# Combining probiotics and an angiotensin-II type 1 receptor blocker has beneficial effects on hepatic fibrogenesis in a rat model of nonalcoholic steatohepatitis

Running title: Effect of probiotics and ARB on liver fibrosis

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ABSTRACT

*Aim*: Intestinal endotoxin is important for the progression of nonalcoholic steatohepatitis (NASH). Circulating endotoxin levels are elevated in most animal models of diet-induced nonalcoholic fatty liver disease (NAFLD) and NASH. Further, plasma endotoxin levels are significantly higher in NAFLD patients, which is associated with small intestinal bacterial overgrowth and increased intestinal permeability. By improving the gut microbiota environment and restoring gut-barrier functions, probiotics are effective for NASH treatment in animal models. It is also widely known that hepatic fibrosis and suppression of activated hepatic stellate cells (Ac-HSCs) can be attenuated using an angiotensin-II (AT-II) type 1 receptor blocker (ARB). We thus evaluated the effect of combination probiotics and ARB treatment on liver fibrosis using a rat model of NASH.

*Methods*: Fisher 344 rats were fed a choline-deficient/L-amino acid-defined (CDAA) diet for 8 weeks to generate the NASH model. Animals were divided into ARB, probiotics, and ARB plus probiotics groups. Therapeutic efficacy was assessed by evaluating liver fibrosis, the lipopolysaccharide (LPS) Toll-like receptor (TLR)4 regulatory cascade, and intestinal barrier function.

*Results*: Both probiotics and ARB inhibited liver fibrosis, with concomitant HSC activation and suppression of liver-specific transforming growth factor (TGF)-β and

TLR4 expression. Probiotics reduced intestinal permeability by rescuing zonula occludens-1 (ZO-1) disruption induced by the CDAA diet. ARB was found to directly suppress Ac-HSCs.

*Conclusions*: Probiotics and ARB are effective in suppressing liver fibrosis via different mechanisms. Currently both drugs are in clinical use; therefore, the combination of probiotics and ARB is a promising new therapy for NASH.

Keywords Nonalcoholic steatohepatitis, hepatic fibrosis, probiotics, angiotensin-2 type 1 receptor blocker (ARB)

## INTRODUCTION

Nonalcoholic steatohepatitis (NASH) is a progressive liver disease characterized by hepatic steatosis that leads to inflammation, fibrosis, and cirrhosis. However, currently, there is no well-established treatment for this disease. Several mechanisms of NASH progression have been reported(1, 2). Regarding the mechanism of NASH pathogenesis, the two-hit theory has become widely accepted. This disease is thought to begin with excessive fat accumulation in the liver (first hit), followed by aggravating factors such as oxidative stress, inflammatory cytokines, and endotoxins (second hit).(3) However, the pathology of NASH is more complicated and recently a theory postulating multiple parallel hits has been presented.(2) The progression of nonalcoholic fatty liver disease (NAFLD) is known to be dependent on both genetic and environmental factors.(1, 2) One such factor is bacterial translocation through intestinal bacterial overgrowth and enhanced intestinal permeability. Lipopolysaccharide (LPS), secreted from the intestinal microbiota, is delivered to the liver via the portal vein.(1, 4) In patients with obesity, diabetes, metabolic disorders, NAFLD, and NASH, endotoxins from intestinal bacteria cause hepatic inflammation.(1, 2) Moreover, in a mouse model of NAFLD, bacterial overgrowth was found to mediate compositional changes and increase intestinal permeability by down-regulating the expression of tight junction proteins.(5) Serum

endotoxin levels were also determined to increase in an animal model of NASH,(6) and were similarly found to be significantly higher in histologically-severe NAFLD patients, which was accompanied by increased intestinal bacterial overgrowth and permeability.(7) Furthermore, systemic inflammation, oxidative stress, and insulin resistance are known to cause cytotoxicity and liver damage.(1, 2, 8) Moreover, obesity, NAFLD, and NASH are associated with changes in the intestinal bacterial flora, and probiotics can interfere with the progression of NASH.

