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ORIGINAL ARTICLE

Lactobacillus plantarum Attenuates Oxidative Stress and Liver Injury in Rats with Nonalcoholic Steatohepatitis

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ABSTRACT

Background: Steatohepatitis is a morphological pattern of liver injury that, in non-alcoholic patients, may represent a form of chronic liver disease currently known as non-alcoholic steatohepatitis (NASH). Probiotics, *Lactobacillus sp.* and *Bifidobacterium sp.*, have been proposed to prevent and treat different inflammatory conditions of the gastrointestinal tract. **Objective:** To examine the effect of *Lactobacillus plantarum* (*L. plantarum*) on the liver damage of non-alcoholic steatohepatitis (NASH) rats. **Materials and Methods:** Male Sprague-Dawley rats were randomly divided into three groups. Group 1 (control, n = 8) was fed with phosphate-buffered saline (PBS) 1 mL/rat. Group 2 (NASH, n = 8) was fed with 100% fat diet for 6 weeks. Group 3 (NASH + *L. plantarum*, n = 8) was fed with 100% fat diet plus *L. plantarum* 1.8×10^9 colony-forming unit/mL was suspended in PBS by gavage twice a day at an interval of 4 h for 6 weeks. All rats were sacrificed to collect blood and liver samples at the end of the treatment period. **Results:** The levels of hepatic malondialdehyde (MDA) and tumor necrosis factor alpha (TNF-α) were increased while the expression of peroxisome proliferator activated receptors gamma (PPAR-γ) was decreased significantly in the NASH group as compared with the control group. Histopathology from the NASH group showed macrovesicular steatosis, hepatocyte ballooning, and lobular inflammation. The NASH + *L. plantarum* group had attenuated the levels of MDA and TNF-α, enhanced PPAR-γ expression, and improved the histopathology. **Conclusion:** *L. plantarum* treatment can attenuate oxidative stress, inflammation, and improvement of histopathology in rats with NASH.

Key words: *Lactobacillus plantarum*, liver injury, nonalcoholic steatohepatitis, oxidative stress, rats

SUMMARY

- The effects of probiotic, L. plantarum attenuated on inflammatory and oxidative mechanisms involved in the pathogenesis of liver damage in NASH rats.

Parameter	MDA	TNF-α	PPAR-γ	Liver histopathology
Control	-	-	-	normal
NASH	Increased	Increased	Decreased	macrovesicular steatosis, hepatocyte ballooning, and lobular inflammation
NASH+*L. plantarum*	Decreased	Decreased	Increased	improved

Abbreviations used: NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; TNF-α: Tumor necrosis factor-alpha; MDA: Malondialdehyde; PPARγ: Peroxisome proliferator-activated receptor gamma; TBARS: Thiobarbituric acid-reactive substances; ELISA: Enzyme-linked immunosorbent assay.

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INTRODUCTION

Non-alcoholic steatohepatitis (NASH) is a liver disease characterized by macrovesicular steatosis, hepatocyte necrosis, inflammation, mallory bodies, and fibrosis.[1-4] NASH is closely associated with the metabolic or insulin resistance syndrome.[5] This is a cluster of disorders, such as obesity, diabetes mellitus, dyslipidemia, arteriosclerosis, and hypertension, with insulin resistance as a common feature.[6] In initial phases, during which fat accumulates in the liver, no clinical symptoms are evident. In advanced stages, fibrosis is detectable, which might progress into cirrhosis in some patients.[7]

There are many models of NASH-like liver injuries in animals such as the genetic model of ob/ob mice,[8] the methionine and choline-deficient diet model,[9,10] and a model with a high-fat liquid diet in which 71% of energy is derived from fat, 11% from carbohydrates, and 18% from protein.[11] The fatty acid excess is converted to triglycerides and stored in the cytoplasm, predisposing the hepatocytes to oxidative stress and to activation of inflammatory pathways.[12] In the last decade, the "2-hit" model has been proposed for the pathogenesis of NASH.[13] Liver fat accumulation and insulin resistance characterize the first hit and are responsible for the development of steatosis. The main factors initiating the second hit are oxidative stress and subsequent lipid peroxidation, together with the production of proinflammatory cytokines, principally tumor necrosis factor-alpha (TNF-α),[14,15] and hormones derived from adipose tissue.[16,17] Peroxisome proliferator-activated receptors-gamma (PPAR-γ), are members of the nuclear hormone receptor subfamily of transcription factors, from heterodimers with retinoid X receptors (RXRs). These heterodimers regulate transcription of genes involved in insulin action, adipocyte differentiation, lipid metabolism, and inflammation. PPAR-γ is implicated in diseases including obesity, diabetes, atherosclerosis, and cancer. PPAR-γ activators include prostanoid, fatty acids, thiazolidinediones, and N-(2-benzoylphenyl) tyrosine analogs. PPAR-γ is a key component in

