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RESEARCH ARTICLE

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Gut microbiota dysbiosis associated with plasma levels of Interferon-γ and viral load in patients with acute hepatitis E infection

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Abstract

Few studies have focused on the effect of hepatitis E virus (HEV) infection on gut microbiota. To explore the relationship between changes in gut microbiota and inflammatory factors and viral load, we conducted a comparative study of 33 patients with acute hepatitis E (AHE) patients and 25 healthy controls (HCs) using high-throughput 16S ribosomal ribonucleic acid gene sequencing. Shannon and Simpson's indices showed no significant differences in bacterial diversity between the AHE and HCs groups. *Proteobacteria*, *Gammaproteobacteria*, and *Enterobacteriaceae* were most abundant in the AHE group, which contributed to the difference between the gut microbiota of the AHE and HCs groups, and the same difference between the HEV-RNA-positive and HEV-RNA-negative groups. Functional prediction analysis showed that ribosome, purine metabolism, and two-component system were the top three pathways. Compared with the AHE group with normal interferon (IFN)-γ, *Proteobacteria*, *Gammaproteobacteria*, *Xanthomonadaceae*, and *Enterobacteriaceae* were more abundant in the high-IFN-γ group. The abundance of *Gammaproteobacteria* was positively correlated with the level of serum alanine transaminase and total bilirubin. The abundance of *Gammaproteobacteria* could discriminate AHE patients from HCs, and could better predict the severity of AHE patients. We believe that our findings will contribute toward a novel treatment strategy for AHE.

KEYWORDS
disease severity, dysbiosis, gut microbiota, hepatitis E virus, interferon-γ, viral load

Abbreviations: AHE, acute hepatitis E; HCs, healthy controls; HEV, hepatitis E virus; IFN-γ, interferon γ; IgM, immunoglobulin M; LEfSe, linear discriminant analysis effect size; OUT, operational taxonomic unit; PCA, principal coordinate analysis.

1 | INTRODUCTION

Hepatitis E is a liver infection caused by the hepatitis E virus (HEV) with single-stranded RNA.[1,2] Hepatitis E is usually self-limiting, but a few patients may develop severe hepatitis, such as pregnant women and elderly people with changes in immune response.[3] There are four types of mammalian HEV; among which, HEV genotypes 1 and 2 are limited to human infection, while genotypes 3 and 4 cause zoonotic disease, with a wide host range.[4] HEV is mainly transmitted through the fecal-oral route. Usually, immunocompetent individuals can spontaneously resolve HEV infection without complications.[5] However, some patients whose immunity is unable to resist the virus have symptoms of acute viral hepatitis such as jaundice, hepatomegaly, vomiting, nausea, abdominal pain, and fever. In patients with low immunity, HEV causes chronic liver infection, liver failure, and extrahepatic symptoms.[6,7] Infection is the most common complication of viral liver disease and one of the main causes of death. Most of the pathogenic bacteria causing the infection of patients come from the normal gut microbiota, which is closely related to various biological mechanisms of the liver.[8] Recently, the relationship between changes in gut microbiota and liver disease has been a hot research topic.

Many studies have confirmed that patients with liver disease have different degrees of gut microbiota disorder, and inflammatory factors and immune cells are involved in a variety of physiological and pathological processes in the liver, which are closely related to the occurrence and development of liver disease.[9–11] Lu et al.[12] detected the main gut microbiota of healthy people, asymptomatic hepatitis B virus carriers, and patients with chronic hepatitis B by 16S ribosomal ribonucleic acid (rRNA) gene sequencing technology, and found that with disease progression, the ratio of *Bifidobacteriaceae* and *Enterobacteriaceae* in the intestine gradually decreased. Wei et al.[13] revealed that compared with the healthy group, the gut microbiota of hepatitis B cirrhosis patients had a lower level of *Bacterobacteriaceae*, and contained a higher level of *Enterobacteriaceae*, *Wei Rong's cocci*, and *streptococci*. The gut microbiota in hepatitis B cirrhosis patients is enriched in the genes and proteins related to the transport and metabolism of the executive substance (mainly amino acids and carbohydrates), indicating that the metabolic activity of gut microbiota in cirrhosis is increased. Our previous research has confirmed that altered gut microbiota is associated with the development and exacerbation of HEV infection.[14] However, the small number of patients and the absence of healthy controls (HCs) made it impossible to confirm the role of gut microbiota in the occurrence of hepatitis E.

To explore further the relationship between the gut microbiota and the occurrence of acute hepatitis E (AHE), we compared 33 AHE patients and 25 HCs using high-throughput 16S rRNA gene sequencing. The study was carried out in response to the changes in gut microbiota, plasma cytokines, and viral load in AHE patients. We believe that our findings will contribute toward a novel treatment strategy for AHE.

2 | MATERIALS AND METHODS

2.1 | Study population

We enrolled 33 AHE patients and 25 HCs who were referred to the First Affiliated Hospital of Zhejiang University School of Medicine, and the First People's Hospital of Yancheng City between 1 September 2018 and 1 June 2019. We followed up with all the AHE patients until 30 January 2020. As in our previous study,[15] the diagnosis of hepatitis E was based on positive serum anti-HEV immunoglobulin M, and/or more than twofold increase in anti-HEV lgG titer, and/or HEV RNA, in combination with clinical manifestations of acute hepatitis. The following exclusion criteria were established: coinfection with hepatitis A virus, hepatitis B virus or other hepatitis viruses; alcoholic liver, fatty liver, and other liver diseases caused by nonviral hepatitis; use of antibiotics, probiotics, prebiotics, or synbiotics during the previous month; active bacterial, fungal, chlamydial or viral infection; irritable bowel syndrome, inflammatory bowel disease or other autoimmune diseases; and patients with incomplete data. The protocol for the present study was approved by the Ethics Committee of the First Affiliated Hospital of Zhejiang University School of Medicine (Approval Number: 2020454). Informed consent was obtained from all participants or their families.

