Single and mixed strains of probiotics attenuated inflammation and oxidative stress in rat model of non-alcoholic steatohepatitis

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Abstract

The development of non-alcoholic steatohepatitis (NASH) involves complex pathophysiological processes. The aim of this study was to determine whether single and mixed strains of probiotics could reduce the severity of NASH induced by high-fat-high-fructose (HFHF) diet through the alleviation of inflammatory cytokines and oxidative stress in rats. Twenty-eight male Sprague-Dawley rats were divided into 4 groups (n=7 each group), control; NASH group fed with HFHF diet; NASH+single strain (1.8×10^9 CFUs/ml of Lactobacillus plantarum B7) 1 ml by gavage once daily; NASH + mixed strains (1.8×10^9 CFUs/ml of Lactobacillus rhamnosus L34 and 1.8×10^9 CFUs/ml of Lactobacillus casei B13) 1 ml by gavage once daily for 6 weeks. Liver histology was used to determine NASH severity. The oxidative stress marker (i.e. hepatic malondialdehyde (MDA) levels), natural antioxidant (i.e. hepatic glutathione (GSH)) and inflammatory marker (i.e. serum interleukin-6 (IL-6) levels) were measured. Gross liver appearance in the NASH group showed enlarged and yellowish livers. Histopathology demonstrated a moderate to severe degree of steatosis, hepatocyte ballooning and lobular inflammation in the NASH group. In both treatment groups, the improvement of liver histology was noted compared with the NASH group. Serum IL-6 and hepatic MDA levels were significantly increased in the NASH group, while GSH levels were significantly decreased compared with the control group. Moreover, in both treatment groups, serum IL-6 and hepatic MDA levels were significantly decreased, while GSH levels were significantly increased compared with the NASH group. In conclusion, single and mixed strains of probiotics were effective in the prevention of NASH, likely through the alleviation of inflammatory cytokines and reduction of oxidative stress. Probiotics could potentially be used for the management of NASH. Clinical studies are needed to confirm their therapeutic effects in human.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is a common liver disorder characterized by fat accumulation and liver inflammation. It is prevalent among obese people and is considered the hepatic manifestation of metabolic syndrome.[1] NAFLD represents a spectrum of liver damage ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), advanced fibrosis, cirrhosis or even hepatocellular carcinoma. Metabolic abnormalities, such as diabetes, hypertension and hyperlipidemia are commonly seen in patients with NASH[2] and increased the risk of cardiovascular death in these patients. The development and progression of NAFLD involve complex pathophysiological processes and are influenced by the "two hit" phenomenon. The primary event is lipid accumulation due to the alteration of lipid homeostasis that is associated with obesity, insulin resistance, and adipokine abnormalities. High fat and high fructose diet have frequently been used to induce NAFLD in animal models. Fructose metabolism leads to the production of intermediates that promote de novo lipogenesis. As a result, high fructose consumption has been shown to increase hepatic fat accumulation.[3] The second "hit" is a combination of oxidative stress, lipid peroxidation, mitochondrial dysfunction, bile acid toxicity, cytokine-mediated recruitment and retention of inflammatory cells and the composition of gut microbiota.[4] Probiotics are live microorganisms that are beneficial to a person's health. They are widely used for the prevention and treatment of gastrointestinal diseases, such as infectious diarrhea, irritable bowel syndrome, inflammatory bowel disease, and others.[5] Recently, probiotics conferring health benefits, e.g. by manipulation of the intestinal microbiota or by affecting the host, have been proven to ameliorate metabolic and infectious diseases.[6] In particular,

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probiotics have been shown to attenuate a variety of liver pathologies, such as alcoholic liver disease and carbon tetrachloride induced liver injury, likely by anti-inflammatory actions and by the stabilization of the intestinal barrier. Lactic acid bacteria have also been shown to have antioxidant properties likely through the production of antioxidant enzymes, the scavenging of reactive oxygen species, and metal ion chelation.[7] These properties would be beneficial in the prevention and treatment of liver disease. For the particular strains of probiotics that we used in this study (*Lactobacillus plantarum B7, Lactobacillus rhamnosus L34,* and *Lactobacillus casei B13*), data are scarce regarding their effects on the development of NAFLD. Therefore, the aim of this study was to investigate whether single and mixed strains of *Lactobacillus spp*. could reduce the severity of NASH induced by high-fat-high-fructose (HFHF) diet through the alleviation of inflammatory cytokines and oxidative stress in rats.