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adipocyte differentiation and fat-specific gene expression.[18-24] These suggest that PPAR-γ may play an important role in the development of hepatocellular inflammation, necrosis, and fibrosis in rats with a high-fat diet.

Probiotics have been proposed to prevent and treat different inflammatory conditions of the gastrointestinal tract.[25,26] These therapeutic effects might be related to a variety of direct and indirect mechanisms, including modulation of local microbiota, epithelial barrier function, and the immune system.[27] Because the probiotic modulatory effect on the intestinal microflora could influence the gut-liver axis, these microorganisms have also been proposed as a possible adjunctive therapy in some types of chronic liver diseases.[28,29] *Lactobacilli* are probiotics which, when administered in adequate amounts, may confer a benefit to the host.[30,31] The most commonly used organisms in probiotics are *Lactobacillus sp.* and *Bifidobacterium sp.*[32] *Lactobacillus plantarum* (*L. plantarum)* is commonly found in the human gastrointestinal tract (GI-tract). It is important in the production of a variety of fermented foods such as sauerkraut, Korean Kimchi, cheese, sausages and stockfish, and is also used as a probiotic. Importantly, *L. plantarum* is acid and bile tolerant, survives passage through the GI-tract, and is safe in humans and animals.

A recent meta-analysis in adult patients suggests that probiotics could be useful in non-alcoholic fatty liver disease (NAFLD) and that further research elucidating the mechanisms of such effects is needed.[30] Preliminary data obtained in rat models of alcohol and NASH showed that the treatment with probiotics could be effective in limiting liver damage,[33-35] but the exact mechanism of these effects is still largely undefined.

Here, we examine the effect of probiotic, *L. plantarum*, on inflammatory and oxidative mechanisms involved in the pathogenesis of liver damage in an experimental model of NASH rats.

MATERIALS AND METHODS

Animal preparation

This study was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University (IRB No. 18/57). Male Sprague-Dawley rats weighing 220–250 g from the National Laboratory Animal Center, Mahidol University, Salaya, Nakorn Pathom were used. The animals were allowed to rest for a week after arrival at the Animal Center, Department of Physiology, Faculty of Medicine, Chulalongkorn University. They were kept at a controlled temperature of 25°C ± 1°C under standard conditions (12 h dark: 12 light cycle) fed with regular dry rat chow *ad libitum*, and had free access to drinking water.

Bacterial strains and culture conditions

L. plantarum, isolated from Thai dyspeptic patients who visited King Chulalongkorn Memorial Hospital, was stored in de Man-Rogosa-Sharp (MRS) broth (Oxoid, Basingstoke, United Kingdom) with 20% glycerol at −80°C. This strain was recovered from frozen stock and cultivated twice on MRS agar anaerobically (10% CO_2, 10% H_2, and 80% N_2) at 37°C in an anaerobic jar for 48 h. A single colony of *L. plantarum* was then inoculated into 10 mL of MRS broth and grown at 37°C under anaerobic conditions for 24 h in a 15 mL conical centrifuge tube (Corning, New York, United States).

Experimental protocol

Rats were randomly divided into three experimental groups (eight rats each) as follows.

- Group 1 (control): Rats were fed *ad libitum* with regular dry rat chow for 6 weeks
- Group 2 (NASH): Rats were fed *ad libitum* with 100% fat diet for 6 weeks to induce NASH
- Group 3 (NASH + *L. plantarum*): Rats were fed *ad libitum* with 100% fat diet for 6 weeks to induce NASH plus *L. plantarum* 1.8×10^9 colony-forming unit (CFUs)/mL suspended in phosphate-buffered saline 1 mL/rat by gavage twice a day at an interval of 4 h for 6 weeks.

