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Anti-inflammatory effect of probiotic *Lactobacillus paracasei* MSMC39-1 on alcohol-induced hepatitis in rats

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ABSTRACT

Probiotics are good microbiota which are able to promote the health of host immunomodulation. In this study, we prepared four antigens of *Lactobacillus paracasei* MSMC39-1 for the detection of tumor necrosis factor-α (TNF-α) secretion in hepatocellular carcinoma cell line (HepG-2). We found that viable cells and heat-killed cells of *L. paracasei* MSMC39-1 exhibited strong TNF-α secretion inhibitors. Viable cells of *L. paracasei* MSMC39-1 treatment significantly reduced serum level of aspartate aminotransferase in hepatitis rats. TNF-α secretion in liver tissues of hepatitis rats in probiotic *L. paracasei* MSMC39-1 treatment was significantly suppressed. Liver histopathology examination of alcohol-induced hepatitis indicated fat accumulation in the hepatocytes. Interestingly, liver damage in hepatitis rats was improved by probiotic *L. paracasei* MSMC39-1. In addition, *L. paracasei* MSMC39-1 alleviated colon inflammation in alcohol-induced hepatitis rats. Administration of *L. paracasei* MSMC39-1 modulated gut microbiota by increasing the number of genera *Lactobacillus* and *Bifidobacterium* in alcohol-induced hepatitis rats. We conclude that *L. paracasei* MSMC39-1 has potent anti-inflammatory effects on HepG-2 cell line and improves hepatitis, colon inflammation, and modulates gut microbiota. Thus *L. paracasei* MSMC39-1 is able to protect against alcoholic hepatitis. It can also be used as an alternative treatment in other inflammation diseases and applied in nutraceutical and dietary supplement products.

INTRODUCTION

Probiotics, beneficial microbiota exhibit several health benefits in human diseases including alcoholic liver disease, obesity, allergy, and inflammatory bowel disease (IBD) (Fang *et al.*, 2019; Huang *et al.*, 2019; Rabah, 2020; Wang *et al.*, 2017). Several studies have utilized probiotics to compensate and reduce side effects from the use of antibiotics and as health supplements. In addition, probiotics are used in animal food products in many countries. Food and Agriculture Organization of the United Nations and World Health Organization (FAO and WHO) defines probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (WHO/FAO, 2002). Selection criteria of probiotics include toleration to acid and bile, adhesion in gut epithelial cell, production of antimicrobial substances, and modulation of immunity. Safety of probiotics follows the criteria of Generally Recognize as Safe by the United States Food and Drug Administration (USFDA) (Shewale *et al.*, 2014). According to their properties, probiotics are considered as a member of good microbiota with health benefits.

Genera of *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Streptococcus*, *Bacillus*, *Lactococcus*, and *Saccharomyces* are used as probiotics. Probiotics have been reported to enhance tight junction, decrease cholesterol level with bile salt hydrolase production, produce gamma-aminobutyric acid (GABA), and modulate proinflammatory cytokines (Bermudez-Brito *et al.*, 2012; Das and Goyal, 2015). Tumor necrosis factor (TNF), a major proinflammatory cytokine, plays a key role in the inflammation

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process. TNF are divided into two forms: tumor necrosis factor-α (TNF-α) and TNF-beta (TNF-β). TNF-α is mainly produced by macrophage. Binding between TNF and TNF receptor 1 leads to transcription of genes encoding proinflammatory cytokines, chemokines, cell survival factors, and growth factors via Ikappa B (IκB) kinase (IKK)/ nuclear factor kappa-B (NF-κB) and mitogen-activated protein kinase /AP-1 pathways (Cheung and Ware, 2013). The level of TNF is alleviated in patients with inflammatory conditions including IBD, rheumatoid arthritis, alcoholic liver disease, atopic dermatitis, and psoriasis (Chu, 2013). Alcoholic hepatitis is a disease caused by chronic alcohol consumption. It causes accumulation of fat and toxin in liver, disruption of intestinal barrier, and stimulation of proinflammatory cytokines leading to liver inflammation and dysbiosis in gastrointestinal (GI) tract. Lipopolysaccharide (LPS) which is released from bad microbiota due to chronic ethanol consumption binds to toll-like receptor 4 (TLR-4) and activates Kupffer cells in liver tissue. Kupffer cells then secrete proinflammatory cytokines such as TNF-α, interleukin (IL)-1β, and IL-6 and generate reactive oxygen species via NF-κB/JNK/AP-1 pathway (Fung and Pyrsopoulos, 2017). Drug is commonly used as a treatment for liver diseases to relieve symptoms of patients, and liver transplantation is the last treatment manner (Leggio and Lee, 2017).

　　　Probiotics are used as an alternative treatment for liver disease. Several pieces of evidence both *in vitro* and *in vivo* reported the usage of probiotics in this disease. Probiotic lactic acid bacteria reduced TNF-α and IL-6 secretion in LPS stimulated human hepatocellular carcinoma cells (HepG2) (Chiu *et al.*, 2013). *Lactobacillus rhamnosus* GG and its component improved TLRs and suppressed proinflammatory cytokine of LPS treated porcine intestinal epithelial cell via NF-kB signaling pathway (Gao *et al.*, 2017). In addition, *Bifidobacterium bifidum*, *Lactobacillus casei*, and *Lactobacillus plantarum* fermented soymilk alleviated liver injury and reduced TNF in liver tissue of rat fed high-fat diet (Zhang *et al.*, 2017). Probiotic *Bifidobacterium animalis* reduced liver injury which reduced mRNA level of TNF-α in mice with alcoholic liver disease (Zhang *et al.*, 2019). Hong *et al.* (2015) reported that *L. rhamnosus* and *Lactobacillus acidophilus* decreased levels of TNF-α, IL-1β, and TLR-4 expression and increased anti-inflammatory cytokine (IL-10) in an alcoholic hepatitis mouse model (Hong *et al.*, 2015). Pretreatment of probiotic *L. casei* reduced TNF-α, IL-1β, endotoxin production, hepatic inflammation, and increasing of genus *Lactobacillus* and *Bifidobacterium* in feces of LPS and D-galactosamine induced acute liver failure rats (Wang *et al.*, 2016). The previous study on mice model showed that *L. rhamnosus* GG supplementation reduced hepatic inflammation and TNF-α production via inhibition of TLR-4 and TLR-5 activation in 5 % alcohol-induced liver injury for 8 weeks (Wang *et al.*, 2013). Likewise, the study on *L. rhamnosus* GG (LGG) supplementation reduced liver inflammation by protected gut microbiota alteration for the treatment of alcoholic liver disease (Bull-Otterson *et al.*, 2013). A recent study on alcoholic liver injury rats showed that *L. plantarum* C88 reduced alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymes in the liver and decreased TNF-α, IL-6, and interferon-gamma (IFN-γ) in serum. Moreover, C88 inhibited NF-κB inflammation signaling pathway (Zhao *et al.*, 2017). Similarly, *L. plantarum* and *Lactobacillus fermentum* treatment reduced ALT and AST levels in serum and