Butyrate is a short chain fatty acid that is produced by microbiota in the colon and distal small intestine from indigestible starch, dietary fiber, and low digestible polysaccharide; it is generated through fermentation by microorganisms in the colon and distal small intestine.(9) This compound is particularly important for colon health, it is the primary energy source for colonic cells, and it has anti-carcinogenic and anti-inflammatory properties. Clinical trials have shown that the administration of butyrate might be a promising treatment option for ulcerative colitis.(10, 11) *Clostridium butyricum* is a gram-positive, anaerobic bacterium that produces butyric acid; it is found in soil and the intestines of healthy animals and humans.(12) MIYAIRI 588, a butyric acid-producing, gram-positive anaerobe, and a *C. butyricum* strain, is used as a probiotic for the treatment and prevention of antibiotic-related diarrhea in humans.(13, 14)

The renin-angiotensin-aldosterone system plays an important role in chronic liver disease.(15-17) We previously reported that blocking angiotensin-II (AT-II) signaling through the AT-II type1 receptor (AT1R) suppresses liver fibrosis in rats.(18, 19) Furthermore, the inhibitory effect of ARB on hepatic fibrosis was consistent with the suppression of activated hepatic stellate cells (Ac-HSCs).(20) In addition, the administration of ARB was found to improve liver fibrosis via AT-II-mediated LPS-Toll like receptor (TLR) 4 signaling and suppress TLR4 signaling in Ac-HSCs.(21)

We thus hypothesized that MIYAIRI 588 might improve the intestinal flora environment and inhibit the progression of NASH by preventing destruction of the intestinal barrier. Moreover, combining MIYAIRI 588 with ARB was predicted to improve NASH via different mechanisms. As such, we examined the effect of MIYAIRI 588 and ARB on NASH progression using animals fed a choline-deficient/L-amino acid-defined (CDAA) diet.

## METHODS

### Animals and regents

Male 6-week-old Fisher 344 (F344) rats were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). Rats were housed in controlled conditions with a temperature of 23 ±

3 °C, a relative humidity of 50 ± 20%, a stainless steel mesh, 10–15 air changes per hour, and 12 hours of light irradiation per day. During the experiment, animals were allowed free access to tap water. MIYAIRI 588 was provided by Miyarisan Pharmaceutical Co. Ltd. (Tokyo, Japan). Losartan, was purchased from Merck (Tokyo, Japan). Conventional chemical reagents were purchased from Nacalai Tesque (Kyoto, Japan). CDAA and choline sufficient /L-amino acid-defined (CSAA) diets were purchased from CLEA Japan Inc. (Tokyo, Japan).

### Experimental design

All experiments were conducted over 8 weeks. Rats were randomly divided into five groups. Groups receiving the CDAA diet, to establish the animal model of diet-induced hepatic steatosis and fibrosis, comprised the CDAA group. The group receiving the CDAA diet containing MIYAIRI 588 ($8.5 \times 10^9$ CFU/g) was designated the probiotics group.(22) Ten percent of the total CDAA diet was replaced with an excipient containing MIYAIRI 588. Animals receiving the CDAA diet containing losartan (30 mg/kg/day) was named the ARB group.(23, 24) Finally, rats receiving the CDAA diet containing MIYAIRI 588 and losartan comprised the combination group of probiotics and ARB. An additional group was administered a CSAA diet. At the end of the experimental period, the rats

were anesthetized with diethyl ether and different parameters were examined. All animal procedures were performed according to Declaration of Helsinki and in compliance with standard recommendations for the proper care and use of laboratory animals. The protocol was approved by the Committee of Nara Medical University.

