Lactobacillus rhamnosus GG supernatant promotes intestinal barrier function, balances T_{reg} and T_{H}17 cells and ameliorates hepatic injury in a mouse model of chronic-binge alcohol feeding

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HIGHLIGHTS

- LGG supernatant ameliorates experimental ALD in a chronic-binge alcohol exposure model.
- LGG supernatant normalizes the balance of T_{reg} and T_{H}17 in peripheral blood of mice exposed to chronic-binge alcohol.
- LGG supernatant decreases the serum level of IL-17 in chronic-binge alcohol mice model.

ABSTRACT

Impaired intestinal barrier function plays a critical role in alcohol-induced hepatic injury, and the subsequent excessive absorbed endotoxin and bacterial translocation activate the immune response that aggravates the liver injury. Lactobacillus rhamnosus GG supernatant (LGG-s) has been suggested to improve intestinal barrier function and alleviate the liver injury induced by chronic and binge alcohol consumption, but the underlying mechanisms are still not clear. In this study, chronic-binge alcohol fed model was used to determine the effects of LGG-s on the prevention of alcoholic liver disease in C57BL/6 mice and investigate underlying mechanisms. Mice were fed Lieber–DeCarli diet containing 5% alcohol for 10 days, and one dose of alcohol was gavaged on Day 11. In one group, LGG-s was supplemented along with alcohol. Control mice were fed isocaloric diet. Nine hours later the mice were sacrificed for analysis. Chronic-binge alcohol exposure induced an elevation in liver enzymes, steatosis and morphology changes, while LGG-s supplementation attenuated these changes. Treatment with LGG-s significantly improved intestinal barrier function reflected by increased mRNA expression of tight junction (TJ) proteins and villus-crypt histology in ileum, and decreased Escherichia coli (E. coli) protein level in liver. Importantly, flow cytometry analysis showed that alcohol reduced T_{reg} cell population while increased T_{H}17 cell population as well as IL-17 secretion, which was reversed by LGG-s administration. In conclusion, our findings indicate that LGG-s is effective in preventing chronic-binge alcohol exposure-induced liver injury and shed a light on the importance of the balance of T_{reg} and T_{H}17 cells in the role of LGG-s application.

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1. Introduction

Alcoholic liver disease (ALD) encompasses a variety of disorders ranging from simple steatosis to steatohepatitis, cirrhosis and hepatocellular carcinoma (Tsukamoto and Xi, 1989; Stewart et al., 2001). ALD is an important cause of morbidity and mortality but no targeted therapy is available. Besides the direct toxic effects on the...
liver, alcohol increases gut permeability and endotoxemia (Purohit et al., 2008; Elamin et al., 2014), and induces immune deregulation during alcohol-induced liver injury (Leftkowitch, 2005).

Lemmers et al. (2009) found that patients with ALD displayed elevated level of IL-17 in plasma compared with healthy counterparts, and higher proportion of IL-17 secreting cells among CD4+ lymphocytes (T_{h17}) was observed in patients with alcoholic cirrhosis. In the presence of cytokines such as TGF-β, IL-6 and IL-21, naïve CD4 T cells differentiate to T_{h17} subset, which is the main source of IL-17. IL-17 is recently reported to play a pivotal role in the stimulation and mobilization of neutrophils (Kolls and Linden, 2004), which exacerbates innate immune responses with the combined effect of IL-6 and TNF-α in mice (Hammrich et al., 2011). T_{reg} cells are characterized by its suppressive function on the proliferation and activation of effector T cells in vitro and in vivo (Guo et al., 2015; Sarris et al., 2008). The T_{reg} subset depleted mice displayed higher levels of proinflammatory cytokines and biochemical indicators related to hepatotoxicity in some models of acute liver injury (Kim et al., 2014; Wang et al., 2015). The balance of different subsets including T_{reg} and T_{h17} cells contributes to the immune homeostasis in gut mucosa. However, the details of derangement of T_{reg} and T_{h17} cells and its contribution to the course in the early stage of ALD remain to be elucidated.

