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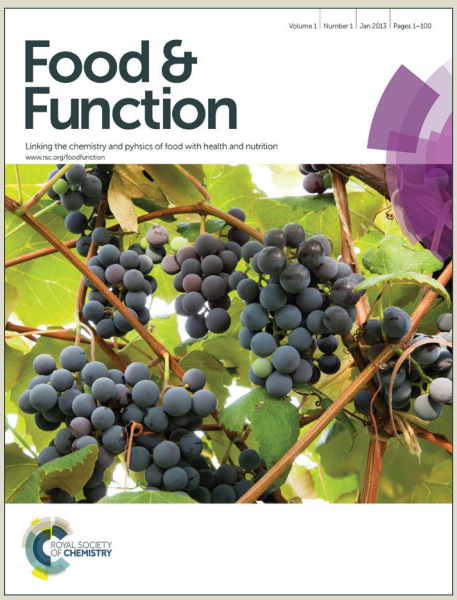

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Title page

Title

Synbiotics reduce ethanol-induced hepatic steatosis and inflammation by improving intestinal permeability and microbiota in rats

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Abstract

Clinical and animal experiments indicated that gut-derived endotoxin and unbalance intestinal microbiota contribute to the pathogenesis of alcoholic liver disease (ALD). In this study, we investigated whether synbiotic supplementation could improve ALD in rats by altering the intestinal microbial composition and improving the intestinal integrity. Male Wistar rats were divided into four groups according to plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities and subjected to either a normal liquid diet (C), a normal liquid diet with synbiotic supplementation (C+S), an ethanol liquid diet (E), or an ethanol liquid diet with synbiotic supplementation (E+S) for 12 weeks. Results revealed that the ethanol-fed group showed increases in plasma AST and ALT activities, the endotoxin level, hepatic triglyceride (TG) level, and hepatic tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 levels, and a decrease in the hepatic IL-10 level. Ethanol-feeding also contributed to an increased the intestinal permeability and decreased fecal bifidobacteria and lactobacilli amounts. However, synbiotic supplementation effectively attenuated the plasma endotoxin, hepatic TG and TNF-α levels, and increased the hepatic IL-10 level. Furthermore, synbiotic supplementation protected the rats against ethanol-induced hyperpermeability of the intestine, and significantly increased amounts of bifidobacteria and lactobacilli in the feces. This study demonstrated that synbiotics possess a novel hepatoprotective function by improving the intestinal permeability and microbiota in rats with ethanol-induced liver injury.

Key words: *synbiotics, ethanol-induced liver injury, endotoxin, intestinal microbiota, intestinal permeability*

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Introduction

Alcoholic liver disease (ALD) is characterized by a spectrum of liver pathologies ranging from fatty liver and steatohepatitis to cirrhosis. Clinically significant liver disease occurs in only a subset (15~30%) of alcoholics,[1,2] indicating that excessive ethanol consumption is necessary but not sufficient to induce liver injury, and therefore, one or more cofactors are required. Gut-derived endotoxin is a required cofactor because removal of the intestinal microflora with antibiotics prevents ALD in animal studies and because an endotoxin-initiated hepatic necroinflammatory cascade causes liver injury in ALD.[3-5] Endotoxins are lipopolysaccharides (LPSs) derived from cell walls of gram-negative bacteria. Endotoxemia in ALD was first recognized by detecting antibodies against *Escherichia coli* in the plasma of patients with ALD.[6] In addition, gut leakiness appears to be the cause of the endotoxemia in ALD.[7] Previous studies showed that intestinal barrier hyperpermeability occurs only in alcoholics with ALD and not in those alcoholics without liver disease.[8] Therefore, these data strongly suggest that ethanol-induced intestinal barrier disruption is the key mechanism in endotoxemia in ALD.

Based on the mechanism of the gut-liver axis, Kirpich et al. found that probiotics (9×10^7 CFU *Bifidobacterium bifidum* and 9×10^8 CFU *Lactobacillus plantarum* 8PA3) restored the bowel flora and improved liver enzyme in human alcohol-induced liver injury.[9] Forsyth et al. also reproted that Lactobacillus GG treatment (2.5×10^7 CFU) ameliorated alcohol-induced intestinal oxidative stress, gut leakiness and liver injury in a rat model of alcoholic steatohepatitis.[10] Keshavarzian et al. used oats as a prebiotic to prevent gut leakiness and ameliorate alcohol-induced liver damage in rats.[11]

The combination of probiotics and prebiotics are called synbiotics. Live microbial additions (probiotics) may be used in conjunction with specific substrates (prebiotics) for growth (e.g., a fructooligosaccharide in conjunction with a bifidobacterial strain or lactitol

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in conjunction with a lactobacillus organism). This combination can improve the survival of the probiotic organism, because its specific substrate is readily available for its fermentation, and result in advantages to a host that the live microorganisms and prebiotics offer. Although synbiotics have been used to modulate the gut flora in patients with infectious diarrhea and inflammatory bowel diseases (IBD), there is little evidences from studies of the relationship between synbiotics and ALD.[12,13]

Therefore, the aim of this study was to investigate whether synbiotic supplementation can reduce the ethanol-induced liver damage by modulating the intestinal permeability and microbiota in rats.

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Materials and methods

Animals

Eight-week-old male Wistar rats were purchased from BioLasco Taiwan (Taipei, Taiwan). Rats were housed individually in cages at 23 ± 2˚C and 55% ± 10% relative humidity with a 12-h light/dark cycle. Animal experiments were approved by the Institutional Animal Care and Use Committee of Taipei Medical University.