decreased TNF-α and IL-1β gene expression in hepatic tissue of carbon tetrachloride-induced acute liver injury mice model (Chen *et al.*, 2018). A recent study on acute liver injury rat model induced by D-galactosamine injection shows that pretreatment of *Lactobacillus helveticus* decreased ALT and AST level in the serum and was downregulated TNF-α gene expression in the hepatic tissue (Wang *et al.*, 2019). Our previous data suggested that *L. paracasei* MSMC39-1 and *L. confusus* MSMC57- 1 strongly inhibited TNF-α secretion in human monocytic cell line (THP-1) (Ladda *et al.*, 2015). Therefore, the aim of this study is to investigate the roles of probiotic *L. paracasei* MSMC39-1 in the suppression of TNF-α secretion in hepatocellular carcinoma cells and alleviated liver inflammation in alcohol-induced hepatitis rats. We hope to search for probiotic *Lactobacillus* which is able to be a potential candidate for ameliorating of liver inflammation.

MATERIALS AND METHODS

Probiotic strains

　　　The probiotic *L. paracasei* MSMC 39-1 and *L. confusus* MSMC57-1 were used in this study. These strains were isolated from newborn feces (SWUEC 37/2551) which inhibited TNF-α production in the THP-1 monocytic cell line (Ladda *et al.*, 2015).

Cultivation of probiotics

　　　Lactobacillus paracasei MSMC39-1 and *L. confusus* MSMC57-1 were cultured in deMan-Rogosa-Sharpe (MRS) broth (Himedia, India) and incubated at 37°C for 48 h under anaerobic condition (Whitley Jar Gassing System, UK) in an anaerobic jar (Mitsubishi, Japan). Subsequently, the probiotic culture was streaked on MRS agar supplemented with 0.3% calcium carbonate (Merck, USA) and incubated at 37°C for 48 h under anaerobic condition. The overnight culture of *L. paracasei* MSMC39-1 and *L. confusus* MSMC57-1 was picked up as a single colony and added to MRS broth and incubated at 37°C for 48 h under anaerobic condition for the next experiments.

Preparation of probiotic antigens

Probiotic culture supernatant and viable cells

　　　Lactobacillus paracasei MSMC39-1 and *L. confusus* MSMC57-1 were cultured on MRS agar and incubated at 37°C under anaerobic condition for 48 h. The single pure colony was picked up and inoculated in MRS broth and incubated at 37°C under anaerobic condition for 48 hours. The two strains were adjusted to a final concentration of 10^9 colony forming units (CFU)/ml (Ladda *et al.*, 2015). The supernatant was separated by centrifugation and filtered with 0.22 μm and concentrated by a vacuum concentrator (Christ, Germany) at 40°C for 4–6 hours. The concentrated supernatant was resuspended in serum-free-medium Dulbecco's modified eagle medium (DMEM) and kept at -20°C until used. For viable cell preparation, the final concentration of viable cell pellet was washed twice with phosphate buffer saline (PBS). The pellets were resuspended with serum-free DMEM and used immediately for TNF inhibition test in cell lines.

Heat-killed cells

　　　Lactobacillus paracasei MSMC39-1 and *L. confusus* MSMC57-1 were cultured on MRS agar, anaerobically incubated

at 37°C for 48 hours. The single colony was cultured in MRS broth and incubated at 37°C in anaerobic condition for 48 hours. The probiotic strains were adjusted to the final concentration of 10^9 CFU/ml (Ladda *et al.*, 2015), centrifuged, and washed twice with PBS. The pellets were resuspended in PBS, heated at 85°C for 1 hour, and kept at −20°C until used. The cell viability was rechecked by streaking on MRS agar and anaerobically incubated at 37°C for 48 hours.

Sonicated cells

Lactobacillus paracasei MSMC39-1 and *L. confusus* MSMC57-1 were cultured on MRS agar incubated at 37°C anaerobic for 48 hours. The single colony was picked up and cultured in MRS broth and incubated at 37°C in anaerobic condition for 48 hours. The probiotic strains were adjusted to the final concentration of 10^9 CFU/ml (Ladda *et al.*, 2015), centrifuged, and washed twice with PBS. The pellets were resuspended in serum-free medium DMEM. Resuspended cells were sonicated at the set speed of 85%–88% for 7.10 minutes by ultrasonic homogenizers (Sonopuls, Bandelin, Germany) and kept at −20°C until used.

Cell culture

HepG-2 cells (HB 8065) and hepatocellular carcinoma cell line were purchased from American Type Culture Collection (ATCC). HepG-2 cells were cultured in DMEM containing 10% fetal bovine serum and 1% penicillin (100 units/ml) streptomycin (100 mg/ml) and incubated in 37°C incubator with 98% humidified and 5% CO_2 (Chiu *et al.*, 2013).

Determination of TNF-α secretion in HepG-2 cells

Hepatocellular carcinoma cells were seeded at 1×10^5 cells/well on a 24-well plate for 3 days (Chiu *et al.*, 2013). Cells were treated with 10% *v/v*, probiotic culture supernatant, 20% *v/v* heat-killed cells, and sonicated cells or viable cells of *L. paracasei* MSMC39-1 and *L. confusus* MSMC57-1 and stimulated with a final concentration of 5 µg/ml purified LPS from *Escherichia coli* serotype O127:B8 (Sigma, USA) for 48 hours. Collection of supernatant was centrifuged at 1,000 g, 4°C, 5 minutes. Levels of TNF-α in the supernatants were detected by human TNF-α sandwich enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Experimental animals

Male Wistar rats (*Rattus norvegicus*), aged 8 weeks and weighing 200–250 g, were purchased from Namura Siam International (Thailand). Housing of the animals was controlled at a temperature of 22 ± 2°C, humidity: 55% ± 5%, and 12:12 hours light/dark cycle at Medical Center Animal Care Laboratory, Srinakharinwirot University, Thailand. The rats were adapted to the environment for 1 week before the experiment. The animal experiment obtained informed license no. 9/2561 from the Animal Ethics Committee of the Faculty of Medicine, Srinakharinwirot University.