At the end of the study, all rats were sacrificed using an intraperitoneal injection of an overdose of thiopental sodium (45 mg/kg) and the abdominal walls were opened. Blood was withdrawn by cardiac puncture for TNF-α determination using ELISA methods. The livers were excised quickly and cleaned in ice-cold nephron-sparing surgery. One lobe of the liver was frozen in liquid nitrogen and stored at −80°C for malondialdehyde (MDA) analysis. The remaining lobes of the liver were fixed in 4% paraformaldehyde in phosphate buffer solution to determine PPAR-γ expression using an immunohistochemistry method and for histological examination.

Determination of serum cytokine level

After the experiment, blood samples were taken by cardiac puncture, allowed to clot for 2 h at room temperature before centrifuging for 20 min at approximately 1000 × g. The serum was then removed and stored at −80°C for determining TNF-α level by ELISA kit (R and D systems, USA).

Hepatic malondialdehyde determination

Gastric MDA level was measured using thiobarbituric acid reactive substances assay kit (Cayman, USA). Basically, principle of the method is the reaction of one molecule of MDA and two molecules of TBA to form a red MDA-TBA complex under high temperature (90°C −100°C) and acidic conditions, which can be quantitated using a spectrophotometer at 532 nm. The assay procedures were performed as per protocol descriptions from the company. The content of MDA was expressed in terms of nmol/mg protein.

Examination of liver histopathology

The remaining liver samples were fixed in 4% paraformaldehyde in phosphate buffer solution at room temperature. They were processed by standard methods. Briefly, tissues were embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin (H and E), and then picked up on glass slides for light microscopy. An experienced pathologist blinded to the experiment evaluated all samples. All fields in each section were examined for grading of steatosis and necroinflammation according to the criteria described by Bacon *et al.*[36]

The severity of steatosis was scored on the basis of the extent of involved parenchyma as 1 if fewer than 33% of the hepatocytes were affected, as 2 if 33%–66% of the hepatocytes were affected, as 3 if more than 66% of the hepatocytes were affected, and as 0 if no hepatocytes were affected.

Hepatic necroinflammation was graded from 0 to 3; score 1 (mild) = sparse or mild focal zone 3 hepatocyte injury/inflammation, score 2 (moderate) = noticeable zone 3 hepatocyte injury/inflammation, score 3 (severe) = zone 3 hepatocyte injury/inflammation, and score 0 = no hepatocyte injury/inflammation. Levels of hepatocytes ballooning degeneration was graded from 0 to 2; score 0 = no ballooning, score 1 = few ballooned hepatocytes, and score 2 = many ballooned hepatocytes.

Immunohistochemistry analysis of proliferator-activated receptors-gamma protein expression in liver

The liver sections were deparaffinized with xylene and ethanol for 10 min. After water washing, antigen (PPAR-γ, Santa Cruz, USA) was retrieved from

the sections with citrate buffer pH 6.0 in a microwave for 13 min. Next, 3% H_2O_2 and 3% normal horse serum were added to the slides to block endogenous peroxidase activity for 5 min and block nonspecific binding for 20 min, respectively. The primary antibody used for PPAR-γ, a monoclonal antibody against the γ subunit of PPAR, was then applied at a dilution of 1:50 for 1 h at room temperature and incubated with the secondary antibody for 30 min. When the development of the color with DAB was detected, the slides were counterstained with hematoxylin. Under light microscopy, the positive stained cells presented dark brown in the nucleus. The results were expressed as the number of positive stained cells per high-power field.

Statistical analysis

All data were presented as mean ± standard deviation. The means were compared by one-way analysis of variance (one-way) followed by LSD *Post hoc* test. All statistical tests were performed using SPSS for Windows version 17.0 (SPSS Inc., Chicago, IL, United States). A $P < 0.05$ was considered statistically significant.

RESULTS

Changes of serum tumor necrosis factor-alpha level

The serum TNF-α level was significantly different between NASH and control groups (3.87 ± 3.46 vs. 0.19 ± 0.30 pg/mL, $P = 0.001$). However, in *L. plantarum* 1.8 × 10^9 CFUs/mL treatment group, there was a significant decrease of serum TNF-α level compared with the NASH group (0.45 ± 0.07 vs. 3.87 ± 3.46 pg/mL, $P = 0.003$). A bar graph of serum TNF-α level of all groups is shown in Figure 1.