Materials and Methods

Animal preparation

Five-week-old male Sprague-Dawley rats weighing 180–200 g from the Nomura Siam International Co., Ltd. (Bangkok, Thailand) were used. The protocol was approved by the Animal Care and Use Committee of the Faculty of Medicine, Chulalongkorn University (IRB No. 022/2563). All animals were kept at the Animal Center, Faculty of Medicine, Chulalongkorn University under a strictly hygienic conventional system in a controlled temperature room at 25 ± 1°C with a normal 12 h light 12 h dark cycle. All rats had free access to purified drinking water. Each group of rats (described in the following paragraph) was housed in a separate stainless-steel cage with solid bottom and open top. The experimental procedure started after one week of acclimatization to the new environment.

Experimental design

Twenty-eight male Sprague-Dawley rats were randomly divided into 4 groups, (1) control: rats were fed ad libitum with standard rat chow diet, (2) NASH: rats were fed ad libitum with HFHF diet containing 55% energy from fat, 10% energy from protein, and 35% energy from carbohydrate (containing 20%fructose) for 6 weeks to induce NASH, (3) NASH+single strain probiotic: rats were fed ad libitum with HFHF diet for 6 weeks. During these 6 weeks, rats were simultaneously treated with *Lactobacillus plantarum B7* (1.8×10^9 CFUs/ml) 1 ml by oral gavage once daily, (4) NASH + mixed strains: rats were fed ad libitum with HFHF diet for 6 weeks. During these 6 weeks, rats were simultaneously treated with *Lactobacillus rhamnosus L34* (1.8×10^9 CFUs/ml) + *Lactobacillus casei B13* (1.8×10^9 CFUs/ml) 1 ml by oral gavage once daily. The number of rats in this study were calculated from the results of hepatic malondialdehyde (MDA levels) (mean and SD) by Werawatganon, et al., 2019[8] using the G Power program with $\alpha = 0.05$, power $(1-\beta) = 0.99$.

Body weights were measured weekly. The treatment groups were sacrificed 24 hours after the last gavage in order to prevent any side effects. Body weights and liver weights were measured at the end of the experiment. Blood samples were obtained by intracardiac puncture. The blood was allowed to clot at room temperature and then centrifuged at 2000 g at 4°C for 20 minutes to obtain serum, which were stored at -80°C until the time of analysis for interleukin 6 (IL-6) levels using ELISA. Liver was rapidly removed and washed several times with ice cold saline. Liver specimens were used for histopathological examination (hematoxylin and eosin (H&E) stained to determine NASH severity score), for the determination of oxidative stress by measuring MDA level using thiobarbituric acid reactive substances (TBARS) assay, and for the measurement of hepatic GSH level by Colorimetric assay.

HFHF diet was prepared according to modified Pickens MK formula.[9] In this study, HFHF diet contained 55% fat (palm oil), 10% protein (egg albumin) and 35% carbohydrate, which was composed of 20% fructose and 15% starch. Standard chow diet contained 6% fat, 25% protein and 47% carbohydrate, which was provided by Perfect Companion Group, Thailand. All rats received food ad libitum.

Lactobacillus plantarum B7 (1.8×10^9 CFUs/ml), *Lactobacillus rhamnosus L34* (1.8×10^9 CFUs/ml), and *Lactobacillus casei B13* (1.8×10^9 CFUs/ml), which were used in this study, was obtained 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 hours. A single colony of these bacteria was then inoculated into 10 ml of MRS broth and grown at 37°C under anaerobic conditions for 24 hours in a 15 ml conical centrifuge tube (Corning, New York, USA).

