Probiotic and glutamine treatments attenuate alcoholic liver disease in a rat model

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Abstract. The pathogenesis underlying alcoholic liver disease (ALD), which is often a result of alcohol abuse, currently remains unclear. Previous studies have reported that enteric dysbiosis serves an important role in the pathogenesis of ALD. The present study aimed to investigate the effects of glutamine and probiotics on a rat model of alcoholic liver disease (ALD). Sixty male Sprague-Dawley rats were randomly divided into 6 groups including control (C), alcohol (M), alcohol + Golden Bifido (T), alcohol + glutamine (G), alcohol + Medilac-S® (N) and alcohol + Golden Bifido + glutamine (L). Histology, body weight (BW), triglycerides (TG), serum aspartate transaminase (AST), alanine aminotransferase (ALT), tumor necrosis factor (TNF-α), interleukin-6 (IL-6), diamine oxidase (DAO), occludin, endotoxin and D-lactate levels were assessed whilst changes in the gut flora were evaluated and compared. Results determined that all probiotic and glutamine treatments elevated the abnormally decreased BW and occludin levels whilst the abnormal elevated serum AST, ALT, TG, IL-6, TNF-α, DAO, endotoxin and D-lactate levels were significantly reduced following chronic ethanol consumption. Histopathological observation of the liver demonstrated that probiotic and glutamine treatments attenuated liver damage induced by alcohol. Moreover, sequencing determined that there was a reduction in *Firmicutes* as well as an increase in *Actinobacteria*, *Proteobacteria* and *Porphyromonadaceae* abundance in the ALD group compared with the healthy controls. However, these changes were prevented by glutamine and probiotic therapy. In conclusion, the present results suggested that probiotics and glutamine ameliorated ALD by suppressing inflammation and regulating the gut microbiota. Therefore, probiotic and glutamine treatments can potentially serve as therapies for the prevention and treatment of ALD.

Introduction

Alcoholic liver disease (ALD) is a major cause of morbidity and mortality worldwide amongst people who abuse alcohol (1). The spectrum of ALD ranges from simple steatosis to alcoholic hepatitis, fibrosis, cirrhosis and ultimately hepato-carcinoma (2). To date, few satisfactory advances have been made in the management of ALD. Thus, novel and more reliable therapeutic approaches are urgently required.

Several factors have been demonstrated to contribute to the progression of ALD in humans, including race, gender, ethnicity and comorbidities such as hepatitis B and C virus infection (3). Amongst these, gut flora serves a pivotal role in the pathogenesis of ALD and is closely associated with the liver in ALD via the gut-liver axis (4). Alcohol induces quantitative and qualitative alterations in the gut microbiota and increases gut permeability. This results in the translocation of endotoxins such as lipopolysaccharide (LPS) into the liver through the portal vein (5) and stimulates the release of pro-inflammatory mediators such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β that ultimately contribute to liver damage in alcohol abuse patients (6).

Probiotics are live microorganisms that have demonstrated promise in ameliorating liver injury by the suppression of hepatic inflammation, regulation of intestinal microbiota and improvement of intestinal barrier integrity (7). Therefore, probiotics likely have therapeutic potential for ALD (8). Therapeutic strategies targeting the intestinal flora may be effective for ALD treatment (9). In addition, as the main source of energy for small intestinal cell metabolism, glutamine regulates intestinal barrier function by reducing its permeability, thereby preventing intestinal bacterial and endotoxin translocation (10). Glutamine also has research and application value in the treatment of diseases related to intestinal barrier damage (11). Most studies report the protective effect of glutamine on the intestinal barrier function based on the study of tight junctions; however, few studies have focused on the effect of the amino acid on intestinal microbiota (12,13).