Although remarkable achievement has been made in the field of ALD in recent years, there is still no effective therapy for ALD. Therefore, there is an urgent need to develop efficient strategies for patients with ALD. Recent studies have revealed that changes in intestinal microbiome play a critical role in ALD development in patients (Mutlu et al., 2012) and mouse model of ALD (Yan et al., 2011). *Lactobacillus rhamnosus* GG (LGG), a widely studied probiotic (Vandenplas et al., 2014), has been shown to restore gut microbiota and the expression of TJ proteins in mice with non-alcoholic fatty liver disease (NAFLD) (Ritze et al., 2014) and alcoholic steatohepatitis (Forsyth et al., 2009). However, it could be difficult for live probiotics to colonize intestine while there are lesions on intestinal mucosa. Moreover, LGG could also cause adverse outcomes like bacteremia (Land et al., 2005). Recently, LGG culture supernatant (LGG-s) generated from LGG culture has been used in ALD. Several studies have indicated that LGG-s is a safe and stable protector against acute and chronic alcohol induced liver injury (Wang et al., 2013, 2012). However, whether LGG-s is effective in chronic-binge alcohol exposure model is unknown. Chronic-binge alcohol exposure mimics the common drinking pattern in patients experiencing an excessive drinking with a history of chronic alcohol consumption (Bertola et al., 2013; Ki et al., 2010).

Recently, probiotic is found to generate T_{h17} cells response, direct the differentiation of T_{reg} cells and even facilitate cytokine expressions (LeCuyer et al., 2014), but mechanisms of LGG protection of ileum permeability and regulation of T_{h17} cells differentiation and cytokine expression in alcohol associated liver diseases have not been understood. Accordingly, we employed the chronic-binge model to investigate the effects of LGG-s on gut permeability and regulation of T cells differentiation in chronic-binge alcohol exposure-induced hepatic injury.

### 2. Material and methods

#### 2.1. Culture of LGG and preparation of LGG-s

LGG was purchased from American Type Culture Collection (ATCC 53103, Rockville, MD) and cultured in MRS broth according to ATCC guidelines. The LGG supernatant (LGG-s) was harvested after filtered through 0.22 μm filters when the bacterial density reached 10^6 colony-forming units/ml (CFU/ml). The supernatant was stored at 0–4 °C for use in a week.

#### 2.2. Animal experiments and sample collection

As shown in the pattern below (Fig. 1), male C57BL/6 mice (10 weeks of age) were supplied with a Lieber–DeCarli liquid diet containing alcohol (5% w/v, AF, n = 6) or isocaloric maltodextrin (PF, n = 6) for 10 days, as previously described (Bertola et al., 2013). An additional group of mice (AF + LGG-s, n = 6) were fed the same diet as AF group supplemented with LGG-s at a dose of equivalent to 10^9 CFU/ml per mouse per day. On Day 11, a bolus of ethanol (5 g/kg body weight) was gavaged. Nine hours later, blood, liver and ileum tissues were collected (Fig. 1). All mice were treated according to the protocols reviewed and approved by the institution ethics committee of Wenzhou Medical University.

#### 2.3. Biochemical analysis

Serum was collected and stored at −80 °C until use. Serum levels of ALT and AST were determined by the clinical laboratory of the First Affiliated Hospital of Wenzhou Medical University using automatic biochemical analyser (AU5800, Beckman Coulter, USA).

#### 2.4. Liver and ileum histology hematoxylin/eosin staining and liver Oil Red O staining

Tissues were harvested after mice were sacrificed. The paraffin-embedded tissue sections after fixation with 4% formalin were processed for staining with hematoxylin/eosin (HE). The frozen
tissue sections for Oil Red O staining were analysed by light microscopy.

2.5. Real-time reverse transcriptase polymerase chain reaction (RT-PCR) assay

Total RNA was isolated from murine ileums using RNA extraction kit (Aidlab Biotechnologies Co, Beijing, China) and reverse-transcribed with RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Rockford, USA) according to the instruction manual. The relevant cDNA was amplified using SYBR Green qPCR kit (TOYOBO, Osaka, Japan). Tight junction protein Zonula Occludens-1 (ZO-1), occludin and claudin-1 were detected to evaluate the intestinal permeability, and the sequences of forward and reverse primers used were listed as follows: ZO-1: 5'-GAGCAGGCTTTGGAGGAGAC-3' (sense) and 5'-TGGGACAAAAGTCCGGGAAG-3' (antisense), 162 bp; occludin: 5'-GTACCCACAGTGACAAACA-3' (sense) and 5'-GGTCCTGAGCTCCAGCCTGT-3' (antisense), 122 bp; claudin-1: 5'-GGCTCTCCTGGGATGGATCG-3' (sense) and 5'-TCTGGCAACCCGAGGACATC-3' (antisense), 134 bp; β-actin: 5'-GTCCCTCACCCTCCCAAAAG-3' (sense) and 5'-GCTGTCCTCACCCTCCAACCA-3' (antisense), 266 bp. RT-PCR was carried out using 7500 Real-Time PCR System (Applied Biosystems, USA). The mRNA expressions were normalized to β-actin and evaluated relatively by the cycle threshold (Ct) using 7500 system SDS software. All samples were run in duplicate.