Histopathological evaluation

Fixed liver samples were cut and stained by H&E. Sections were examined under a light microscope for grading of steatosis, inflammation, and hepatocellular ballooning according to the criteria described by Brunt, et al.[10] Histological assessment was performed by an experienced pathologist who was blinded to the experimental groups.

Hepatic MDA and GSH measurements

Liver tissue (0.1 g) was homogenized on ice for 30 min in 1 ml of RIPA buffer (Cell Signaling Technology®, Danvers, MA, USA) with protease inhibitor cocktails (Sigma Aldrich, DS, Germany). Bicinchoninic acid (BCA) assay was performed to determine protein concentration in the supernatant by

BCA protein assay kit (Pierce®, Thermo scientific, Rockford, IL, USA). The supernatant was used for both MDA and GSH measurement.

Lipid peroxidation was determined by measuring MDA level detected by the level of production of thiobarbituric acid-reactive components (TBARS). Liver tissue was homogenized and centrifuged to obtain the supernatant liquid**.** Two milliliter of stock solution containing 20% acetic acid, 0.8% thiobarbituric acid, and 8.1% sodium dodecyl sulfate was added to the homogenized liver and then boiled in a water bath at 95ºC for 1 hour. The solution was centrifuged for 10 min at 1600g and the absorbance of the supernatant fraction was measured at wavelength of 530 nm. The MDA was expressed as nmol/mg protein.

Hepatic GSH levels were measured using Glutathione Assay Kit (Cayman Chemical, USA). Liver tissue was washed and homogenized before being centrifuged to obtain the supernatants which were then deproteinated. The sulfhydryl group of glutathione reacted with DTNB to form TNB, the absorbance of which was measured at 405 nm. The results were expressed in micromolar (μM).

Serum IL-6 measurement

Serum levels of interleukin-6 were measured using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, USA). The optical density of each well was determined using a microplate reader set at 450 nm. The values were read off the standard curve.

Statistical analysis

The data were presented as mean ± standard error of mean (SEM). Continuous variables in each group were compared using one-way analysis of variance (one-way ANOVA) with Tukey *post hoc* test using the Statistics Package for the Social Sciences (SPSS) software version 18.0 for Windows. Probability value of less than 0.05 was considered statistically significant. In addition, descriptive statistics were used for histological examination of the liver.

Results

Gross liver appearance and histopathological changes

Results showed enlarged and yellowish livers in the NASH group and normal gross liver appearance in the control and both treatment groups (Figure 1). In the NASH group, liver histopathology demonstrated a higher degree of fat accumulation, ballooning and lobular inflammation than in the control group (Figure 2). In both treatment groups, the degree of fat accumulation, ballooning and inflammation was less severe than in the NASH group (Figure 2).

Liver histology of the NASH group demonstrated a higher degree of steatosis, ballooning and lobular inflammation than those of the control group (Table 1). In both treatment groups, the degree of steatosis, ballooning and inflammation was less than in those of the NASH group**.** Both treatments, however, did not normalize liver histology when compared to the control group as some degree of liver steatosis and hepatocyte ballooning were still seen after treatment.

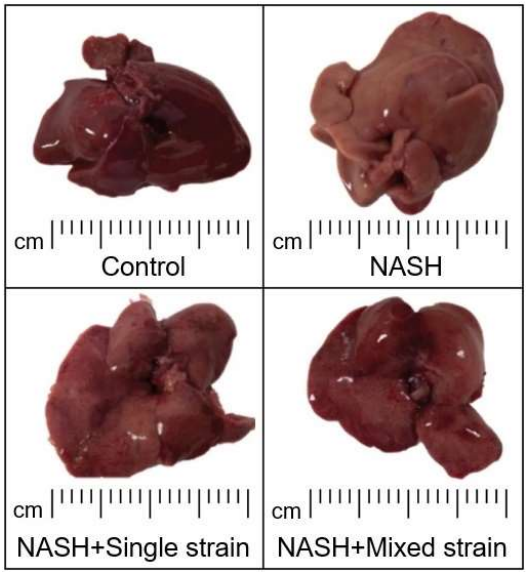

Figure 1 Gross liver appearance of rats in all experimental groups.