Study protocol

After 1 week of acclimation, thirty two rats were divided into four groups (8 rats for each group) according to plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in order to reduce the variation among groups at the baseline. Four groups were fed a normal liquid diet (C group), a normal liquid diet with synbiotic supplementation (C+S group), an ethanol liquid diet (E group), or an ethanol liquid diet with synbiotic supplementation (E+S group) for 12 weeks. Diets were modified from Lieber and DeCarli.[14] The liquid ethanol diet contained 35% energy as ethanol, while the control liquid diet provided an isocaloric diet in which the ethanol was replaced with maltodextrin. The control and ethanol liquid diets provided 16.6% of total calories as protein and 35.6% of total calories as fat. Components of synbiotic powder are shown in Table 1 and the synbiotic powder (FloraGuard®) was provided by Viva Life Science (Costa Mesa, CA, USA). Synbiotic supplementation was provided as the synbiotic powder in 10 mL of distilled water (1.5 g/kg body weight (BW)/day) in the C+S and E+S group. At the same time, 10 mL of distilled water was given to the C and E groups. The synbiotic solution was provided to rats with drinking bottles from 10am to 3pm and the liquid diet was provided to rats with drinking tube from 4pm to the next day 9am. We measured the bottle weight before and after feeding to each rat and calculated the daily intake of synbiotic powder and recorded the calibration on the drinking tubes to calculate the daily

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intake of the liquid diet and ethanol. At the 11 week, all rats underwent an intestinal permeability test and microbial culture of feces. At the 12 week, all rats were sacrificed. Blood and liver were collected for analysis. Blood samples were collected in heparin-containing tubes and centrifuged (1200×*g* for 15 min at 4°C) to obtain plasma samples. All plasma samples were stored at -80°C until being assayed. Liver tissues were rapidly excised. Parts of the liver tissues were fixed in 10% formaldehyde and embedded in paraffin for a histopathological analysis. Other liver tissues were stored at -80°C for further analysis.

Liver function

Plasma AST and ALT activities were measured as biochemical indicators of liver function. Blood samples were collected in heparin-containing tubes and centrifuged (1200 ×*g* for 15 min at 4 °C) to obtain plasma samples. Plasma AST and ALT activities were measured using SYNCHRON CX System Hitachi 7170 (Hitachi High-Technologies, Tokyo, Japan).

Histopathological analysis

Formalin-fixed liver tissues were processed hematoxylin-eosin (H&E) stain and Masson's trichrome staining. The H&E stain was used to evaluate chronic liver damage including the hepatocyte inflammatory response, hepatocyte necrosis and degeneration, fatty change and bile duct hyperplasia. Masson's trichrome stain was used to evaluate collagenous fibers. A semiquantitative histological evaluation was carried out by a pathologist blinded to the treatment groups to assess the degree of the tissue inflammatory response, fatty change, necrosis of hepatocytes and bile duct hyperplasia. The grading ranged from 0 to 4 where 0 = absent, 1 = trace, 2 = mild, 3 = moderate, and 4 = severe.

Hepatic triglyceride contents

Liver lipids were extracted by the method of Folch et al.[15] Hepatic triglyceride (TG)

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and concentrations in the liver were determined with diagnostic kits (Randox Laboratories, Antrim, UK) with TG as a standard.

Inflammatory response

Hepatic cytokine concentrations

Hepatic tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-10 levels were measured as inflammatory response. Liver tissue (0.5 g) was homogenized in 1.5 ml ice-cold buffer [50 mM Tris (pH 7.2), 150 mM NaCl, and 1% Triton-X] plus 0.1% of a protease inhibitor. The homogenate was then shaken on ice for 90 min. After shaking, the homogenates were centrifuged at 3000 ×*g* and 4 ℃ for 15 min. The supernatant was analyzed with a DuoSet® rat TNF-α kit, a rat IL-1β/IL-1F2 kit, a rat IL-6 kit, and a rat IL-10 kit (R&D Systems, Minneapolis, MN, USA). Assays of samples were carried out according to the assay kit instructions. The optical density (OD) was read at 450 nm for all cytokines using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Plasma endotoxin level

Plasma endotoxin levels were measured using the Limulus amebocyte lysate assay kit (Pyrochrome® Cape Cod, East Falmouth, MA, USA) and the procedure followed the manufacturer's instructions. The OD was read at 405 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Intestinal permeability

An oral sugar test was used to assess intestinal permeability.[11,16] Briefly, rats were intragastrically administered 2mL of a sugar solution containing lactulose (100 mg/kg BW), mannitol (6 mg/kg BW), and sucrose (200 mg/kg BW). To promote urine output, each rat was subcutaneously injected with 10 ml of lactated Ringer's solution prior to sugar administration. Then, rats were housed individually in metabolic cages and urine samples were collected for 5 h. Urinary sugar levels were measured using liquid

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chromatography/tandem mass spectrometry (LC-MS/MS, AB SCIEX QTRAP® 5500, Framingham, MA, USA). An increased urinary lactulose/mannitol (L/M) ratio indicates that the intestinal permeability is elevated.