2.6. Western blotting analysis

Liver tissues were lysed in ice-cold RIPA buffer and the total liver protein was obtained from all mice. The protein samples

![Fig. 2. Effect of LGG-s on serum transaminase and hepatic histological change. (A) Serum ALT and AST levels. Data were mean ± SEM, n = 6, *P < 0.05, **P < 0.01 and #P < 0.001. (B) The representative photomicrographs of HE stained liver sections (original magnification: ×200, scale bar = 100 μm). (C) The representative photomicrographs of Oil Red O stained liver sections (original magnification: ×400, scale bar = 100 μm).]
were added to lanes and electrically transferred to PVDF membranes (Bio-Rad, CA, USA). The membranes were blocked by TBS/T (Tris Buffered Saline + 0.1% Tween 20), subsequently incubated with primary antibodies against GAPDH (Santa Cruz, CA, USA) and E. coli proteins (Abcam, MA, USA) overnight at 4 °C and with secondary antibodies for 90 min at room temperature, respectively. All bands were detected and analysed with Image Lab 4.1 software (Bio-Rad).

2.7. Flow cytometry analysis

The peripheral blood mononuclear cells (PBMCs) were isolated from fresh whole blood samples with lymphocyte separation medium (TBDscience, Tianjin, China) and stained with anti-mouse CD4 FITC (eBioscience, CA, USA) and CD25 PE (eBioscience), and incubated with fixation/permeabilization reagent for 40 min and then stained with anti-mouse Foxp3

Fig. 3. Effect of LGG-s on gene expression of intestinal TJ proteins. Fluorescence quantitative reverse transcription PCR was used to determine the mRNA levels of TJ proteins. Data are expressed as fold changes relative to the control group. Results are means ± SEM; n = 6. *P < 0.05, **P < 0.01, #P < 0.001.

Fig. 4. Effect of LGG-s on villus-crypt junction in ileum. (A) Typical micrographs for HE stained ileum sections. Original magnification, >200. (B) Length measurements for villi and crypts from 3 sections per group were conducted. Ratios of villus length to crypt depth were illustrated as means ± SEM, P < 0.05.
PE-Cyanine5.5 (eBioscience). For analysis of T_h17 cells, lymphocytes were first labeled with CD4 FITC, and then incubated with the combination of PMA/Ionomycin mixture (eBioscience) and BFA/Monensin mixture (eBioscience) for 10 h followed by intracellular staining with anti-mouse/rat IL-17 PE (eBioscience). Rat IgG2a K isotype control PE-cyanine5.5 and Rat IgG2a K isotype control PE were used to avoid nonspecific binding. T17 cells were defined as CD4^+IL-17^+ T cells, and T_reg cells were defined as CD4^+CD25^+Foxp3^+ T cells. Cells labeled with fluorescent conjugated antibody were performed with BD FACSCalibur platform (BD Bioscience, CA, USA) following manufacturer’s instruction, and ratios of T17 or T_reg cells to total CD4^+ T cells were analysed by FlowJo software.

2.8. ELISA assay

The concentrations of IL-17 were measured in serum using ELISA kit (eBioscience) according to the manufacturer’s instruction. All the samples were analysed in duplicate, and the concentrations of IL-17 were calculated according to the standard curve based on optical density.

2.9. Statistical analysis

One-way analysis of variance (ANOVA) and LSD test were used for comparison between multiple groups. Data were expressed as median with standard deviation. P < 0.05 was considered statistically significant. SPSS 19.0 and GraphPad Prism 5 were used for statistical analysis.

3. Results

3.1. Effect of LGG-s treatment on the alcohol induced liver injury and steatosis

Pilot study using LGG-s supplemented in the pair-fed group showed that LGG-s did not produce significant change in the liver of mice evaluated by HE staining. Therefore, we did not include this group in the following studies.