Induction of hepatitis

Alcoholic hepatitis was induced in each rat by oral gavage with 30% ethanol dissolved in reverse osmosis water at a dose of 5 mg/kg of body weight for 8 weeks (Forsyth *et al.*, 2009; Zhao *et al.*, 2017).

Experimental designs

Lactobacillus paracasei MSMC39-1 was the strongest TNF-α inhibition. Thus, *L. paracasei* MSMC39-1 was selected for the animal experiment. The rats were divided into four groups of six animals each. Standard diet and distilled water were given to rats in the normal control group (group 1). Viable cells of probiotic *L. paracasei* MSMC39-1 with standard diet were administrated to rats in the probiotic control group (group 2). Hepatitis rats (group 3) received a standard diet with 30 % alcohol. Probiotic-treated rats (group 4) received a standard diet with 30 % alcohol and viable cells of *L. paracasei* MSMC39-1. Viable cells of *L. paracasei* MSMC39-1 were administered before induction of hepatitis for 2 weeks and treated continuously until 8 weeks; the standard diet was *ad libitum*. Viable cells of *L. paracasei* MSMC 39-1 were given to rats in groups 2 and 4 at 1×10^9 CFU/ml/rat/day. At the end of the experiment, body and liver weight were measured in each rat. Then rats were anesthetized with isoflurane, and blood was collected from cardiac puncture.

Detection of liver function enzymes

Rats were anesthetized with isoflurane, and blood was collected from cardiac puncture at the end of the experiment (Wang *et al.*, 2016). Serum was separated and centrifuged at 1,500 g, 4°C for 10 minutes. AST and ALT levels were detected in the serum (Zhao *et al.*, 2017).

Detection of TNF-α level in the liver

After rats were euthanasia, liver tissue was removed, weighed, and homogenized by ultrasonic homogenizers (Sonopuls, Bandelin, Germany) at 35% power and centrifuged at 800 g, 5 minutes, 4°C. Levels of TNF-α in the supernatant of liver homogenate were detected by sandwich ELISA kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN) (Ladda *et al.*, 2015).

Histology evaluation of hepatitis rat

After treatment, rats were anesthetized with isoflurane and sacrificed. Liver and colon were removed, weighed, and excised from the tissue (Wang *et al.*, 2016). The pieces of tissue were fixed in 4% paraformaldehyde at 4°C and dehydrated in a graded alcohol series (10%, 20%, 50%, 95%, and 100%). The liver and colon specimens were embedded in paraffin and sectioned at 5–7 µm. The section slides were deparaffinized, cleared in xylene, and rehydrated in a graded series of ethanol (100%, 95%, and 70%) before staining with hematoxylin and eosin (H&E). Histology of each tissue section was evaluated under a light microscope (Olympus UC50, Japan).

Detection of fecal microbiota by standard plate count

Stool was collected at the end of the experiment for detection of gut microbiota alteration. One gram of stool was diluted in normal saline solution and spread in MRS agar, *Bifidobacterium* agar, and Eosin Methylene Blue Agar (EMB agar). The plates were incubated at the optimal condition of each

bacterial growth. Colonies were counted and calculated as the percentage of fecal microbiota alteration.

Detection of gut microbiota by next-generation sequencing

Stool was collected at the end of the experiment for detection of gut microbiota alteration by next-generation sequencing. QIAamp Fast DNA Stool Mini Kit was used for stool DNA extraction according to the manufacturer's instructions (Qiagen, Germany). DNA concentration was measured by Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA). The primers used in this study were as followed: forward primer: 5′TCGTCGGCAGCGTCAGATGTGTATAAGA

GACAGCCTACGGGNGGCWGCAG3′ and reverse primer: 5′GTCTCGTGGGCTCGGAGATG

TGTATAAGAGACAGGACTACHVGGGTATCTAAT CC3′. Two variable regions of 16S rDNA (V3 and V4) of 16S metagenome were sequenced by Illumina Miseq sequencer (Illumina, San Diego, CA) (Pérez-Burillo *et al.*, 2020).

Statistical analysis

Statistical significances or differences were evaluated by GraphPad Prism version 8.00. For the *in vitro* study, Student's *t*-test with one-tailed distribution was used. A ρ-value ≤ 0.05 was considered to be statistically significant. For the animal study, one-way analysis of variance (ANOVA) comparing each group with the control group was used and expressed as mean ± SD.

RESULTS AND DISCUSSION

The effect of *L. paracasei* MSMC39-1 on the TNF-α secretion in HepG-2 cells

Probiotic lactic acid bacteria mostly genus *Lactobacillus* and *Bifidobacterium* have been used to promote human health. Probiotic *Lactobacillus* isolated from human, fermented foods, and dairy products exhibited antimicrobial, anticancer, antioxidant, and cholesterol-lowering properties as well as immunomodulation (Choi and Chang, 2015; Kim *et al.*, 2019; Miao *et al.*, 2020; Pan *et al.*, 2020). Alleviation of TNF-α can be found in inflammatory diseases such as IBD, rheumatoid arthritis, alcoholic liver disease, atopic dermatitis, and psoriasis. TNF-α is also detected in alcohol liver disease and plays a key role in liver inflammation (Niederreiter and Tilg, 2018). Inhibition of TNF-α production by probiotics has been reported. *L. rhamnosus*, 4B15, and *Lactobacillus gasseri* 4M13 isolated from human feces with antioxidant and cholesterol reduction properties suppressed the production of proinflammatory cytokine including TNF-α, IL-6, and IL-1β in RAW 264.7 macrophage cell line (Oh *et al.*, 2018). In addition, surface layer protein of *L. acidophilus* decreased inflammatory cytokine secretions including TNF-α, IL-10, IL-1β, interferon-alpha (IFN-α), and interferon-beta (IFN-β) in LPS-activated RAW 264.7 cells (Cai *et al.*, 2018).