Microbiota composition of feces

Rats were anesthetized by ethyl ether inhalation and fecal samples were collected in an anaerobic dilution solution (4.5 g/L KH_2PO_4, 6 g/L Na_2HPO_4, 0.5 g/L L-cysteine: HCl, 2 g/L gelatin, and 1 mL/L Tween-20). Fecal samples were followed by 10-fold serial dilutions (10^{-1} to 10^{-6}) to acquire different concentrations and 50 μL of solution was inoculated onto agar (Oxoid, Basingstoke, Hampshire, England) by the spread plate method for plate counts. Certain microorganisms were isolated from fecal samples using different isolation media.[17] CDC anaerobe blood agar plates (A01-12, Creative Media Products, Taiwan) were used to detect the total aerobic bacterial flora. Endo agar plates (Difco™ & BBL™, Becton, Dickinson and Company, Sparks, MD, USA) were used to detect the coliform organisms (*Escherichia coli*). *Lactobacillus* anaerobic MRS with bromocresol green (modified MRS agar) (Difco™ & BBL™, Becton, Dickinson and Company, Sparks, MD, USA) was used to detect lactobacilli. Modified *Bifidobacterium* iodoacetate medium-25 (BIM-25) (Difco™ & BBL™, Becton, Dickinson and Company, Sparks, MD, USA) was used to detect bifidobacteria. The number of colony forming units (CFU) of bacteria was quantified. Endo plates were incubated for 24 h at 37 ˚C to count colonies of *E. coli*. CDC plates, modified MRS agar plates, and BIM-25 plates were incubated in anaerobic chambers for 48 h at 37 ˚C to respectively count colonies of total aerobic bacterial flora, lactobacilli, and bifidobacteria.

Statistical analysis

All data are presented as the mean ± standard deviation (SD). SPSS vers. 18 (PASW Statistics for Windows, vers. 18.0. SPSS. Chicago, IL, USA) was used to analyze the

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difference in different groups. A two-way analysis of variance (ANOVA) was used to compare multiple groups with Duncan's multiple-range test. When $p<0.05$ was regarded as statistically significant.

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Results

Food intake, growth performance and liver weight

The average liquid diet intake levels of the C, C+S, E and E+S groups were 62±9, 62±10, 63±10 and 63±11 g/rat/day, respectively. Average ethanol intake levels of rats in the E and E+S groups were 3.2±0.5 and 3.1±0.5 g/day. In addition, average synbiotics intake levels in C+S and E+S groups were 0.52±0.09 and 0.46±0.07 g/day.

The BWs and relative hepatic weights are given in Table 2. Initial BWs showed no significantly difference among groups. However, the final BWs of the E and E+S groups were significantly lower than that of the C group ($p < 0.05$). The relative liver weight of the E and E+S groups were significantly higher compared to that of the C groups ($p < 0.05$).

Biochemical indicators of liver function

Plasma AST and ALT activities of each group are presented in Figure 1 and 2. There were no significant differences in plasma AST or ALT activities of rats in any group at the baseline of the experiment. It was found that plasma AST and ALT activities in the E group were significantly increased compared to those of the C group after 4weeks of ethanol feeding and maintained this phenomenon until to the end of the experiment ($p < 0.05$). However, plasma AST and ALT activities in the E+S group were significantly lower than those in the E group only at the 4 weeks. There were trends toward lower plasma AST and ALT activities in the E+S group than those of the E group at the 8 and 12 weeks.

Hepatic TG content

The hepatic TG content of each group is given in Table 3. Compared to the C group, the C+S group showed a significantly decreased hepatic TG content ($p < 0.05$). However,

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chronic ethanol consumption (E group) caused significantly higher hepatic TG content. Conversely, the TG content in the ethanol-challenged rats with synbiotic supplementation (E+S group) was significantly lower than that in group E ($p < 0.05$).

Histopathological analysis

The effects of synbiotics on hepatic histopathological analysis scores in rats under long-term ethanol feeding are given in Table 4. Scores of hepatic fatty change, inflammation, necrosis and bile duct hyperplasia were significantly higher in the E group ($p < 0.05$), while the hepatic fatty change, inflammation and necrosis scores in the E+S group were significantly lower than those in the E group ($p < 0.05$). Although hepatic fibrosis was found in this ethanol-challenged rat model, there was no difference in each group. As shown in photomicrographs of the liver of each group, fatty change, inflammation and necrosis were observed in the E group (Figure 3). Furthermore, collagenous fibers were stained in several biopsy specimens of the E group (Figure 4).

Inflammatory response

Effects of synbiotics on inflammatory cytokines in the liver of rats under long-term ethanol feeding are show in Table 5. Chronic consumption of ethanol (E group) led to a significant ($p < 0.05$) increase in hepatic TNF-α, IL-1β, and IL-6 levels. In addition, hepatic IL-10 was significantly lower in the E group compared to the C group ($p < 0.05$). The hepatic TNF-α level in ethanol-treated rats with synbiotic supplementation (E+S grouop) was significantly lower, and the hepatic IL-10 level was significantly higher compared to those of the E group ($p < 0.05$).

Plasma endotoxin concentrations of each group are presented in Table 6. The serum endotoxin concentration was significantly higher in the E group than in the C group ($p <$

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0.05); however, the plasma endotoxin concentration of the E+S group was significantly lower compared to that of the E group ($p < 0.05$).

Intestinal permeability

Intestinal permeability was measured by the urinary L/M ratio as shown in Table 7. The E group showed the highest urinary L/M ratio among all groups ($p < 0.05$).

Microbial composition of feces

The fecal microbial culture may also reflect the status of the intestinal integrity. Results of the fecal microbial culture in rats of each group are given in Table 8. The C+S, E and E+S groups presented reduced numbers of anaerobe compared to the C group ($p < 0.05$). Numbers of *E. coli* were significantly higher; conversely, numbers of lactobacilli were significantly lower in the E groups than in the C group ($p < 0.05$). There was no significant difference in the number of bifidobacteria between the C and E groups. On the other hand, numbers of lactobacilli and bifidobacteria in the E+S group were significantly higher than those in the E group ($p < 0.05$).