### Histological and immunohistochemical analyses

For all experimental groups, 5-μm thick sections of formalin-fixed and paraffin-embedded liver specimens were routinely subjected to hematoxylin and eosin staining, oil red O staining to evaluate liver steatosis, Sirius Red (S-R) staining to evaluate liver fibrosis, and immunohistochemical staining probing for alpha smooth muscle actin (α-SMA; DAKO, Kyoto, Japan) as previously described (25, 26) and for glutathione S-transferase placental form (GST-P) as hepatic preneoplastic lesions. The stained sections were analyzed using Image-J software (National Institutes of Health).

### Quantitative RT-PCR analysis

mRNA was extracted from pulverized frozen liver and intestinal tissues using the RNeasy Mini Kit (QIAGEN, Tokyo, Japan). Total RNA (1 μg) from each sample was reverse transcribed into complementary DNA (cDNA) using a high capacity RNA-to-

cDNA kit (Applied Biosystems Inc., Foster City, Calif., USA). The expression levels of mRNA encoding liver tissue-derived TGF-β1, LPS-binding protein (LBP), and liver-derived TLR4 were analyzed using SYBR Green and the Step One Sequence Detection System (Applied Biosystems Inc., Foster City, Calif., USA) by polymerase chain reaction (PCR). The PCR procedure was as follows: the sample was heated at 95 °C. for 20 s and subjected to 40 cycles of denaturation at 95 °C for 3 s and annealing at 60 °C for 30 s. For this experiment, β-Actin was used as an endogenous control. Primer sequences used were as follows: TGF-β1, forward 5 -CGG CAG CTG TAC ATT GAC TT-3′ and reverse 5′-AGC GCA CGA TCA TGT TGG AC-3′; α1(I)-procollagen, forward 5′-AGC TCC TGG GCC TAT CTG ATG A-3′ and reverse 5′-AAT GGT GCT CTG AAA CCC TGA TG-3′; TLR4, forward 5′-CCG CTC TGG CAT CAT CTT CA-3′ and reverse 5′-CCC ACT CGA GGT AGG TGT TTC TG-3′; LBP, forward 5′- AAC ATC CGG CTG AAC ACC AAG-3′ and reverse 5′-CAA GGA CAG ATT CCC AGG ACT GA-3′; β-actin, forward 5′-GGA GAT TAC TGC CCT GGC TCC TA- 3′ and reverse 5′-GAC TCA TCG TAC TCC TGC TTG CTG-3′.

**Enzyme-linked immune sorbent assay (ELISA) analysis**

Mmp-9 concentrations were measured in the supernatant of the snap-frozen liver tissue using ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

**Immunofluorescence analysis**

Frozen sections of intestinal tissue (7-μm thick) were prepared and fixed with 4% paraformaldehyde at 4 °C for 10 min. After blocking with 10% normal goat serum in PBS, frozen sections were incubated with rabbit polyclonal anti-mouse ZO-1 antibody (1: 100, Invitrogen Life Technologies, Carlsbad, Calif., USA) overnight at 4 °C and then donkey anti rabbit secondary antibody associated with DyLight 488 for 1 h at room temperature with antibody fluorescent dye (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania USA). Sections were mounted using Vectashield loaded medium with 4',6-diamidino-2-phenylindole Fluoromount-G (DAPI) for fluorescent nucleic acid staining (Vector Laboratories, Burlingame, CA, USA). The stained specimens were examined using a confocal scanning laser microscope equipped with a digital camera (Leica TCSNT, Leica microsystems, Wetzlar, Germany). Green fluorescence corresponding to ZO-1 localized to the intestinal epithelial cell tight junctions (excitation light wavelength, 490 nm; emission wavelength, 510 nm). For each sample, the mean fluorescence intensity (MFI) values from areas of equal size were measured for each image using ImageJ Software. The MFI of negatively-stained sections was subtracted from the MFI of positively-stained sections.

### Statistical analyses

Student's t-test or one-way ANOVA followed by Bonferroni's multiple-comparison test were performed. Statistical analyses were performed using GraphPad Prism version 6.04 (GraphPad Software, Inc., La Jolla, CA, USA). All tests were two-tailed and p values < 0.05 were considered statistically significant.

