



Effect of Additional *Clostridium butyricum* on the Intestinal Flora of Chronic Hepatitis B Patients Treated with Entecavir

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ABSTRACT

Introduction: To explore the influence of intestinal flora on the occurrence, development and antiviral therapy of chronic hepatitis B (CHB), 16S rDNA amplification sequencing was performed to investigate the intestinal flora in CHB patients treated with entecavir (ETV) and *Clostridium butyricum* (CB).

Methods: CHB patients were divided into the ETV group (treatment with ETV alone) and ETV + CB group (treatment with ETV and CB). After 8-week treatment, feces samples were collected and processed for 16S rDNA amplicon sequencing; blood samples were collected for the biochemical, immunologic and virologic evaluations, which were compared between groups.

Results: ETV treatment for 8 weeks significantly decreased the serum levels of alanine

aminotransferase (ALT), interleukin-6 (IL-6), IL-8, tumor necrosis factor- α (TNF- α) and HBV DNA compared to those before treatment, but there were no marked differences between the ETV group and ETV + CB group. The intestinal flora changed significantly in the CHB patients after ETV + CB treatment: there were marked differences in 13 unique species before treatment and 4 unique species after ETV + CB treatment; at the phylum level, the top five bacteria with significant difference between patients before treatment and ETV + CB patients were Firmicutes, Actinobacteria, Cyanobacteria, Euryarchaeota and Synergistetes. There were significant differences in 25 unique species in the ETV group and 4 unique species in the ETV + CB group; at the phylum level, the top five bacteria with significant difference between ETV patients and ETV + CB patients were Actinobacteria, Fusobacteria, Proteobacteria, Saccharibacteria and Synergistetes.

Conclusion: ETV treatment improves the serum biochemical, immunologic and virologic variables, but additional CB fails to further improve these variables. Of note, additional CB affects the intestinal flora in the CHB patients treated with ETV.

Keywords: Chronic viral hepatitis B; *Clostridium butyricum*; Entecavir; Intestinal microflora

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Key Summary Points

ETV treatment for 8 weeks significantly decreased the serum levels of ALT, IL-6, IL-8, TNF- α and HBV DNA compared to those before treatment, but there were no marked differences between the ETV group and ETV + CB group

The healthy control group had the highest abundance of intestinal flora species, followed by the untreated CHB patients and the ETV group, and the ETV + CB group had the lowest abundance of species

Each group had unique species. Additional CB affected the intestinal flora in the CHB patients treated with ETV

DIGITAL FEATURES

This article is published with digital features, including a summary slide, to facilitate understanding of the article. To view digital features for this article go to <https://doi.org/10.6084/m9.figshare.14637825>.

INTRODUCTION

Chronic hepatitis B (CHB) is caused by hepatitis B virus (HBV) infection and has been one of the most common infectious diseases in the world. It is estimated that there are 350 million HBV carriers worldwide [1]. An epidemiologic survey in 2006 reported that the proportion of HBsAg + carriers was 7.18% in the general population in China; thus, it was estimated that there were about 93 million patients with chronic HBV infection, of whom about 20 million patients had CHB [2]. Therefore, HBV infection has been a heavy burden in China. The existing antiviral drugs can inhibit the replication of HBV, but cannot remove the cccDNA in the nucleus of hepatocytes. Thus,

most patients with HBV infection require long-term or even lifelong treatment. Exploring better strategies to enable CHB patients to obtain functional cure has been the goal pursued by researchers.

Our previous study [3] indicated that the healthy controls had the highest abundance of intestinal flora species, followed by the CHB group after entecavir (ETV) treatment, and CHB patients before treatment had the lowest abundance of species. Increasing studies (references in the Discussion) have shown that intestinal flora disorder is involved in the development and progression of CHB, and probiotic supplementation may be important for the prevention and management of chronic liver disease induced by HBV. Fecal bacteria transplantation may be a potential treatment for CHB-related diseases in the future. *Clostridium butyricum* (CB) [4] is a probiotic approved for humans. It can tolerate gastric acid and then enter the intestine to secrete butyric acid (an important nutrient for the regeneration and repair of intestinal mucosa), eliminate inflammation, inhibit the growth of harmful bacteria, restore the balance of intestinal flora, and rebuild intestinal immune function and normal physiologic function. Up to now, no studies have been conducted to investigate the effects of CB in CHB patients.

In the present study, CHB patients were recruited and treated with ETV and CB. 16S rDNA amplicon sequencing was used to investigate the intestinal flora of these patients, and the influence of intestinal flora on the occurrence and development of CHB was further explored.

