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Altered faecal microbiota on the expression of Th cells responses in the exacerbation of patients with hepatitis E infection

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Abstract

Fulminant hepatitis E may lead to acute liver failure (ALF). Perturbations of intestinal microbiota are related to severe liver disease. To study the correlations between faecal microbiota and the occurrence and exacerbation of hepatitis E virus (HEV) infection, we characterized 24 faecal samples from 12 patients with acute hepatitis E (AHE) and 12 patients with HEV-ALF using high-throughput sequencing. We found both the alpha and beta diversity indices showed no significant differences between the AHE and HEV-ALF groups. Several predominant taxa were significantly different between the AHE and HEV-ALF groups. Most notably, the HEV-ALF group had increased levels of *Gammaproteobacteria*, *Proteobacteria*, *Xanthomonadceae* and *Stenotrophomonas*, but reduced levels of *Firmicutes*, *Streptococcus*, *Subdoligranulum* and *Lactobacillus*, compared with the AHE group. The levels of *Lactobacillaceae* and *Gammaproteobacteria* could be used to distinguish patients with HEV-ALF from those with AHE. In addition, the level of Th lymphocytes was significantly lower in the HEV-ALF group than in the AHE group. The relative abundances of *Lactobacillaceae* and *Gammaproteobacteria* were positively correlated with Th lymphocytes, serum international normalized ratio (INR) and hepatic encephalopathy severity. Moreover, surviving patients had higher levels of *Lactobacillus mucosae* than deceased patients. Our study demonstrated that the presence of altered faecal microbiota is associated with exacerbation of HEV infection; this finding may be useful for exploring the interactions among faecal microbiota, immune responses, mechanisms of infection and progression in patients with HEV, as well as for the development of novel diagnostic and therapeutic strategies.

KEYWORDS

acute hepatitis E, acute liver failure, exacerbation, faecal microbiota, T helper (Th) lymphocytes

Abbreviations: ACLF, acute on chronic liver failure; AHE, acute hepatitis E; ALF, acute liver failure; HE, hepatic encephalopathy; HEV, hepatitis E virus; Ig M, immunoglobulin M; LEfSe, linear discriminant analysis effect size; MELD, model for end-stage liver disease; OTU, operational taxonomic Unit; PBMCs, peripheral blood mononuclear cells; PCoA, principal coordinate analysis; Th lymphocytes, T helper lymphocytes.

Jian Wu and Fen Huang contributed equally to this work.

1 | BACKGROUND

Acute hepatitis E (AHE) is caused by hepatitis E virus (HEV) infection, which is endemic in many developing countries because of poor sanitation.[1] The virus is predominantly transmitted by the faecal-oral route.[2] In some developing countries, HEV infection causes the majority of cases of acute viral hepatitis. The global burden of HEV infection-related hepatitis cases has been estimated to be 20.1 million.[3] Although HEV typically causes asymptomatic and self-limiting disease with a low fatality rate (<0.1%), possibility of fulminant hepatitis leading to acute liver failure (ALF) or acute on chronic liver failure (ACLF) always exists.[4] AHE can cause severe jaundice and liver failure, leading to death. Approximately 20%-50% of patients with AHE, especially older people or pregnant women, may progress to ALF.[5] The mortality rate can reach 20% among elderly or pregnant patients with AHE. Thus far, minimal data are available regarding the mechanism of exacerbation in patients with HEV infection.

Based on recent studies, gut microbiota is presumed to play important roles in the pathophysiology of many intestinal and extra-intestinal diseases.[6–8] The liver is the organ most closely connected with the intestine and is exposed to substantial quantities of bacterial components and metabolites. There is increasing evidence that alterations in human gut microbiota are related to liver disease. For example, the gut microbiota of patients with cirrhosis are affected by multiple gut and systemic alterations.[9] These changes cause microbial imbalances in different parts of the body, leading to inflammation.[10] Some biological disorders caused by continuous immune stimulation are related to the incidence of cirrhosis. The gut microbiota can also affect the prognosis of patients with hepatitis B virus–related cirrhosis. Chou et al[11] revealed that the establishment of gut microbiota in adult mice can stimulate liver immunity, thus rapidly clearing hepatitis B virus. Preveden et al[12] revealed that changes in gut microbiota may be related to the pathogenesis of chronic liver disease caused by hepatitis C. In addition, other liver diseases (eg alcoholic liver disease,[13] nonalcoholic liver disease[14] and primary sclerosing cholangitis,[15]) are related to altered gut microbiota. These alterations may affect the degree of fatty degeneration, inflammation and fibrosis of the liver through multiple interactions with host immune cells and other cell types.

Currently, very little is known regarding the roles of gut microbiota in patients with HEV infection. To investigate whether gut microbiota play important roles in the occurrence, development and exacerbation of HEV infection, we studied whether faecal microbiota was altered in patients with AHE and those with HEV-related acute liver failure (HEV-ALF). We characterized 24 faecal samples from 12 patients with AHE and 12 patients with HEV-ALF by using high-throughput sequencing. To the best of our knowledge, this is the first study to characterize the faecal microbiota in patients with HEV infection.