## RESULTS

### Inhibitory effect of probiotics and ARB on hepatic fibrosis, carcinogenesis and steatosis

First, we examined the effect of clinically comparable doses of probiotics and ARB on liver fibrosis. Liver fibrosis was suppressed in both the probiotic and ARB group, compared to that in the CDAA group (Fig. 1A, B). A more potent inhibitory effect on hepatic fibrogenesis was observed in the combination group of probiotics plus ARB, compared to that with either drug alone. We then performed immunohistochemistry for α-SMA, which correlates with activated hepatic stellate cell (HSCs). In both the probiotics and ARB groups, a significant decrease in the number of α-SMA immunopositive Ac-HSCs was observed (Fig. 2A). Computer-assisted semi-quantitative analysis of α-SMA immunohistochemistry showed a reduced area of α-SMA staining in parallel with the inhibition of liver fibrosis (Fig. 2B). A significant inhibition in TGF-β

expression was also observed in both the probiotic and ARB groups, compared to that in the CDAA group (Fig. 3). Similarly, the combination of probiotics and ARB resulted in a stronger inhibitory effect than either drug alone. We performed immunohistochemistry for GST-P, as a significant factor in carcinogenesis (Fig. 4). Treatment with either probiotics or ARB resulted in a marked inhibitory effect on hepatic GST-P expression compared to the CDAA group. Combination treatment with both agents was equivalent to single administration. No GST-P cells were observed in liver sections from the CSAA group. On the other hand, Liver steatosis was suppressed in the probiotic group, compared to that in the CDAA group, but not in ARB group (Fig. 5).

**Inhibitory effect of probiotics and ARB on TLR4 signaling**

Next, we evaluated the effect of probiotics and ARB on hepatic TLR4. Hepatic TLR4 expression increased in the CDAA group compared to that in the CSAA group (Fig. 6). In the probiotic and ARB groups, hepatic TLR4 expression decreased as compared to that in the CDAA group. The combination of probiotics and ARB resulted in a significant attenuation of CDAA-induced hepatic TLR4 expression compared to that with probiotics or ARB alone ($p < 0.01$). Notably, the inhibitory effect of probiotics and ARB on liver TLR4 expression was proportional to the inhibition of liver fibrosis.

### Inhibitory effect of probiotics and ARB on LBP

The direct detection of LPS is difficult, and thus we evaluated hepatic LBP, as this marker directly correlates with LPS.(27) We found that liver LBP mRNA was increased after 8 weeks of CDAA administration ($p < 0.05$). However, in the probiotics group, LBP mRNA decreased compared to that in the CDAA group ($p < 0.05$; Fig. 7). In contrast, no significant effect on this liver LBP mRNA was observed in the ARB group. Regarding the combination group of probiotics and ARB, LBP mRNA was decreased compared to that in the CDAA group; however, there was no additional benefit compared to probiotics alone. These data suggest that the inhibitory effect on hepatic fibrosis with respect to LPS is not associated with ARB, but rather probiotics.

### Correlation mmp-9 concentrations and liver fibrosis

Mmp-9 levels were higher in CDAA group compared to the CSAA group ($p < 0.01$; Fig. 8). CDAA induced increase in mmp-9 expression was not reduced in probiotics group, ARB group, and both probiotics and ARB group.

### Semi-quantitative determination of intestinal tight junction protein expression

Intestinal epithelial permeability is regulated by the intercellular tight junction protein (TJP) complex consisting of many components including zonula occuludens-1 (ZO-1). We thus evaluated the effect of probiotics and ARB on ZO-1 expression to identify the potential mechanism associated with intestinal permeability. Semi-quantitative immunofluorescence microscopy revealed a marked reduction in ZO-1 expression in the CDAA group compared to that in the CSAA group (Fig. 9A). MIYAIRI 588 significantly improved ZO-1 expression compared to that in the CDAA group. In contrast, this was not significantly changed with ARB administration compared to that in the CDAA group. Upon combining probiotics with ARB, ZO-1 expression significantly improved compared to that in the CDAA group; however, there was no difference compared to expression in the probiotics only group (Fig. 9B). These results indicate that the inhibitory effect of probiotics, but not ARB, on liver fibrosis in CDAA-induced NASH potentially occurs through the restoration of TJP expression, which could contribute to the regulation of endotoxin influx.

