Role of probiotics in the treatment of minimal hepatic encephalopathy in patients with HBV-induced liver cirrhosis

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Abstract

Objective: This study was performed to investigate the role of probiotics (*Clostridium butyricum* combined with *Bifidobacterium infantis*) in the treatment of minimal hepatic encephalopathy (MHE) in patients with hepatitis B virus (HBV)-induced liver cirrhosis.

Methods: Sixty-seven consecutive patients with HBV-induced cirrhosis without overt hepatic encephalopathy were screened using the number connection test and digit symbol test. The patients were randomized to receive probiotics (n = 30) or no probiotics (n = 37) for 3 months. At the end of the trial, changes in cognition, intestinal microbiota, venous ammonia, and intestinal mucosal barriers were analyzed using recommended systems biology techniques.

Results: The patients' cognition was significantly improved after probiotic treatment. The predominant bacteria (*Clostridium* cluster I and *Bifidobacterium*) were significantly enriched in the probiotics-treated group, while *Enterococcus* and Enterobacteriaceae were significantly decreased. Probiotic treatment was also associated with an obvious reduction in venous ammonia. Additionally, the parameters of the intestinal mucosal barrier were obviously improved after probiotic treatment, which might have contributed to the improved cognition and the decreased ammonia levels.

Conclusion: Treatment with probiotics containing *C. butyricum* and *B. infantis* represents a new adjuvant therapy for the management of MHE in patients with HBV-induced cirrhosis.

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Introduction

Long-term chronic hepatitis B virus (HBV) infection, which can evolve into liver cirrhosis and carcinoma, affects an estimated 350 million people worldwide and results in more than 0.5 to 1.0 million deaths per year.[1] Growing evidence has shown that an altered gut microbiota profile is associated with cirrhosis and its complications, such as hepatic encephalopathy (HE), spontaneous bacterial peritonitis, and other infections, which might be correlated with disruption of the intestinal barrier.[1] HE is a serious neuropsychiatric syndrome that causes central nervous system dysfunction due to severe liver dysfunction, especially decompensated liver cirrhosis.[1] According to the West-Haven criteria, minimal HE (MHE) is the mildest form of HE, affecting about one-third of patients with cirrhosis.[1] Patients with MHE have no recognizable clinical symptoms of HE; instead, they have mild cognitive and psychomotor deficits that negatively affect their daily functioning and quality of life.[1] The prevalence of MHE varies from 30% to 84% in patients with liver cirrhosis.[2] No standardized criteria for a diagnosis of MHE have yet been established; the diagnosis depends on the patient's history and physical examination findings, mental status, and alteration of cognition and/or neurophysiological function as well as the exclusion of concomitant neurological disorders.[3] Previous studies have shown that ammonia is a crucial factor in the pathogenesis of MHE: hyperammonemia induces abnormalities such as brain edema and intracranial hypertension in patients with liver cirrhosis, and the blood ammonia levels are correlated with the severity of MHE in patients with cirrhosis.[4]

The human intestinal microbiota plays vital roles in maintaining intestinal homeostasis, which is central to human general health and wellbeing. The gut–brain axis is characterized by complex bidirectional communication between the gut and brain to maintain intestinal homeostasis and influence cognitive functions. Dysbiosis of the intestinal microbiota alters the microenvironment of the colonic lumen, especially the pH, which contributes to increased production of ammonia from the gut microbiota and increased ammonia absorption from the colonic lumen to the blood. Several studies have confirmed that probiotics are effective and safe, are minimally toxic, and have few adverse effects in the treatment of MHE.[5–9] Compared with lactulose, probiotics are associated with no major adverse events and are better tolerated. The present study was performed to evaluate the effects of a probiotic mixture containing *Clostridium butyricum* combined with *Bifidobacterium infantis* in patients with MHE with the goal of developing a new adjuvant therapy for MHE in clinical practice.