Changes of hepatic malondialdehyde level

The level of gastric MDA increased significantly in the NASH group compared with the control group (12.41 ± 7.98 vs. 6.48 ± 4.03 nmol/mg protein, $P = 0.032$). After treatment for 6 weeks with 1.8 × 10^9 CFUs/mL of *L. plantarum*, there were significant decreases in elevated gastric MDA level in the NASH + *L. plantarum* group compared with the NASH group (1.66 ± 0.19 vs. 12.41 ± 7.98 nmol/mg protein, $P = 0.000$). A bar graph of hepatic MDA level of all groups is shown in Figure 1.

Histopathology

There were no steatosis, hepatocyte ballooning, or lobular inflammation revealed on histology in the control group. In the NASH group, steatosis is predominantly macrovesicular, with ballooned hepatocytes and lobular inflammation noted as compared with the control group. *L. plantarum* 1.8 × 10^9 CFUs/mL treatment resulted in a significant improvement in liver histopathology of the NASH + *L. plantarum* group when compared with the NASH group. Liver sections from this group showed mild steatosis, hepatocyte ballooning, and lobular inflammation. Most rats in the NASH group developed steatosis and necroinflammation scores, while the NASH + *L. plantarum* group improved. Histological scores of steatosis and necroinflammation are summarized in Table 1 and photomicrograph of liver histopathology is shown in Figure 2.

Proliferator-activated receptors-gamma protein expression

The percentage of PPAR-γ positive stained cells using the immunohistochemistry method was significantly decreased in the NASH group when compared with the control group (36.11% ± 13.57% vs. 54.34% ± 5.78%, $P = 0.001$). After treatment for 6 weeks with 1.8 × 10^9 CFUs/mL of *L. plantarum*, the percentage of PPAR-γ positive stained cells were significantly increased in the NASH + *L. plantarum* group when compared with the NASH group (75.04% ± 7.57% vs. 36.11% ± 13.57%, $P = 0.000$). The average percentage of PPAR-γ of all groups is shown in Figure 1 and the immunohistochemical staining of PPAR-γ is shown in Figure 3.

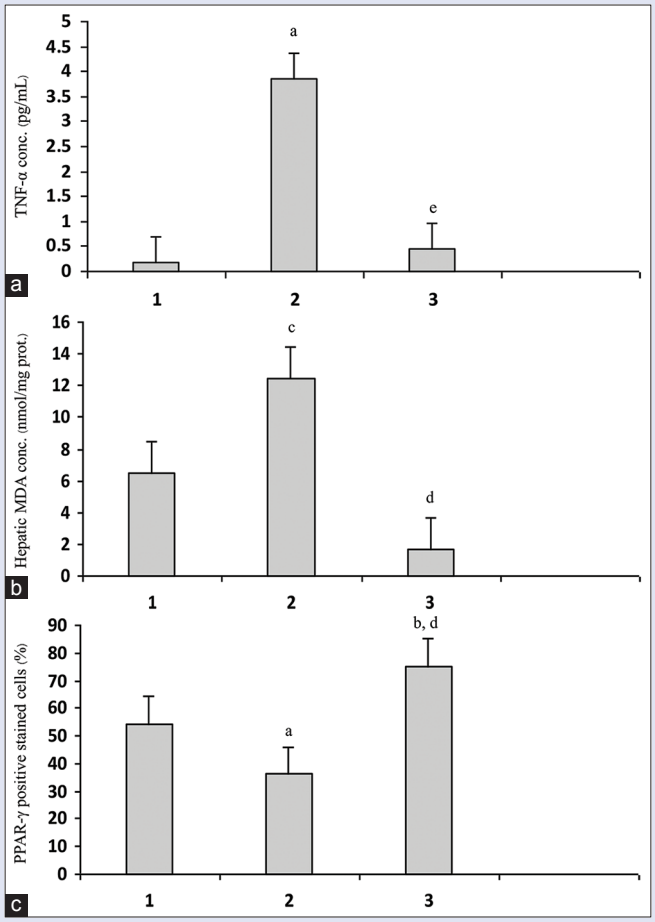

Figure 1: (a) Serum level of tumor necrosis factor alpha (b) Hepatic malondialdehyde level (c) Peroxisome proliferator activated receptors gamma positive stained cells ($^aP = 0.001$; $^bP = 0.000$; $^cP = 0.032$ *vs* control group. $^dP = 0.000$; $^eP = 0.003$ *vs* non-alcoholic steatohepatitis (NASH). Group 1: Control; 2: NASH; 3: NASH + *L. plantarum*)