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It is therefore important to analyze intestinal flora to assess the effects of glutamine and probiotics, and clarify the mechanisms associated with ALD. The present study investigated alterations in the gut microbiota in response to chronic alcohol feeding followed by glutamine, Golden Bifido (probiotic mixture containing live *Lactobacillus bulgaricus*, *Bifidobacterium* and *Streptococcus thermophilus*) and Medilac-S® (probiotic mixture containing live *Bacillus subtilis* and *Enterococcus faecium*) treatment in ALD rats. The present results may help further understanding of glutamine and probiotics in ALD as well as the complexity of the interplay amongst probiotics, the gut flora, inflammation and ALD.

Materials and methods

Animals and treatments. A total of 60 male Sprague-Dawley rats were obtained from the Laboratory Animal Center of Fuzhou Wushi Animal Center (Fuzhou, China). Rats weighing 140±10 g and aged 8 weeks were housed at three per cage in an specific pathogen free animal room. Rats were housed in controlled conditions at a temperature of 20±2°C, a relative humidity of 55±5% and 12-h of light/dark cycle with free access to food and water.

For the experiment, following one week of adaptive feeding with a normal chow diet, the 60 rats were randomly divided into six groups as follows (n=10; Fig. 1): i) C group: Normal chow diet and 1 ml/kg/day saline via intragastric administration for 20 weeks; ii) M group: Normal chow diet and intragastric ethanol (8 ml/kg/day; 56% ethanol) on the first day, followed by a gradual increase to 18 ml/kg/day until the end of the experiment with a 2 ml/kg/day interval; iii) T group: Same method as the M group with intragastric administration of 1 ml/kg/day Golden Bifido suspension (total concentration, 500 mg/1 ml saline; cat. no. S1998004; Inner Mongolia Shuang Qi Pharmaceutical Co., Ltd) for the last 8 weeks; iv) G group: Same method as the M group with the intragastric administration of 1 ml/kg/day glutamine suspension (total concentration of 1 g/1 ml saline; cat. no. 62010836; Sinopharm Chemical Reagent Co., Ltd.) for the last 8 weeks; v) N group: Same method as the M group with the intragastric administration of 1 ml/kg/day Medilac-S® suspension (total concentration of 140 mg/1 ml saline; cat. no. S20030087; Beijing Hanmei Pharmaceutical Co., Ltd.) for the last 8 weeks; and vi) the L group: Same method as the M group with the intragastric administration of a Golden Bifido suspension (500 mg/kg/day) and a glutamine suspension (140 mg/kg/day) for the last 8 weeks. Doses of the treatment agents were chosen on the basis of previous studies (14,15).

Subjects were weighed and then anesthetized using an intraperitoneal injection of 10% chloral hydrate (300 mg/kg) at the end of 20 weeks then rats were sacrificed by cervical dislocation. Blood, liver, intestinal tissues and feces were collected for subsequent biochemical analysis. All procedures were approved by the Institutional Animal Care and Use Committee of Fujian Medical University (registration number: 2015-CX-1).

Serum biochemical estimation. Blood samples were kept at room temperature for 1 h and then centrifuged at 1,500 x g for 15 min at 4°C. The serum was stored at -80°C. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and triglyceride (TG) levels were measured using an automatic biochemical analyzer (Konelab 20; Thermo Fisher Scientific, Inc.). Tumor necrosis factor (TNF)-α (cat. no. SEA133Ra), interleukin (IL)-6 (cat. no. SEA079Ra), diamine oxidase (DAO) (cat. no. SEA656Ra), endotoxin (cat. no. CEB526Ge), occludin (cat. no. SEC228Ra) and D-lactate (cat. no. CEV643Ge) levels were detected by the relevant ELISA kits according to the manufacturer's instructions (All from Wuhan USCN Business Co., Ltd.). Each experiment was performed at least three times.

Pathologic evaluation. Each liver was fixed in 10% formalin solution for 36 h at 37°C, embedded in paraffin, sectioned into 4 μm-thick slices and stained with hematoxylin for 10 min at 37°C. Stained sections were imaged using a light microscope (Leica DM200; Leica Microsystems Ltd.) using a 40X objective lens and a color camera.