2.2 | Fecal sample collection and microbial DNA extraction

Participants' fecal samples (2 g) were collected in sterile plastic cups and stored at −80°C for further extraction of bacterial genomic DNA within 15 min. Agarose gel (1%) was used to determine DNA concentration and purity. CTAB method was used to extract total genomic DNA from fecal samples. A total of 1000 μl CTAB lysate was sucked into a 2.0 ml EP tube, and 20 μl lysozyme was added to the EP tube. An appropriate amount of the sample was added to the lysate and placed in a water bath at 65°C (2–3 h). During this time, the mixture was reversed several times to allow the sample to fully decompose. After centrifugation, the supernatant of 950 μl was sucked out, and the mixture of phenol (PH 8.0), chloroform, and isoamyl alcohol (25:24:1) was added to the supernatant in equal volume. A total of 12 000 rpm for 10 min, the supernatant was absorbed and an equal volume of chloroform:isoamyl alcohol (24:1) was added. A total of 12 000 rpm for 10 min, then we removed the supernatant into a 1.5 ml centrifuge tube, added 3/4 of the supernatant volume of isopropyl alcohol, shook it up and down, and precipitated at −20°C. A total of 12 000 rpm for 10 min, and pour out the liquid. The centrifuge tube was washed twice with 1 ml of 75% ethanol. The remaining small amount of liquid could be collected again by centrifugation and then sucked out with a micropipettor. We dried the samples at room temperature. A total of 51 μl of ddH2O was added to dissolve the DNA sample, which could be incubated at 55–60°C for 10 min. Added 1 μl of RNase A to digest RNA, and placed at 37°C for 15 min.

2.3 | Amplicon library construction

All genes in different regions of 16S rRNA (16S V4/16S V3/16S V3-v4/16S v4-v5-16s V4-V5) were amplified by specific primers (e.g., 16S V4: 515F-806R) and barcodes. PCR was performed using Phusion® High-Fidelity PCR Master Mix (New England Biolabs). A total of 0.2 μM of forward and reverse primers, and about 10 ng template DNA. The thermal cycle consisted of denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and elongation at 72°C for 30 s, with a final extension at 72°C for 5 min. PCR products were mixed in an equal density ratio. Qiagen gel was purified from the mixed PCR products using an extraction kit (Qiagen). TruSeq®DNA PCR-free sample preparation kit (Illumina) was used to generate the sequencing library, following the manufacturer's recommendations and index code. The Qubit@2.0 fluorometer (Thermo Fisher Scientific) and an Irelen Bioanalyzer 2100 system were used to evaluate the quality of the library.

2.4 | 16S rRNA sequencing

After the constructed library passed qubit quantification and library detection on the Qubit@ 2.0 Fluorometer (Thermo Fisher Scientific), 400/600 bp single products were sequenced on an Ion S5 TM XL platform.

2.5 | HEV RNA detection

Internally controlled quantitative real-time reverse transcription PCR was used to detect HEV RNA, as described previously.[14] Total RNA was extracted from serum according to the manufacturer's instructions (Aikang). The 348-nucleotide fragment of the HEV open reading frame 2 was amplified by nested PCR and sequenced to determine the genotype. The diagnostic kit for HEV RNA (Aikang) was used to estimate the viral load of each sample by quantitative PCR.

2.6 | Plasma cytokines measurements

The levels of plasma cytokines (interferon [IFN]-γ, tumor necrosis factor [TNF]-α, interleukin [IL]−4 and IL-10) were tested by ProcartaPlex (eBioscience). The value of samples was ≤0.2 pg/ml, indicating undetectable concentrations with Milliplex Map Kit.[16]

2.7 | Bioinformatics analysis

The 16S rRNA gene sequence data set generated by the Illumina MiSeq platform was imported into QIIME2 (version 2020.11), and the steps of sequence processing and quality control all adopted default parameters. Multiple estimates were used to evaluate the diversity and abundance of bacteria, including the level of operational taxonomic units (OTUs), the Chao1, Shannon, and Simpson indices, and so on. The KEGG pathway analysis was conducted using Picrust2 software. The principal coordinates analysis was employed to investigate the relationship between the AHE group and the HCs group. The effect size method of linear discriminant analysis was applied to the OTU table to determine the abundances of different bacterial taxa. The output files were further analyzed using Metagenomic profiling (STAMP) software.

2.8 | Statistical analysis

GraphPad Prism 9 was used for the analysis. The receiver operating characteristic (ROC) curve analysis was used to assess the predictive ability of *Gammaproteobacteria*. Continuous data were expressed as mean ± *SD* and were analyzed using the Student *t* test. A $p < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Altered bacterial diversity in AHE and HCs groups

There were no significant differences noted in gender, age, body mass index (BMI), or race between the AHE and HCs groups ($p > 0.05$, Table 1). From 58 fecal samples, we obtained 4 442 263 high-quality sequences, accounting for 95.1% of the 4 671 149 valid sequence reads (Table S1). The average read length was 412 bp (range: 394–427 bp). The total number of sequences that were unique in the two groups was 14 073, and all phylotypes were represented. The HCs group had 5632 OTUs, and the AHE group had 8441. In the two groups, the coverage of all sequences was about 99.8%, which indicated that the study of HEV-related gut microbiota had good sequencing depth.

There were no significant differences in Shannon and Simpson indices between the two groups (both $p > 0.05$, Figure S1 in Supporting Information). According to the rarefaction analysis Estimates (Figure S2 in Supporting Information), the trend of species richness in HCs was lower than that of the AHE group. In the rank abundance curves, there was a long tail in the OTU analysis, which indicated that the majority of OTUs were present at low abundance (Figure S3 in Supporting Information). PCA plot showed that there was no obvious separation between the two groups (Figure S4 in Supporting Information). A Venn diagram was used to analyze the bacterial richness using the number of shared and unique OTUs and compare the OTUs between the two groups (Figure S5 in Supporting Information).

3.2 | Altered gut microbiota composition in AHE and HCs groups

We used Metastats to investigate the alterations of gut microbiota composition between the AHE and HCs groups. At the phylum level,

TABLE 1 Characteristics of the enrolled subjects

Variables	AHE group ($n = 33$)	HCs group ($n = 25$)	p
Clinical characteristics			
Age (year)	51.21 ± 12.28	47.68 ± 12.14	0.280
Gender (F/M)	12/21	9/16	0.977
BMI	22.52 ± 1.22	22.35 ± 1.38	0.624
Laboratory parameters			
WBC (10^9/L)	6.30 (4.85–8.25)	5.70 (4.45–8.75)	0.808
PLT (10^9/L)	192.00 (141.50–253.00)	198.00 (132.00–236.50)	0.505
ALT (U/L)	691.00 (264.50–849.00)	24.00 (15.50–33.00)	<0.001
AST (U/L)	698.00 (388.00–1373.00)	27.00 (18.00–35.00)	<0.001
TP (g/L)	69.82 ± 5.02	70.62 ± 4.47	0.532
ALB (g/L)	41.75 ± 4.62	42.82 ± 4.78	0.389
GGT (U/L)	710.00 (463.00–1052.00)	24.00 (17.50–32.50)	<0.001
TBIL (umol/L)	174.30 (112.95–262.35)	10.70 (6.45–13.65)	<0.001
CHE (U/L)	4858.00 (2916.50–7167.50)	6891.00 (5013.50–9699.50)	0.003
TCH (mmol/L)	4.93 (3.70–6.20)	4.02 (3.35–4.46)	0.031
UREA (mmol/L)	4.01 (3.04–5.47)	4.46 (3.20–5.44)	0.689
CR (umol/L)	88.45 ± 26.75	92.08 ± 28.14	0.619
INR	1.14 (1.06–1.26)	0.94 (0.84–1.07)	<0.001
GLU(mmol/L)	4.70 (4.40–5.60)	4.90 (4.45–5.65)	0.609
AFP (ng/ml)	54.10 (10.50–219.05)	11.40 (3.65–17.45)	<0.001
IFN-γ (pg/ml)	27.48 (21.93–29.64)	2.42 (1.74–4.60)	<0.001
TNF-α (pg/ml)	2.50 (1.18–4.46)	0.05 (0.05–0.27)	<0.001
IL-4 (pg/ml)	9.96 (7.52–13.26)	8.27 (6.13–9.85)	0.016
IL-10 (pg/ml)	1.31 (0.50–2.56)	1.65 (1.17–1.90)	0.361
HEV-RNA	20 (60.61)	0 (0.00)	<0.001
IgM (+)	33 (100.00)	0 (0.00)	<0.001
Pregnant women	0 (0.00)	0 (0.00)	–

