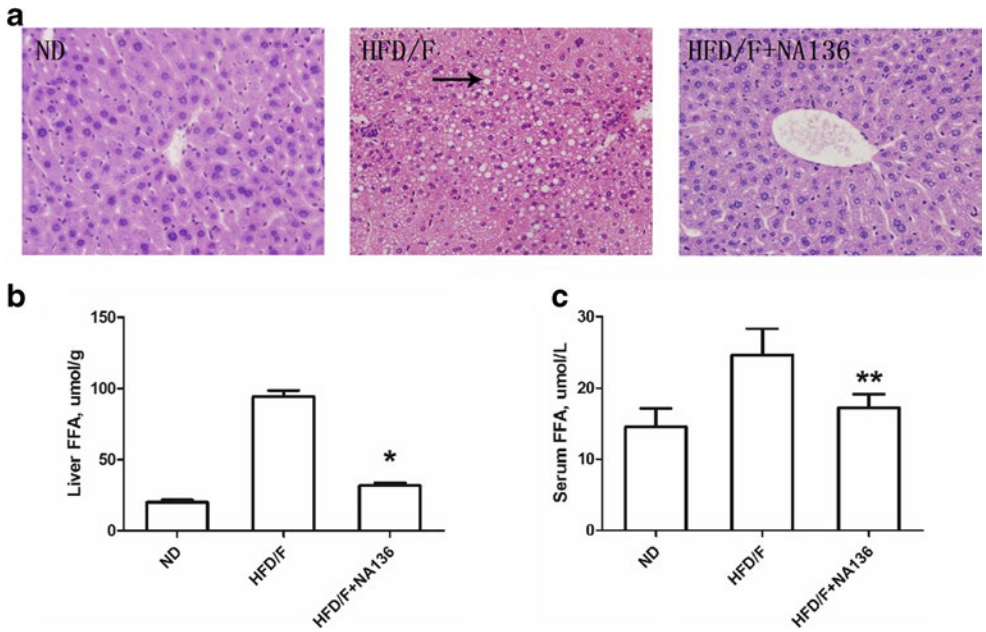

Fig. 1 Effect of *L. plantarum* NA136 treatment on hepatic lipid accumulation. Typical images of representative liver pathology for HE staining (**a**), liver FFA (**b**), and plasma FFA (**c**). Data represent the mean±SD of each group. $^{\#\#}P<0.01$ versus ND group; $^{*}P<0.05$ and $^{**}P<0.01$ versus HFD/F group

the HFD/F group markedly decreased more than that in the ND and HFD/F+NA136 groups (Fig. 4a). As shown in Fig. 4b, the activities of SOD increased evidently in the HFD/F+NA136 group compared with those in the HFD/F group. Liver MDA contents are shown in Fig. 4c. There was a marked increased content of hepatic MDA in the HFD/F group. As expected, the treatment with *L. plantarum* NA136 reduced the content of MDA.

Nrf2 and its repressor Keap1 regulate numerous cellular functions (Ludtmann et al. 2014). When exposed to oxidative stress, the Nrf2 pathway was adaptively activated to alleviate cell damage. As shown in Fig. 4d, the total Nrf2 levels displayed no obvious changes in the three groups. Furthermore, the expressions of HO-1 and the contents of nuclear Nrf2 were markedly increased and the expression of Keap1 was decreased significantly in the ND and HFD/F+NA136 groups as compared to that of the HFD/F group.

Discussion

Probiotics therapy is a novel, safe, and effective approach to reverse the metabolic abnormalities observed in obesity and become a specific liver therapy for NAFLD (Leung et al. 2016). In the present study, treatment with *L. plantarum* NA136 could ameliorate HFD and fructose-induced NAFLD by altering the blood lipid levels, inhibiting hepatic lipid accumulation, protecting the liver function, decreasing the lipogenesis via AMPK activation, and regulating the oxidative stress through Nrf2 pathway.

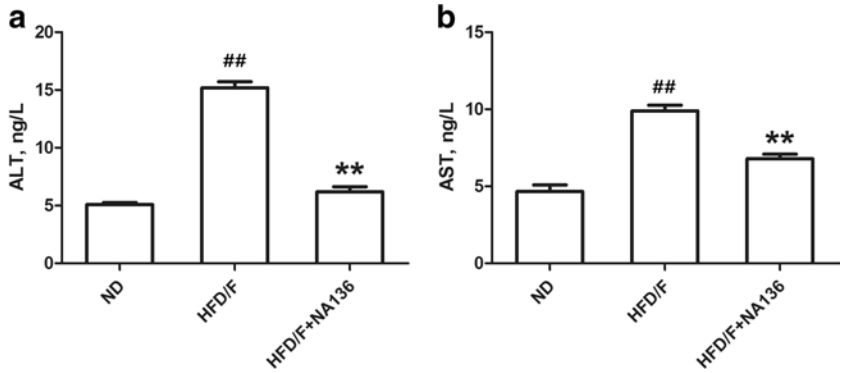

Fig. 2 Effect of *L. plantarum* NA136 on serum ALT (**a**) and AST (**b**) levels. Data represent the mean±SD of each group. $^{\#\#}P<0.01$ versus ND group; $^{**}P<0.01$ versus HFD/F group

