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Original Article

Effects of fecal microbiota transplantation and joint application of probiotics on rats with alcoholic liver disease

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Abstract: The present study investigated the effects of fecal microbiota transplantation (FMT) and a combination of golden bifid and Medilac-S on rats with alcoholic liver disease (ALD). Sixty Sprague-Dawley male rats were randomly divided into 6 groups (n = 10): control (C), alcohol (M, ALD model), alcohol + FMT(F), alcohol + golden bifido (T), alcohol + Medilac-S (B), and alcohol + golden bifido + Medilac-S (L). Blood was collected for the detection of triglycerides (TG), serum transaminase (AST), alanine aminotransferase (ALT), tumor necrosis factor α (TNF-α), interleukin-6 (IL-6), interleukin-4 (IL-4), diamine oxidase (DAO), endotoxin, and D-lactate. Liver tissues were obtained for histologic observation. Fecal samples were collected to detect changes in the gut flora by 16S rDNA sequencing analysis. Results showed that FMT and probiotics reduced abnormally elevated serum levels of ALT, IL-4, IL-6, TNF-α, DAO, endotoxin, and D-lactate in chronic ethanol consumption ($P < 0.05$). Histopathologic observation of the livers of rats treated with the combination of probiotics confirmed the biochemical data. Sequencing results showed a reduction in Bacteroidetes and Lactobacillus, as well as an increase in Actinobacteria, Proteobacteria, and Prevotellaceae in the ALD group, compared with healthy controls. However, these shifts were prevented after therapy. Therefore, present results suggest that FMT and a combination of probiotic compounds can protect rats from ALD by suppressing inflammation and restoring intestinal homeostasis.

Keywords: Fecal microbiota transplantation, probiotics, alcoholic liver

Introduction

Alcoholic liver disease (ALD), resulting from excessive alcohol consumption, is a major cause of morbidity and mortality worldwide. Progression of ALD involves a series of liver diseases, ranging from steatosis and inflammation to fibrosis and cirrhosis, ultimately resulting in hepatocellular carcinoma in some circumstances [1]. Numerous factors, such as endotoxins, cytokines, mitochondrial damage, and oxidative stress, have been closely associated with ALD [2-5]. To date, effective therapies for ALD patients remain unavailable [6-8]. Therefore, a safe and reliable therapeutic approach is urgently needed.

Although the underlying pathogenesis of ALD remains unclear, numerous studies have suggested that intestinal barrier function and intestinal endotoxemia are involved in occurrence, development, and prognosis of ALD [9]. Alcohol can increase intestinal mucosa permeability and cause enterogenous endotoxin leakage, which ultimately leads to the formation of ALD and aggravation of liver injuries [10, 11]. Fecal microbiota transplantation (FMT) is the introduction of feces from a healthy donor to a patient with a disease related to disturbances in intestinal flora [12]. In its longstanding history, FMT was refocused clinically since it can restore gut microbiota [13]. It is currently being considered as an effective alternative for treatment of gut microbiota-related diseases, such as asthma [14] inflammatory bowel disease [12] and *Clostridium difficile* infection [15]. However, only a few studies have reported the application of FMT in ALD treatment. Thus, it was hypothesized that FMT is a promising therapeutic technique for treatment of ALD. As live