Four antigens including probiotic culture supernatant, viable cells, heat-killed cells, and sonicated cells were prepared from *L. paracasei* MSMC39-1 and *L. confusus* MSMC57-1. The effects of these four antigens on TNF-α were investigated in LPS-induced TNF-α secretion in HepG-2 cells. Although probiotic culture supernatant of MSMC57-1 had no effect on TNF-α secretion, a slight inhibition of TNF-α secretion can

be observed in HepG-2 cells treated with probiotic culture supernatant of *L. paracasei* MSMC39-1 (Fig. 1A). Viable cells of *L. paracasei* MSMC39-1 and *L. confusus* MSMC57-1 decreased TNF-α secretion in LPS-activated HepG-2 cells with 53% and 23% reduction, respectively (Fig. 1B). More than 50% of TNF-α secretion was suppressed by heat-killed cell of *L. paracasei* MSMC39-1 when compared to MRS controls (*p < 0.05) (Fig. 1C). TNF-α secretion was comparable in HepG-2 cells treated with heat-killed cells of *L. confusus* MSMC57-1 when compared with the MRS control (Fig. 1C). Sonicated cells of *L. confusus* MSMC57-1 significantly suppressed TNF-α secretion in HepG-2 cells (*p < 0.05) (Fig. 1D). Slight inhibition of TNF-α secretion was observed in HepG-2 cells treated with *L. paracasei* MSMC39-1 (Fig. 1D). In this study, four antigens generated from probiotic lactic acid bacteria, *L. paracasei* MSMC39-1 and *L. confusus* MSMC57-1, were used to investigate the TNF-α secretion in HepG-2 cells. The supernatant of *L. paracasei* MSMC39-1 slightly inhibited TNF-α in LPS-activated HepG-2 cells. This result is in agreement with previous reports that supernatant of *L. plantarum* and *L. fermentum* inhibited TNF-α production in LPS-induced THP-1 monocytic cells (Aoudia *et al.*, 2016). Moreover, extracellular metabolites of *L. plantarum* prepared from supernatant suppressed TNF-α secretion via attenuation of NF-kB activation in LPS-activated RAW 264.7 macrophage cell line (Chon *et al.*, 2010). TNF-α secretion in HepG-2 cells was dramatically inhibited by viable cells, heat-killed cells, and sonicated cells of *L. paracasei* MSMC39-1. This may be due to the activity of probiotic cells but not the substances produced by the cells. Our results were similar to previous studies reporting that heat-killed cells and sonicated cells of probiotic *Lactobacillus* reduced TNF-α, and IL-6 secretion in LPS stimulated HepG-2 cells (Chiu *et al.*, 2013). A recent study revealed that peptidoglycan extracts of *L. plantarum* isolated from human feces modulated TNF-α and IL-6 in LPS-induced murine RAW264.7 cell (Choi *et al.*, 2019).

Effect of alcohol and *L. paracasei* MSMC39-1 on body weight and liver weight

To examine the hepatoprotective role of *L. paracasei* MSMC39-1, we used an ethanol-induced hepatitis rat model. Ethanol was administrated by oral gavage into the rats for 8 weeks. Although alcohol administration for 8 weeks decreased body weight, *L. paracasei* MSMC39-1 treatment significantly enhanced body weight in these rats (Table 1). There was no statistically significant difference in initial body weight, liver weight, and liver-to-body weight ratio of rats in probiotic, alcohol-induced hepatic steatosis, and probiotic treatment groups when compared with the normal control (Table 1). In the hepatitis rat model of this study, body weight and liver weight were examined. After administration of alcohol for 8 weeks, body weight was decreased, liver weight was increased, and liver-to-body weight ratio (%) exhibited an upward trend; in accordance with Huang *et al.* (2019), probiotics increased body weight, reduced AST, ALT, IL-6, and TNF-α, and modulated intestinal microbiota in alcohol liver disease model (Huang *et al.*, 2019). Moreover, administration of probiotics increased body weight and decreased ALT, AST, IL-1β, and TNF-α compared to the alcohol group of a normal diet and high-fat diet in mice (Hong *et al.*, 2015).
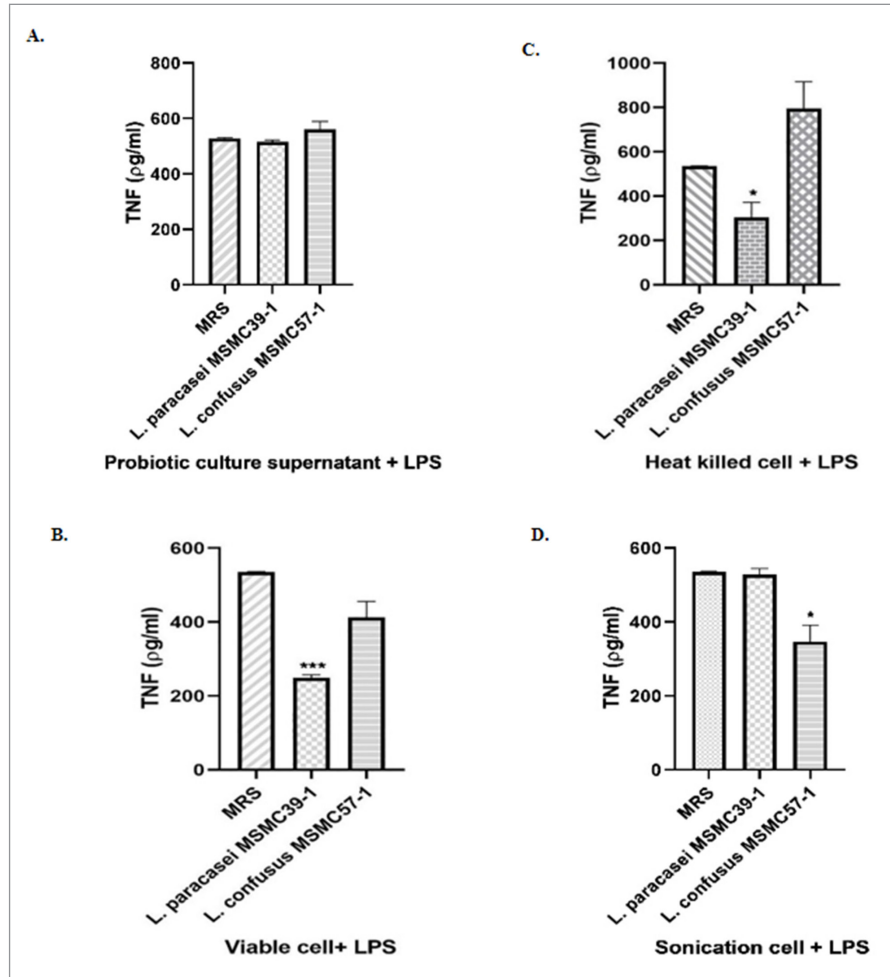
Figure 1. Suppression of TNF-α secretion of (A) probiotic culture supernatant, (B) viable cell, (C) heat-killed cell, and (D) sonication cell of *L. paracasei* MSMC39-1 and *L. confusus* MSMC57-1 in HepG-2 cells. Experiments were performed three times, MRS control. Statistical analysis used Student's *t*-test with one-tailed distribution. Error bars indicated standard deviations. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Table 1. Effect of alcohol and *L. paracasei* MSMC39-1 on body weight and liver weight.