Methods

Patient recruitment

In the present study, patients with HBV-induced liver cirrhosis without overt encephalopathy were screened for MHE, and those patients with MHE were

enrolled. HBV-induced liver cirrhosis was diagnosed on a clinical basis involving laboratory tests, ultrasound imaging, and liver histology, if available. Patients with MHE were diagnosed according to the consensus on the diagnosis and treatment of HE in China,[10] which involved the performance of two psychometric tests: the number connection test A (NCT-A) and digit symbol test (DST). Patients with abnormal NCT-A and DST results were diagnosed with MHE. All patients were treated with the same conventional therapy, while a portion was randomized to probiotic treatment and another portion was randomized to no probiotic treatment. The following exclusion criteria were applied: a history of overt HE in the past 2 months; the use of probiotics, prebiotics, synbiotics, or antibiotics during the previous 4 weeks; the presence of any other neurological or psychiatric diseases; a history of spontaneous bacterial peritonitis or gastrointestinal bleeding in the past 2 months; known active microbial infections; and a high-protein diet. The protocols utilized in the present study were approved by the Ethics Committee of the Changxing People's Hospital (Huzhou, China) and were carried out according to the approved guidelines. Written informed consent was obtained from each patient prior to enrollment.

Probiotics

The probiotic mixture containing *Clostridium butyricum* (CGMCC0313-1) combined with *B. infantis* (CGMCC0313-2) used in the present study was produced by Shandong Kexing Bioproducts Co., Ltd. (Shandong, China). The freeze-dried probiotic mixture had more than 1.0×10^7 CFU/g viable *C. butyricum* and more than 1×10^6 CFU/g viable *B. infantis* per capsule. The patients treated with the probiotics received a dose of 1500 mg three times daily for 3 months.

Quantitative assessment of predominant fecal bacteria

At the end of the treatment, a fresh fecal sample (approximately 2 g) was collected from each patient for bacterial analysis. Bacterial genomic DNA was extracted using a QIAamp® DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to our previous study.[11] The amount of bacterial genomic DNA was analyzed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the integrity and size of bacterial genomic DNA were checked by electrophoresis. All bacterial genomic DNA was stored at $-20°C$ for further use.

For analysis of the predominant gut bacteria, quantitative polymerase chain reaction (PCR) was performed on an ABI Prism 7900HT real-time PCR system (Applied Biosystems, Carlsbad, CA, USA) with Power SYBR Green PCR Master Mix (Vazyme Biotech, Nanjing, China). The bacterial primer sets and reaction conditions were in accordance with our previous studies.[11,12] SDS 2.4 software (Applied Biosystems) was used for data analysis. Triplicate repeats were carried out for all reactions in every analysis with inclusion of a nontemplate. The quantity of these bacteria was presented as log10 bacteria per gram of feces (wet weight).

Measurement of venous ammonia levels

Blood was sampled for blood ammonia analysis at the end of the therapy using a commercial ammonia test kit (ARKRAY, Tokyo, Japan) within 30 minutes after sample collection with a detection range of 10 to 400 µmol/L.

Intestinal mucosal barrier function analysis

At the end of the treatment, serum from each patient was collected for intestinal

mucosal barrier function analysis. Three parameters of the intestinal mucosal barrier function [D-lactate, endotoxin (LPS), and diamine oxidase (DAO)] were detected by a dry chemical method using the Intestinal Mucosal Barrier Biochemical Index Analysis System (JY-DLT; Beijing Zhongsheng Jinyu Diagnostic Technology Co., Ltd., Beijing, China).

Statistical analysis

The quantitative data of gut bacteria and other parameters in our study are presented as mean ± standard deviation, and differences between the two groups were evaluated by Student's t test using IBM SPSS Statistics, version 20.0 (IBM Corp., Armonk, NY, USA). A p value of <0.05 was considered statistically significant for all analyses.

Results

Patients' characteristics

Sixty-seven patients were enrolled in this study (probiotic treatment, n = 30; no probiotic treatment, n = 37). The characteristics of all patients after 3 months of probiotic treatment are shown in Table 1. All participants were diagnosed with HBV-induced liver cirrhosis. No obvious differences in age or sex were found between the two groups, while there were significant improvements in the serum alanine aminotransferase, aspartate aminotransferase, total bilirubin, and albumin levels after probiotic treatment at the end of the trial (p < 0.05). In addition, the probiotics improved the results of the psychometric tests for MHE (NCT-A and DST). These data suggest that probiotics containing *C. butyricum* and *B. infantis* can improve the MHE status and be used as an effective adjuvant therapy for MHE.