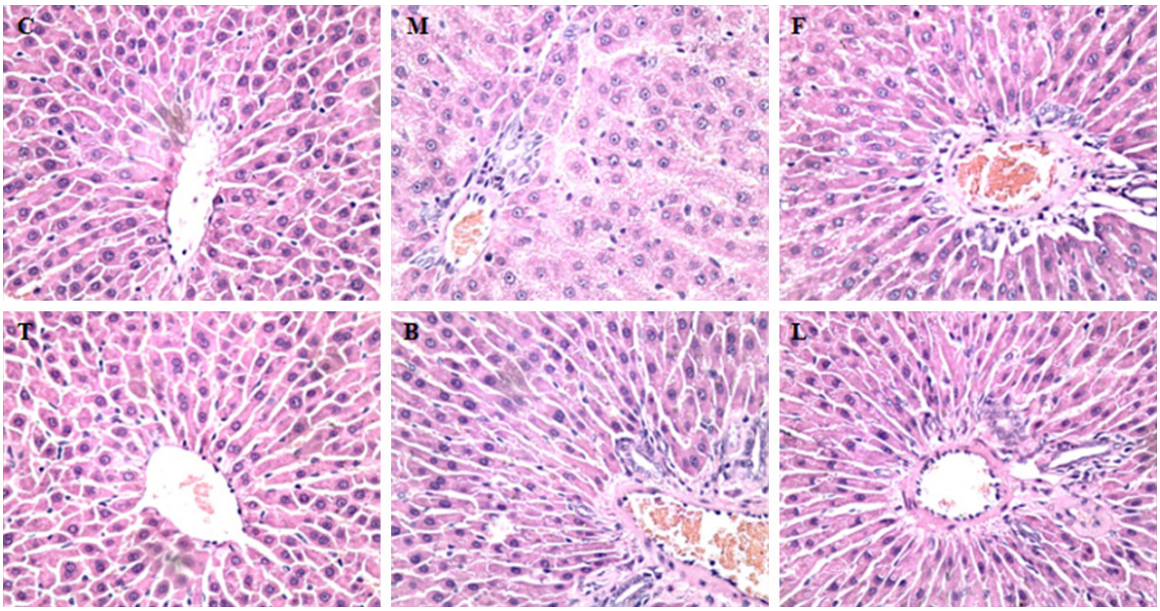

Figure 1. Hepatic histopathological analysis. Representative photomicrographs of H&E staining for observing the morphology of livers from different groups (×200).

microorganisms, probiotics prevent intestinal disorders, poison absorption, and inflammatory response [16]. Thus, they could have potential application in ALD treatment [17, 18]. The most common probiotics are lactose-fermenting *Lactobacilli* and *Bifidobacteria*. Golden bifid is a preparation containing *Bifidobacteria*, *Lactobacilli*, and *Streptococcus thermophiles* [19]. Medilac-S is a probiotic capsule containing live *Bacillus subtilis* and *Enterococcus faecalis* [20]. The release of these bacteria into the intestines can reduce intestinal inflammation and increase the number of beneficial bacteria. Although many studies have explored the therapeutic effects of single probiotic compounds, no work has established whether the combined use of two probiotic compounds for ALD treatment is better than single-use application.

The current study aimed to investigate the protective effects of FMT and a combination of probiotics against ALD, further exploring the underlying mechanisms of FMT and probiotics as promising therapeutic agents.

Methods

Animals and treatments

Male Wistar rats, weighing 140 ± 10 g, were purchased from Fuzhou Wushi Animal Center. All rats were housed in cages in an SPF animal room with a temperature of 20 ± 2°C and a humidity of 55 ± 5%. Rats had free access to water. After a week of adaptive feeding with a normal chow diet, 60 rats were randomly divided into six groups, each with 10 animals: (1) C group, normal chow diet and 1 mL saline via intragastric administration for 20 weeks; (2) M group, normal chow diet and intragastric ethanol at 8 mL/kg/day and 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; (3) F group, same method as the M group with intragastric fecal suspension (1 mL) for the last 8 weeks (1 g/mL/day); (4) T group, same method as the M group with intragastric golden bifid suspension for the last 8 weeks (500 mg/kg/day); (5) B group, same method as the M group with intragastric Medilac-s suspension for the last 8 weeks (140 mg/kg/day); and (6) L group, same method as the M group with intragastric golden bifid suspension (500 mg/kg/day) and Medilac-s suspension (140 mg/kg/day) for the last 8 weeks. At the end of the experiment, all rats were weighed and sacrificed. Blood, liver, and feces were collected.