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Discussion

The average ethanol intake of rats in both groups E and E+S was 3.1~3.2 g/day and this is the equivalent to 73.6 g/day in a human according to the conversion of animal doses to human equivalent which is based on the body surface area. A heavy alcohol intake is more than 50~60 g/day, 31~50 g/day is considered to be moderate, and 21~30 g/day is considered to be mild, whereas 1~20 g/day is considered to be minimal.[18] Therefore, the ethanol intake of rats in this study was similar to that of a heavy human drinker.

Although each group had the same caloric intake, significantly lower final BWs were observed in both the E and E+S groups (Table 2). This indicates that long-term ethanol feeding may affect nutrition intake and absorption in rats, and rats that experienced chronic ethanol feeding were less able to effectively utilize the calories.[19] The relative liver weight in E group was significantly higher compared to that in the C group, which is consistent with finding from our previous study.[20] The current study further indicated that combined treatment with synbiotics had no effect on hepatomegaly due to chronic ethanol feeding.

Plasma AST and ALT activities in the E group were significantly higher than those in the C group after 12 weeks of ethanol feeding (Figures 1 and 2). In addition, the hepatic TG level was significantly higher in the E group (Table 3). Similarly, fat accumulation was observed in group E from the light micrograph of the liver (Table 4 and Figure 3), which might have been due to enhanced mobilization of free fatty acids from adipose tissue and increased hepatic biosynthesis of lipids, as previously suggested.[21] Furthermore, an inflammatory response also occurred in the E group (Table 4 and Figure 3). The present results indicated that 12 weeks of ethanol consumption led to liver injury. On the other hand, the plasma AST and ALT activities showed significant lower only at the 4 weeks in E+S group than those in the E group. According to our previous studies, the plasma AST and ALT activities usually significantly increased after 4 weeks of ethanol feeding, and then slightly declined until to the end of the experiment (8 or 12 weeks).[20,22-23] We

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speculated that there was a compensatory hepatic response lessening ethanol-induced liver injury, which seemed to be a reason why synbiotics didn't show any significant beneficial effect on the plasma AST and ALT activities. In addition, Gressner et al also indicated that single measurements of biochemical markers in serum, plasma or even urine are presently not valid enough to replace liver biopsy.[24] In this study, we measured the hepatic TG content and observed the liver histopathology (Table 4 and Figure 3) and found that the ethanol-induced fat accumulation and inflammatory response in the liver were prevented by synbiotics supplementation. Therefore, we speculated that synbiotics supplementation may alleviate ethanol-induced liver damage by eliminating fat accumulation and decreasing inflammation in the liver.

Proinflammatory cytokines, such as TNF-α, IL-1, and IL-6, which are secreted by Kupffer cells and peripheral blood monocytes, play key roles in the inflammatory response under chronic ethanol exposure. This inflammatory response was triggered in several steps, including enhanced intestinal gram-negative bacterial LPS, increased gut permeability, endotoxemia, and Kupffer cell activation.[25,26] Proinflammatory cytokines lead to cell death by apoptosis and necrosis, which precipitate liver injury.[1,27] On the other hand, IL-10 is an anti-inflammatory cytokine, and it participates in the initiation and progression of ALD.[28] Like other anti-inflammatory cytokines, IL-10 is secreted by Kupffer cells and peripheral blood monocytes and protects the liver against injury. Anti-inflammatory hepatoprotective cytokines are secreted with or after the secretion of proinflammatory cytokines, which in turn maintains homeostasis.[26] In this study, hepatic TNF-α, IL-1, and IL-6 concentrations significantly increased, and the IL-10 concentration significantly decreased in rats under long-term ethanol feeding (E group) compared to group C (Table 5), which indicates that an imbalance exists between pro- and anti-inflammatory cytokines during chronic ethanol exposure. On the contrary, synbiotic supplementation (E+S group) produced lower hepatic

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proinflammatory and higher anti-inflammatory cytokine concentrations than those in the E group. In this study, we showed for the first time that supplementation with synbiotics may normalize hepatic cytokine secretions in rats under long-term ethanol feeding.

In order to investigate the relationship between intestinal health and ethanol-induced liver damage, the plasma endotoxin level and intestinal permeability were measured. Both the plasma endotoxin level and urinary L/M ratio were significantly higher in the E group (Tables 6 and 7). Possible mechanisms for the high intestinal permeability caused by ethanol feeding were reported in a previous review article.[29] First, ethanol exposure promotes the growth of gram-negative bacteria in the intestines which may result in accumulation of endotoxins. Second, ethanol metabolism by gram-negative bacteria and intestinal epithelial cells can result in an accumulation of acetaldehyde, which in turn can increase intestinal permeability to endotoxin by increasing tyrosine phosphorylation of tight junction and adherens junction proteins. Third, ethanol-induced generation of nitric oxide may also contribute to the increased permeability to endotoxin by reacting with tubulin, which may cause damage to the microtubule cytoskeleton and subsequently disrupt the intestinal barrier function. Lastly, ethanol may also increase the intestinal permeability to peptidoglycan which can initiate an inflammatory response in the liver and other organs. Thus, taken together with the results in this study, we surmised that the increased intestinal permeability induced by chronic ethanol intake may lead to increased transfer of endotoxin from the intestine to the liver and general circulation where where it can trigger inflammatory changes in the liver. However, ethanol exposure with synbiotic supplementation (E+S group) showed a similar plasma endotoxin level and urinary L/M ratio, compared to the C group (Tables 6 and 7). That is, synbiotics may normalize the plasma endotoxin level by inhibiting the high intestinal permeability caused by chronic ethanol ingestion.