Gut microbiota analysis. Fresh feces (3-5 g) was obtained by a sterile swab, placed 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; 1 ml/l Tween-20) and analyzed by Hangzhou Jinghang Biotechnology Technology Co., Ltd. (Hangzhou, China) for 16S rDNA sequencing.

Western blot analysis. A total of 0.1 g of intestinal tissue sample were ground, homogenized and then treated with radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.). Protein content was determined by the bicinchoninic acid method. Equal amounts of protein (30 μg) were separated via 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. PVDF membranes were blocked with 5% fat-free milk powder diluted in TBS-T (0.05% Tween 20) at 37°C for 2 h. The membranes were incubated with the primary antibodies against occludin (1:1,000; cat. no. ab167161) and β-actin (1:1,000; cat. no. ab8227; both Abcam, Cambridge, UK) at 4°C overnight. After rinsing three times with Tris-buffered saline and Polysorbate 20, the membranes were incubated with the corresponding horseradish peroxidase-labeled secondary antibody (1:1,000; cat. no. 31470; Thermo Fisher Scientific, Inc.) at 37°C for 2 h in the dark. Protein bands were detected using the Pierce™ ECL Western Blotting Substrate (cat. no. 32209; Thermo Fisher Scientific, Inc.) and Image lab software 3.0 (Bio-Rad Laboratories, Inc.). Relative protein expression levels were normalized to that of β-actin. Each experiment was performed at least three times.

Statistical analysis. Data are expressed as the mean ± standard deviation. SPSS 16.0 (SPSS, Inc.) statistical software was used for statistical analysis. One-way ANOVA was used to compare differences amongst multiple groups followed by Dunnett's post hoc test. $P<0.05$ was considered to indicate statistical significance.

Results

Glutamine and probiotic treatments elevate the body weight (BW) of rats with ALD. As shown in Fig. 2, the BW of ALD
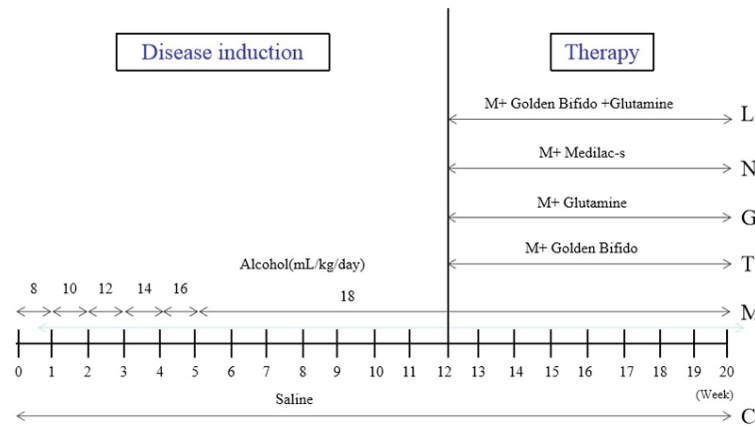
Figure 1. The study design and timeline. Schematic representation of the timeline for feeding rats with different concentrations of alcohol, Golden Bifido, glutamine, and Medilac-S®. Treatments were as follows: i) C group: Normal chow diet and 1 ml saline via intragastric administration for 20 weeks; ii) M group: Normal chow diet and intragastric ethanol (8 ml/kg/day; 56% ethanol) on the first day, followed by a gradual increase to 18 ml/kg/day until the end of the experiment with a 2 ml/kg/day interval; iii) T group: Same method as the M group with intragastric administration of 1 ml/kg/day Golden Bifido suspension (total concentration of 500 mg/1 ml saline) for the last 8 weeks; iv) G group: Same method as the M group with the intragastric administration of 1 ml/kg/day glutamine suspension (total concentration of 1 g/1 ml saline) for the last 8 weeks; v) N group: Same method as the M group with the intragastric administration of 1 ml/kg/day Medilac-S® suspension (total concentration of 140 mg/1 ml saline) for the last 8 weeks; and vi) the L group: Same method as the M group with the intragastric administration of a Golden Bifido suspension (500 mg/kg/day) and a glutamine suspension (140 mg/kg/day) for the last 8 weeks.
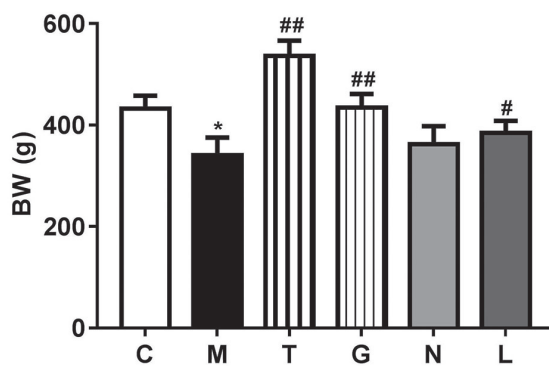