## DISCUSSION

We demonstrated that treatment with clinically equivalent doses of losartan (30 mg/kg/day) and MIYAIRI 588 successfully ameliorated hepatic fibrosis and suppressed

Ac-HSCs in a rat model of NASH. In this model, combining MIYAIRI 588 with losartan treatment resulted in a synergistic inhibitory effect and almost completely attenuated hepatic fibrogenesis. Various factors have been reported to cause NASH. Two of these include inflammation and endotoxins. Liver inflammation causes the activation of HSCs, which is a central event during hepatic fibrosis. This involves a complex network of autocrine/paracrine fibrogenic signals that promote the transdifferentiation of quiescent HSCs to Ac-HSCs (myofibroblastic phenotypes), which are characterized by abundant expression of αSMA.

The interaction between AT-II and vascular endothelial growth factor (VEGF) plays an important role in liver fibrogenesis and carcinogenesis. ARB was previously shown to significantly suppress the development of liver fibrosis along with VEGF expression and neovascularization in the liver. (28) We previously reported that ARB directly inhibits Ac-HSC activation, and that AT-II is important for the up-regulation of TLR4 expression through the stimulation of AT1R in Ac-HSCs. (21) Crosstalk between AT-II and TLR4 signaling plays a substantial role in the development of liver fibrosis by regulating TGF-β1 production in Ac-HSCs. Our results showed that ARB administration in a rat model of NASH reduces α-SMA-positivity, TLR4, and TGF-β, which leads to an improvement in hepatic fibrogenesis and carcinogenesis.

As probiotics control intestinal bacteria and endotoxin production, it was expected to be effective for suppressing NASH. There are numerous studies on the effectiveness of probiotics for NASH,(29) however, sufficient effectiveness has not been established using probiotics. Long term administration of butyric acid-producing probiotic *C. butyricum* strain MIYAIRI 588 was shown to decrease hepatic fibrous deposition and the development of GST-P positive foci in CDAA-induced NAFLD.(22) The TJP has been reported to localize to the apical plasma membrane of epithelial cells to maintain epithelial barrier integrity.(30) Endotoxins derived from intestinal bacteria can cause liver inflammation via TLR4 activation. TLR4 is a pattern recognition receptor that recognizes endotoxin and signals through myeloid differentiation primary-response protein 88 (MyD 88) and Toll/interleukin1 receptor-domain-containing adaptor protein inducing interferon-β (TRIF) to activate transcription factors involved in innate immunity.(31) TLR4 is expressed in various hepatic cells including liver vascular endothelial cells, Kupffer cells, and HSCs. (32, 33) In human and animal studies, NASH was shown to be associated with portal LPS levels via mechanisms involving bacterial translocation.(22, 34) In addition, endotoxin produced by gut microbiota might cause inflammation in patients with obesity, diabetes mellitus, metabolic disorder, NAFLD, and NASH.(1, 35) Moreover, plasma LPS levels are associated with small intestinal

bacterial overgrowth, changes to the composition of the microbiota, and increased intestinal permeability.(7) As such, the disruption of intestinal bacteria contributes to the pathogenesis of NAFLD. A methionine-choline deficient diet and fructose uptake was shown to cause NAFLD in mice. In these models, intestinal endotoxin levels increased in the portal vein(36-38) An imbalance between proliferation and apoptosis, in addition to intestinal mucosal atrophy and edema, which is associated with portal hypertension or the absence of bile acids, results in increased production of inflammatory cytokines and enhanced oxidative stress in the liver. (27, 39, 40) Accordingly, we performed immunohistochemistry for the intestinal tight junction protein ZO-1. The expression of intestinal ZO-1 was reduced in the CDAA model compared to that in the CSAA group; however, it was increased with the administration of MIYAIRI 588. In contrast, ARB did not influence intestinal ZO-1 expression. Taken together, this suggests that MIYAIRI 588, but not ARB, has the potential to improve intestinal tight junction integrity. The inhibitory effect of MIYAIRI 588 on hepatic fibrogenesis and carcinogenesis was mediated by preventing intestinal permeability through the restoration of TJP expression and inhibition of the systemic inflammatory response caused by LPS translocation.