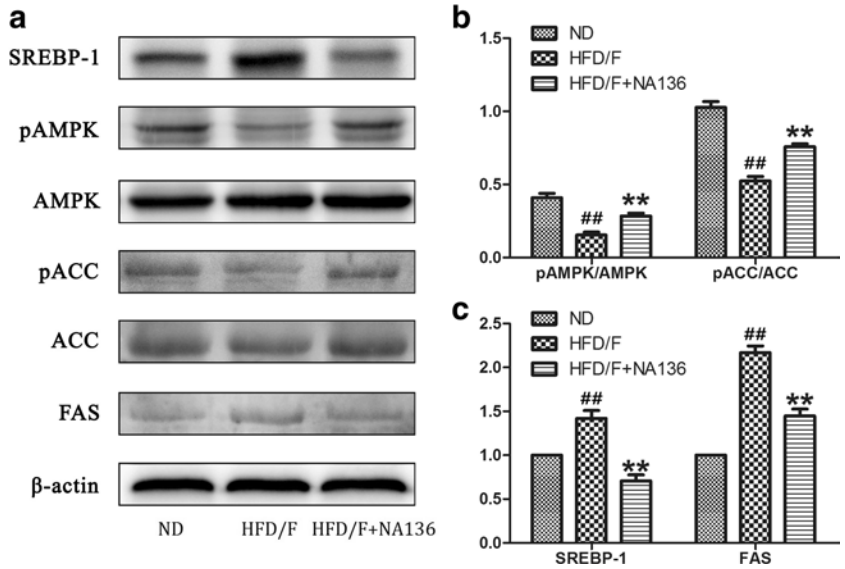

Fig. 3 Effect of *L. plantarum* NA136 on hepatic AMPK signaling. **a** SREBP-1, pAMPK, AMPK, pACC, ACC, and FAS protein expressions in liver. β-actin was used as a control for the protein blots. **b** Quantification of AMPK and ACC phosphorylation. **c** Relative protein levels of SREBP-1 and FAS. Data represent the mean±SD of each group. $^{\#\#}P<0.01$ versus ND group; $^{**}P<0.01$ versus HFD/F group