Serum biochemical estimation

Blood samples were stored at room temperature for 1 hour and then centrifuged at 3500 r/

Table 1. Effects of FMT and probiotics on serum ALT, AST, and TG in ALD rats

Groups	ALT (U/L)	TG (mmol/L)	AST (U/L)
C	121 ± 20.785	0.377 ± 0.114	107 ± 2.645
M	177 ± 18.193[a]	0.777 ± 0.522	44 ± 10.536[a]
F	142.667 ± 9.291[b]	0.513 ± 0.031	66 ± 2.646[c]
T	144.333 ± 7.559[b]	0.713 ± 0.452	67 ± 8.66[c]
B	121 ± 20.785[c]	0.667 ± 0.049	70.667 ± 20.214[c]
L	119.333 ± 31.770[c]	0.750 ± 0.216	84.333 ± 5.033[c]

[a]*P* < 0.01, vs. C group. [b]*P* < 0.05, [c]*P* < 0.01, vs. M group.

Table 2. Effects of FMT and probiotics on serum IL-6, TNF-α, and IL-4 in ALD rats

Group	IL-6	TNF-α	IL-4
C	4.149 ± 0.423	1.751 ± 0.079	1.798 ± 0.164
M	6.881 ± 0.821[a]	2.835 ± 0.178[a]	2.978 ± 0.272[a]
F	5.731 ± 0.413[b]	2.399 ± 0.203[c]	2.435 ± 0.477[b]
T	5.241 ± 0.518[c]	2.222 ± 0.182[c]	2.838 ± 0.071
B	5.277 ± 0.246[c]	2.446 ± 0.069[c]	2.810 ± 0.219
L	4.117 ± 0.337[c]	1.961 ± 0.067[c]	1.892 ± 0.111[c]

[a]P < 0.01, vs. C group. [b]P < 0.05, [c]P < 0.05, vs. M group.

Table 3. Effects of FMT and probiotics on serum DAO, endotoxins, and D-lactate in ALD rats

Groups	DAO (mg/ml)	Endotoxin (EU/ml)	D-lactate (mg/L)
C	5.898 ± 0.487	0.719 ± 0.032	8.069 ± 0.113
M	8.585 ± 0.191[a]	0.944 ± 0.061[a]	14.911 ± 0.094[a]
F	6.761 ± 0.277[b]	0.814 ± 0.018[b]	11.298 ± 0.942[b]
T	7.351 ± 0.270[b]	0.819 ± 0.047[b]	12.165 ± 0.322[b]
B	6.785 ± 0.057[b]	0.783 ± 0.188[b]	13.006 ± 0.236[b]
L	6.392 ± 0.207[b]	0.750 ± 0.019[b]	9.289 ± 0.814[b]

[a]P < 0.01, vs. C group. [b]P < 0.01, vs. M group.

min for 15 minutes. Serum was stored at -80°C. Levels of triglycerides (TG), alanine aminotransferase (ALT), and serum transaminase (AST) were measured with an automatic biochemical analyzer (Konelab 20, Thermo Fisher Scientific, Waltham, Finland). Levels of TNF-α, IL-6, DAO, endotoxin, and D-lactate were measured using relevant ELISA assay kits, in accordance with manufacturer instructions (Wuhan USCN Business Co., Ltd., WuHan, China).

Pathologic evaluation

Livers were fixed in 10% formalin, embedded in paraffin, sectioned at 4 μM, and stained with hematoxylin and eosin.

Intestinal flora analysis

Fresh feces (3-5 g) of the mice were obtained with a sterile swab and analyzed by Hangzhou Jinghang Biotechnology Co., Ltd. (Hangzhou, China) for 16S rDNA sequencing.

Statistical analysis

Data are expressed as mean ± SD. Student's t-test was used to examine differences between the two groups. One-way ANOVA was used to compare the groups. All statistical analyses were performed using SPSS software (version 17, SPSS Inc., Chicago, USA).