The mechanism through which MIYAIRI588 improves intestinal barrier function has

been suggested; it was postulated that butyrate enhances this property by activating adenosine monophosphate-activated protein kinase (AMPK) signaling.(41) Dietary supplementation of butyrate induces the activation of AMPK, thereby preventing and inhibiting high-fat-diet-induced obesity and insulin resistance in mice.(42) AMPK regulates energy homeostasis through its effects on glucose and lipid metabolism,(43) controls fatty acid oxidation by regulating mitochondrial biogenesis, and suppresses lipogenic gene expression by reducing the activity of the transcription factor sterol-regulatory element-binding protein 1c (SREBP-1c).(44) Hepatic AMPK also decreases hepatic lipogenesis, and its activity can inhibit reactive oxidative stress and inflammation.(45) *In vitro* experiments have shown that NaB treatment can increase AMPK activity and accelerate the assembly of TJPs in the Caco-2 colonic epithelial cell line.(41)

In this report, the combined use of probiotics and ARB resulted in a stronger anti-fibrotic effect as compared to that with either drug alone. Several investigators including our group have shown that ACE-I and ARB possess strong anti-angiogenic activity, and that these agents could inhibit the growth of several types of tumors including HCC at clinically comparable low doses.(46, 47) MIYAIRI 588 was shown to decrease the development of GST-P positive foci in CDAA-induced NAFLD.(22) Probiotics and ARB

have the inhibitory effect on hepatic carcinogenesis, but in combination there was no better effect than single administration. In our experiments, the period given CDAA was 8 weeks, which was short, so GST-P positive foci was small. If we give CDAA for a long period of time we may have seen the combined effect of probiotics and ARB.

MIYAIRI 588 improved the integrity of intestinal tight junctions, whereas ARB suppressed the activation of HSCs. In other words, these agents improved hepatic fibrosis through two different mechanisms, one targeting the intestinal tract and the other affecting HSCs.

We investigated the involvement of fibrolysis in clarifying the mechanism of improvement of fibrosis. In this experiment, there was no change in mmp-9 expression by probiotics and ARB administration. It seems that fiblolysis is not involved in the mechanism of improvement of fibrosis in this experiment.

In conclusion, using our experimental models, simultaneous administration of MIYAIRI 588 and losartan exerted a more potent and synergistic inhibitory effect on hepatic fibrogenesis than either agent alone by alleviating endotoxin-induced gut barrier dysfunction and suppressing Ac-HSC proliferation, respectively (Fig. 10). Since both drugs are clinically safe, the combination of probiotics and ARB could be useful for slowing NASH progression in future clinical applications.

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Figure legends

Fig.1 (A) Microphotographs of liver sections with Sirius red. No fibrosis was observed in choline-sufficient amino acid (CSAA)-fed control rats. Liver fibrosis was observed in the choline-deficient L-amino acid (CDAA) treatment group. Monotherapy with Probiotics demonstrated a little inhibitory effect (p=0.08). Monotherapy with angiotensin II type I receptor blocker (ARB) demonstrated a significant inhibitory effect. Combined probiotics and ARB (Probiotics+ARB) exerted a more inhibitory effective than either monotherapy. (B) Semiquantitative analysis confirmed histological findings. Values represent mean ± SD. *P< 0.05, **P< 0.01.