METHODS

Subjects

A total of 60 patients with CHB in the immune clearance phase and reactivation phase were recruited from the Department of Infectious Diseases and Department of Gastroenterology and Hepatology of Tongji Hospital in Shanghai between January 2017 and December 2018. These patients were randomly divided into two

groups (Group A and Group M; $n = 30$ per group). In addition, 30 healthy subjects were recruited as controls (Group C). This study was approved by the Institutional Review Board of Tongji Hospital, Tongji University. Informed consent was obtained from all subjects, and the protocols conformed to the ethical guidelines of the Declaration of Helsinki.

Inclusion criteria: subjects were aged 18–65 years; patients were diagnosed with CHB according to the “Guideline for the Prevention and Treatment of Chronic Hepatitis B” (2015 Update), which was developed by the Chinese Society of Liver Diseases and the Chinese Society of Infectious Diseases of the Chinese Medical Association [2]. According to the serum alanine aminotransferase (ALT), HBV DNA, severity of liver disease, age and family history, the risk of disease progression was comprehensively assessed. Patients meeting the standard of antiviral treatment were recruited.

Exclusion criteria were: (1) subjects were pregnant or breast feeding; (2) patients had psychiatric diseases; (3) patients were diagnosed with cancer; (4) patients were diagnosed with hepatic cirrhosis; (5) patients had other liver diseases or infectious diseases; (6) patients had concomitant severe disease of the heart, lung, kidney, central nervous system or gastrointestinal tract; (7) patients were treated with antiviral drugs or had a history of antiviral therapy before inclusion; (8) patients received treatment with antibiotics or microecologic drugs 1 month before enrollment; (9) patients had poor compliance, could not cooperate with treatment or were lost to follow-up.

Inclusion criteria for healthy controls were: subjects were aged 18–65 years; physical examination showed no abnormalities; subjects had no treatment with antibiotics or microecologic drugs 1 month before enrollment; patients were not virus carriers.

The patients and healthy controls were all Han Chinese and lived in Shanghai. During the study period, their eating habits remained unchanged. The staple food included rice and wheat, supplemented with vegetables, fruits, meat, fish and shrimp; there were no dietary supplements or drugs that could have affected

the microbial ecology, and they were non-smokers and did not drink.

Methods

In Group A, 30 patients were orally treated with entecavir tablets (0.5 mg, qd; Runzhong, Chia Tai Tianqing Pharmaceutical Group Co., Ltd., H20100019, 0.5 mg/tablet) for 8 weeks, and then blood and stool samples (Group N) were collected; in Group M, patients were orally treated with entecavir tablets (0.5 mg, qd) and viable *Clostridium butyricum* capsules (0.4 g tid) (Missan, Chongqing Taiping Pharmaceutical Co., Ltd., S20040054, 0.2 g/tablet) for 8 weeks, and then blood and stool samples (Group Y) were collected.

Biochemical examinations: (1) Enzyme method was employed to detect the liver function variables (AU5800; Beckman automatic biochemical analyzer); (2) chemiluminescence method was used to detect the serum levels of interleukin-6 (IL-6), IL-8, tumor necrosis factor- α (TNF- α) (Immulite1000; Siemens chemiluminescence immunoassay analyzer, with corresponding kits); (3) HBV DNA was detected by real-time fluorescence quantitative PCR (ABI 7300 Real-time Fluorescence Quantitative PCR Instrument) with corresponding kit (Shanghai Zhijiang Biotechnology Co., Ltd).

The 16S rDNA amplicon sequencing was performed to detect the flora of stool samples. The procedures included: DNA extraction and quality assessment, amplification of the 16S rDNA variable region by PCR, product purification, library preparation and detection, high-throughput sequencing, etc. The quality was strictly controlled, and the library volume was adjusted according to the target data volume. Then, multiple libraries were mixed for Illumina MiSeq sequencing. The FloraPrep™ sample collection tubes and primers were from Suzhou Admera Health Medical Technology Co., Ltd.

Statistical Analysis

Statistical analysis was performed with Statistical Product and Service Solutions (SPSS), version 20.0. Sex was compared between the two groups

with chi-square test; age was compared with *t*-test between the two groups. The quantitative data with normal distribution are expressed as mean \pm standard deviation ($X \pm SD$) and those without normal distribution as medians (quartiles). Variables before and after treatment were compared with paired *t*-test if normal distribution was present or Wilcoxon signed rank test if normal distribution was not observed. $P < 0.05$ was considered statistically significant.

RESULTS

General Characteristics

A total of 60 patients with CHB in the immune clearance phase or reactivation phase were recruited. Of them, 35 were positive for HBeAg and 25 negative for HBeAg. Patients were randomly divided into two group (Group A and Group M; $n = 30$ per group). Healthy subjects ($n = 30$) were also recruited as controls in Group C (Table 1). There were no marked differences in the gender and age between patients and controls ($P > 0.05$).