Abbreviations: AFP, alpha fetoprotein; ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CHE, cholinesterase; CR, creatinine; GGT, γ-Glutamyl Transpeptidase; GLU, glucose; INR, international normalized ratio; PLT, platelets; TBIL, total bilirubin; TCH, total cholesterol; UREA, urea nitrogen; WBC, white blood cell.

Proteobacteria was significantly more abundant in the gut microbiota in the AHE group than the HCs group (Figure 1A). Compared with the HCs group at the class level, the abundance of *Gammaproteobacteria* was significantly increased in the AHE group (Figure 1B). At the family level, *Xanthomonadaceae* and *Enterobacteriaceae* were significantly more abundant in the gut microbiota in the AHE group than the HCs group, while the abundance of *Bifidobacteriaceae* was lower (Figure 1C).

The key phylotypes responsible for the difference between the AHE and HCs groups were identified by metagenome analysis LEfSe. *Proteobacteria*, *Gammaproteobacteria*, and *Enterobacteriaceae* were most abundant in the AHE group, which contributed to the difference between the gut microbiota of the AHE and HCs groups (Figure 1D,E).

3.3 | HEV-associated microbial functional prediction

PiCRUSt analysis showed that there were 32 KEGG pathways related to gut microbiota between the AHE and HCs groups, including 18 KEGG pathways enriched in the AHE group and 14 enriched in the HCs group. Ribosome ($P_{FDR} < 0.001$), purine metabolism ($P_{FDR} < 0.001$), and two-component system ($P_{FDR} < 0.001$) were the top three

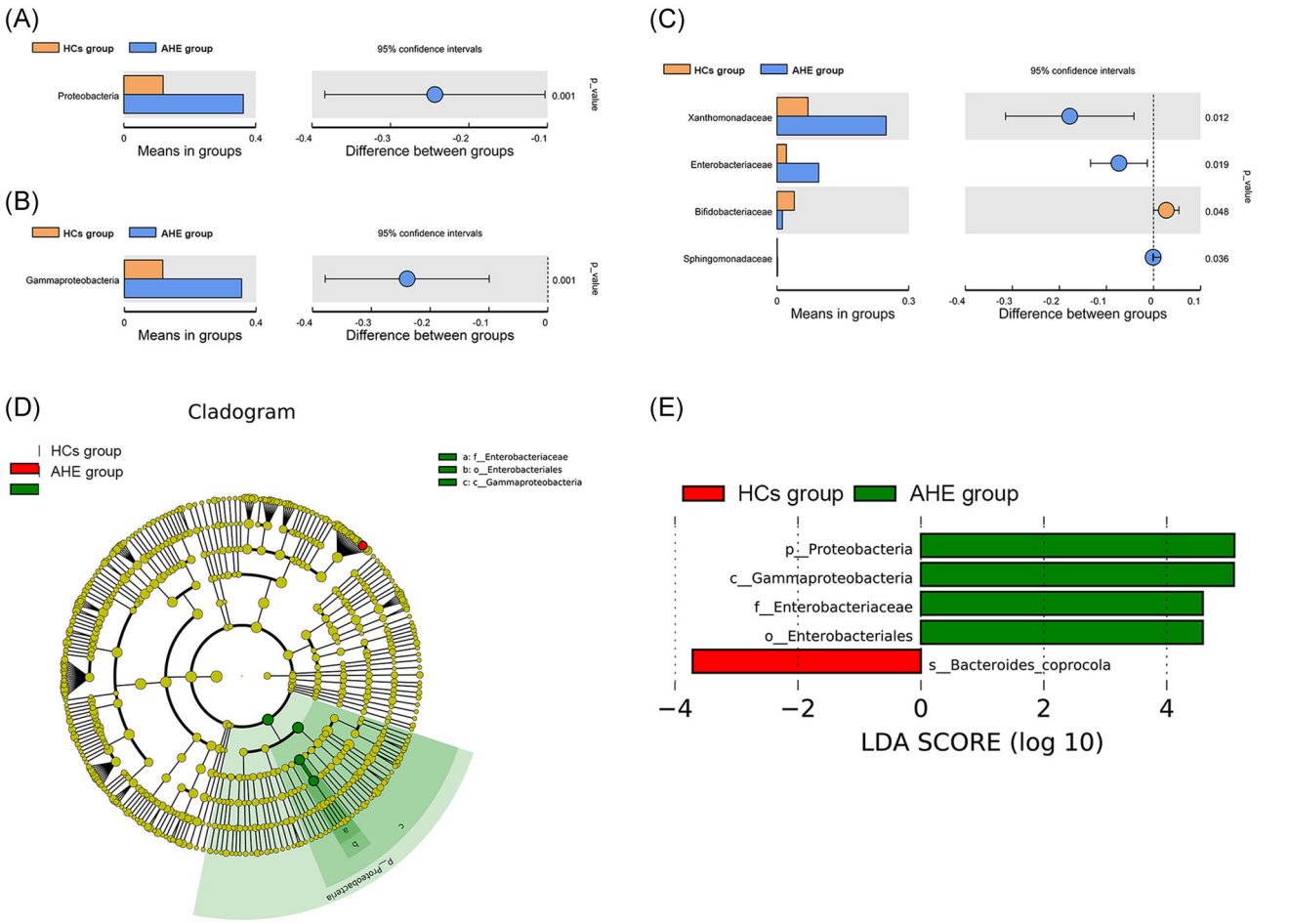

FIGURE 1 Taxonomic differences in gut microbiota composition between AHE and HCs groups. The relative abundance of gut microbiota at bacterial phylum (A), class (B), and family (C) levels between the two groups; Cladogram representing features of bacterial hierarchies determined using the LDA-based model (D) LDA Effect Size showed the different microbiota from the kingdom level to the species level between AHE group and HCs group (LDA score >4 and $p < 0.05$) (E). We perform the analysis by number of sequences 39601 per sample. AHE, acute hepatitis E; HC, healthy control

pathways, which may play crucial roles during the process of AHE infection (Figure 2).