DISCUSSION

Steatohepatitis is a morphological pattern of liver injury that, in non-alcoholic patients, may represent a form of chronic liver disease currently known as NASH.[36] It is accepted that this pattern may occur in a variety of clinical settings including, but not limited to, diabetes and obesity, but in many cases, the etiology is unknown.[37-41] The distinctive morphological features of steatohepatitis, regardless of the clinical background, include some "alcohol hepatitis-like" finding: steatosis; lobular inflammation, which includes polymorphonuclear leukocytes; and perisinusoidal fibrosis in zone 3 of the acinus. Other common features are hepatocellular ballooning, pooly formed Mallory's hyaline, and glycogenated nuclei.[37,42,43]

To study the pathogenesis of or therapeutic options for NASH, there are many models that can be used, including a genetic model (obese rats), a model of methionine and choline deficient diet, a model of a high-fat liquid diet, and a 100% fat diet.[1,5,40-45] We showed that a 100% fat-diet fed rat was able to induce NASH; the hepatic lesions of NASH were apparent within 6 weeks. Histopathological examination showed macrovesicular steatosis, hepatocyte ballooning, and lobular inflammation.

Table 1: Summary of scores of steatohepatitis and necroinflammation levels in all experimental groups and graded using Bacon *et al.*[36]

Groups	*n*	Steatosis				Inflammation				Ballooning		
		0	1	2	3	0	1	2	3	0	1	2
Control	8	8	-	-	-	8	-	-	-	8	-	-
NASH	8	-	4	3	1	-	4	3	1	-	2	6
NASH + *Lactobacillus plantarum*	8	1	7	-	-	5	3	-	-	1	6	1

NASH: Nonalcoholic steatohepatitis

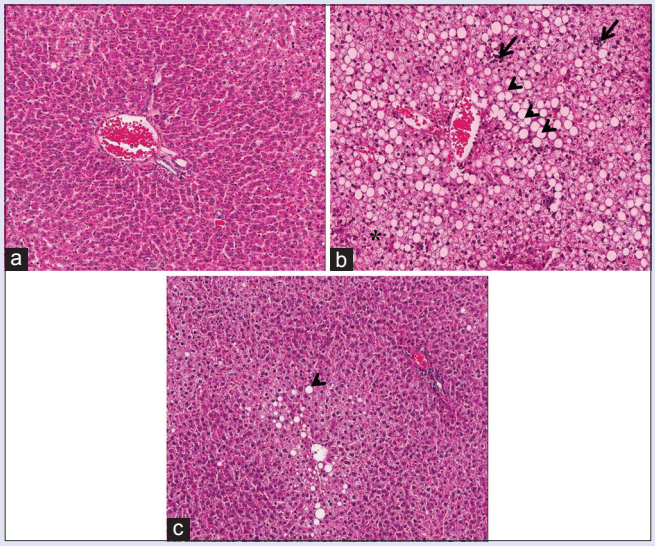

Figure 2: Liver histopathology in rats with non-alcoholic steatohepatitis (H and E, ×20). (a) Control group showed normal histopathology; (b) nonalcoholic steatohepatitis group showed predominantly macrovesicular steatosis (arrowheads), hepatocytes ballooning (asterisks), and lobular inflammation (arrows); (c) nonalcoholic steatohepatitis + *Lactobacillus plantarum* group showed improvement of histopathology

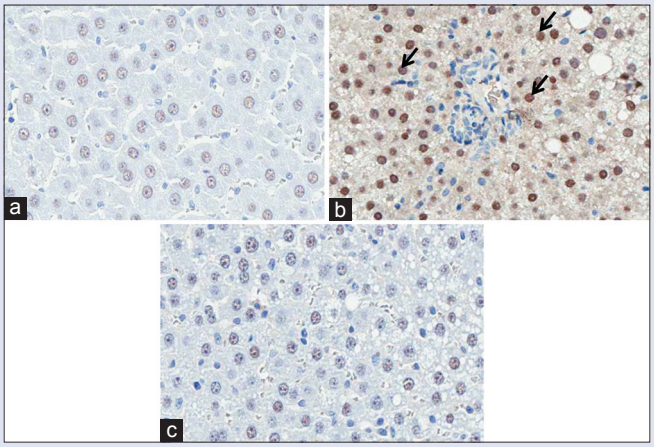

Figure 3: Peroxisome proliferator-activated receptors-gamma positive stained cells in rats with non-alcoholic steatohepatitis (Immunohistochemistry, ×20). (a) Control group; (b) non-alcoholic steatohepatitis group showed dark brown stain in their nuclei (arrows); (c) non-alcoholic steatohepatitis + *Lactobacillus plantarum* group showed improvement of immunohistochemistry