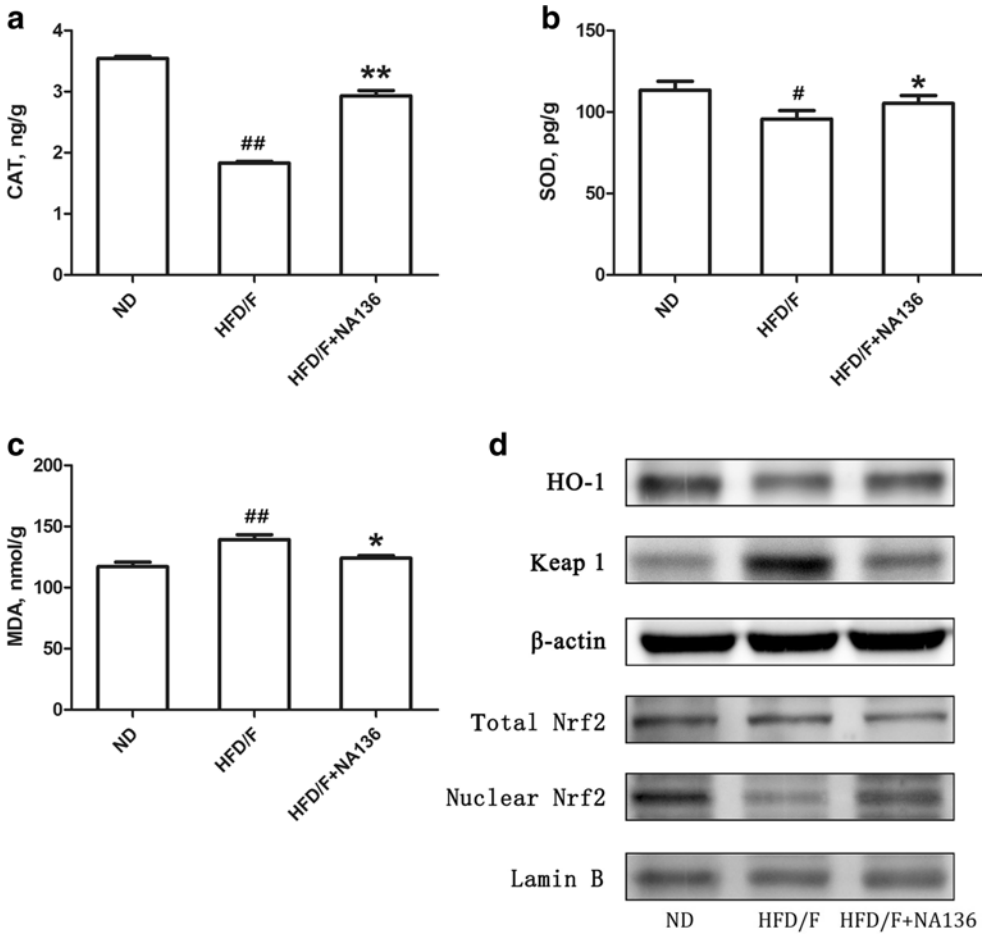

Fig. 4 Effect of *L. plantarum* NA136 on hepatic antioxidants. **a** CAT levels, **b** SOD levels, and **c** MDA levels. **d** HO-1, Keap-1, total Nrf2, and nuclear Nrf2 protein expressions in liver. β-actin and Lamin B were used as controls for the protein blots. Data represent the mean±SD of each group. $^{\#}P<0.05$ and $^{\#\#}P<0.01$ versus ND group; $^{*}P<0.05$ and $^{**}P<0.01$ versus HFD/F group

It is well established that increased TC, TG, and LDL levels and decreased HDL levels are primary features of NAFLD induced by a HFD, ultimately resulting in hyperlipidemia (Nabavi et al. 2014). Previous studies showed that NAFLD can be effectively alleviated by lowering the levels of TC, TG, and LDL (Ivanovic et al. 2016; Ye et al. 2017). In our present study, the HFD/F significantly decreased the HDL levels and increased the serum levels of TC, TG, and LDL (Table 1), resulting in lipid metabolism disorder. This might be due to the increased FFA accumulation in the liver. Meanwhile, H&E staining showed that HFD/F mice demonstrated obvious hepatic steatosis. In the HFD/F+NA136 group, *L. plantarum* NA136 intake could lower the serum TG, TC, and LDL levels and increase HDL concentration significantly. As expected, the HFD/F+NA136 group showed lower fat deposition than that of the HFD/F group. These data demonstrated that *L. plantarum* NA136 might protect the individuals from hepatic steatosis by lowering the lipid content in NAFLD mice. Furthermore, lipid deposition and lipid toxicity could induce inflammation in hepatic parenchymal cells and liver injury. The contents of ALT and AST are considered as essential markers for estimating the degree of liver injury (Lv et al. 2014). We found that, after treatment with *L. plantarum* NA136, AST levels were decreased and ALT level was decreased significantly, indicating that *L. plantarum* NA136 could attenuate HFD/F-induced liver injury and protect liver function.

AMPK is a well-established cellular energy charge sensor that maintains cellular energy homeostasis including lipid metabolism (Long and Zierath 2006). AMPK is activated by stress conditions, which in turn rises the intracellular AMP/ATP ratio (Hardie 2003). Once activated, AMPK modulates fatty acid oxidation and switches off anabolic pathways by phosphorylating multiple downstream substrates to conserve ATP levels (Hardie and Pan 2002). Furthermore, the expression levels of ACC and FAS could alter lipid metabolism in the liver such as fatty acid synthesis in hepatocytes and accumulation of fatty acids in the liver (Kohjima et al. 2008). Interestingly, AMPK phosphorylation of ACC could block ACC dimerization and in turn reduce the ACC activity which inhibits the de novo lipogenesis and switches on fatty acid oxidation in the mitochondria (Smith et al. 2016). Thus, we believed that AMPK activation might be viewed as a therapeutic target to alleviate the effects of NAFLD (Kim et al. 2013). In the present study, we found that HFD and fructose significantly attenuated AMPK phosphorylation in the HFD/F group. As expected, *L. plantarum* NA136 supplementation increased the attenuated AMPK phosphorylation, which subsequently increased the levels of ACC phosphorylation. These data suggested that HFD/F induced the removal of AMPK phosphorylation which resulted in increased levels of liver FFA and serum FFA through de novo lipogenesis. Treatment with *L. plantarum* NA136 could increase AMPK activity and block the activity of ACC which may improve HFD/F-induced NAFLD by suppressing de novo lipogenesis and increasing fatty acid oxidation. Furthermore, it is well documented that AMPK activation inhibits SREBP-1 through mTOR and LXRα, resulting in the attenuation of NAFLD (Quan et al. 2013). SREBP-1 could upregulate the lipogenic genes including ACC and FAS. Many previous studies showed that HFD induced up-expression of hepatic SREBP-1 (Kohjima et al. 2008; Yang et al. 2014). Consistent with the previous studies, the present study indicated that the decreased production of TC and FFA in the HFD/F+NA136 group may be due to the decreased levels of SREBP-1 and FAS. Therefore, our results indicated that *L. plantarum* NA136 supplementation is mediated by AMPK pathway to phosphorylate ACC and suppress SREBP-1/FAS signaling to achieve the effect of lipid-lowering.