3.4 | Dysbiosis of the gut microbiota associated with viral load in patients with AHE

HEV RNA detection showed 20 (60.61%) of the 33 AHE patients were positive (Table 1 and Table S2). Genome sequencing showed that all enrolled AHE patients had HEV genotype 4. To study the relationship between viral load and gut microbiota, 33 patients were divided into HEV-RNA-positive ($n = 20$) and HEV-RNA-negative ($n = 13$) groups.

At the phylum level, *Proteobacteria* in the HEV-RNA-positive group was significantly more abundant in the gut microbiota than in the HEV-RNA-negative group (Figure 3A). The abundance of *Firmicutes* in the HEV-RNA-negative group was significantly higher than in the HEV-RNA-positive group (Figure 3A). Compared with the HEV-RNA-negative group, the abundance of *Gammaproteobacteria* was increased in the HEV-RNA-positive group; however, the abundance of *Clostridia* was significantly reduced (Figure 3B). At the family level, *Enterobacteriaceae* in the HEV-RNA-positive group were significantly more abundant in the gut microbiota than in the HEV-RNA-negative group, while the abundance of *Ruminococcaceae* and *Lachnospiraceae* in the HEV-RNA-positive group was lower than in the HEV-RNA-negative group (Figure 3C).

The metagenomic analysis LEfSe approach showed that *Proteobacteria*, *Gammaproteobacteria*, and *Enterobacteriaceae* were more abundant in the HEV-RNA-positive group, which contributed to the difference between the gut microbiota of the HEV-RNA-positive and HEV-RNA-negative groups (Figure 3D,E).

3.5 | Dysbiosis of the gut microbiota associated with plasma levels of IFN-γ in patients with AHE

Our previous study found that IFN-γ showed a gradual upward trend from the HCs to the AHE group, but a gradual downward trend during progression of disease severity.[16] In this study, we measured the plasma levels of Th1 (IFN-γ and TNF-α) and Th2 (IL-4 and IL-10)

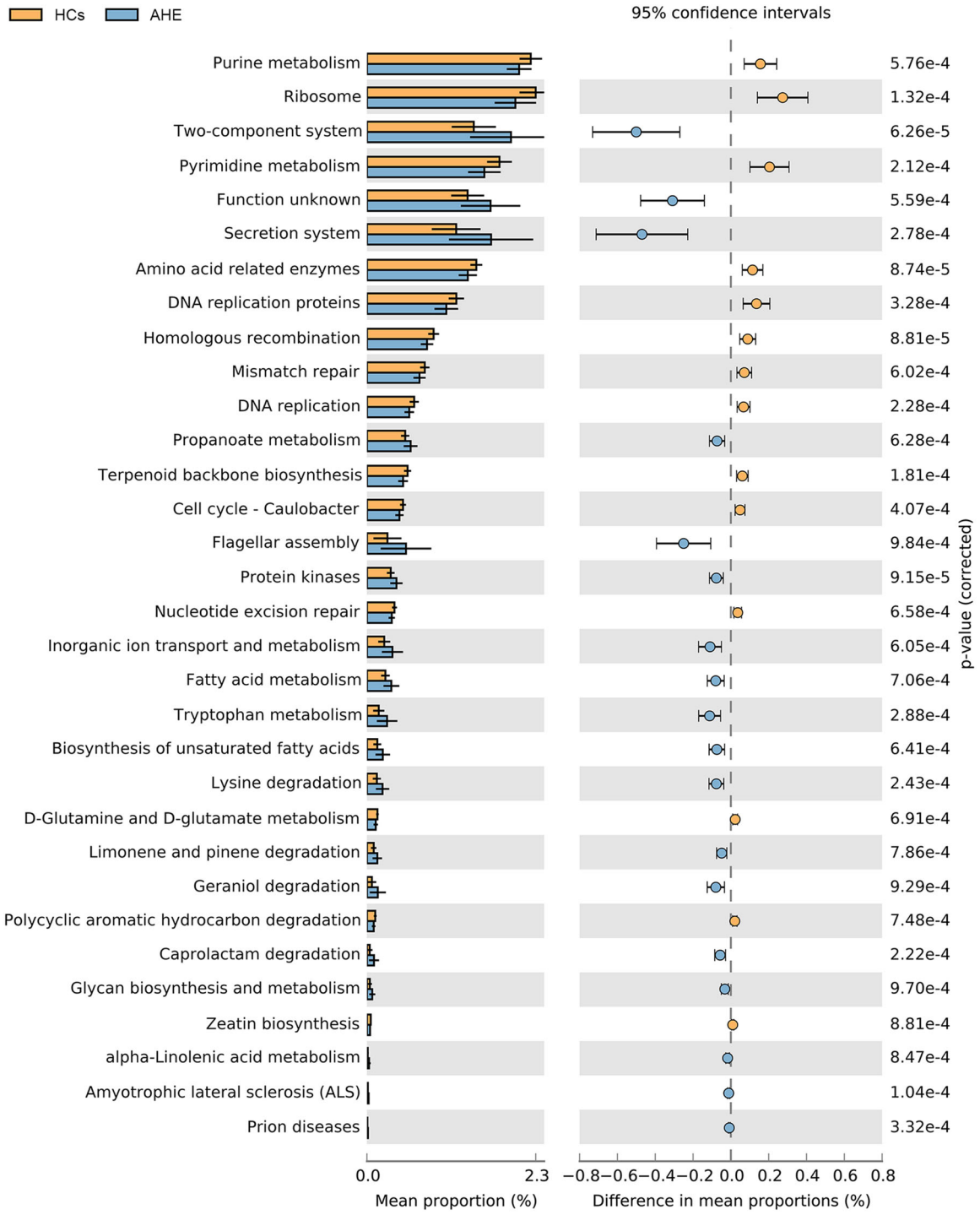

FIGURE 2 KEGG pathway analysis of the gut microbiota. A total of 18 pathways were enriched in the AHE group and 14 pathways were enriched in the HCs group. AHE, acute hepatitis E; HC, healthy control

cytokines in the AHE and HCs groups. Compared with Th1/Th2 cytokines among the HCs group, the plasma levels of IFN-γ, TNF-α, and IL-4 all showed gradual upward trends from the HCs to the AHE group (all $p < 0.001$), while there were no significant differences noted in IL-10 between the AHE and HCs groups ($p = 0.361$; Table 1 and Table S2). There were still eight (24.24%) AHE patients whose plasma levels of IFN-γ were not increased. To study the relationship between the high levels of IFN-γ and gut microbiota, we regrouped the 33 patients in the AHE according to whether the level of IFN-γ was increased [IFN-γ-high ($n = 25$) and IFN-γ-normal ($n = 8$) groups].