Results

Appearance of rats

In the normal control group (C), the rats had smooth fur, sensitive reactions, lively posture, normal eating and drinking behavior, and normal tendency of weight change. Three weeks later, rats in the M group became rough and dull and exhibited a rigid body, slow movement, and malaise after drinking. These characteristics disappeared after 2-3 hours. Compared with control rats, rats from the five other groups suffered from poor appetite and low body weights.

Pathological changes in the livers of rats

Under a light microscope, cell swelling, obvious steatosis, cytoplasmic vacuolization, inflammatory cell infiltration, and necrosis were observed in the ALD group. In contrast, treatment with FMT and probiotics distinctly alleviated alcohol-induced hepatic histopathological injuries (**Figure 1**).

Effects of FMT and probiotics on serum biochemical parameters

As presented in **Table 1**, the M group showed higher serum ALT and lower AST levels than the C group (P < 0.01). F, T, B, and L groups showed a significant increase in AST levels and a decrease in ALT levels in the serum relative to

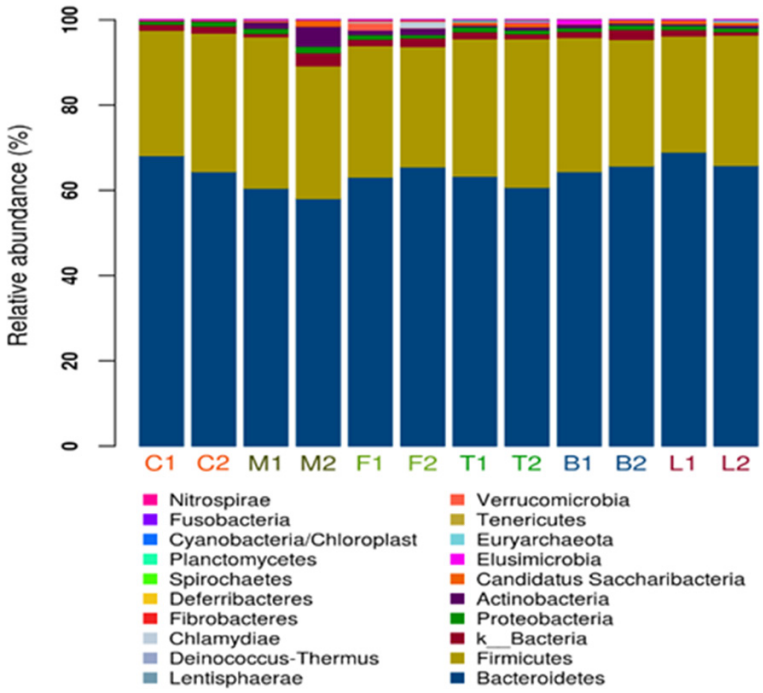

Figure 2. Bacterial community structures at the phylum level. The abundance is presented in terms of the percentage of the total effective bacterial sequences in the sample.

the M group ($P < 0.01$, $P < 0.05$). No changes in TG levels were observed among the groups ($P > 0.05$).

Effects of FMT and probiotics on serum levels of inflammatory factors

As shown in **Table 2**, serum IL-6, TNF-α, and IL-4 levels of the M group dramatically increased relative to those of the control group ($P < 0.01$). Compared with the M group, the F and L groups showed decreases in serum IL-6, TNF-α, and IL-4 levels ($P < 0.05$), while T and B groups showed decreases in serum IL-6 and TNF-α levels ($P < 0.05$). The concentration of IL-4 was not altered using golden bifid or Medilac-S ($P > 0.05$).

Effects of FMT and probiotics on serum DAO, endotoxin, and D-lactate in ALD rats

As shown in **Table 3**, ALD rats had significantly higher serum DAO, endotoxin, and D-lactate levels than controls ($P < 0.01$). After 8 weeks of treatment, however, serum DAO, endotoxin, and D-lactic acid levels in the F, T, B, and L groups markedly decreased, compared with those in the M group ($P < 0.01$).