Table 1. Patients' characteristics

	Control (n = 37)	Treatment (n = 30)
Age, y	43.8 ± 10.3	41.5 ± 12.9
Sex, male/female	20/17	18/12
TBIL, μmol/L*	48.3 ± 6.7	17.6 ± 13.5
ALT, U/L*	89.1 ± 26.4	35.9 ± 22.5
AST, U/L*	95.6 ± 101.7	28.5 ± 18.7
Albumin, g/L*	55.3 ± 25.9	62.3 ± 22.5
Child–Pugh score		
B	15	18
C	22	12
Ascites	15 (40.54)	8 (26.67)
INR	1.4 ± 0.6	1.3 ± 0.4
NCT-A, s*	72.4 ± 11.5	41.2 ± 8.9
DST, points/90 s*	12.5 ± 8.3	29.8 ± 10.7

Data are presented as mean ± standard deviation, n, or n (%).
ALT, alanine aminotransferase; AST, aspartate aminotransferase; DST, digit symbol test; INR, international normalized ratio of prothrombin time; NCT-A, number connection test A; TBIL, total bilirubin. *p < 0.05

Quantification and comparison of the predominant fecal microbiota between the two groups

The differences in the predominant fecal bacteria in patients with MHE were detected by quantitative PCR (Figure 1). After treatment, the total bacteria of the gut microbiota were similar between the probiotics-treated group and control group. Nine other fecal bacteria were also analyzed to reveal the specific changes in the fecal microbiota. Our data indicated that *Clostridium* cluster I and *Bifidobacterium* were significantly increased after probiotic treatment (p < 0.05). Because *C. butyricum* belongs to *Clostridium* cluster I and *B. infantis* belongs to the genus *Bifidobacterium*, the colonization of the probiotic strains might have contributed to the increase in these two abundant bacteria. In addition, *Enterococcus* and Enterobacteriaceae were significantly decreased after probiotic treatment (p < 0.05); these microorganisms
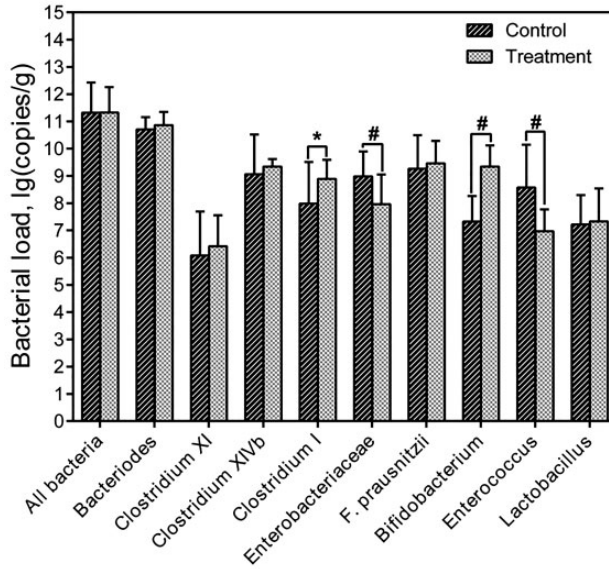
Figure 1. Quantification of the predominant intestinal bacteria in patients with minimal hepatic encephalopathy treated by probiotics (log10 copies per gram of fresh feces). *p < 0.05; #p < 0.01.

might be the sources of the opportunistic infections that translocate from the gut to other tissues. There were no significant differences in the other bacteria between the two groups.