Figure 2. Final BW of ALD rats in each group after 20 weeks of treatment. *P<0.05 vs. C group; #P<0.05 and ##P<0.01 vs. M group. BW, body weight; ALD, alcoholic liver disease; C, control group; M, ALD group; T, Golden Bifid treatment group; G, Glutamine treatment group; N, Medilac-S® treatment group; L, Golden Bifido and glutamine treatment group.

Table I. AST, ALT and TG levels in each group.

Group	ALT (U/l)	TG (mmol/l)	AST (U/l)
C	19.92±4.98	0.74±0.03	59.26±6.23
M	43.59±0.61[a]	1.95±0.06[a]	65.38±2.24[a]
T	36.66±1.12[b]	1.62±0.36[b]	52.94±2.92[b]
G	34.56±4.05[b]	1.52±0.04[b]	47.62±4.56[b]
N	35.42±3.53[b]	1.47±0.04[b]	49.62±1.22[b]
L	23.38±4.91[b]	0.90±0.14[b]	37.61±3.37[b]

Data are reported as mean ± SD, n=10. [a]P<0.01 vs. C group; [b]P<0.01 vs. M group. ALT, alanine aminotransferase; TG, triglycerides; AST, aspartate transaminase. C, control group; M, ALD group; T, Golden Bifid treatment group; G, Glutamine treatment group; N, Medilac-S® treatment group; L, Golden Bifido and glutamine treatment group.

rats was obviously lower than the control (P<0.05). However, the BW of rats in the T, G and L groups was significantly higher compared with rats in the M group (P<0.01, P<0.01 and P<0.05, respectively). There was no difference in body weight between the N and M groups (P>0.05).

Glutamine and probiotic treatments attenuate hepatic histopathological injury in rats with ALD. H&E staining of rat livers demonstrated serious hepatic fatty changes, necrosis and inflammation in ALD rats. However, rat liver tissues from the T, G, N and L groups exhibited distinctly reduced alcohol-induced hepatic histopathological injury in ALD rats (Fig. 3).

Glutamine and probiotic treatments reduce serum ALT, TG and AST levels in rats with ALD. ALD rats had significantly higher ALT, TG and AST serum levels compared with the normal controls (P<0.01; Table I). In contrast, serum AST,

ALT, and TG levels in T, G, L and N groups were significantly lower compared with the ALD group (P<0.01; Table I).

Glutamine and probiotic treatments decrease serum DAO, endotoxin and D-lactic acid levels in rats with ALD. As shown in Table II, the ALD rats had significantly higher serum DAO, endotoxin and D-lactate levels compared with the controls (P<0.01). Following 20 weeks of various glutamine and probiotic treatments, the serum DAO, endotoxin and D-lactic acid levels in the T, G, N and L groups were significantly decreased compared with the M group (P<0.01; Table II).