Characteristics of CHB Patients Before Grouping

There were no marked differences in the blood biochemical, immunologic and virologic variables of CHB patients between Groups A and M ($P > 0.05$; Table 2).

Blood Biochemical, Immunologic and Virologic Variables in CHB Patients Before and After Treatment

In Group A, patients were treated with ETV for 8 weeks, and blood and stool samples were

collected as Group N; in Group M, patients were treated with ETV + CB for 8 weeks, and blood and stool samples were collected as Group Y (Tables 3, 4 and 5).

As shown in Table 3, the serum levels of ALT, IL-6, IL-8, TNF and HBV DNA in Group A reduced significantly (Group N) after 8-week treatment ($P < 0.001$). As shown in Table 4, the serum levels of ALT, IL-6, IL-8, TNF and HBV DNA in Group M reduced significantly (Group Y) after 8-week treatment ($P < 0.001$). As shown in Table 5, the serum levels of ALT, IL-6, IL-8, TNF and HBV DNA in Group Y were lower than in Group N although there were no marked differences ($P > 0.05$).

Effect of CB on the Operational Taxonomic Unit of Intestinal Microflora in the CHB Patients

After de-linking and low-quality filtering of the original data of each sequence, the chimera sequences were removed and valid sequences were obtained for clustering analysis. Each cluster was assigned as an operational taxonomic unit (OTU). At a similarity level of 97%, all sequences were classified according to the OTUs, followed by bioinformatics analysis. Based on the OTU clustering analysis, the shared and unique OTUs were analyzed in CHB patients before and after ETV + CB treatment and in healthy controls.

The common and unique OTU of each sample was analyzed, and the Venn diagram (Venn) is shown in Fig. 1. There were 470 OTUs in Groups M, Y and C; 65 OTUs were unique in Group M, 28 OTUs were unique in Group Y, and 129 OTUs were unique in Group C. This indicated that the healthy controls had the most abundant OTUs, and the abundance of OTU in the CHB patients decreased after treatment with ETV and CB.

Table 1 General characteristics of subjects in the present study

Variables	CHB patients (A + M) ($n = 60$)	Controls (C) ($n = 30$)	Statistics	<i>P</i>
Gender (M/F)	35/25	18/12	0.023	0.880
Age (years)	42.35 \pm 11.04	41.70 \pm 11.46	0.260	0.795

Table 2 Characteristics of CHB patients before grouping

Variables	Group A (n = 30)	Group M (n = 30)	Statistics	P
Gender (M/F)	17/13	18/12	0.069	0.793
Age (years)	42.30 ± 11.35	42.40 ± 10.91	– 0.035	0.972
ALT (U/l)	211.00 (152.00, 319.00)	226.50 (156.00, 313.00)	– 0.044	0.965
IL-6 (pg/ml)	3.34 (2.00, 6.32)	4.09 (2.00, 6.89)	– 0.432	0.666
IL-8 (pg/ml)	45.62 (13.21, 132.55)	38.55 (11.30, 148.90)	– 0.148	0.882
TNF (pg/ml)	12.19 (8.30, 17.87)	13.21 (8.51, 18.71)	– 0.384	0.701
HBV DNA (IU/ml)	5.11 × 10 ⁷ (0.51 × 10 ⁷ , 10.00 × 10 ⁷)	5.06 × 10 ⁷ (5.70 × 10 ⁶ , 1.00 × 10 ⁸)	– 0.127	0.899

Table 3 Blood biochemical, immunologic and virologic variables of CHB patients in Group A before and after treatment

Variables	Before (n = 30)	After (Group N) (n = 30)	Statistics	P
ALT (U/l)	211.00 (152.00, 319.00)	90.50 (57.25, 125.25)	7.927	< 0.001
IL-6 (pg/ml)	3.34 (2.00, 6.32)	2.00 (2.00, 4.74)	– 3.622	< 0.001
IL-8 (pg/ml)	45.62 (13.21, 132.55)	22.23 (10.46, 57.30)	– 4.453	< 0.001
TNF (pg/ml)	12.19 (8.30, 17.87)	7.69 (6.44, 12.51)	– 4.703	< 0.001
HBV DNA (IU/ml)	5.11 × 10 ⁷ (5.09 × 10 ⁶ , 1.00 × 10 ⁸)	1.54 × 10 ³ (1.00 × 10 ² , 5.32 × 10 ⁴)	– 4.783	< 0.001

Table 4 Blood biochemical, immunologic and virologic variables of CHB patients in Group M before and after treatment