Analysis of the abundance of intestinal flora at the phylum level

Sequencing results showed that, at the phylum level, the intestinal flora mainly belonged to seven phyla, with the predominant phyla being Firmicutes, Bacteroidetes, Bacteria, Actinobacteria, and Proteobacteria. In the M group, the relative abundance of Bacteroidetes obviously declined and the proportion of Proteobacteria ($P = 0.049$) and Actinobacteria ($P = 0.02$) notably increased, compared with that in the control group ($P = 0.011$). The abundance of Bacteroidetes increased and that of Proteobacteria and Actinobacteria decreased after treatment with FMT and probiotics, compared with the control group. However, no significant differences were noted (**Figure 2**).

Analysis of the abundance of intestinal flora at the genus level

At the genus level, the relative abundance of Prevotellaceae (28 ± 0.28 vs. 18.15 ± 2.61) was higher and the proportion of *Lactobacillus* (8.17 ± 2.31 vs. 11.71 ± 0.72) was lower in the ALD group than in the normal group. After treatment, the abundance of Prevotellaceae markedly decreased in the ALD group, compared with the M group ($P < 0.01$). Moreover, the proportion of *Lactobacillus* was significantly higher in the F group (18.50 ± 4.52), B group (26.25 ± 9.97 vs. 8.17 ± 2.31), and L group (38.2 ± 6.51 vs. 8.17 ± 2.31) than in the ALD group. No alterations were observed in the T Group, compared with the ALD group (**Figure 3**).

Discussion

Long-term excessive alcohol use can destroy the integrity of the intestinal barrier and disturb intestinal flora. Consequently, intestinal

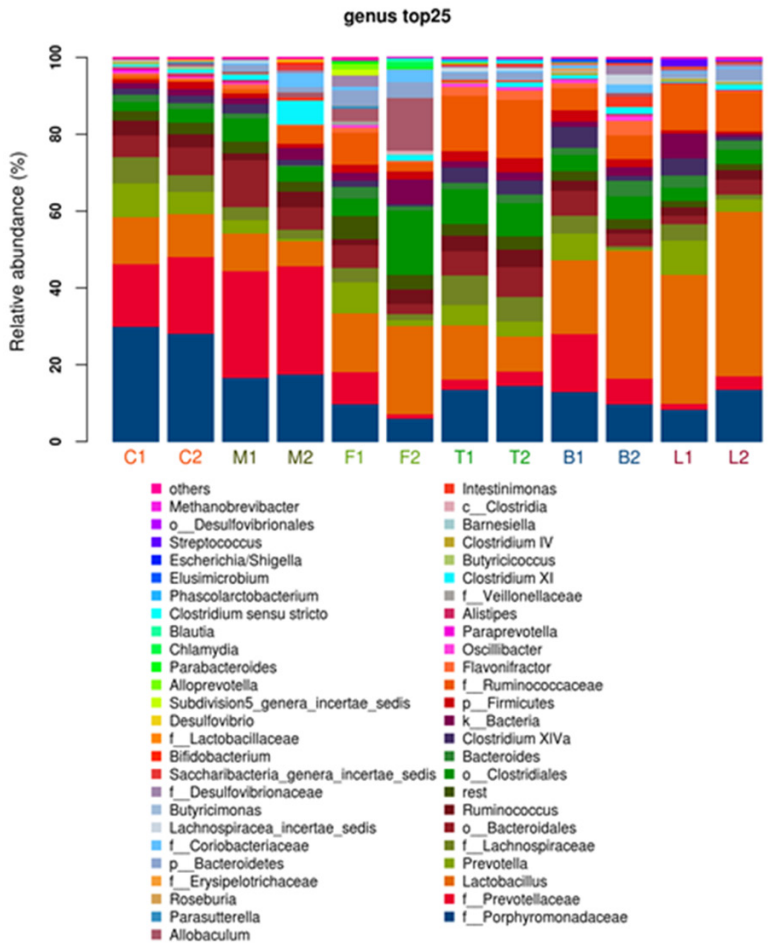

Figure 3. Bacterial community structures at the genus level. The abundance is presented in terms of the percentage of the total effective bacterial sequences in the sample.

toxicants, such as endotoxins, are produced excessively and imported into the liver through the portal vein. This effect ultimately results in ALD [21-23]. Therefore, restoring normal bowel flora serve as a potential therapy for ALD.