Serum ALT and AST levels were elevated by alcohol exposure, which were reduced by LGG-s supplementation (Fig. 2A). Alcohol ingestion increased hepatic steatosis determined by histological staining and Oil Red O staining of liver tissues. Importantly, the alcohol-induced hepatic fat accumulation was decreased by LGG-s administration (Fig. 2B and C). In PF group, the photomicrographs of Oil Red O stained liver samples showed limited microsteatosis (Fig. 2C), while liver sections from the AF group showed obvious macrosteatosis, widespread deposition of microsteatosis, swelling hepatocyte and cytoplasm rarefaction (Fig. 2B and C). In AF + LGG-s group, microsteatosis and hepatocyte swelling were still observed but obviously attenuated on the number and size compared with those in AF mice (Fig. 2B and C).

3.2. Effect of LGG-s treatment on intestinal permeability

Intestinal barrier function is dependent on TJ integrity. To evaluate the effect of LGG-s on the intestinal permeability, we detected the gene expression of three key TJ proteins in the intestinal tissues of mice exposed to alcohol and LGG-s treatment. Chronic-binge alcohol consumption caused a significant reduction of mRNA levels of claudin-1, occludin and ZO-1 (Fig. 3). However, LGG-s treatment attenuated the negative effects of alcohol on claudin-1 and ZO-1 mRNA expression. Occludin mRNA expression was not significantly affected by LGG-s (Fig. 3).

To determine intestinal integrity by alcohol and LGG-s, 10 well-oriented villus-crypt junctions from 3 sections for each group were analyzed. HE staining showed that the ratio of height of villus to depth of crypt was also reduced in AF group (Fig. 4). Treatment of LGG-s significantly ameliorated the alcohol-induced disruption on villus-crypt junctions (Fig. 4).

3.3. Influence of LGG-s treatment on E. coli protein in liver tissues

Alcohol exposure-induced intestinal barrier dysfunction enables gut bacteria and its products to translocate into liver through portal circulation. To determine the effects of LGG-s on the bacterial translocation, we measured E. coli protein in the livers by immunoblotting. Alcohol exposure led to an increase of E. coli protein level, which was attenuated by LGG-s supplementation (Fig. 5), indicating that LGG-s supplementation improved gut barrier function.

3.4. Effect of LGG-s treatment on the balance of T_reg and T_h17 cells

The balance of T_reg and T_h17 cells is a marker of alcohol-induced liver damage. To evaluate the change of T_reg and T_h17 cells in response to bacterial translocation from intestine, we used flow cytometry to determine the subpopulation of T_reg and T_h17 cells of CD4^+ T cells. The total number of events each time recorded by flow cytometry analysis was 20,000. Alcohol exposure significantly reduced the proportion of T_reg cells in peripheral blood, which was markedly elevated by LGG-s treatment (Fig. 6). However, the frequency of T_h17 in the alcohol treated group was higher compared to the control group, but it was reduced by the treatment of LGG-s.

Fig. 5. Western blotting of hepatic tissue for E. coli. The E. coli proteins were detected by western blotting and normalized to GAPDH. Data were expressed as fold changes relative to PF group, and results are means ± SEM, n = 6, #P < 0.001.
3.5. Effect of LGG-s treatment on IL-17 secreting

To further investigate the effect of LGG-s on TH17 function, we compared serum levels of IL-17 in AF and PF groups. Serum IL-17 level was significantly higher in mice in AF group than that in PF group. However, LGG-s treatment reduced the serum IL-17 level. (Fig. 7).

4. Discussion

Previous studies have demonstrated that alcohol-induced liver steatosis and injury are reduced by LGG-s treatment in acute and chronic alcohol exposure mice models (Wang et al., 2013, 2012). In this study, we further showed that LGG-s was also effective in attenuation of chronic-binge alcohol exposure model in mice.

Fig. 6. Effect of LGG-s on balance of Treg and TH17 cells. (A) Representative flow cytometric plots of CD4+CD25+Foxp3+ Treg and CD4+IL-17+ TH17 cells labeled with the corresponding percentage of CD4+ T cells. (B, C) The ratios of Treg or TH17 to CD4+ T cells were expressed in the form of scatter diagram. Events > 20,000. Results are means ± SEM, n = 6, *P < 0.05 and #P < 0.001.
LGG-s supplementation decreased alcohol exposure-induced intestinal barrier dysfunction, bacterial translocation and subsequent liver steatosis and injury. Importantly, our study showed that the ratio of Treg and Th17 cells was corrected by LGG-s treatment in response to alcohol exposure, implying that the effects of LGG-s treatment in ALD are likely mediated, at least in part, by Treg and Th17 cell-regulated immune response.