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Normal gut microbes and the intestine permeability play important roles in intestinal health. And fecal microbes may reflect gut microbes. In this study, significantly higher numbers of *E. coli* and lower numbers of lactobacilli in the E group were found (Table 8). Our results are consistent with our previous study[23] and a clinical pilot study which showed that an alcoholic group had significantly reduced numbers of fecal lactobacilli with a trend towards increased *E. coli*.[9] In this study, we also further found that synbiotic supplementation produced higher numbers of lactobacilli and bifidobacteria. Therefore, we showed for the first time that the synbiotics supplementation increased numbers of lactobacilli and bifidobacteria in stools and may tend to restore the bowel flora in rats under chronic ethanol feeding.

There were some limitations in this study. First, the hepatic cytokines should be enriched with the evaluation of gene expression in order to comparison with the cytokine levels in the future study. Moreover, the analysis of the fecal microbiota should be more accurate not only in quantitative technology but also in flora species.

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Conclusion

In conclusion, the increased intestinal permeability and unbalanced fecal microbial composition may cause the high plasma endotoxin level in rats under chronic ethanol feeding, which then contributes to liver damage such as fatty change, necrosis and an inflammatory response. However, synbiotic supplementation during ethanol exposure inhibited the elevation of plasma endotoxin levels by means of normalizing the intestinal permeability and fecal microbial composition, which provided ameliorating effects on ethanol-induced liver injuries.

Acknowledgment

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Table 1 Components of the synbiotic powder[1,2]

Component	Amount/1.5 g	
Calories	4	kcal
Protein	1	g
Fat	0	g
Trans fat	0	g
Saturated fat	0	g
Carbohydrates	80	mg
Sodium	14	mg
Inulin	50	mg
Vitamin B_1	75	mg
Vitamin B_2	0.85	μg
Niacinamide	1	mg
Vitamin B_6	100	μg
Vitamin B_{12}	0.3	μg
Biotin	15	μg
Folate	20	μg
Pantothenic acid	0.5	mg
Proprietary blend culture count	2×10^9	CFU
Lactobacillus acidophilus and *L. bulgaricus*	5.4×10^8	CFU
Bifidobacterium bifidum and *B. longum*	1.3×10^9	CFU
Streptococcus thermophilus	1.2×10^8	CFU

[1] Synbiotic powder (FloraGuard) was provided by Viva Life Science (Costa Mesa, CA, USA)

[2] CFU, colony forming unit

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Table 2 Effects of synbiotics on the initial body weight (BW), final BW and relative hepatic weight in rats after 12 weeks of ethanol feeding[1, 2, 3, 4]

Group	Initial BW (g)	Final BW (g)	Relative hepatic weight (%)
C	252 ± 12	417 ± 12	2.0 ± 0.1[a]
C+S	248 ± 7	427 ± 14	2.0 ± 0.1[a]
E	245 ± 8	384 ± 12[ab]	2.5 ± 0.2[b]
E+S	252 ± 14	372 ± 12 [ab]	2.4 ± 0.2[b]
Ethanol effect	0.6749	< 0.0001	< 0.0001
Synbiotic effect	0.6268	0.7903	0.1377
Ethanol × Synbiotics	0.1704	0.0211	0.4847

[1] Data are expressed as the mean ± SD for n=8. [a] $p < 0.05$ versus C group, [b] $p < 0.05$ versus C+S group.

[2] C, control group; C+S, control diet with synbiotic supplementation (1.5 g/kg BW/day); E, ethanol group; E+S, ethanol diet with synbiotic supplementation (1.5 g/kg BW/day).

[3] Relative hepatic weight: (hepatic weight/final BW) × 100 %

[4] Significance difference analyzed by using two-way ANOVA and *post hoc* test with Duncan's multiple range test.

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Table 3 Effects of synbiotics on hepatic triglycerides (TGs) in rats after 12 weeks of ethanol feeding[1, 2, 3]

Group	Hepatic TGs (mg/g liver)
C	14.8 ± 7.2^{b}
C+S	7.0 ± 3.4^{a}
E	20.4 ± 5.2^{ab}
E+S	9.9 ± 3.1^{c}
Ethanol effect	0.0211
Synbiotic effect	< 0.0001
Ethanol × Synbiotics	0.4478

[1] Data are expressed as the mean ± SD for n=8. [a] $p < 0.05$ versus C group, [b] $p < 0.05$ versus C+S group, [c] $p < 0.05$ versus E group.

[2] C, control group; C+S, control diet with synbiotic supplementation; E, ethanol group; E+S, ethanol diet with synbiotic supplementation.

[3] Significance difference analyzed by using two-way ANOVA and *post hoc* test with Duncan's multiple range test.

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Table 4 Effects of synbiotics on the histopathology of liver tissue in rats after 12 weeks of ethanol feeding[1, 2, 3]

Group	Fatty changes		Inflammation	Degeneration and necrosis	Bile duct hyperplasia	Fibrosis
	Macrosteatosis	Microsteatosis				
C	0.6 ± 0.2	0.0 ± 0.0	1.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.4	0.3 ± 0.5
C+S	0.7 ± 0.3	0.0 ± 0.0	1.3 ± 0.7	0.0 ± 0.0	0.5 ± 0.5	0.4 ± 0.5
E	1.3 ± 0.7^{ab}	1.8 ± 0.8^{ab}	2.4 ± 0.5 ^{ab}	2.8 ± 0.5^{ab}	1.1 ± 0.6^{ab}	0.6 ± 0.7
E+S	0.6 ± 0.2^{c}	1.3 ± 0.5^{abc}	1.5 ± 0.5^{c}	1.8 ± 0.5^{abc}	0.9 ± 0.6^{a}	0.5 ± 0.5
Ethanol effect	0.0499	< 0.0001	0.0001	< 0.0001	0.0016	0.2288
Synbiotic effect	0.0499	0.0745	0.0961	0.0002	0.7525	1.0000
Ethanol × Synbiotics	0.0184	0.0745	0.0044	0.0002	0.1225	0.5434

[1] Data are expressed as the mean ± SD for n=8. [a] $p < 0.05$ versus C group, [b] $p < 0.05$ versus C+S group, [c] $p < 0.05$ versus E group.