Changes of hepatic malondialdehyde levels

As shown in Figure 3A, hepatic malondialdehyde **(**MDA**)** levels were higher in the NASH group than in the control group (3.34 ± 1.34 *vs* 1.14 ± 0.37 nmol/mg protein, respectively; $P < 0.05$). After treatment with single strain and mixed strains of probiotics, MDA levels decreased when compared with the NASH group (0.82 ± 0.39 and 0.99 ± 0.25 *vs* 3.34 ± 1.34 nmol/mg protein, respectively; $P < 0.05$).

Changes of serum IL-6 levels

As shown in Figure 3B, serum levels of interleukin-6 **(**IL-6**)** were higher in the NASH group than in the control group (262.71 ± 12.49 *vs* 199.57 ± 23.69

Table 1 Steatosis, inflammation and hepatocyte ballooning scores of liver histology in each group.

Group	n	Steatosis				Inflammation				Hepatocyte ballooning		
		0	1	2	3	0	1	2	3	0	1	2
Control	7	7	0	0	0	6	1	0	0	6	1	0
NASH	7	0	0	1	6	0	0	6	1	0	0	7
NASH +Single strain	7	1	2	3	1	3	4	0	0	0	0	7
NASH +Mixed strain	7	0	3	3	1	1	6	0	0	0	2	5

Data are expressed as the number of rats in each of histology grading score. Steatosis grade; 0 = < 5%, 1 = < 33%, 2 = 33–66%, 3 = > 66%. Inflammation grade; 0 = normal, 1 = mild, 2 = moderate, 3 = severe. Hepatocyte ballooning grade; 0 = no ballooning, 1 = few balloon cells, 2 = many balloon cells.