Improvement in venous ammonia level

Venous ammonia is thought to play vital roles in the pathogenesis of HE. At the end of the trial, the mean venous ammonia level was significantly lower in the probiotics-treated group than control group (76.4 ± 37.3 vs. 152.0 ± 48.3 μmol/mL, respectively; p = 0.032) (Figure 2). This decrease in venous ammonia might have been associated with the improvement in the NCT-A and DST results for these patients with MHE after probiotic treatment.
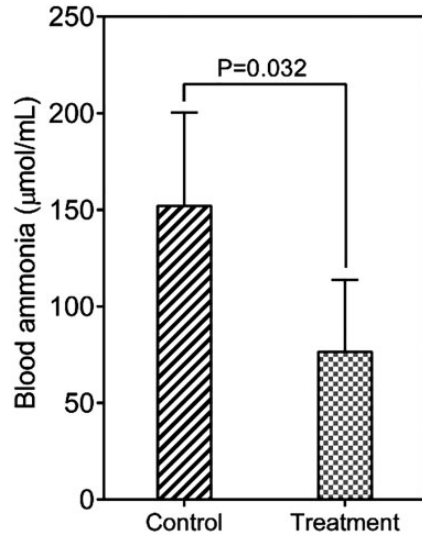
Restoration of intestinal mucosal barrier function

Dysfunction of the intestinal mucosal barrier is associated with the development of

Figure 2. Changes in the blood ammonia level after treatment with probiotics.

liver disease and is more relevant to clinical complications such as HE. Increased intestinal permeability is reportedly associated with bacterial translocation from the

lumen to extraintestinal sites, resulting in the development of various complications.[13] At the end of the trial, the parameters of the intestinal mucosal barrier were significantly improved. Specifically, after 3 months of probiotic treatment, the LPS level was significantly decreased (0.27 ± 0.16 EU/mL in treatment group vs. 1.38 ± 0.32 EU/mL in control group, p = 0.002) (Figure 3(a)), as was the concentration of D-lactate (5.38 ± 1.23 mg/mL in treatment group vs. 25.76 ± 6.14 mg/mL in control group, p = 0.014) (Figure 3(b)). These findings indicate that the probiotics improved the intestinal permeability. In addition, the level of DAO, an indicator of mucosal damage, was significantly decreased after probiotic treatment (1.84 ± 0.46 U/mL in treatment group vs. 7.35 ± 1.29 U/mL in control group, p = 0.000) (Figure 3(c)). Our data indicate that probiotics can restore the intestinal mucosal barrier in patients with MHE.

Discussion

MHE is related to poorer quality of life, and the liver must work harder in affected patients. MHE is present in one-third of patients with cirrhosis depending on liver function. The pathogenesis of MHE is thought to be similar to that of overt HE, and higher blood ammonia plays a key role. Dysbiosis of the gut microbiota has recently been observed in patients with liver cirrhosis,[14] and such dysbiosis plays a critical role in the generation of ammonia. In patients with HBV-induced cirrhosis, the alterations in the intestinal microbiota are characterized by an obvious increase in potentially pathogenic bacteria and a decrease in commensal bacteria, which may contribute to the elevation of fecal ammonia.[15] A previous study demonstrated that the gut microbiota plays a prominent role in gut–brain interactions,[16] and perturbations of the intestinal microbiota can impact the gut–brain axis to alter behavioral responses. The bidirectional crosstalk between the gut and brain is critical for maintaining host homeostasis and is regulated at the neural, hormonal, and immunological levels. Alterations in the gut microbiota are associated with disruption of the intestinal barrier, which is related to the development of HE. Increased intestinal permeability can increase the translocation of bacterial products and/or bacteria into the blood. By targeting modulation of the intestinal microbiota, previous randomized controlled trials (RCTs) have estimated the
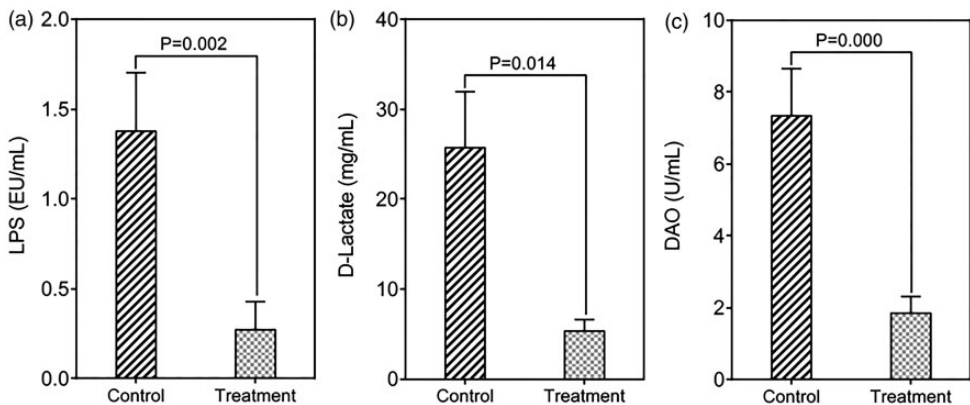