[2] C, control group; C+S, control diet with synbiotic supplementation; E, ethanol group; E+S, ethanol diet with synbiotic supplementation.

[3] Significance difference analyzed by using two-way ANOVA and *post hoc* test with Duncan's multiple range test.

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Table 5 Effects of synbiotics on hepatic tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and IL-10 concentrations in rats after 12 weeks of ethanol feeding[1, 2, 3]

Group	TNF-α (pg/μg protein)	IL-1β (pg/μg protein)	IL-6 (pg/μg protein)	IL-10 (pg/μg protein)
C	22.0 ± 8.3	3.9 ± 1.7	97.7 ± 45.4	148.1 ± 31.7
C+S	17.1 ± 5.4	3.2 ± 1.5	132.8 ± 57.0	146.6 ± 29.4
E	47.6 ± 10.1^{ab}	13.2 ± 5.0^{ab}	166.6 ± 54.8^{a}	95.4 ± 48.7^{ab}
E+S	36.5 ± 5.3^{abc}	11.6 ± 4.0^{ab}	188.6 ± 68.3^{a}	136.6 ± 46.7^{ac}
Ethanol effect	< 0.0001	< 0.0001	0.0044	0.0354
Synbiotic effect	0.0060	0.3344	0.1666	0.1718
Ethanol × Synbiotics	0.2518	0.7049	0.7472	0.1434

[1] Data are expressed as the mean ± SD for n=8. [a] $p < 0.05$ versus C group, [b] $p < 0.05$ versus C+S group, [c] $p < 0.05$ versus E group.

[2] C, control group; C+S, control diet with synbiotic supplementation; E, ethanol group; E+S, ethanol diet with synbiotic supplementation.

[3] Significance difference analyzed by using two-way ANOVA and *post hoc* test with Duncan's multiple range test.

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Table 6 Effects of synbiotics on serum endotoxin concentrations in rats after 12 weeks of ethanol feeding[1, 2, 3, 4]

Group	Endotoxin (EU/mL)
C	4.0 ± 1.0
C+S	4.5 ± 1.0
E	12.6 ± 2.3[ab]
E+S	5.7 ± 0.6[c]
Ethanol effect	< 0.0001
Synbiotic effect	< 0.0001
Ethanol × Synbiotics	< 0.0001

[1] Data are expressed as the mean ± SD for n=8. [a] $p < 0.05$ versus C group, [b] $p < 0.05$ versus C+S group, [c] $p < 0.05$ versus E group.

[2] C, control group; C+S, control diet with synbiotic supplementation; E, ethanol group; E+S, ethanol diet with synbiotic supplementation.

[3] EU, endotoxin unit

[4] Significance difference analyzed by using two-way ANOVA and *post hoc* test with Duncan's multiple range test.

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Table 7 Effects of synbiotics on intestinal permeability in rats after 12 weeks of ethanol feeding[1, 2, 3]

Group	Relative intensity of lactulose (%)
C	5.7 ± 2.3
C+S	8.1 ± 3.6
E	23.4 ± 0.8[ab]
E+S	8.4 ± 9.7[c]
Ethanol effect	0.0055
Synbiotic effect	0.0374
Ethanol × Synbiotics	0.0067

[1] Data are expressed as the mean ± SD for n=8. [a] $p < 0.05$ versus C group, [b] $p < 0.05$ versus C+S group, [c] $p < 0.05$ versus E group.

[2] C, control group; C+S, control diet with synbiotic supplementation; E, ethanol group; E+S, ethanol diet with synbiotic supplementation.

[3] Significance difference analyzed by using two-way ANOVA and *post hoc* test with Duncan's multiple range test.

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Table 9 Effects of synbiotics on fecal microbiota composition in rats after 12 weeks of ethanol feeding[1, 2, 3]

Group	Anaerobe	*Escherichia coli*	Lactobacilli	Bifidobacteria
C	9.89 ± 0.54	6.59 ± 0.79	8.16 ± 0.38	8.05 ± 0.49
C+S	9.04 ± 0.16[a]	6.60 ± 0.47	8.45 ± 0.19	9.21 ± 0.19[a]
E	8.80 ± 0.15[a]	7.22 ± 0.27[ab]	7.42 ± 0.35[ab]	8.14 ± 0.32[b]
E+S	8.90 ± 0.13[a]	6.97 ± 0.47	8.01 ± 0.33[bc]	8.51 ± 0.11[abc]
Ethanol effect	< 0.0001	0.0129	< 0.0001	0.0111
Synbiotic effect	0.0013	0.5409	0.0006	< 0.0001
Ethanol × Synbiotics	0.0001	0.4871	0.2004	0.0012

[1] Data are expressed as the mean ± SD for n=8. [a] $p < 0.05$ versus C group, [b] $p < 0.05$ versus C+S group, [c] $p < 0.05$ versus E group.