Metastats showed that *Proteobacteria* in the IFN-γ-high group were significantly more abundant in the gut microbiota than in the IFN-γ-normal group at the phylum level, while the abundances of *Firmicutes* was lower (Figure 4A). At the class level, compared with the IFN-γ-normal

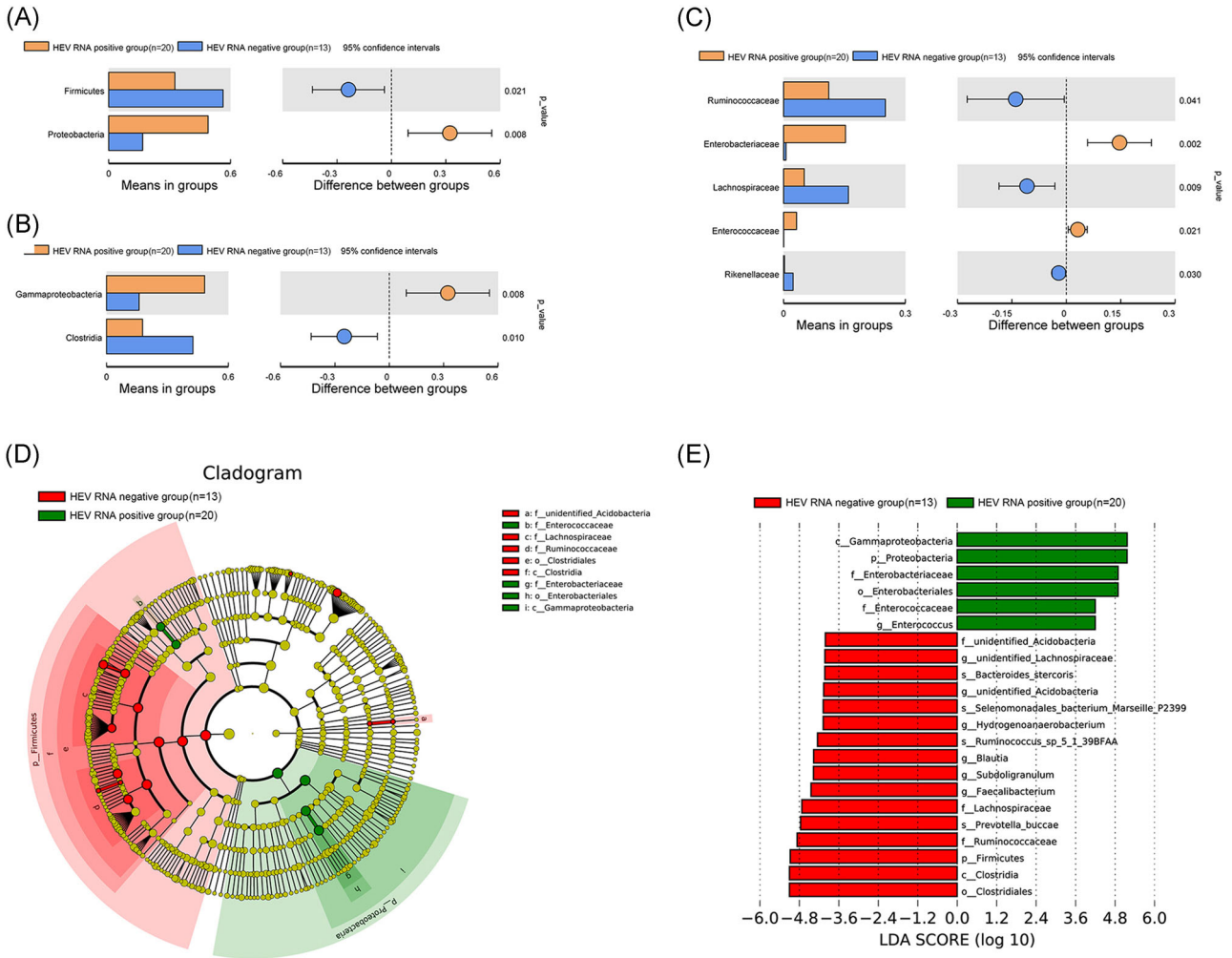

FIGURE 3 Dysbiosis of the gut microbiota associated with viral load in patients with AHE. The relative abundance of gut microbiota at bacterial phylum (A), class (B), and family (C) levels between the HEV-RNA-positive group and HEV-RNA-negative group; Comparisons of difference between the gut microbiota of the HEV-RNA-positive and HEV-RNA-negative groups with the metagenomic analysis LEfSe approach (D and E). AHE, acute hepatitis E; HEV, hepatitis E virus

group, *Gammaproteobacteria* in the IFN-γ-high group were significantly more abundant, while the abundance of *Clostridia* was lower (Figure 4B). At the family level, both *Xanthomonadaceae* and *Enterobacteriaceae* in the IFN-γ-high group were significantly more abundant in the gut microbiota than in the IFN-γ-normal group, while the abundance of *Ruminococcaceae* and *Lachnospiraceae* was lower (Figure 4C).

The metagenomic analysis LEfSe approach showed that *Proteobacteria*, *Gammaproteobacteria*, *Xanthomonadaceae*, and *Enterobacteriaceae* were more abundant in the IFN-γ-high group, which contributed to the difference between the gut microbiota of the IFN-γ-high and IFN-γ-normal groups (Figure 4D,E).

3.6 | Associations between gut microbiota and AHE-related laboratory parameters

The levels of alanine transaminase (ALT), aspartate transaminase, γ-glutamyltranspeptidase, and total bilirubin (TBIL) in the AHE group were significantly higher than in the HC group (Table 1). We evaluated correlations between the relative abundances of bacterial taxa using *Gammaproteobacteria*, and AHE-related laboratory parameters (ALT and TBIL). Abundance of *Gammaproteobacteria* was positively correlated with serum level of ALT ($r = 0.744$, $p < 0.001$; Figure 5A) and TBIL ($r = 0.732$, $p < 0.001$; Figure 5B).

ROC curves were constructed and the area under the curve (AUC) was calculated to assess the diagnostic performance of the *Gammaproteobacteria*. The *Gammaproteobacteria* could discriminate AHE from HCs with an AUC of 0.85 (95% confidence interval [CI]: 0.68–1.0) (Figure 5C).