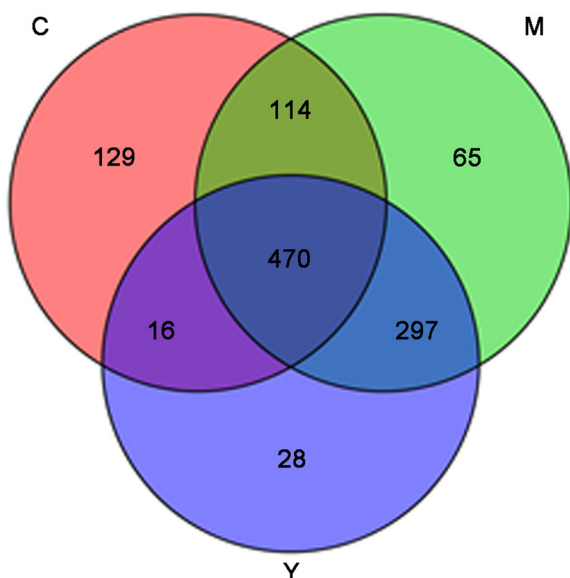
Variables	Before (n = 30)	After (Group Y) (n = 30)	Statistics	P
ALT (U/l)	226.50 (156.00, 313.00)	85.47 ± 34.07	– 4.782	< 0.001
IL-6 (pg/ml)	4.09 (2.00, 6.89)	2.00 (2.00, 3.80)	– 3.823	< 0.001
IL-8 (pg/ml)	38.55 (11.30, 148.90)	21.44 (9.78, 56.46)	– 4.782	< 0.001
TNF (pg/ml)	13.21 (8.51, 18.71)	7.16 (5.43, 9.46)	– 4.782	< 0.001
HBVDNA (IU/ml)	5.06 × 10 ⁷ (5.70 × 10 ⁶ , 1.00 × 10 ⁸)	8.67 × 10 ² (1.00 × 10 ² , 6.81 × 10 ³)	– 4.785	< 0.001

The top ten unique OTUs in Group M were: OTU854 (p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Prevotella 9), OTU764 (p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__Coprococcus 2), OTU843 (p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Prevotellaceae; g__Alloprevotella), OTU252 (p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales;

f__Bacteroidaceae; g__Bacteroides), OTU719 (p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__Lachnospiraceae UCG-003), OTU311 (p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__Ruminococcus 1), OTU716 (p__Firmicutes; c__Clostridia; o__Clostridiales; f__Ruminococcaceae; g__[Eubacterium] coprostanoligenes), OTU906 (p__Firmicutes; c__Clostridia;

Table 5 Blood biochemical, immunologic and virologic variables of CHB patients in Group M and Group A after treatment

Variables	Group A after treatment (Group N) (n = 30)	Group M after treatment (Group Y) (n = 30)	Statistics	P
ALT (U/l)	90.50 (57.25,125.25)	85.47 ± 34.07	– 0.939	0.348
IL-6 (pg/ml)	2.00 (2.00,4.74)	2.00 (2.00,3.80)	– 1.040	0.298
IL-8 (pg/ml)	22.23 (10.46,57.30)	21.44 (9.78,56.46)	– 0.414	0.679
TNF (pg/ml)	7.69 (6.44,12.51)	7.16 (5.43,9.46)	– 1.375	0.169
HBVDNA (IU/ml)	1.54×10^3 (1.00×10^2 , 5.32×10^4)	8.67×10^2 (1.00×10^2 , 6.81×10^3)	– 0.794	0.427

**Fig. 1** Venn diagram of OTUs in different groups

o_Clostridiales; f_Ruminococcaceae; g_Ruminococcaceae UCG-010), OTU855 (p_Firmicutes; c_Clostridia; o_Clostridiales; f_Ruminococcaceae; g_Ruminococcaceae UCG-014), OTU887 (p_Bacteroidetes; c_Bacteroidia; o_Bacteroidales; f_Bacteroidaceae; g_Bacteroides).

The top ten unique OTUs in Group Y were: OTU999 (p_Firmicutes; c_Negativicutes; o_Selenomonadales; f_Veillonellaceae; g_Megasphaera), OTU1154 (p_Proteobacteria; c_Betaproteobacteria; o_Burkholderiales; f_Alcaligenaceae; g_Sutterella), OTU1022 (p_Firmicutes; c_Clostridia; o_Clostridiales; f_Ruminococcaceae; g_[Eubacterium] coprostanoligenes group), OTU939 (p_Proteobacteria; c_Betaproteobacteria; o_Burkholderiales; f_Alcaligenaceae; g_Sutterella), OTU1106 (p_Actinobacteria; c_Coriobacteriia; o_Coriobacteriales; f_Coriobacteriaceae; g_Collinsella), OTU1108 (p_Bacteroidetes; c_Bacteroidia; o_Bacteroidales; f_Bacteroidales S24-7 group), OTU1093 (p_Firmicutes; c_Clostridia; o_Clostridiales; f_Ruminococcaceae; g_[Eubacterium] coprostanoligenes group), OTU1098 (p_Firmicutes; c_Clostridia; o_Clostridiales; f_Ruminococcaceae; g_Ruminococcaceae UCG-008), OTU1129 (p_Firmicutes; c_Clostridia; o_Clostridiales; f_Peptostreptococcaceae; g_Peptoclostridium), OTU957 (p_Firmicutes; c_Clostridia; o_Clostridiales; f_Lachnospiraceae; g_Anaerostipes).