In the present study, rat models with ALD were constructed to evaluate the potential therapeutic efficacy of FMT and probiotics. Various inflammatory cytokines, such as TNF-α, IL-6, and IL-4, are involved in the occurrence and development of ALD [24, 25]. Serum ALT and AST levels are used to index liver injuries [26]. DAO, endotoxins, and D-lactate can reflect the severity of intestinal mucosa injuries [27]. These parameters are increased in rats subjected to chronic alcohol feeding [28, 29]. In the present study, serum levels of ALT, AST, TG, DAO, endotoxins, D-lactate, TNF-α, IL-4, and IL-6 were elevated in alcohol-fed rats. Present results indicated that the ALD models were successfully established and that alcohol intake could increase intestinal permeability and cause inflammatory response and liver damage. Liver injuries and steatosis at the histopathological level were completely reversed after the joint application of golden bifid and Medilac-S. In addition, serum levels of ALT, DAO, endotoxins, D-lactate, TNF-α, IL-4, and IL-6 significantly decreased after therapy. This inhibitory effect became increasingly obvious after treatment using the combination of Medilac-S and golden bifid. A reasonable explanation for this result is that joint application of these probiotic compounds could not only supply the nutrients for intestinal epithelial cells but also regulate and balance the intestinal flora. In this manner, the combination of these probiotic compounds could protect the intestinal mucosal barrier more efficiently than an individual application of golden bifid and Medilac-S. These findings indicate that therapy with FMT and probiotics may protect the integrity of the intestinal mucosa and prevent inflammatory cytokine production.

To further investigate the protective mechanisms of FMT and the joint application of golden bifid and Medilac-S in the intestinal barrier and liver injuries, this study employed 16S rDNA high-throughput sequencing for analysis of intestinal flora in rats. An increasing number of studies have indicated that chronic alcohol feeding induces alterations in the gut microbiota [30, 31]. Bull-Otterson et al. [32] found that chronic alcohol consumption decreases the abundance of Bacteriodetes and increases those of Gram-positive Actinobacteria and Gram-negative Proteobacteria in mouse models. Similar results were obtained by Mutlu et al. [33]. They noted a low proportion of Bacteroidetes and high abundance of Proteobacte-

ria in a mouse model of ALD. Chung et al. [34] reported that Prevotellaceae was more abundant in patients with abnormal gut microbiota than in healthy people. Yan et al. [35] found that the population of *Lactobacillus* was depleted in alcohol-treated mice and suggested that various probiotic *Lactobacillus* strains exert beneficial effects. These decreased beneficial bacteria and elevated pathogenic bacteria promote the growth of other pathogenic bacteria, causing enteric flora disturbance, intestinal barrier dysfunction, and liver disease. Consistent with prior studies, the abundance of Bacteroidetes and *Lactobacillus* was reduced and those of Actinobacteria, Proteobacteria, and Prevotellaceae were increased in the ALD group, compared with healthy controls. These changes were inhibited after therapy, suggesting that FTM and probiotics can restore gut microbiota [30, 36]. These results show that chronic alcohol consumption causes quantitative and qualitative alterations in the gut flora, but the imbalance in intestinal flora benefits from probiotics and FMT.

In conclusion, FMT and the joint application of golden bifid and Medilac-S can protect rats from ALD by suppressing inflammation and regulating intestinal flora. The combined use of probiotics protects the intestinal mucosal barrier more efficiently than single-use treatment. Thus, FMT and the joint application of golden bifid and Medilac-S show therapeutic potential for ALD treatment.

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Disclosure of conflict of interest

None.

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