In the context of NAFLD, increasing FFA load could affect β-oxidation that improves the reactive oxygen species (ROS) (Dowman et al. 2010). The accumulation of ROS causes oxidative stress which results in protein carbonylation and damage to DNA (Zhao et al. 2017). MDA is produced by the product of unsaturated lipid peroxidation and considered as toxic molecule and biological marker of oxidative stress. In addition, SOD acts as the first line of defense oxidative stress that converts oxygen radicals (O^{2-}) to hydrogen peroxide (H_2O_2) and dioxygen (Padurariu et al. 2010). In our results, the activity of SOD decreased and the production of MDA increased in the HFD/F group which might damage the liver cells. In contrast, *L. plantarum* NA136 treatment markedly increased the activity of SOD and decreased the level of MDA. It was also known that the HO-1 expression was an easy method for evaluating oxidative stress (Wang et al. 2017). As expected, *L. plantarum* NA136 treatment increased the expression of HO-1. Overall, our data suggested that *L. plantarum* NA136 treatment could regulate cellular antioxidant elements to ameliorate HFD and fructose-induced oxidative stress.

Nrf2 plays a pivotal role in cellular defenses against oxidative stress by regulating the gene expression of antioxidant and detoxication enzymes (Chen et al. 2017). Normally, Nrf2 binds to Keap1 to sequester Nrf2 in the cytoplasm. Once cells are exposed to the oxidative stress, Nrf2 is released from Keap1 and moves to the nucleus. After this movement, Nrf2, with its partner Maf, will form a heterodimer and then trigger the cellular antioxidant defense reaction by stimulating the antioxidant stress genes (Kensler et al. 2007). Furthermore, previous study showed that CAT triggered Nrf2 translocation and further targeted downstream genes, such as HO-1 gene, to trigger Nrf2 defense system (Chang et al. 2012). In the present study, treatment with *L. plantarum* NA136 increased CAT expression which was attenuated in the HFD/F group. This in turn triggers the Nrf2 translocation to the nucleus to regulate cellular antioxidant enzymes, including SOD and

HO-1. Similar results indicated that *Bacillus amyloliquefaciens* SC06 is a kind of probiotics that increased the level of CAT to activate Nrf2/Keap1 signaling pathway, elevating antioxidant status (Wang et al. 2017). Thus, we suggested that *L. plantarum* NA136 could activate Nrf2 signaling pathway to attenuate oxidative stress in NAFLD. In addition, Nrf2 not only controls the intracellular levels of glutathione and ROS, but also negatively regulates lipid biosynthesis (Liu et al. 2011; Ludtmann et al. 2014). Recent studies demonstrated AMPK regulated Nrf2 nuclear translocation to induce HO-1 gene expression (Choi et al. 2015; Qu et al. 2016). Taken together, we suggested that NAFLD could be attenuated by treatment with *L. plantarum* NA136 through regulating lipid metabolism and oxidative stress, which are the roles of AMPK and Nrf2 pathways, respectively. Furthermore, we believe that the changes of gut microbiota by treatment with *L. plantarum* NA136 must play a critical role in improving NAFLD, and detailed follow-up work will be reported in due course.

In conclusion, our findings in the present study suggest that *L. plantarum* NA136 has protective effect on NAFLD. We propose that the beneficial effects of treatment with *L. plantarum* NA136 are partly related to the activation of AMPK/Nrf2 pathway, which will significantly improve hepatic lipid metabolic disorders and ameliorate hepatic oxidative stress response in NAFLD. Therefore, our findings show the possibility of improving NAFLD by treatment of *L. plantarum* NA136.

Author's contributions Z.Z. designed the experiments. Z.Z., C.W., Y.Z., L.G., and C.D. contributed to the experimental work. Z.Z. performed the data analysis and wrote the manuscript. L.Z., X.Z., and S.L. revised the manuscript.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest.

Ethical approval All procedures involving animals were approved by the Animal Care and Ethic Committee of Jilin Academy of Agricultural Sciences.

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