Table 6 OTU distribution of CHB patients in different groups

Variables	M Group	Y Group	A Group	N Group	C Group
OTU (n)	204.62 ± 75.00	188.12 ± 48.20	216.81 ± 70.91	204.03 ± 69.70	230 ± 57.08

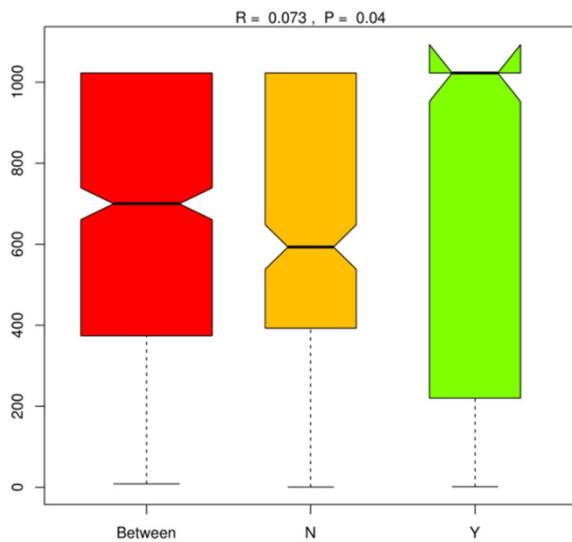


Fig. 2 ANOSIM analysis of OTU in CHB patients after ETV treatment (N) and after ETV + CB treatment (Y)

The abundance of OTU is shown in Table 6. The healthy controls had the most abundant OTU, followed by the CHB patients before treatment and the CHB patients after treatment with ETV, and the abundance of OTU in the CHB patients after treatment with ETV + CB was the least. ANOSIM analysis is shown in Fig. 2. There was significant difference in the

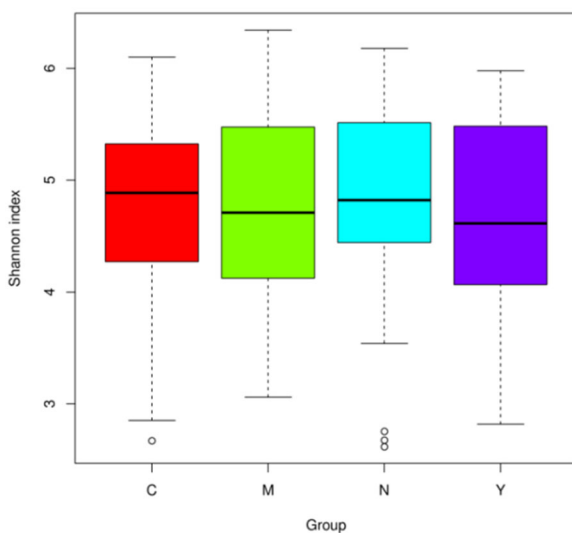


Fig. 3 Shannon index analysis of intestinal flora in different groups

abundance of OTUs between Groups N and Y ($P = 0.04$, $R = 0.073$).

Effects of CB on the Diversity of Intestinal Flora in the CHB Patients

Shannon-Wiener diversity index analysis is shown in Fig. 3. The median Shannon index was 4.886, 4.819, 4.710 and 4.614 in Groups C, N, M and Y, respectively. This indicated that controls had the highest abundance, followed by the CHB patients after ETV treatment and CHB patients before treatment; patients treated with ETV + CB had the lowest abundance of intestinal flora.

Effect of CB on the Abundance of Intestinal Flora in the CHB Patients

The intestinal flora was investigated at the genus level in different groups, the top 30 bacteria with high abundance were determined, and the histogram was delineated (Fig. 4).