[2] C, control group; C+S, control diet with synbiotic supplementation; E, ethanol group; E+S, ethanol diet with synbiotic supplementation.

[3] Significance difference analyzed by using two-way ANOVA and *post hoc* test with Duncan's multiple range test.

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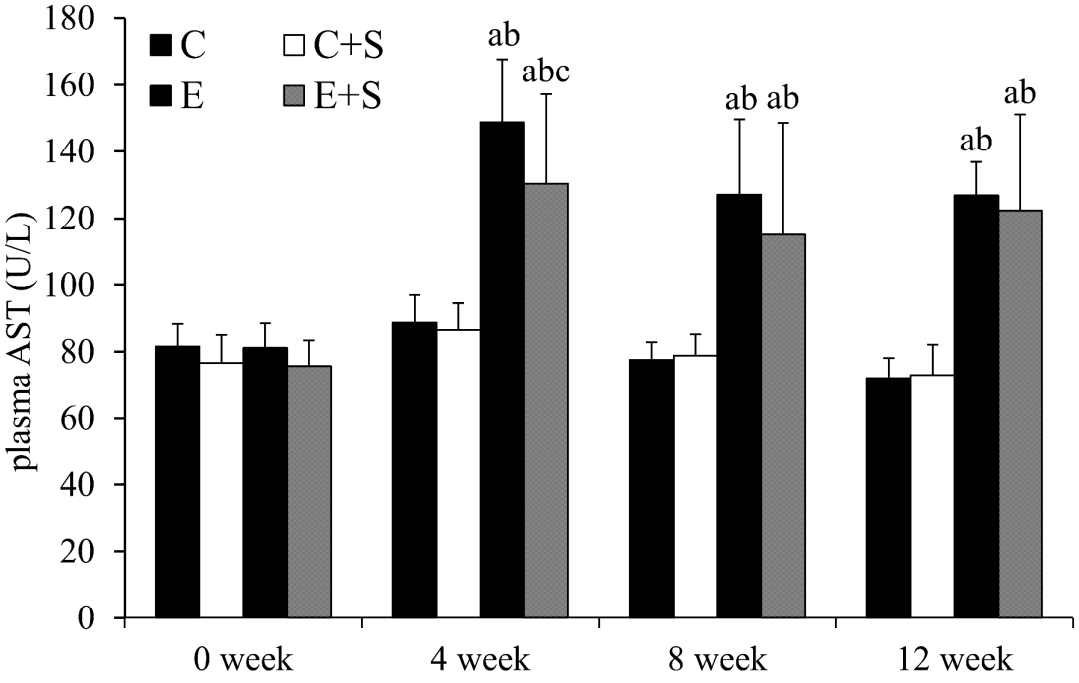

Fig. 1 Effects of synbiotics on plasma AST activity in rats after 12 weeks of ethanol feeding
Data are expressed as the mean ± SD for n=8. [a] $p < 0.05$ versus C group, [b] $p < 0.05$ versus C+S group, [c] $p < 0.05$ versus E group. C, control group; C+S, control diet with symbiotic supplementation; E, ethanol group; E+S, ethanol diet with synbiotic supplementation. Significance difference analyzed by using two-way ANOVA and *post hoc test* with Duncan's multiple range test.

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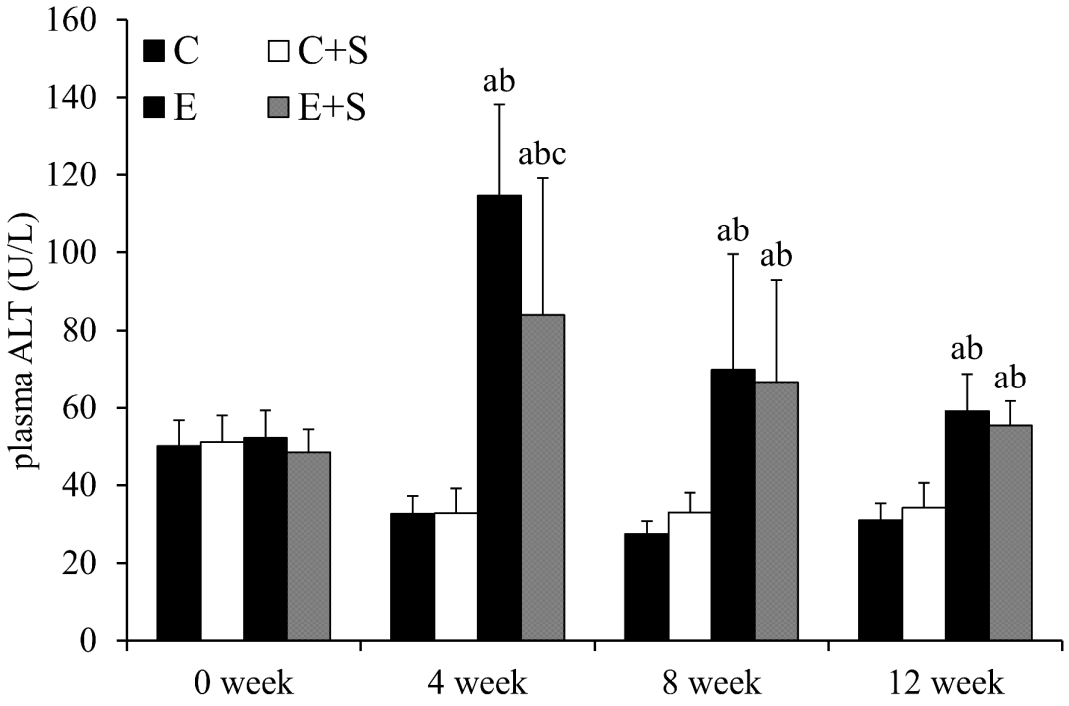

Fig. 2 Effects of synbiotics on plasma ALT activity in rats after 12 weeks of ethanol feeding
Data are expressed as the mean ± SD for n=8. [a] $p < 0.05$ versus C group, [b] $p < 0.05$ versus C+S group, [c] $p < 0.05$ versus E group. C, control group; C+S, control diet with symbiotic supplementation; E, ethanol group; E+S, ethanol diet with synbiotic supplementation. Significance difference analyzed by using two-way ANOVA and *post hoc test* with Duncan's multiple range test.