Parameters	Group			
	Normal	Probiotic	Alcohol	Probiotic treatment
Initial body weight (g)	237.5 ± 4.03	239.8 ± 2.40	239.5 ± 7.85	239.4 ± 10.71
Body weight (g)	486.4 ± 6.93	462.5 ± 25.14	403.1 ± 10.39*	479.3 ± 16.5**
Liver weight (g)	15.03 ± 1.41	14.27 ± 0.43	15.83 ± 4.86	15.1 ± 1.28
Liver-to-body weight ratio (%)	3.08 ± 0.25	3.09 ± 0.08	3.94 ± 1.3	3.15 ± 0.05

The data are shown as mean ± SD. Statistical analysis used one-way ANOVA.
*$p < 0.05$ compared with the normal groups; **$p < 0.05$ compared with the alcohol group.

Effect of *L. paracasei* MSMC39-1 treatment on liver enzyme activity in rats

      In this study, hepatitis rats were treated with viable cells of *L. paracasei* MSMC39-1 for 8 weeks. The level of AST in the serum of the alcohol-treated group was elevated (Fig. 2A). AST level significantly reduced in probiotic-treated hepatitis rat (*$p < 0.05$) (Fig. 2A) when compared to the alcohol group. The level of ALT in serum of alcohol-induced hepatitis rats was elevated but reduced when *L. paracasei* MSMC39-1 was given to these rats (Fig. 2B). ALT levels in the normal control and *L. paracasei*

MSMC39-1 groups were lower than that off alcohol group (Fig. 2B). These results suggest the protective role of *L. paracasei* MSMC39-1 on liver disease. AST and ALT are biomarker enzymes of liver function. The ratio of AST and ALT level which is greater than or equal to two is considered as alcoholic hepatitis (Torruellas *et al.*, 2014). In this study, AST levels of alcohol hepatitis rats were two times more than ALT levels in serum. In addition, the probiotic-treated group with viable cells of *L. paracasei* MSMC39-1 showed reductions of AST and ALT levels in serum of hepatitis rats. Similar to other studies, *L. plantarum* C88, *L.*
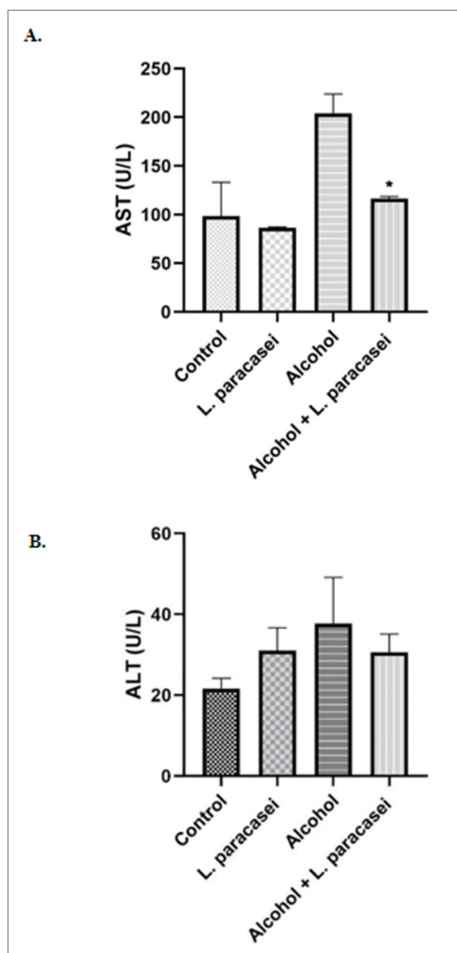
Figure 2. Effect of *L. paracasei* MSMC39-1 on liver enzyme functional test. (A) AST level; (B) ALT. Error bar indicated standard deviation. Statistical analysis used one-way ANOVA and *p < 0.05; significant differences from the control (n = 6 rats).

plantarum, *L. fermentum*, *L. helveticus*, and *L. casei* reduced ALT and AST enzymes in the serum (Chen *et al.*, 2018; Wang *et al.*, 2019; Zhao *et al.*, 2014, 2017). Likewise, in the clinical trial study, the levels of AST and ALT were reduced in alcoholic liver disease patients who received probiotics *B. bifidum* and *L. plantarum* 8PA3 supplementation (Kirpich *et al.*, 2008). *L. paracasei* MSMC39-1 improved AST and ALT function which may be due to suppression of proinflammatory cytokine production such as TNF-α in the liver resulting in less inflammation. Similar mechanism, *L. plantarum*, *L. fermentum*, and *L. plantarum* C88 improved liver function enzyme caused by decreasing of TNF-α expression (Chen *et al.*, 2018) and oxidative stress (Zhao *et al.*, 2017).

Effect of *L. paracasei* MSMC39-1 treatment on TNF-α secretion in the liver tissues

A high level of TNF-α secretion was observed in hepatic tissues of hepatitis rats administered with 30% alcohol for 8 weeks (Fig. 3). TNF-α level in hepatic tissue of rat in probiotic *L. paracasei* MSMC39-1-treated group was lower than that of the alcohol control group (*p < 0.05) (Fig. 3). A similar level of TNF-α was observed in liver tissues of the normal control and probiotic
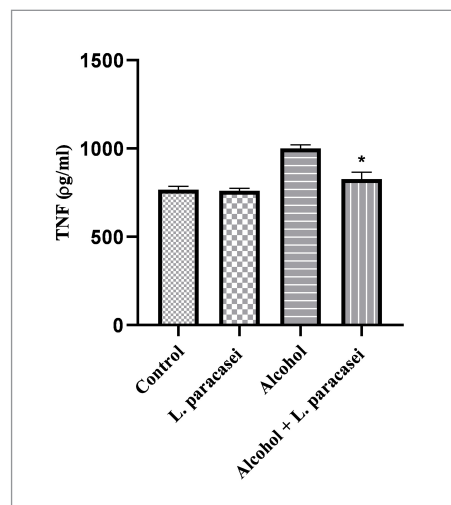

Figure 3. Effect of *L. paracasei* MSMC39-1 suppresses TNF-α secretion in liver tissue of hepatitis rat. Error bar indicated standard deviation. Statistical analysis used one-way ANOVA and *p < 0.05; significant differences from the control (n = 6 rats).