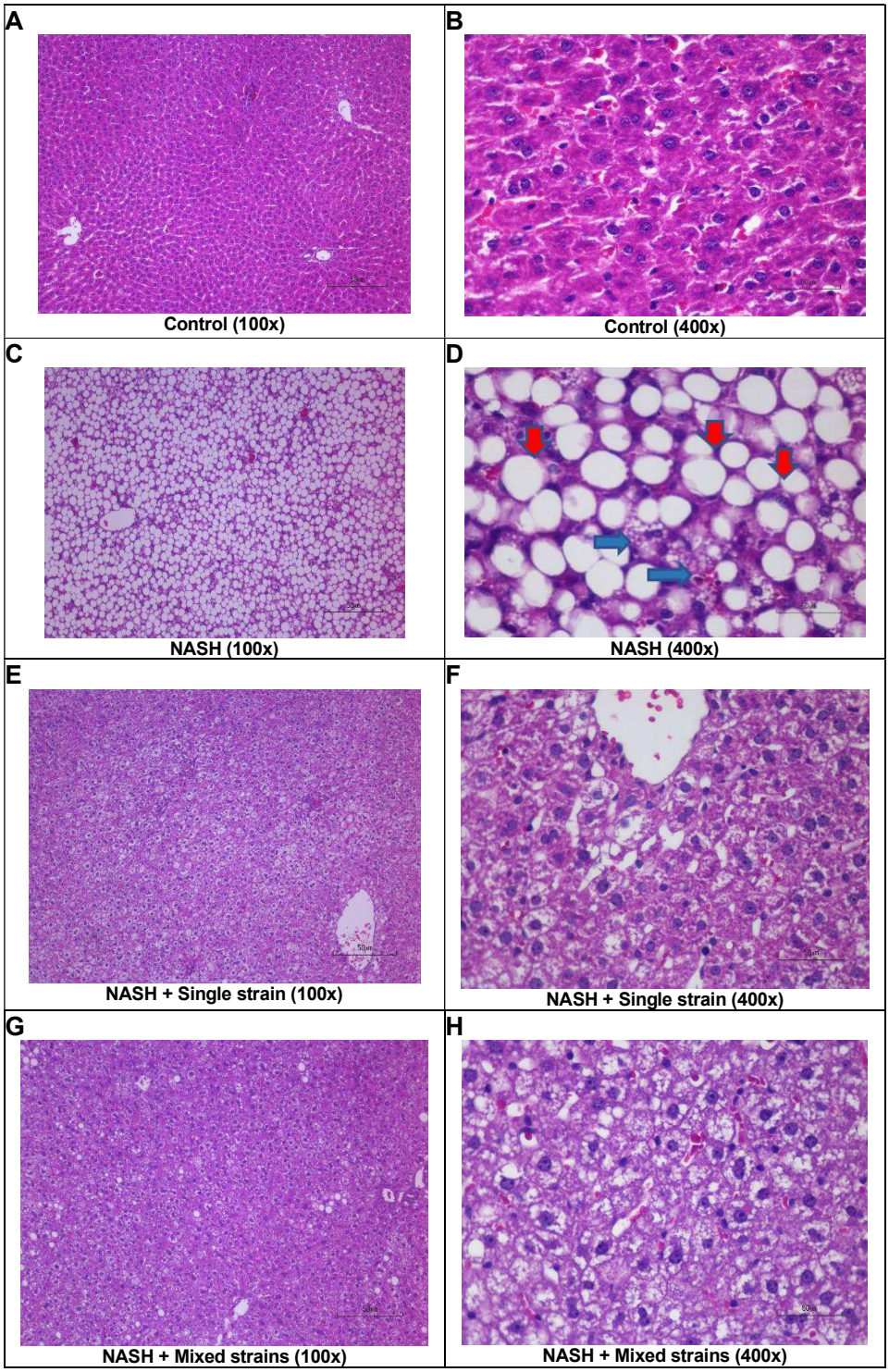

Figure 2 Representative images of liver histopathology (H&E stained) in each group. Red arrows indicate steatosis and blue arrows indicate hepatocyte ballooning.

pg/ml, respectively; $P < 0.05$). After treatment with single strain and mixed strains of probiotics, IL-6 levels decreased comparing with those of the NASH group (209.42 ± 17.55 and 214.57 ± 6.53 *vs* 262.71 ± 12.49 pg/ml, respectively; $P < 0.05$).

Changes of glutathione levels

As shown in Figure 3C, glutathione **(**GSH**)** levels decreased in the NASH group when compared with the control group (3.72 ± 0.21 *vs* 4.87 ± 0.44 nmol/mg protein, respectively; $P < 0.05$). After treatment with single and mixed strains of probiotics, GSH levels were higher than that the NASH group (5.11 ± 0.35 and 5.08 ± 0.18 *vs* 3.72 ± 0.21 nmol/mg protein, respectively; $P < 0.05$).

Discussion

This study demonstrated that HFHF diet could induce NASH as evidenced by the presence of steatosis, lobular inflammation, and hepatocellular ballooning on liver histology.[11] Fructose and saturated fat intake are associated with fat accumulation in the liver, insulin resistance and obesity. They also play a major role in hepatic inflammation and thus NASH development.[12] Fructose is an important lipogenic and adipogenic nutrient. It is sweeter than glucose and can be absorbed easily. High fructose diet also accelerates adipocyte hypertrophy.[13] Our results confirmed that a high fructose diet could induce NASH in this rat model. A high-calorie diet induces inflammatory stress in white fat tissue through immunomodulatory cytokines, such as tumor necrosis factor (TNF) and IL-6.[14] A previous animal study reported the increases in hepatic MDA, TNF-α and IL-6 levels in rats with high fat diet induced NASH.[15,16] Similarly, our results showed that hepatic MDA and IL-6 levels were significantly higher in the NASH group than in the control group.