3.7 | Predictive ability of *Gammaproteobacteria* for the severity of AHE

We followed up with 33 AHE patients and found that 28 recovered rapidly after treatment, five developed liver failure, and none developed a chronic infection.

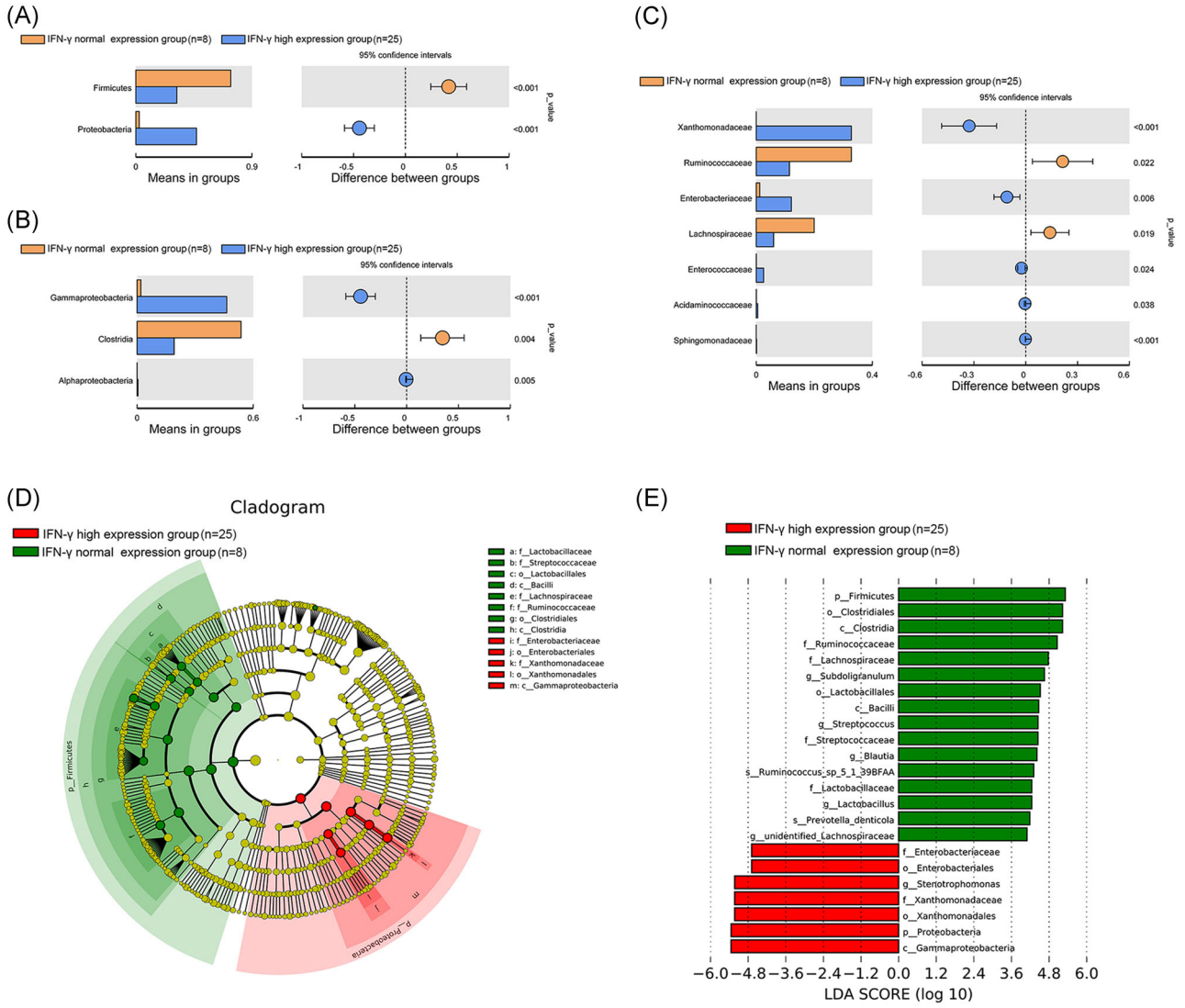

FIGURE 4 Dysbiosis of the gut microbiota associated with plasma levels of IFN-γ in patients with AHE. The relative abundance of gut microbiota at bacterial phylum (A), class (B), and family (C) levels between the IFN-γ-high group and IFN-γ-normal group; Comparisons of difference between the gut microbiota of the IFN-γ-high group and IFN-γ-normal group with the metagenomic analysis LEfSe approach (D and E). AHE, acute hepatitis E; IFN, interferon

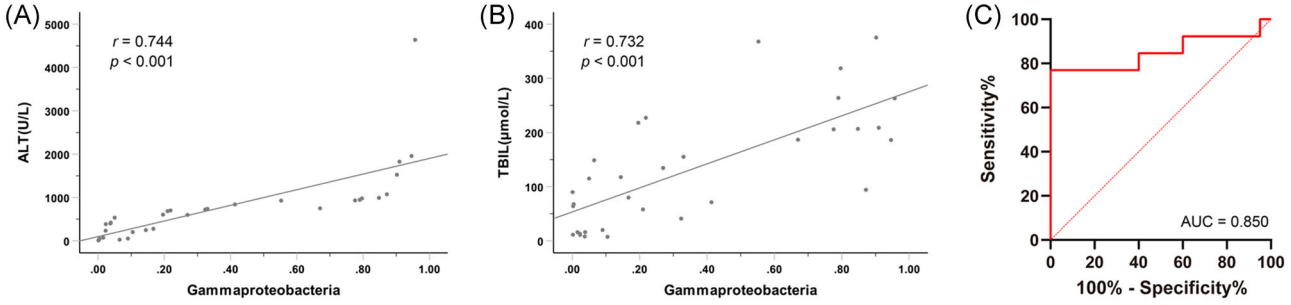

FIGURE 5 Associations between gut microbiota and AHE-related laboratory parameters. The associations between *Gammaproteobacteria* and ALT (A), and TBIL (B); The *Gammaproteobacteria* could discriminate AHE from HCs (C). ALT, alanine aminotransferase; AHE, acute hepatitis E; TBIL, total bilirubin

TABLE 2 The predictive ability of *Gammaproteobacteria* for severity of AHE patients

Variable	AHE patients (n = 33)
AUROC	0.89 (0.74–0.97)
Cutoff value	0.79
Sensitivity, %	80.0 (28.4–99.5)
Specificity, %	89.3 (71.8–97.7)
Positive predictive value, %	57.1 (18.4–90.1)
Negative predictive value, %	96.2 (80.4–99.9)
Positive likelihood ratio	7.47 (2.4–23.7)
Negative likelihood ratio	0.22 (0.04–1.3)

Abbreviation: AHE, acute hepatitis E.