control group (Fig. 3). In this study, *L. paracasei* MSMC39-1 significantly suppressed TNF-α secretion (*p < 0.05) in liver tissue of alcohol hepatitis rats. Our result is similar to previous studies which reported that *L. plantarum*, *L. fermentum*, and *L. helveticus* decreased TNF-α and IL-1β gene expression in hepatic tissues of acute liver injury models (Chen *et al.*, 2018; Wang *et al.*, 2019). Moreover, *Bifidobacterium longum* LC67 and *L. plantarum* LC27 reduced TNF-α levels in serum and liver of ethanol-induced acute liver damage in mice (Kwon *et al.*, 2017). Wang *et al.* (2013) revealed that *L. casei* Zhang pretreatment reduced TNF-α expression in LPS/D-galactosamine induced liver injury rat. Moreover, in liver fibrosis rats, *Lactobacillus* sp. and α-lipoic acid reduced TNF-α expression in liver tissue (Zoheir *et al.*, 2014). Ethanol consumption destroys gut barrier function leading to elevation of LPS in the GI tract. This LPS then binds to TLR-4 on Kupffer cells resulting in proinflammatory cytokine production and liver inflammation (Meroni *et al.*, 2019). A study by Xu *et al.* (2019) reported the role of probiotics on proinflammatory cytokine production. Exopolysaccharides produced by *L. buchneri* TCP016 reduced proinflammatory cytokine production such as TNF-α, IL-1β, and IL-6 leading to liver protection in LPS/D-galactosamine-induced liver damage in mice. In addition, supernatant of *L. reuteri* reduced liver inflammation by inhibition of IL-6 and TNF-α production in acute liver injury mice (Cui *et al.*, 2019).

Effect of *L. paracasei* MSMC39-1 treatment on the liver and colon histopathology in alcohol-induced hepatitis rats

Probiotic *L. paracasei* MSMC39-1 at 1×10^9 CFU/ml was administered in alcohol-induced hepatitis rats for 8 weeks and histopathology of liver tissues was examined by hematoxylin and eosin staining. Normal hepatic architectures of liver tissues were observed in normal and probiotic control groups as shown in Figure 4. High levels of fat accumulation in cytoplasm of hepatocytes, swollen hepatocytes, hydropic degeneration, and infiltration of inflammatory cells were prominent in liver tissues of the alcoholic

hepatitis group. Interestingly, treatment of probiotic *L. paracasei* MSMC39-1 improved liver tissue condition of hepatitis rats as indicated by normal hepatic architecture and slight fat accumulation in the hepatocytes as shown in Figure 4. Alcoholic hepatitis rats were treated with probiotic *L. paracasei* MSMC39-1 for 8 weeks and histopathology of colon tissues was examined by hematoxylin and eosin staining. Normal intestinal architectures of colon tissues were observed in normal and probiotic control groups as shown in Figure 5. Crypt edema and infiltration of inflammatory cells were found in alcoholic hepatitis colon tissues of rats administered with 30% alcohol for 8 weeks. Interestingly, probiotic *L. paracasei* MSMC39-1 treatment improved colon tissues of hepatitis rats with slight crypt edema and fewer inflammatory cells as shown in Figure 5. Chronic alcohol consumption induced lipid accumulation via the AMP-activated protein kinase (AMPK) signaling pathway, resulting in hepatic steatosis (Gu *et al.*, 2019). Hepatic steatosis and inflammatory cell infiltration can be observed in liver histology of alcohol hepatitis rats. On the other hand, feeding viable cells of *L. paracasei* MSMC39-1 reduced hepatic steatosis and the number of inflammatory cells. The reduction of hepatic steatosis by probiotics may be due to the reduction of sterol regulatory element-binding protein 1 (SREBP-1), which plays a key role in lipogenesis and fatty acid oxidation (Gu *et al.*, 2019). Previous animal models showed that

oral gavage of *L. rhamnosus* GG improved leaky gut and reduced colonic and liver inflammation in alcohol-induced liver injury in rats (Forsyth *et al.*, 2009). Similarly, Wang *et al.* (2012) indicated that cell-free supernatant of *L. rhamnosus* GG improved liver damage and intestinal inflammation via tight junction protection. In contrast, administration of probiotics *L. casei*, *L. plantarum*, *L. Acidophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *B. longum*, *Bifidobacterium breve*, *Bifidobacterium Infantis*, and *Streptococcus salivarius* subsp. *thermophilus* (VSL#3) for 6 months reduced hepatic encephalopathy in liver cirrhosis Indian patients (Dhiman *et al.*, 2014).