Comparisons between Groups Y and M: the unique species in Group M included *Lactococcus*, *Campylobacter*, *Catenisphaera*, *Coprobacter*, Lachnospiraceae UCG-003, Lachnospiraceae UCG-004, Oxalobacter, Senegalimassilia, Defluviitaleaceae UCG-011, Oscillibacter, *Eubacterium*

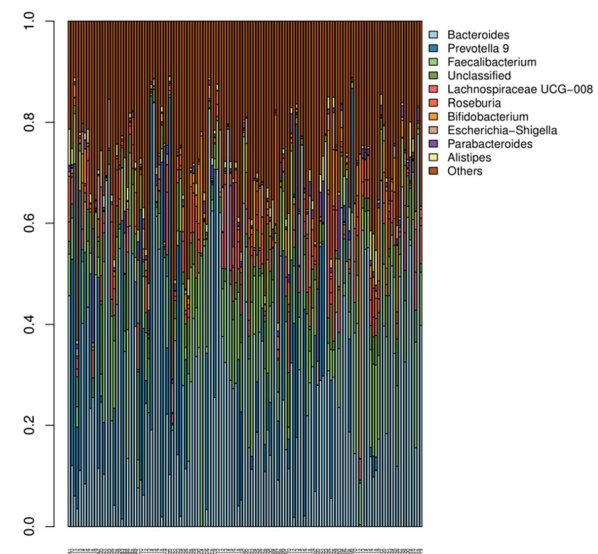


Fig. 4 Top 30 species with high abundance in different groups

rectale group, *Proteus*, and *Lachnoclostridium* 5; the unique species in Group Y included *Acidaminococcus*, *Ruminococcaceae* UCG-008, *Sellimonas* and *Methanobrevibacter*; the abundance of the following species in Group M was significantly higher than in Group Y ($P < 0.05$): *Coprococcus* 2, *Haemophilus*, *Pseudobutyribrio*, *Prevotella* 9, *Gemella*, *Olsenella*, *Weissella* and *Peptococcus*; the abundance of the following species in Group Y was slightly higher than in Group M: *Peptostreptococcus*, *Bifidobacterium*, *Murdochiella* and *Ruminiclostridium* 9.

For comparisons between Groups Y and N, the unique species in Group N included *Howardella*, *Lactococcus*, *Oxalobacter*, *Eubacterium rectale* group, *Campylobacter*, *Coprobacter*, *Dysgonomonas*, *Faecalicoccus*, *Lachnoclostridium* 5, *Lachnospiraceae*, *Lachnospiraceae* UCG-003, *Mobilitalea*, *Oscillibacter*, *Prevotellaceae* UCG-001, *Proteus*, *Rikenellaceae* RC9 gut group, *Senegalimassilia*, *Slackia*, *Succinivibrio*, *Defluviitaleaceae* UCG-011, *Mesorhizobium*, *Gluconobacter*, *Plesiomonas*, *Acinetobacter* and *Morganella*. The unique species in Group Y included *Ruminococcaceae* UCG-008, *Sellimonas*, *Phaseolus acutifolius* (tepyary bean) and *Prevotellaceae* NK3B31 group. The abundance of the following species in Group Y was significantly higher than in Group N ($P < 0.05$): *Erysipelotrichaceae* UCG-003, *Aeromonas*, *Fusicatenibacter*, *Murdochiella*, *Pseudobutyribrio*, *Eggerthella*, *Bifidobacterium*, *Coprococcus* 1 and *Faecalibacterium*. The abundance of the following species in Group N was significantly higher than in Group Y ($P < 0.05$): *Prevotella* 9, *Gemella*, *Olsenella*, *Coprococcus* 2, *Escherichia-Shigella*, *Haemophilus*, *Ruminococcaceae* UCG-009, *Alloprevotella*, *Clostridium sensu stricto* 1 and *Parasutterella*.

Effect of CB on the Intestinal Flora of CHB Patients

The results of Metastats analysis of intestinal flora at the phylum level between Groups Y and M are shown in Fig. 5. There was significant difference in the following top five species between Groups M and Y ($P < 0.05$): Firmicutes,

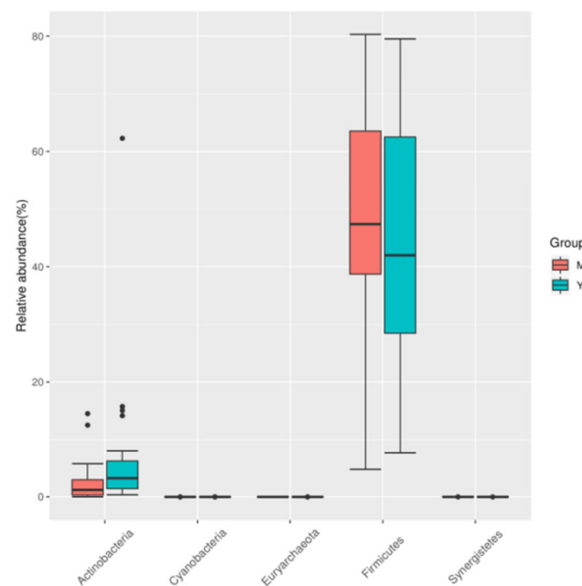


Fig. 5 Metastats analysis of intestinal flora in CHB patients before treatment (M) and after ETV + CB treatment (Y)