In general, probiotic *Lactobacilli* strains promote beneficial effects in attenuating liver pathologies likely through anti-inflammatory actions and the stabilization of the intestinal barrier. Most studies focused on particular *Lactobacillus* strains, such as *Lactobacillus paracasei*, *Lactobacillus rhamnosus GG* (LGG) and their anti-inflammatory mechanisms of action. LGG is also known to prevent intestinal barrier impairment caused by inflammatory reactions and reduce intestinal infection and diarrhea.[17] Most studies used single and mixed strains of probiotics to improve NASH. Karahan et al., 2012,[18] investigated the preventive effect of two probiotic mixtures (Pro-1 and Pro-2) on the methionine choline-deficient diet (MCD)-induced NASH model in rats. In that study, MCD diet caused an elevation of serum ALT, which was slightly reduced by Pro-1 and Pro-2. Receiving the MCD diet for 2 and 6 weeks resulted in steatosis and inflammation, but not fibrosis, in all rats. Pro-1 and Pro-2 alleviated steatohepatitis in rats partly due to the modulation of apoptosis and their anti-

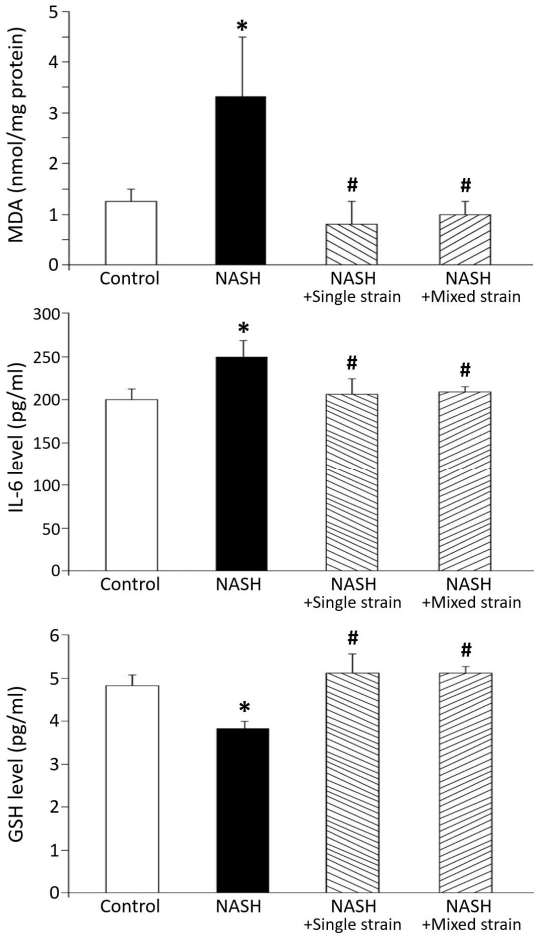

Figure 3 (A-C) Bar graphs representing hepatic MDA (A), serum IL-6 (B), and hepatic GSH levels (C). $^{*}P < 0.05$ compared with Control, $^{\#}p < 0.05$ compared with NASH

inflammatory activity. Those probiotic mixtures included *Lactobacillus plantarum* in the mix along with other strains. *Lactobacillus plantarum* markedly suppressed NASH development, along with the reduction of MDA in the liver.[8] Similar to other studies, our results showed that single and mixed strains of *Lactobacillus* could improve NASH histopathology, probably by the reduction in inflammation and oxidative stress. We also hypothesized that probiotics reduce inflammation and oxidative stress through the improvement of gut dysbiosis, the reduction of intestinal permeability, bacterial translocation, and endotoxin exposure. Further studies are needed to confirm this hypothesis.

Conclusion

Single and mixed strains of probiotics could reduce fat accumulation, liver inflammation and oxidative stress, thus improving histological changes of NASH. Probiotics could potentially be used for the management of NASH. Clinical studies are warranted to confirm their therapeutic effects in human.

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Conflict of Interest

None to declare.

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