Fecal gut microbiota alteration in *L. paracasei* MSMC39-1 treatment

Stool was collected after the end of experiments for the detection of gut microbiota alteration. The normal control and probiotic control showed a similar number of genera *Lactobacillus* and *Bifidobacterium* as shown in Figure 6. The numbers of *E. coli* and *Proteus* were increased in alcohol hepatitis rats, whereas the number of *Lactobacillus* was decreased in this group as shown in Figure 6. Although administration of *L. paracasei* MSMC39-1 increased the number of genus *Lactobacillus* and *Bifidobacterium* alcohol-induced hepatitis rats, a decrease in the number of *E. coli* and *Proteus* was observed in these rats when compared with the
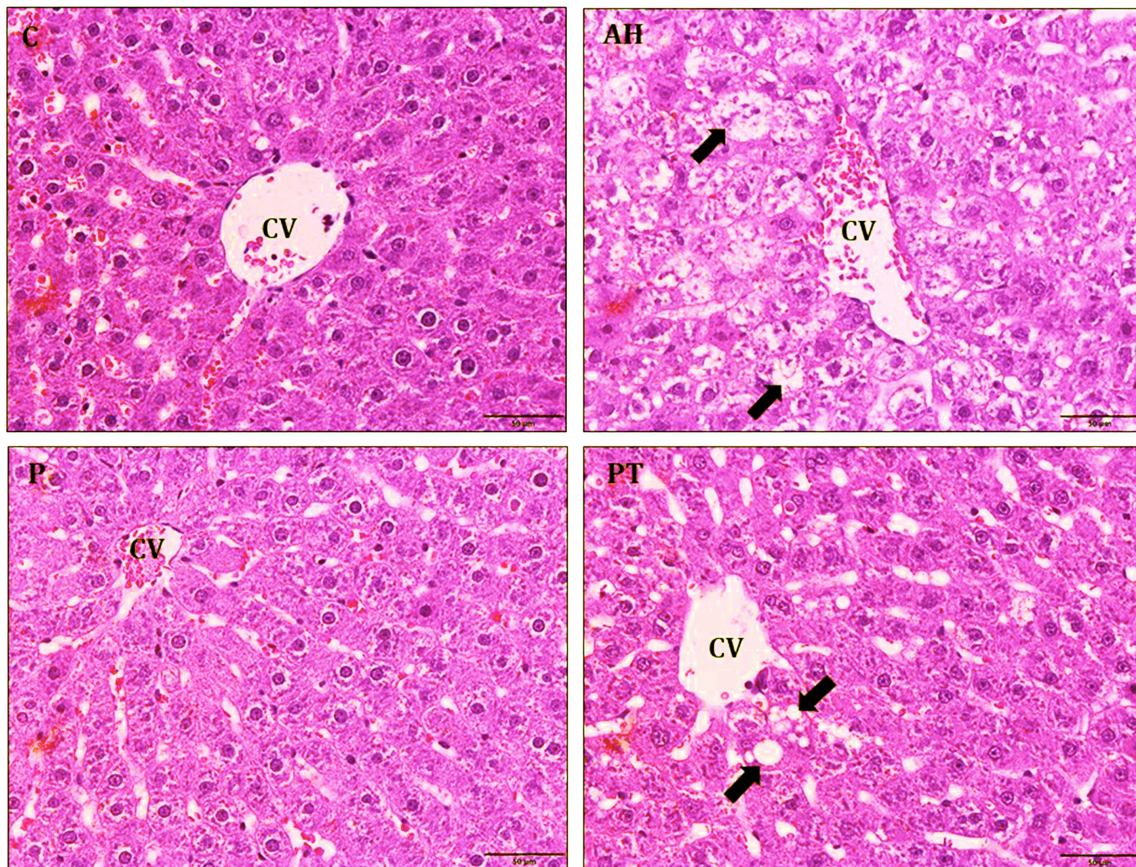

Figure 4. Histology of liver tissue was estimated by hematoxylin and eosin staining in hepatitis rats (*n* = 6 rats). Normal control (C) and probiotic control (P) showed normal hepatocyte and regularity hepatocyte arrangement. Alcoholic hepatitis (AH) indicated that fat accumulation was in the hepatocyte (black arrow) and improved by probiotic *L. paracasei* MSMC39-1 treatment (PT). 400× magnification and scale bar 50 μm. The tip of portal lobules was the central vein (CV).
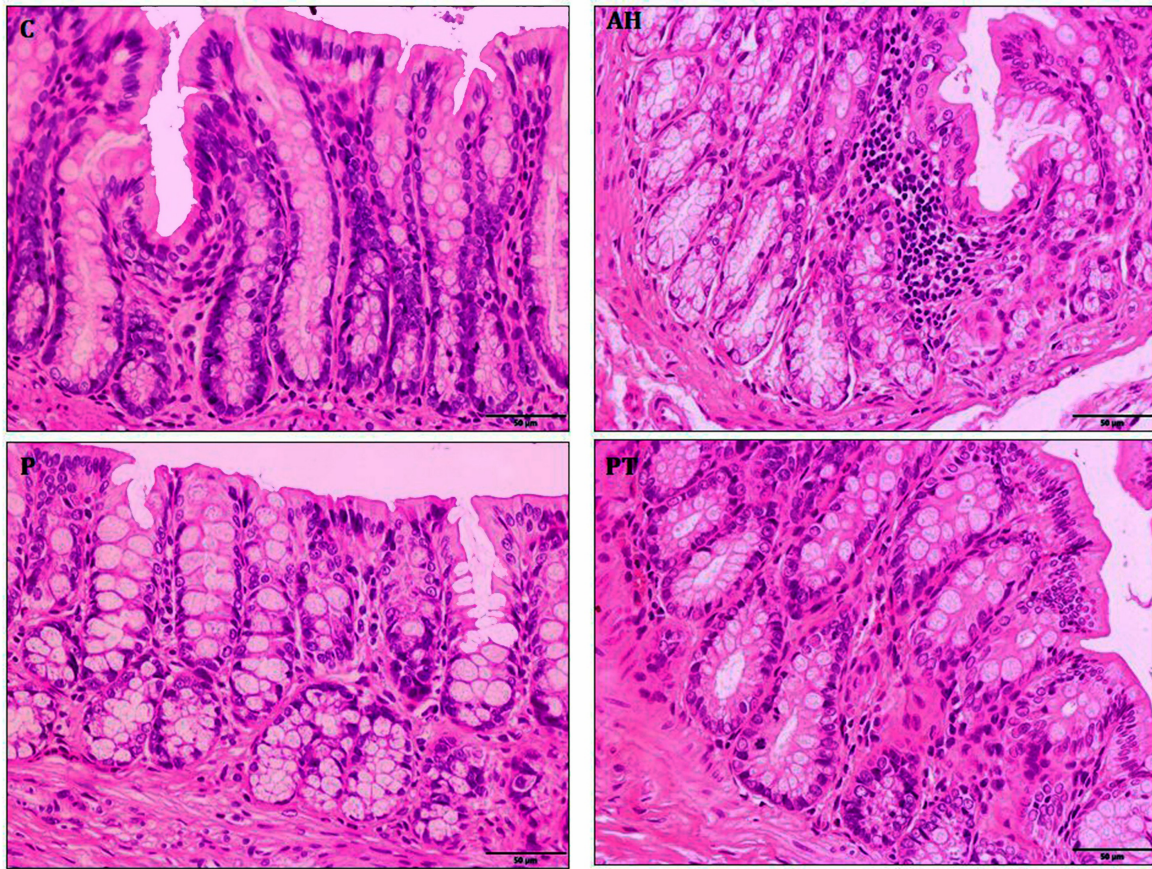
Figure 5. Histology of colon tissue was an estimate by hematoxylin and eosin staining in hepatitis rats. Normal control (C) and probiotic control (P) showed normal crypt. Alcoholic hepatitis (AH) indicated intense inflammatory cell and crypt edema and was improved by probiotic *L. paracasei* MSMC39-1 treatment (PT). 400× magnification.
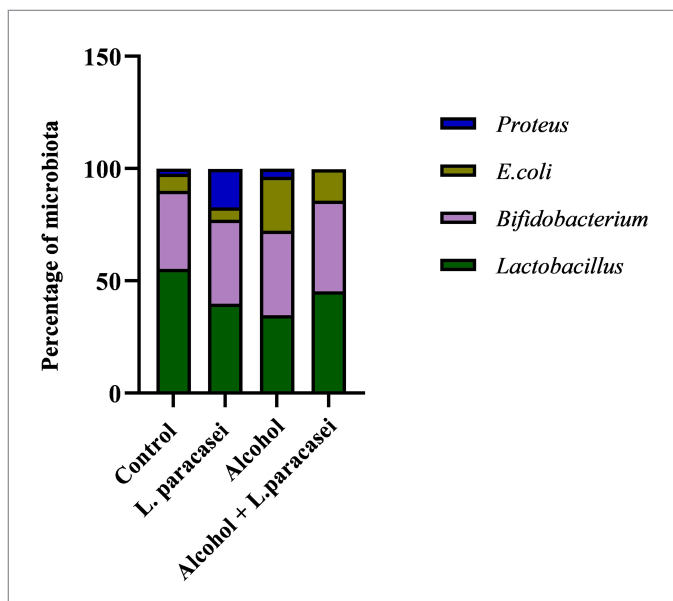