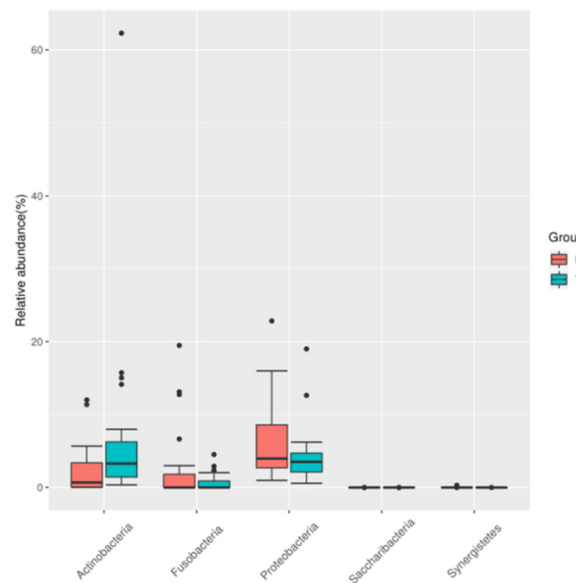


Fig. 6 Metastats analysis of intestinal flora in CHB patients after ETV treatment (N) and after ETV + CB treatment (Y)

Actinobacteria, Cyanobacteria, Euryarchaeota and Synergistetes. The abundance of Firmicutes in Group M was higher than in Group Y, and

that of Actinobacteria in Group M was lower than in Group Y.

The results of Metastats analysis of intestinal flora at the phylum level between Groups Y and N are shown in Fig. 6. There was significant difference in the following top five species between Groups N and Y ($P < 0.05$): Actinobacteria, Fusobacteria, Proteobacteria, Saccharibacteria and Synergistetes. The abundances of Fusobacteria and Proteobacteria in Group N were higher than in Group Y, and that of Actinobacteria in Group N was lower than in Group Y.

DISCUSSION

There are about 10–100 trillion bacteria in the human gut, and they can be divided into 500–1500 species. The intestinal flora may affect the development and physiology of the host and plays an important role in the metabolism and immune regulation. The intestinal flora is in a dynamic equilibrium under normal conditions. In pathologic conditions, the intestinal microenvironment may facilitate the overgrowth of certain bacteria, which manifests as a decline in flora diversity, leading to diseases.

CHB is one of the common infectious diseases; it is difficult to cure CHB, and most patients require long-term or even lifelong treatment. Thus, CHB has become a challenge for global public health [5]. The intestine and liver share an embryonic origin: the foregut. Intestinal lymphocytes originate from the developing liver. After maturation, these cells communicate with each other through the portal vein and the biliary system, and there is an inseparable connection between anatomical and biologic functions [6, 7]. Marshall formally proposed the concept of the “gut-liver axis” in 1998 [8]. Intestinal bacterial translocation may cause the release of bacterial endotoxins (such as bacterial lipopolysaccharide, LPS, peptidoglycan, lipoprotein, etc.) into the circulation, which leads to the production of some inflammatory factors, resulting in liver damage [9–11]. In recent years, advances in science and technology such as metagenomics and

metabolomics have revealed that the metabolites of intestinal flora can enter the systemic circulation and then affect the functions of the liver and other organs. Studies have summarized the role of intestinal flora in the regulation of physiology and pathology of the host, and a majority of studies have focused on the role of the intestinal-liver axis in the pathogenesis of some diseases [12–25]. It has been reported that the change in the composition of intestinal microbiota, bacterial translocation and subsequent immune-mediated injury will affect the occurrence and development of inflammation in the liver. In addition, intestinal flora may become an important target for the prevention or management of HBV-induced chronic liver disease. Fecal microbiota transplantation (FMT) has been proposed as an effective treatment for CHB-related diseases in the future. However, data in this area are still limited.

Some investigators have conducted a case-control, open-label prospective study in which CHB patients who were still positive for HBeAg after long-term NA treatment received FMT treatment [19]. Eighteen patients with CHB who were still positive for HBeAg after treatment with ETV or tenofovir for > 3 years were included. Of these patients receiving antiviral therapy, 5 received additional FMT treatment and 13 served as controls. At the end of study, the HBeAg titer in the FMT group significantly reduced compared to that at baseline. Two patients achieved HBeAg clearance after FMT treatment, and being negative for HBeAg was noted after two FMT treatments in one patient. In contrast, no controls were negative for HBeAg. Although the sample size was small in this study, it suggests that the intestinal microecologic environment affects the efficacy of antiviral therapy in CHB patients.