Glutamine and probiotic treatments elevate occludin levels and reduce serum inflammatory factor levels in rats with ALD. After chronic alcohol feeding, the M group exhibited decreased occludin expression and elevated serum TNF-α and IL-6 levels compared with those in the C group (P<0.01; Fig. 4; Table III). However, rats in the T, G, L, and N groups
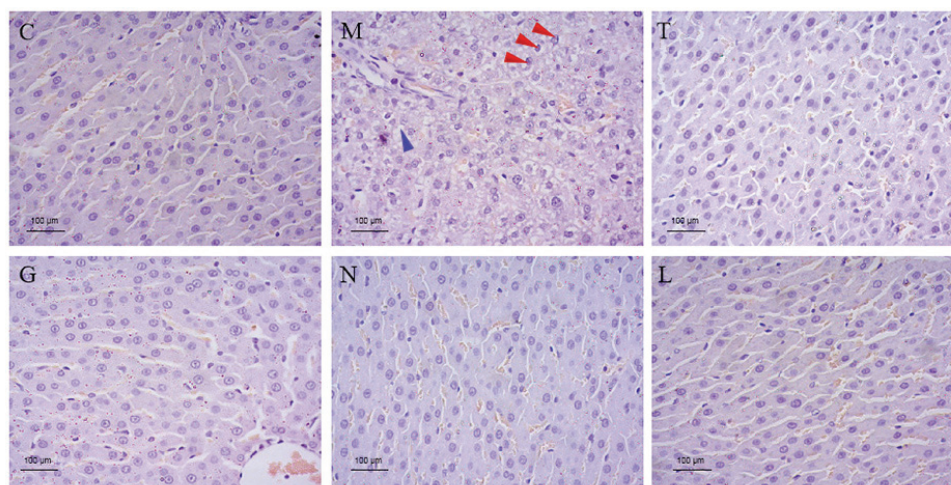
Figure 3. Hepatic histopathological analysis of ALD rats following various probiotic and glutamine treatments. Representative photomicrographs following hematoxylin and eosin staining to observe the morphology of livers from different groups. Red arrows represented necrosis and inflammation and blue arrow represented fatty change. Scale bar, 100 μm. ALD, alcoholic liver disease; C, control group; M, ALD group; T, Golden Bifid treatment group; G, Glutamine treatment group; N, Medilac-S® treatment group; L, Golden Bifido and glutamine treatment group.

Table II. Serum DAO, endotoxin and D-lactic acid levels in each group.

Group	DAO (mg/ml)	Endotoxin (EU/ml)	D-lactic acid (mg/l)
C	6.11±0.07	0.66±0.018	8.73±0.04
M	7.88±1.10[a]	0.86±0.05[a]	15.46±0.31[a]
T	6.51±0.59[b]	0.76±0.01[b]	13.47±0.13[b]
G	6.72±0.19[b]	0.74±0.02[b]	13.86±0.07[b]
N	6.54±0.40[b]	0.77±0.004[b]	14.20±0.17[b]
L	6.34±1.21[b]	0.70±0.01[b]	10.43±0.15[b]

Data are reported as mean ± SD, n=10. [a]P<0.01 vs. C group; [b]P<0.01 vs. M group. DAO, diamine oxidase. C, control group; M, ALD group; T, Golden Bifid treatment group; G, Glutamine treatment group; N, Medilac-S® treatment group; L, Golden Bifido and glutamine treatment group.
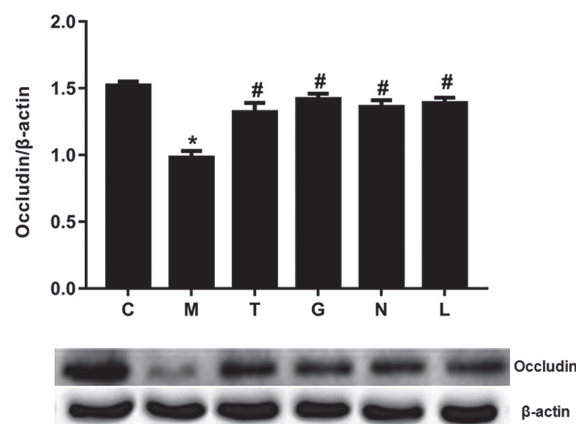

Figure 4. Western blot analysis of the occludin protein expression level in intestinal tissue of ALD rats following various probiotic and glutamine treatments. *P<0.05 vs. C group; #P<0.05 vs. M group. C, control group; M, ALD group; T, Golden Bifid treatment group; G, Glutamine treatment group; N, Medilac-S® treatment group; L, Golden Bifido and glutamine treatment group.