Free fatty acid (FFA) causes oxidative stress that has the potential to induce NASH.[5] FFA in the body is increased and this is associated with state of starvation.[5] Stored FFA can be mobilized from adipose tissue through lipolysis.[5] FFA metabolism increases the production of reactive oxygen species, which activated lipid peroxidation. Consequences are, the disruption of membranes and the production of reactive metabolites such as MDA.[46] Peroxidation of phospholipids generates MDA and other MDA-like aldehydes and ketones, however, MDA is the major product that reacts with thiobarbituric acid. A high-fat diet-induced an increase in the amount of hepatic MDA.[44,45,47-50] This study found high hepatic MDA levels in 100% fat-diet fed rats in accordance with studies by others.[44,45,47-50]

Among inflammatory cytokines, TNF-α, interleukin-6, and interleukin-1 β plays a major role in the pathogenesis of the disease, contributing to systemic and hepatic insulin resistance and cellular injury, and hepatic stellate cell activation.[35,51-53] In this study, TNF-α was chosen to study inflammation in rats with 100% fat diet in the NASH model. Here, we found a significant increase in TNF-α in serum from this group.

Peroxisome PPARs-γ, members of the nuclear hormone receptor subfamily of transcription factors, form heterodimers with RXRs. These heterodimers regulate the transcription of genes involved in insulin action, adipocyte differentiation, lipid metabolism, and inflammation. PPAR-γ is implicated in diseases including obesity, diabetes, atherosclerosis, and cancer. PPAR-γ activators include prostanoid, fatty acids, thiazolidinediones, and N-(2-benzoylphenyl) tyrosine analogs. PPAR-γ is a key component in adipocyte differentiation and fat-specific gene expression.[1,5,6,8-11] These suggest that PPAR-γ may play an important role in the development of hepatocellular inflammation, necrosis, and fibrosis in rats with a high-fat diet model. We found a significant decrease in PPAR-γ expression in rats with NASH. As previously reported, in a mouse model of steatohepatitis, the activation of another PPAR subtype, PPAR-α, prevented the induction of Cyclooxygenase-2 expression.[54]

Lactobacilli are probiotics which, when administered in adequate amounts, may confer a benefit to the host.[31] The most commonly used organisms in probiotics are *Lactobacillus sp.* and *Bifidobacterium sp.*[32] *L. plantarum* is commonly found in the human GI-tract. It is important in the production of a variety of fermented foods such as sauerkraut, Korean Kimchi, cheese, sausages and stockfish, and is also used as a probiotic. Importantly, *L. plantarum* is acid and bile tolerant, survives passage through the GI-tract, and is safe in humans and animals.

A recent meta-analysis in adult patients suggests that probiotics could useful in NAFLD and that further research elucidating the mechanisms of such effects is needed.[30] Preliminary data obtained in rat models of alcohol and NASH showed that treatment with probiotics could be effective in limiting liver damage,[33-35] but the exact mechanism of these effects is still largely undefined. Interestingly, all of these studies were concordant with our results. In 100% fat-diet fed rat model, we found that *L. plantarum* treatments resulted in improving liver pathology, PPAR-γ expression, decreasing serum TNF-α level and hepatic MDA level. However, the mechanisms of action were unclear; these need further investigations. Our results strongly support the anti-inflammatory and anti-oxidative activity of *L. plantarum* probiotic, which is responsible for the preventive effect of the early onset of NASH. Another possible

mechanism of the protective effect of *L. plantarum* may include the maintenance of gut integrity.

CONCLUSION

The high-fat diet induced NASH accompanied by an increased oxidative stress, inflammation, and liver histopathology. Probiotic, *L. plantarum*, treatment in rats with NASH could attenuate oxidative stress, inflammation, and liver histopathology.

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Conflicts of interest

There are no conflicts of interest.

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