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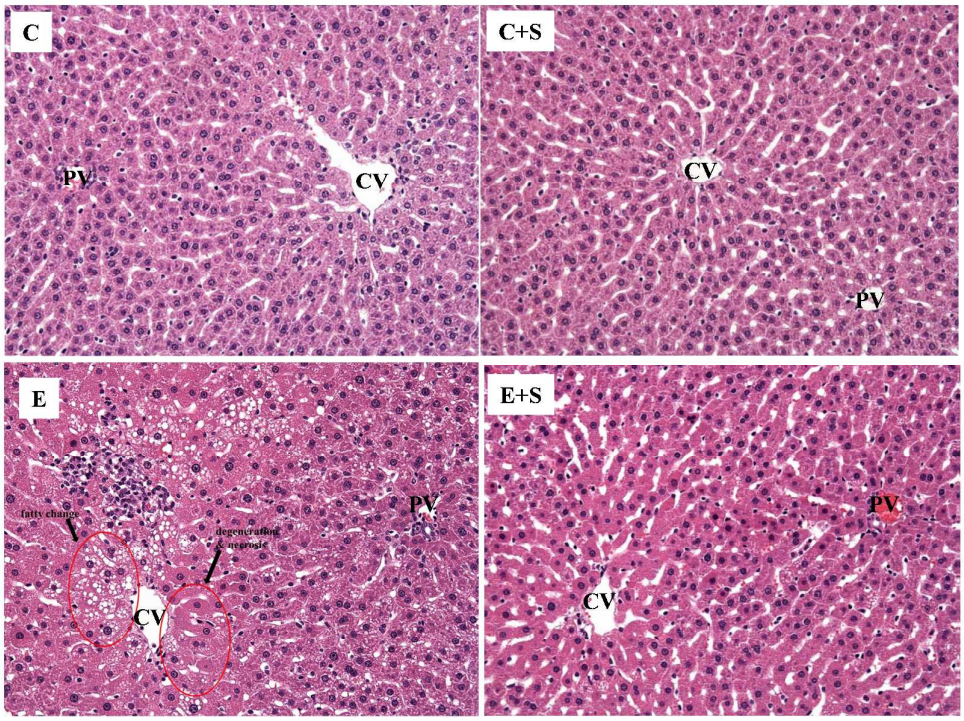

Fig. 3 Representative photomicrographs of liver tissue (H&E stain, magnification: ×200).

C, control group; C+S, control diet with synbiotic supplementation; E, ethanol group; E+S, ethanol diet with synbiotic supplementation. CV, central vein; PV, portal vein; Fatty change, degeneration and necrosis (arrows) occurred in E group while there were few histopathological change in the other groups.

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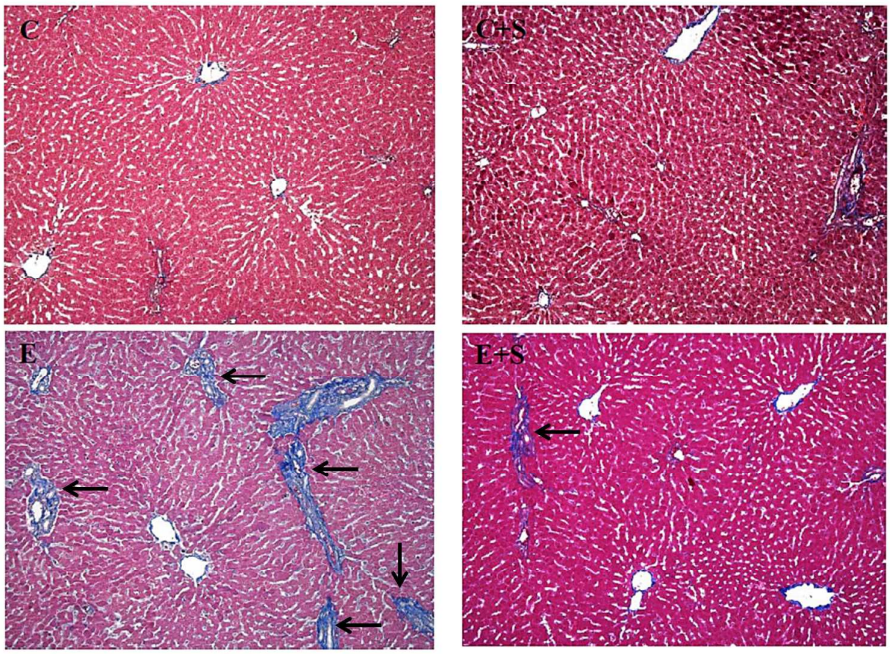

Fig. 4 Representative photomicrographs of liver tissue (Masson's trichrome stain, magnification: ×200).

C, control group; C+S, control diet with synbiotic supplementation; E, ethanol group; E+S, ethanol diet with synbiotic supplementation. Collagenous fibers were stained in several biopsy specimens of the E group; however, few collagenous fibers were found in the other groups.

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