Table III. IL6, occludin and TNF-α levels in each group.

Group	IL-6 (ng/l)	Occludin (ng/l)	TNF-α (ng/l)
C	3.47±0.07	48.22±5.58	1.92±0.12
M	5.53±0.13[a]	35.24±9.55[a]	3.70±0.14[a]
T	4.82±0.17[b]	39.03±3.23[b]	3.07±0.20[b]
G	4.64±0.39[b]	40.51±1.32[b]	2.74±0.10[b]
N	4.91±0.37[b]	41.07±8.39[b]	2.69±0.14[b]
L	3.86±0.48[b]	41.76±4.55[b]	2.22±0.12[b]

Data are reported as mean ± SD, n=10. [a]P<0.01 vs. C group; [b]P<0.01 vs. M group. IL-6, interleukin-6; TNF-α, tumor necrosis factor. C, control group; M, ALD group; T, Golden Bifid treatment group; G, Glutamine treatment group; N, Medilac-S® treatment group; L, Golden Bifido and glutamine treatment group.

demonstrated significantly reduced TNF-α and IL-6 levels (P<0.01; Table III) and increased occludin protein expression (P<0.05; Fig. 4) compared with the M group.

Glutamine and probiotic treatments modulate gut microbiota in rats with ALD. At the phylum level, the abundance of *Firmicutes* was notably decreased in the ALD group compared with the healthy controls (P<0.05; Fig. 5), whilst the proportion of *Bacteroidetes* was not significantly different in ALD and control rats. The proportion of *Proteobacteria* (P<0.05; Fig. 5) and Actinobacteria (P<0.01; Fig. 5) in the ALD group was significantly higher compared with the control group. However, treatment with glutamine and probiotics increased the abundance of *Firmicutes* (P<0.05) and decreased the abundance of *Proteobacteria* (P<0.05) and *Actinobacteria* (P<0.05 P<0.01, P<0.05 and P<0.01, respectively) in T, G, L, and N groups compared with the M groups (Fig. 5).
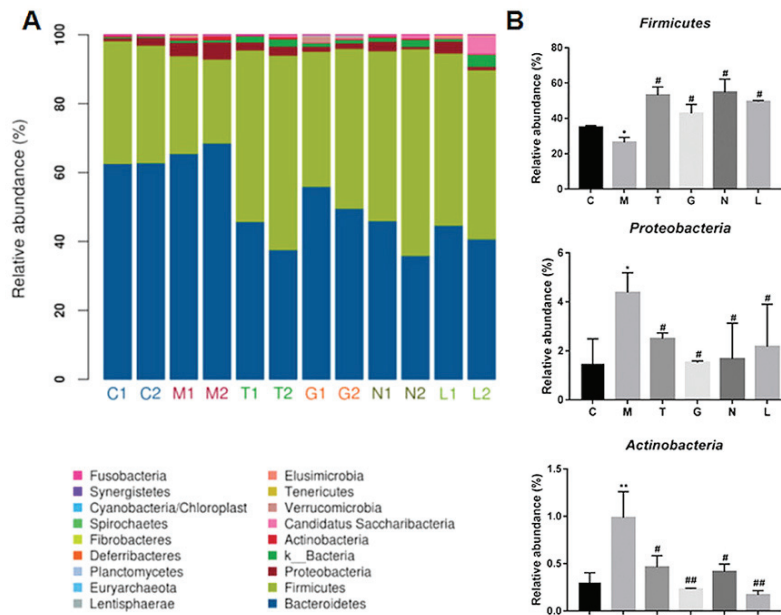
Figure 5. Bacterial community structures at the phylum level. (A) The abundance of each bacteria genus is presented as the percentage of the total effective bacterial sequences in the sample. (B) The relative abundance of *Firmicutes*, *Proteobacteria* and *Actinobacteria* in each group. $^*P<0.05$ and $^{**}P<0.05$ vs. C group; $^#P<0.05$ and $^{##}P<0.01$ vs. M group. C, control group; M, ALD group; T, Golden Bifid treatment group; G, Glutamine treatment group; N, Medilac-S® treatment group; L, Golden Bifido and glutamine treatment group.
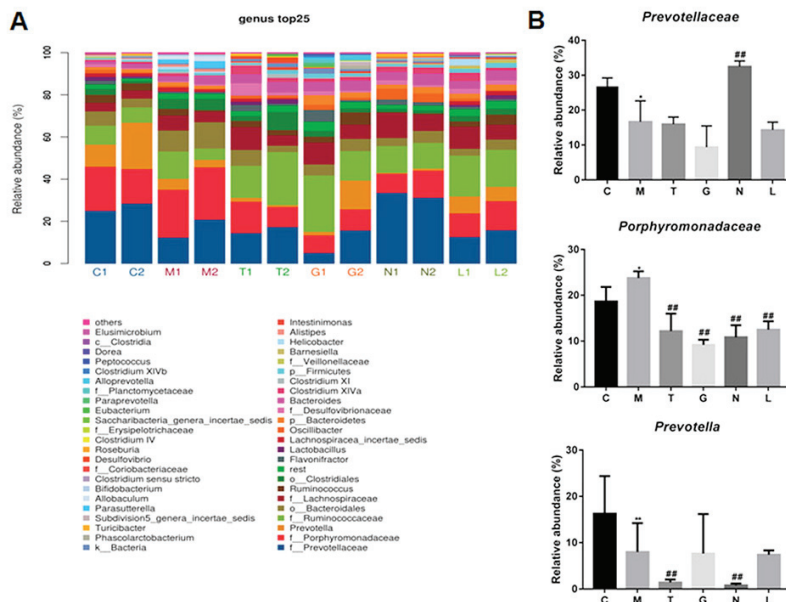