Progression of alcoholic liver injury in heavy drinkers varies substantially depending vitally on various factors such as gender, obesity, diet, smoking and drinking patterns (Stickel and Setz, 2010; Gao and Bataller, 2011; O’Shea et al., 2010). A binge drinking could be more harmful than a long period of habitual alcohol consumption at low levels in human (Li, 2008). As previously reported, chronic-binge alcohol exposure resulted in marked elevations of ALT and AST with peak levels of about 150 IU/L ALT and 390 IU/L AST at the ninth hour after intragastric administration. Severe steatosis and necrosis were also observed in this model (Fig. 2).

Our current studies on this chronic-binge alcohol exposure model confirmed that the intestinal barrier function can be ameliorated by LGG-s oral administration. It has been reported that alcohol consumption lead to a decreased expression of TJ associated proteins claudin-1 and ZO-1 and an increased intestinal permeability (Wang et al., 2011). Tight junction proteins are the essential determinants of paracellular permeability in intestine under normal conditions. The destruction of TJ proteins leads to the penetration of intestinal bacteria and its metabolites into circulation. Our results demonstrated that the gene expressions of claudin-1 and ZO-1 were reduced by alcohol, but restored by LGG-s treatment, which was in line with previous studies (Forsyth et al., 2009; Wang et al., 2011; Yuhua Wang et al., 2012). The disruption of villus-crypt structure was also inhibited by LGG-s (Fig. 4). As a result, gut bacterial translocation was inhibited by LGG-s treatment, confirming that LGG-s has a protective effect on intestinal barrier function.

Leaked bacteria and the products (Purohit et al., 2008) stimulate Th17 proliferation indirectly via activation of macrophages, dendritic cells and Kupffer cells (Ma et al., 2007; Kim et al., 2007) or probably directly by TLR4 in liver (Park et al., 2015), which initiates liver inflammation (Takeuchi and Akira, 2010). Th17 was a newly found T cell subset with proinflammatory function. We are the first to notice that proportion of Th17 cells was elevated by alcohol and decreased by LGG-s in peripheral blood of mice exposed to chronic-binge alcohol (Fig. 6). Analogously, it was found that livers of patients with alcoholic hepatitis were infiltrated by Th17 cells correlating to the hepatic damage (Lemmers et al., 2009), which is consistent with our findings. Inhibition of translocated bacterial represses Th17 differentiation and the population growth subsequently. Associated with the alcohol treatment, IL-17 protein in serum increased significantly, but markedly reduced by LGG-s treatment (Fig. 7). Previous studies demonstrated that IL-17 secreted by Th17 cells exacerbates steatosis along with an increased IL-6 production in HepG2 cells in presence of free fatty acid (Tang et al., 2011). IL-6 produced by hepatocytes was suggested to increase IL-21 expression in naïve CD4+ T cells and aggravate the differentiation of Th17 with the combination of TGF-β in turn (Zhou et al., 2007). A positive feedback loop is formed during the liver inflammation. In our study, hepatic steatosis was induced in AF group and significantly improved by LGG-s treatment along with an elevated IL-17 serum level (Fig. 2C).

To date, few studies investigated reduced frequency of Treg cells in peripheral blood in ALD. Almeida (Almeida et al., 2013) and the colleagues have demonstrated that patients with alcoholic hepatitis showed a decreased frequency of Treg cells in peripheral blood with increased levels of inflammatory cytokines (IL-1β, IL-6, IL-12, and TNF-α) in comparison with the healthy counterparts. Depletion of Treg cells aggravates immune response to LPS resulting in an increased production of pro-inflammation cytokines (Okeke et al., 2013, 2014). Our results indicate that LGG-s treatment improved the proportion of Treg cells in peripheral blood compared with the AF group, which is likely a mechanism of the beneficial effects of LGG-s in ALD.

In summary, our study demonstrate that LGG-s is effective in the prevention of chronic-binge alcohol exposure-induced hepatic steatosis and injury through mechanisms involving improvement of intestinal barrier function and normalization of the balance of Treg and Th17 cells.

Conflict of interest

The authors declare that there is no actual or potential conflict of interests.

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References


![Graph showing serum IL-17 levels](image-url)

**Fig. 7.** Effect of LGG-s on serum IL-17 levels. The serum levels of IL-17 were detected by ELISA. The data were expressed as means ± SEM, *n* = 6, *p* < 0.05.