We evaluated the predictive ability of *Gammaproteobacteria* for the severity of AHE (Table 2). The AUC for severity of AHE was 0.89 (95% CI: 0.74–0.97), with a sensitivity of 80.0% (95% CI: 28.4%–99.5%) and specificity of 89.3% (95% CI: 71.8%–97.7%). The cutoff value was 0.79. The positive predictive value and positive likelihood ratio were 57.1 (95% CI: 18.4–90.1) and 7.47 (95% CI: 2.4–23.7), respectively. The negative predictive value and negative likelihood ratio were 96.2 (95% CI: 80.4–99.9) and 0.22 (95% CI: 0.04–1.3), respectively.

4 | DISCUSSION

The gastrointestinal tract has the largest accumulation of bacteria and endotoxin storage in the human body and contains 10^{13}–10^{14} bacteria; most of which are Gram-negative or facultative anaerobic bacteria.[17–19] As the predominant gut microbiota, they can provide energy for the activities of intestinal epithelial cells by fermenting dietary fiber. They can antagonize the colonization of intestinal pathogens by competing for living space and activating the immune function of the intestinal mucosa, to participate in a series of physiological functions such as nutrition, immunity, and metabolism.[20] In recent years, with the establishment of the concept of the gut–liver axis, the relationship between the anatomy and biological function of the gut and liver has become increasingly close.[21–23] Under physiological conditions, due to the blocking effect of the gut mucosal barrier, only a small number of bacteria and their metabolites enter the liver through the portal vein, and maintain the liver reticuloendothelial system in a state of continuous activation, thus inducing immune tolerance to harmful substances.[24] However, in a state of liver injury, the gut barrier function is destroyed, the microbiota in the gut are out of control, and there is large-scale intestinal endotoxin translocation to the liver, which leads to runaway of the liver immune response, and the release of a large number of inflammatory media that aggravate the liver injury.[25,26] To determine the role of gut microbiota in the occurrence and development of liver diseases, especially those caused by HEV infection, could provide new ideas for clinical prevention and treatment of AHE.

In this study, we used a variety of indices based on the OTU level to evaluate the ecological features of the gut microbiota communities in the AHE and HCs groups. Shannon and Simpson's indices showed that there were no significant differences between the AHE and HCs groups. There were no significant differences noted in gender, age, BMI, or race between the two groups. Our previous study also showed that there were no significant differences between the AHE and HEV-ALF groups. We speculate that the change in gut microbiota diversity in patients with HEV infection is not obvious. This conclusion needs more samples for verification.

In the present study, we used Metastats to investigate the alterations of gut microbiota composition between the AHE and HCs groups. At the phylum level, *Proteobacteria* were significantly more abundant in the gut microbiota in the AHE group than in the HCs group. At the class level, the abundance of *Gammaproteobacteria* was significantly increased in the AHE group compared with the HCs group. As for the family level, both *Xanthomonadaceae* and *Enterobacteriaceae* were significantly more abundant in the gut microbiota in the AHE group than in the HCs group, while the abundance of *Bifidobacteriaceae* was lower. The metagenomic analysis LEfSe approach showed that *Proteobacteria*, *Gammaproteobacteria*, and *Enterobacteriaceae* were more abundant in the AHE group, which contributed to the difference between the gut microbiota of the AHE and HCs groups. Wang et al.[27] revealed that patients with the alcohol-induced chronic liver disease showed a significant increasing trend in abundance of *Proteobacteria*. Fernández et al.[28] demonstrated that patients with decompensated cirrhosis and acute-on-chronic liver failure in Europe showed a significant increasing trend in abundance of *Enterobacteriaceae*. Our previous study found that the abundance of *Proteobacteria*, *Gammaproteobacteria*, and *Enterobacteriaceae* was also increased as AHE developed into HEV-ALF.

Different from previous studies, we also studied the functional and metabolic changes in microbial communities in patients with AHE and HCs, which showed that 32 KEGG pathways associated with intestinal microbiota were significantly different between the groups. Eighteen of them were enriched in the AHE group and 14 in the HCs group. Ribosome, purine metabolism, and two-component system are the top three pathways with significant differences between the groups.

To study the relationship between viral load and gut microbiota, we compared the dysbiosis of the gut microbiota between the HEV-RNA-positive and HEV-RNA-negative groups. At the phylum level, *Proteobacteria* in the gut microbiota in the HEV-RNA-positive group were significantly more abundant than in the HEV-RNA-negative group, while the abundance of *Firmicutes* in the HEV-RNA-negative group was significantly increased compared with the HEV-RNA-positive group. At the class level, compared with the HEV-RNA-negative group, the abundance of *Gammaproteobacteria* was increased in the HEV-RNA-positive group; however, the abundance of *Clostridia* was significantly reduced. At the family level, *Enterobacteriaceae* were significantly more abundant in the gut microbiota in the HEV-RNA-positive group than in the HEV-RNA-negative group, while the abundance of *Ruminococcaceae* and *Lachnospiraceae*

in the HEV-RNA-positive group was lower than in the HEV-RNA-negative group. *Proteobacteria*, *Gammaproteobacteria* and *Enterobacteriaceae* were more abundant in the HEV-RNA-positive group, which contributed to the difference in the gut microbiota between the two groups. We also studied the relationship between plasma levels of IFN-γ and gut microbiota, which showed that *Proteobacteria*, *Gammaproteobacteria*, *Xanthomonadaceae*, and *Enterobacteriaceae* were most abundant in the IFN-γ-high group, which contributed to the difference in gut microbiota between the two groups.

The relationship between gut microbiota and degree of liver injury showed that the abundance of *Gammaproteobacteria* was positively correlated with the level of serum ALT and TBIL. The abundance of *Gammaproteobacteria* in fecal microbiota samples could discriminate AHE from HCs with an AUC of 0.850, meanwhile, *Gammaproteobacteria* also could be used to predict the severity of AHE with an AUC of 0.89.

In conclusion, our study showed that dysbiosis of the gut microbiota is associated with HEV infection, plasma levels of IFN-γ, and viral load in AHE patients. The top three top pathways were ribosome, purine metabolism, and the two-component system. The abundance of *Gammaproteobacteria* could be used to could discriminate AHE patients from HCs, and also had a high predictive value for the severity of AHE. We believe that our findings will contribute toward a novel treatment strategy for AHE.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Hongcui Cao: is the guarantor of this work; **Jian Wu and Jiong Yu:** experimental design, acquisition of data, analysis, and interpretation of data, drafting of the manuscript, statistical analysis; **Jian Wu, Guanghua Zhai, Zongxin Ling, Xiaochen Shen, and Anquan Shang:** conducted the main experiments; **Jiong Yu, Anquan Shang, Yiwen Yao, Jiong Yu, and Xiaochen:** Shen collected serum and completed the follow-up; **Mariza Bortolanza, and Bin Jiang:** acquisition of data, statistical analysis, critical revision of the manuscript; **Hongcui Cao, and Lanjuan Li:** obtained financial support for this study, study concept and design, study supervision, and critical revision of the manuscript for important intellectual content.