Figure 6. Bacterial community structures at the genus level. (A) The abundance of each bacteria genus is presented as the percentage of the total effective bacterial sequences in the sample. (B) The relative abundance of *Prevotellaceae*, *Porphyromonadaceae* and *Prevotella* in each group. $^*P<0.05$ and $^{**}P<0.05$ vs. C group; $^{##}P<0.01$ vs. M group. C, control group; M, ALD group; T, Golden Bifid treatment group; G, Glutamine treatment group; N, Medilac-S® treatment group; L, Golden Bifido and glutamine treatment group.

At the genus level, *Prevotellaceae* was the most prevalent genus in healthy controls and significantly decreased in the ALD group (P<0.05; Fig. 6). Moreover, the proportion of *Prevotellaceae* in the N group was significantly higher compared with the ALD group (P<0.01; Fig. 6). *Porphyromonadaceae* was the second most prevalent genus in the control group. Alcohol infusion induced a significant increase in *Porphyromonadaceae* in comparison with the control group (P<0.05; Fig. 6). However, *Porphyromonadaceae* abundance was reduced in T, G, L, and N groups when compared with the ALD group (P<0.01; Fig. 6). *Prevotella* was the third most prevalent genus in healthy controls, whereas their relative abundance was reduced by alcohol treatment (P<0.01; Fig. 6). Following glutamine and probiotic treatments, *Prevotella* abundance in the T and N groups were significantly decreased compared with the M group (P<0.01; Fig. 6).

Discussion

Chronic ethanol feeding destroys the integrity of the intestinal barrier and disturbs the gut microbiota (16,17). These alterations increase the translocation of endotoxins such as LPS from the intestinal lumen to the portal blood (18). LPS then binds to toll like receptor 4 on the surface of hepatic Kupffer cells to produce inflammatory cytokines which ultimately results in ALD (19). Therefore, the restoration of intestinal homeostasis could be a potential therapy for ALD. Many studies have reported that alcohol exposure decreased final BW in chronic ALD (20,21). The present study identified that BW was lower in the ALD group compared with the control group at 20 weeks. However, treatments with Golden Bifido and glutamine attenuated the reduction in BW induced by alcohol feeding.