Figure 3. Improvement in the indicators of the intestinal mucosal barrier after treatment with probiotics.

efficacy of probiotics, prebiotics, and synbiotics in patients with MHE and revealed that probiotic treatment is effective in decreasing endotoxin and serum ammonia levels, improving MHE, and preventing overt HE development in patients with liver cirrhosis.[17–19] Several RCTs have compared probiotic treatment with placebo or no treatment, and others have compared the therapeutic effect of probiotics and lactulose.[5,7,20–23] These RCTs used different bacterial strains as probiotics for MHE treatment, including *Lactobacillus*, *Bifidobacterium*, *Pediococcus*, *Streptococcus*, *Enterococcus*, and others. Sharma *et al.*[5] used *C. butyricum* in combination with other bacteria such as *Streptococcus faecalis*, *Bacillus mesentericus*, and lactic acid bacillus as probiotics for MHE treatment and reported improvement in abnormal psychometric parameters and a significant reduction of venous ammonia and the P300 auditory event-related potential. Although the beneficial effects of probiotics on MHE have been verified, the efficacy of the combination of *C. butyricum* and *B. infantis* on MHE in patients with HBV-induced liver cirrhosis remains unclear.

The probiotic mixture of *C. butyricum* combined with *B. infantis* has been used to modulate the intestinal microbiota for many years. *Clostridium butyricum* (belonging to *Clostridium* cluster I) is a typical butyric acid-producing, endospore-producing, gram-positive anaerobe that has been used to modulate the intestinal microbiota and treat intestinal disorders.[24–26] As a commensal bacteria of the gut microbiota, *B. infantis* can produce lactic acid, which is frequently associated with health-promoting effects. A previous study showed that *B. infantis* can specifically relieve various symptoms of irritable bowel syndrome.[27] However, this probiotic has not been used for MHE treatment. In the present study, probiotics were used to treat patients with MHE for a relatively long period of time. Interestingly, the NCT-A and DST psychometric test results were significantly improved in the probiotic-treated group after 3 months of treatment. Compared with the control group, these patients' clinical symptoms were obviously controlled and their quality of life was significantly improved. In addition, beneficial bacteria such as *Clostridium* cluster I and *Bifidobacterium* were significantly increased, while opportunistic pathogens such as *Enterococcus* and Enterobacteriaceae were significantly decreased after probiotic treatment. The two beneficial bacteria produce wide range of metabolites, mainly short-chain fatty acids such as butyrate and acetate. The production of short-chain fatty acids can alter bacterial adhesion and improve tight-junction integrity, indicating that these metabolites play an important role in the maintenance of the intestinal mucosal barrier function.[28,29] After probiotic treatment, the parameters of intestinal permeability (LPS and D-lactate) and the indicator of mucosal damage (DAO) were significantly improved. In addition, the venous ammonia level was obviously decreased after probiotic treatment, which might have been due to restoration of the intestinal mucosal barrier. The decrease in the venous ammonia level effectively relieved the clinical symptoms of MHE.

The present study has several limitations. First, the sample size was relatively small; the treatment of more patients with MHE using this probiotic mixture will validate our conclusions. Second, this was a case-control study involving only one probiotic mixture. Future RCTs with more probiotics, prebiotics, and synbiotics will help to select the best therapy for patients with MHE. Third, the long-term effects of the probiotic treatment should be observed to establish a suitable intervention course.

In summary, the present data have important implications for MHE treatment in clinical practice. Probiotics that contain *C. butyricum* and *B. infantis* can modulate the

intestinal microbiota, which plays a vital role in re-establishing the intestinal mucosal barrier function. Restoration of the intestinal barrier contributes to a decrease in the blood ammonia level. The present study indicates that probiotics containing *C. butyricum* and *B. infantis* can be used as an effective adjuvant therapy for MHE in clinical practice.

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