DATA AVAILABILITY STATEMENT

All data relevant to the study are included in the article.

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REFERENCE

1. Wu J, Guo N, Zhu L, et al. Seroprevalence of AIH-related autoantibodies in patients with acute hepatitis E viral infection: a prospective case-control study in China. *Emerg Microbes Infect*. 2020;9:332-340.
2. Kobayashi T, Takahashi M, Ohta S, et al. Production of capsid proteins of rat hepatitis E virus in Escherichia coli and characterization of self-assembled virus-like particles. *Virus Res*. 2021;302:198483. https://doi.org/10.1016/j.virusres.2021.198483
3. Yang Y-L, Nan Y-C. Open reading frame 3 protein of hepatitis E virus: multi-function protein with endless potential. *World J Gastroenterol*. 2021;27:2458-2473.
4. Ji H, Chen S, He Q, et al. The different replication between nonenveloped and quasi-enveloped hepatitis E virus. *J Med Virol*. 2021;93:6267-6277. https://doi.org/10.1002/jmv.27121
5. Niendorf S, Harms D, Hellendahl K, et al. Rattus norvegicuspresence and diversity of different enteric viruses in Wild Norway Rats. *Viruses*. 2021:13(6):992. https://doi.org/10.3390/v13060992
6. Primadharsini PP, Nagashima S, Okamoto H. Mechanism of cross-species transmission, adaptive evolution and pathogenesis of hepatitis E Virus. *Viruses*. 2021;13:909. https://doi.org/10.3390/v13050909
7. Smith I, Said B, Vaughan A, et al. Case-control study of risk factors for acquired hepatitis E virus infections in blood donors, United Kingdom, 2018-2019. *Emerg Infect Dis*. 2021;27:1654-1661.
8. Maslennikov R, Ivashkin V, Efremova I, et al. Gut dysbiosis is associated with poorer long-term prognosis in cirrhosis. *World J Hepatol*. 2021;13:557-570.
9. Lv L, Yao C, Yan R, et al. Lactobacillus acidophilus LA14 alleviates liver injury. *mSystems*. 2021;6:e0038421.
10. Wang Y, Tian Y, Zhang N, et al. Pediococcus pentosaceus PP04 improves high-fat diet-induced liver injury by the modulation of gut inflammation and intestinal microbiota in C57BL/6N mice. *Food Funct*. 2021;12:6851-6862. https://doi.org/10.1039/d1fo00857a
11. Rao BC, Lou JM, Wang WJ, et al. Human microbiome is a diagnostic biomarker in hepatocellular carcinoma. *Hepatobiliary Pancreat Dis Int*. 2020;19:109-115.
12. Lu H, Wu Z, Xu W, Yang J, Chen Y, Li L. Intestinal microbiota was assessed in cirrhotic patients with hepatitis B virus infection. Intestinal microbiota of HBV cirrhotic patients. *Microb Ecol*. 2011;61:693-703.
13. Wei X, Yan X, Zou D, et al. Abnormal fecal microbiota community and functions in patients with hepatitis B liver cirrhosis as revealed by a metagenomic approach. *BMC Gastroenterol*. 2013;13:175.
14. Wu J, Huang F, Ling Z, et al. Altered faecal microbiota on the expression of Th cells responses in the exacerbation of patients with hepatitis E infection. *J Viral Hepat*. 2020;27:1243-1252.
15. Wu J, C, Sheng X, et al. Prognostic nomogram for patients with hepatitis E virus-related acute liver failure: a multicenter study in China. *J Clin Transl Hepatol*. 2021. https://doi.org/10.14218/JCTH.2020.00117
16. Jian Wu, Yurong Guo, Xuan Lu, et al. Th1/Th2 cells and associated cytokines in acute hepatitis E and related acute liver failure. *J Immunol Res*. 2020;2020:6027361.
17. Nelson MT, Charbonneau MR, Coia HG, et al. Characterization of an engineered live bacterial therapeutic for the treatment of phenylketonuria in a human gut-on-a-chip. *Nat Commun*. 2021;12:2805.
18. Newman TM, Shively CA, Register TC, et al. Diet, obesity, and the gut microbiome as determinants modulating metabolic outcomes in a non-human primate model. *Microbiome*. 2021;9:100.
19. Chopyk DM, Grakoui A. Contribution of the intestinal microbiome and gut barrier to hepatic disorders. *Gastroenterology*. 2020;159:849-863.

20. Vich Vila A, Collij V, Sanna S, et al. Impact of commonly used drugs on the composition and metabolic function of the gut microbiota. *Nat Commun*. 2020;11:362.
21. Schalk VM, Shilpa C, Christine B, et al. The multifactorial mechanisms of bacterial infection in decompensated cirrhosis. *J Hepatol*. 2021;75:S82-S100.
22. Sharpton SR, Schnabl B, Knight R, Loomba R. Current concepts, opportunities, and challenges of gut microbiome-based personalized medicine in nonalcoholic fatty liver disease. *Cell Metab*. 2021;33:21-32.
23. Lou JM, Ren ZG, Li A, Rao BC, Yu ZJ. Fecal microbiota transplantation has therapeutic effects on chronic hepatits B patients via altering composition of gut microbiota. *Hepatobiliary Pancreat Dis Int*. 2020;19:486-487.
24. Yoshiyuki G, Hiroshi K. Epithelial barrier: an interface for the cross-communication between gut flora and immune system. *Immunol Rev*. 2012;245:147-163.
25. Zhang H, Liu M, Liu X, et al. Bifidobacterium animalis ssp. Lactis 420 mitigates autoimmune hepatitis through regulating intestinal barrier and liver immune cells. *Front Immunol*. 2020;11:569104.
26. Sun J, Zhang J, Wang X, et al. Gut-liver crosstalk in sepsis-induced liver injury. *Crit Care*. 2020;24:614.
27. Wang H, Yan Y, Yi X, et al. Histopathological features and composition of gut microbiota in rhesus monkey of alcoholic liver disease. *Front Microbiol*. 2019;10:165.
28. Javier F, Salvatore P, Michele B, et al. Management of bacterial and fungal infections in cirrhosis: The MDRO challenge. *J Hepatol*. 2021;75:S101-S117.

SUPPORTING INFORMATION

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