Epithelial tight junctions are the primary component of the intestinal mucosal barrier (12). Occludin is an integral membrane protein localized at tight junctions (22) that has an important role in the maintenance of the basic structure and function of tight junctions. Chaudhry *et al* (23) reported that chronic alcohol consumption caused the redistribution of occludin from the intercellular junctions of the colonic epithelium and disrupted colonic epithelial tight junctions and intestinal epithelial barrier function; however, Glutamine supplementation protected tight junctions in the colonic epithelium of mice fed with alcohol and thus maintained intestinal epithelial barrier function. Moreover, serum ALT and AST levels can be used to indicate liver injury (24). D-Lactate, endotoxin and DAO levels reflect the severity of intestinal mucosa injury (25). Li *et al* (26) identified that an increase in these parameters occurred in rats that underwent chronic alcohol feeding. In the present study, abnormally decreased occludin levels and abnormally elevated plasma ALT, AST, TG, DAO, endotoxin and D-lactate levels were detected in the ALD group, indicating alcohol-induced liver injury and intestinal mucosa injury. Significant changes in these indexes in the sera of rats in all intervention groups revealed that probiotics and glutamine alleviated liver damage and intestinal mucosa injury caused by chronic alcohol use. Moreover, various inflammatory cytokines, such as TNF-α and IL-6, have been reported to be involved in the occurrence and development of ALD (27,28). Previous studies have shown that glutamine and probiotics reduce inflammation, promote the secretion of anti-inflammatory cytokines and maintain intestinal barrier functions (29,30). The present results demonstrated that probiotic and glutamine treatments reduced the abnormally elevated serum IL-6 and TNF-α levels following chronic ethanol consumption, demonstrating that probiotics and glutamine likely alleviated hepatic inflammation via the suppression of inflammatory cytokines. Moreover, liver injury induced by alcohol at the histopathological level was completely reversed after probiotic and glutamine treatments, indicating the protective effect of probiotics and glutamine on liver damage.

Alcohol-induced intestinal dysbiosis is involved in the pathogenesis of ALD (4,31). In the present study, at the phylum level, *Firmicutes* was the most dominant phyla in healthy controls, which was consistent with previous studies (32).

The present results demonstrated decreased *Firmicutes* and higher *Actinobacteria* and *Proteobacteria* abundance at the phylum level in the ALD group, which were in agreement with the results obtained by Bull-Otterson *et al* (33). At the genus level, increased *Porphyromonadaceae* and decreased *Prevotellaceae* and *Prevotella* abundance were observed in ALD mice compared with the controls. The increase in the abundance of *Porphyromonadaceae*, elevated plasma endotoxin levels and hepatic inflammation are strongly associated with complications in chronic liver disease (34).

Previous studies have demonstrated that probiotics ameliorate alcohol-induced gut dysbiosis and prevent alcoholic liver injury (7,35). In the present study, probiotics and glutamine notably elevated the abundance of *Firmicutes* and reduced *Actinobacteria*, *Proteobacteria* and *Porphyromonadaceae* abundance following continuous alcohol consumption, indicating that probiotic and glutamine treatments may attenuate gut dysbiosis.

In conclusion, the present study demonstrated that probiotic and glutamine treatments ameliorated ALD in rats via the suppression of inflammation and the regulation of intestinal microbiota. Results suggested that these interventions may potentially serve as inexpensive therapies for the prevention and treatment of ALD.

Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

ZL and YZ designed the study; HH, ZL and YZ performed the experiments; HH and XL collected the data; HH, XL and YZ analyzed the data; and YZ and XL prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures were approved by the Institutional Animal Care and Use Committee of Fujian Medical University (registration number: 2015-CX-1).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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