Fig.2 (A) Immunohistochemical analysis of hepatic -α-smooth muscle actin (α-SMA) expression. (B) Semiquantitative analysis of the α-SMA immunohistochemistry were performed by using image analysis software. Treatment with either Probiotics or ARB resulted in a significant inhibitory effect on hepatic α-SMA expression compared to the CDAA-fed group. Combined probiotics and ARB (Probiotics+ARB) exerted a stronger inhibitory effect. No α-SMA cells were observed in liver sections from the CSAA-fed group. Values represent mean ± SD. *P< 0.05, **P< 0.01.

Fig.3 Semiquantification of RT-PCR of hepatic TGF-ß mRNA. Inhibitory effect of probiotics and ARB on TGF-ß. Hepatic TGF-ß content were markedly increased in CDAA-fed rats compared to CSAA-fed rats. Treatment with Probiotics and ARB significantly suppressed both hepatic TGF-ß content compared to CDAA group. The combination treatment was more effective than either agent alone. Values represent mean ± SD. *P< 0.05, **P< 0.01.

Fig.4 (A) Microphotographs of liver sections with GST-P. Immunohistochemical analysis of GST-P expression. (B) Cells stained by GST-P immunohistochemistry were measured using image analysis software. Treatment with either probiotics or ARB resulted in a marked inhibitory effect on hepatic GST-P expression compared to the CDAA-fed group. Combination treatment with both agents was equivalent to single administration. No GST-P cells were observed in liver sections from the CSAA-fed control group. Values represent mean ± SD. **P< 0.01.

Fig.5 (A) Microphotographs of liver sections with Oil red O staining. Lipid accumulation was observed in the choline-deficient L-amino acid (CDAA) treatment group . (B) Cells stained by Oil red O were measured using image analysis software. Treatment with probiotics resulted in a marked inhibitory effect on hepatic oil red O positive area compared to the CDAA-fed group. However, no significant reduction in oil red O positive

area was observed in ARB-treated rats. Values represent mean ± SD. **P< 0.01.

Fig.6 Semiquantification of RT-PCR of hepatic TLR-4 mRNA. Inhibitory effect of probiotics and ARB on hepatic TLR4 mRNA expression. Hepatic TLR4 expression was significantly increased in the CDAA-fed group compared to the CSAA-fed group. Probiotics and ARB were found to suppress hepatic TLR4 expression. And the combination treatment was more effective than either agent alone. Values represent mean ± SD. *P< 0.05, **P< 0.01.

Fig.7 Semiquantification of RT-PCR of hepatic LBP mRNA. Inhibitory effect of Probiotics and ARB on hepatic LPS binding protein (LBP) expression. LBP expression were significantly increased in CDAA-fed rats compared to CSAA-fed rats. CDAA induced increase in LBP expression was significantly reduced in Probiotics group and both Probiotics and ARB treated group. No significant reduction in LBP expression was seen in ARB group. Values represent mean ± SD. *P< 0.05, **P< 0.01.

Fig.8 Using ELISA, mmp-9 levels in the liver was significantly increased in CDAA rat. Adding probiotics and ARB did not show any effect. Values represent mean ± SD. **P< 0.01.

Fig.9 (A) Effect of probiotics and ARB on intestinal ZO-1 expression assessed by fluorescence microscopy. An immunofluorescence microscopy was used to evaluate the effect of probiotics and ARB on ZO-1 expression in intestinal tissues. (B) Semi-quantitative immunofluorescence microscopy revealed ZO-1 expression was increased in CDAA group compared to CSAA group. Similar to LBP expression, CDAA induced increases in ZO-1 expression were significantly reduced in Probiotics group and Probiotics and ARB treated group. No significant reduction in liver fibrosis was observed in ARB group. Values represent mean ± SD. *P< 0.05, **P< 0.01.

Fig.10 Schematic representation of the mechanisms showing that probiotics and ARB prevent the progression of NAFLD through the gut-liver axis. Probiotics improves intestinal permeability and ARB controls hepatic stellate cells. Combining probiotics and ARB suppresses liver fibrosis from two different sides.