Figure 6. Effect of *L. paracasei* MSMC39-1 on gut microbiota alteration in hepatitis rats. Statistical analysis used one-way ANOVA and *$p < 0.05$; significant differences from the control ($n = 6$ rats).

alcohol control group (Fig. 6). The data from next-generation sequencing of stool revealed an increase in Bacteroidetes and a decrease in Firmicutes in alcohol hepatitis rats as shown in Figure 7A. After *L. paracasei* MSMC39-1 treatment in alcohol-induced hepatitis rats, the number of phylum Firmicutes (*Lactobacillus* spp.) increased whereas the number of Bacteroidetes (*Prevotella* spp.) decreased when compared to alcohol control as shown in Figure 7A and B. Furthermore, in this study, *L. paracasei* MSMC39-1 was a potent gut microbiota modulation which increased phylum Firmicutes and decreased Bacteroidetes in alcohol-induced hepatitis rats. In the alcohol alone group, the number of phylum Firmicutes decreased whereas Bacteroidetes increased resulting in dysbiosis in the GI tract (Tian *et al.*, 2020). Modulation of microbiota by probiotics has been reported. Administration of *L. buchneri* TCP016 modulated gut microbiota in LPS/D-galactosamine-induced liver damage in mice (Xu *et al.*, 2019). A recent study by Tian *et al.* (2020) showed that administration of live cells of *B. breve* for 6 weeks was able to modulate gut microbiota in alcohol liver disease mice model via protection of intestinal tight junction and liver inflammation. Likewise, supplementation of LGG modulated intestinal microbiota and alleviated liver injury in mice (Gu *et al.*, 2020).
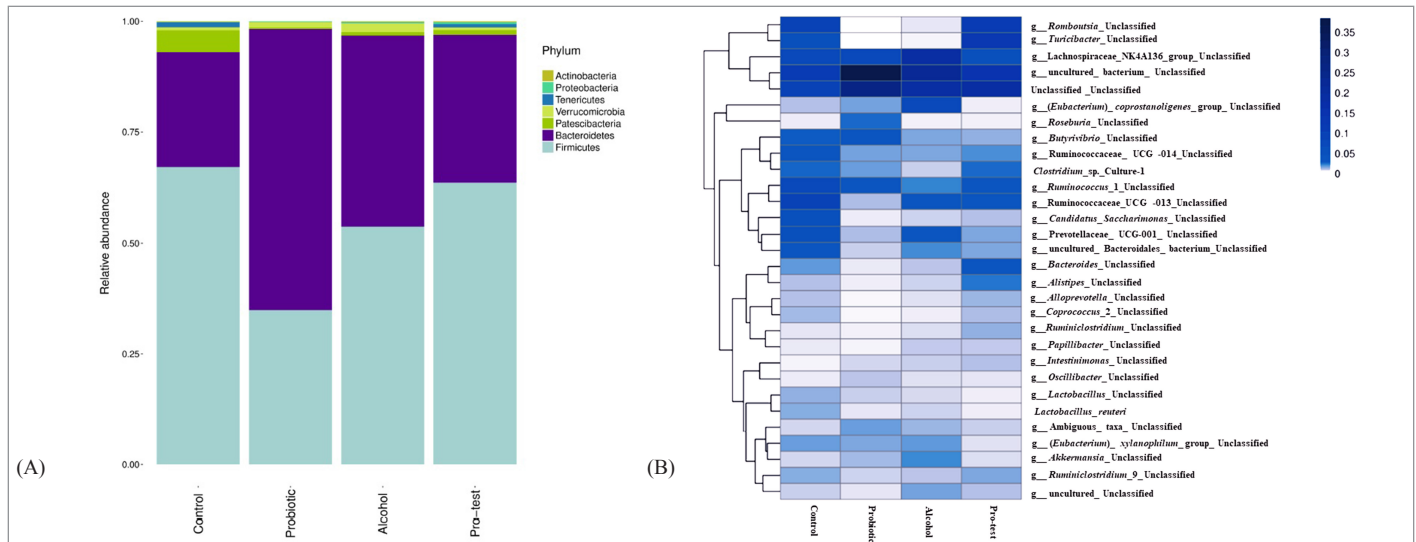
Figure 7. Effect of *L. paracasei* MSMC39-1 on gut microbiota alteration in alcohol-induced hepatitis rats. (A) Relative abundance of phylum in the fecal microbiota of four groups of rats; normal control, probiotic control, alcohol control, and probiotic test. (B) Heat map of fecal microbiota species distribution in the four groups of rats. The color code indicates the differences in the relative abundance ranging from dark blue (highest) to light blue (the mean) to white (lowest). Statistical analysis used one-way ANOVA and *p < 0.05; significant differences from the control (n = 6 rats).

CONCLUSION

In conclusion, this finding suggested that probiotic *L. paracasei* MSMC39-1 inhibited TNF-α production in HepG-2 cell lines and improved liver and colon inflammation. Administration of *L. paracasei* MSMC39-1 ameliorated the number of beneficial microbes including *Lactobacillus* and *Bifidobacterium* in alcohol-induced hepatitis rats. Further studies would confirm the potential roles of *L. paracasei* MSMC39-1 to protect other liver functions in alcohol liver diseases and inflammation condition diseases.

CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

ETHICAL APPROVALS

The animal experiment protocol was approved (license no. 9/2561) by the Animal Ethics Committee of the Faculty of Medicine, Srinakharinwirot University.

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