Although many studies have shown that probiotics are beneficial for the chronic liver disease, it is still controversial. A study investigated the efficacy of probiotic therapy in alleviating the small intestinal bacterial overgrowth (SIBO) and intestinal permeability of chronic liver disease patients [26]. Fifty-three patients with chronic liver disease were randomly divided into probiotic therapy or placebo therapy group, and six species of bacteria were used:

Bifidobacterium bifidum, *Bifidobacterium lactis*, *Bifidobacterium longum*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* and *Streptococcus thermophilus*. After 4-week treatment, the stool samples were collected, and the SIBO, intestinal permeability and clinical symptoms were assessed. The results showed that the abundance of three of six probiotics in the stool increased in the probiotic treatment group, while there was no change in the fecal microbiota of the placebo group. Some SIBO disappeared in the probiotic treatment groups, but none disappeared in the placebo group (24–0%, $P < 0.05$); the general gastrointestinal symptoms were also improved in the probiotic group, and the intestinal permeability was also improved in the probiotics group compared to the placebo group (50–31.3%, $P = 0.248$); the number of lactobacillus in the stool was negatively related to the intestinal permeability (P trend < 0.05); the hepatic chemistry was comparable between the two groups. These results suggest that short-term probiotic treatment in chronic liver disease patients is effective to improve SIBO and clinical symptoms, but has little influence on the intestinal permeability and liver function.

The traditional microbial identification methods often have difficulty identifying many microorganisms with complex growth habits. In the present study, 16S rDNA amplification subsequent sequencing was employed to investigate the diversity of intestinal flora of CHB patients, and the intestinal flora were compared between CHB patients and healthy controls. In addition, ETV and CB (bacteria that can produce butyric acid) were administered to CHB patients. The biochemical, immunologic and virologic variables were assessed, and the intestinal flora were compared in patients before and after treatment and between groups.

In our study, the serum levels of ALT, IL-6, IL-8, TNF and HBV DNA decreased significantly in CHB patients after ETV treatment, suggesting that the blood biochemistry, immunity and virology are improved in CHB patients after ETV treatment. The additional CB treatment further improves the blood biochemistry, immunity and virology of these patients although no statistical difference was observed. Long-term

investigation is needed to confirm our findings. There is evidence showing that probiotic supplementation helps improve liver inflammation, which is consistent with our findings [27].

Clustering analysis of OTU in this study showed that each group had a unique OTU, the abundance of the unique OTU was the highest in the healthy control group, and the abundance of OTU decreased in the CHB patients after ETV + CB treatment. Anosim analysis showed that the OTU of intestinal flora was significantly different between groups. There was marked difference in the OTU between the ETV group and ETV + CB group. Shannon index analysis showed that the abundance of species was the highest in the healthy control group, followed by the ETV group and then untreated group, and patients treated with ETV + CB had the lowest abundance of species of intestinal flora. There was marked difference in the intestinal flora between the ETV group and ETV + CB group. The ETV group and ETV + CB group had their own unique species. At the phylum level, the top five species with significant differences in abundance between the untreated group and ETV + CB group included Firmicutes, Actinobacteria, Cyanobacteria, Euryarchaeota and Synergistetes. At the phylum level, the top five species with significant difference in the abundance between the ETV group and ETV + CB group included Actinobacteria, Fusobacteria, Proteobacteria, Saccharibacteria and Synergistetes.

With the in-depth investigations of the gut-liver axis, increasing attention has been paid to the role of gut microecology in the occurrence and development of CHB. A supplement with the unique or abundant species of intestinal flora observed in healthy people and inhibition of the unique or abundant species in patients may provide potentially effective therapy targeting intestinal microorganisms. More studies with large sample size, refined disease stratification, extended time of intervention, and screening and functional analysis of key differential bacteria are needed to reach an in-depth understanding of the impact of long-term intervention on the intestinal flora of CHB patients, which may provide evidence for the individualized treatment of CHB.

CONCLUSION

ETV treatment improves the serum biochemical, immunologic and virologic variables, but additional CB fails to further improve these variables. Of note, additional CB affects the intestinal flora in the CHB patients treated with ETV.

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Compliance with Ethics Guidelines. Approval was obtained from the Ethics Committee of the Tongji Hospital, Tongji University School of Medicine (K-2016–056). The procedures used in this study adhere to the Declaration of Helsinki. We thank the participants of the study.

Data Availability. The datasets generated during and analyzed during the current study are available from the corresponding author on reasonable request.

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