2 | PATIENTS AND METHODS

2.1 | Patients

We enrolled 12 patients with HEV-ALF and 12 patients with AHE who were referred to the First Affiliated Hospital (College of Medicine, Zhejiang University) between 1 May 2018 and 30 May 2019. HEV infection was diagnosed by testing for anti-HEV immunoglobulin (Ig) (IgM and IgG) using enzyme-linked immunosorbent assays and HEV RNA quantification. Hepatitis E cases were defined based on positive serum anti-HEV IgM, and/or a greater than two-fold increase in the anti-HEV IgG titre, and/or detectable HEV RNA, combined with clinical presentation of acute hepatitis (eg elevated liver enzymes and/or jaundice and/or nonspecific symptoms, such as fatigue, itching, and nausea). The selection criteria for patients with HEV-ALF were based on the King's College criteria,[16] which were as follows: (a) evidence of abnormal liver synthetic function (prothrombin activity ≤ 40% or international normalized ratio [INR] ≥1.5), jaundice and hepatic atrophy over a 2-week period; (c) the presence of stage 2 or 3 encephalopathy complicating end-stage disease manifestations; (c) no chronic liver disease.

The following exclusion criteria were established: (a) use of antibiotics, probiotics, prebiotics or synbiotics during the previous month; (b) known active bacterial or fungal infections; (c) co-infection with hepatitis A virus, hepatitis B virus, or hepatitis C virus, or the presence of alcoholic or nonalcoholic fatty liver disease; (d) drug-induced liver disease; (e) autoimmune liver disease; (f) liver cancer; (g) co-infection with cytomegalovirus or Epstein-Barr virus; (h) presence of metabolic liver disease; (i) approval for liver transplantation; (j) incomplete data; and (k) loss to follow-up.

We collected all enrolled patients' clinical, demographic, and laboratory data, including age, sex, coagulation parameters, hepatic encephalopathy, laboratory parameters, length of hospitalization, intensive care unit stay and prognosis. The diagnosis of hepatic encephalopathy met the West Haven Criteria.[17] Patients were followed up every 7 days; survival data were collected through medical records or by direct contact with the patients or their families, with death or liver transplantation as a composite endpoint.

The present study was performed in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the First Affiliated Hospital, Zhejiang University (approval number: 2011013). Informed consent to participate in this study was obtained from all participants or their families.

2.2 | Faecal sample collection and DNA extraction

Fresh faecal samples were collected in a sterile plastic cup and stored at −80°C until use; they were then thawed and prepared for bacterial genomic DNA extraction within 15 minutes of thawing. The cetyltrimethylammonium bromide/sodium dodecyl

sulphate method was used to extract total genomic DNA. DNA concentration and purity were monitored on 1% agarose gels.

2.3 | Polymerase chain reaction (PCR) and pyrosequencing

16S rRNA genes of distinct regions (16S V4/16S V3/16S V3-V4/16S V4-V5) were amplified by specific primers (eg 16S V4: 515F-806R). All PCR reactions were implemented using Phusion® High-Fidelity PCR Master Mix, 0.2 μmol/L of forward and reverse primers and approximately 10 ng of template DNA. Thermal cycling consisted of initial denaturation at 98°C for 60 seconds, 30 cycles of denaturation at 98°C for 10 seconds, annealing at 50°C for 30 seconds and elongation at 72°C for 30 seconds. PCR products were mixed in equidensity ratios. Next, PCR products were purified in accordance with the instructions of the Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany). Sequencing libraries were generated using TruSeq®DNA PCR-Free Sample Preparation Kits (Illumina, San Diego, CA, USA). The Qubit@ 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and Agilent Bioanalyzer 2100 systems (Agilent, Santa Clara, CA, USA) were used to assess library quality. Finally, the library was sequenced on an Ion S5 TM XL platform (Thermo Fisher Scientific).

2.4 | HEV-specific antibody detection

All serum samples were tested for the presence of anti-HEV IgM and IgG antibodies using commercially available HEV enzyme-linked immunosorbent assay (ELISA) kits (Wantai, Beijing, China), in accordance with the manufacturer's instructions. Samples with optical density >1.1 were considered positive. Samples with optical density ≤1.1 were considered negative.

2.5 | HEV RNA detection

HEV RNA was tested by means of internally controlled quantitative real-time reverse transcription PCR, as previously described.[18] Total RNA was extracted from serum using a viral nucleic acid purification kit (Aikang, Hangzhou, China), in accordance with the manufacturer's instructions. A 348-nucleotide fragment of the HEV open reading frame 2 was amplified using nested PCR and sequenced to identify the genotype. The viral load of each sample was estimated via quantitative PCR, using a diagnostic kit for hepatitis E virus RNA (Aikang, Hangzhou, China), in accordance with the manufacturer's instructions.

2.6 | Isolation of peripheral blood mononuclear cells

Peripheral blood samples (10 mL) were collected with venipuncture in ethylenediaminetetraacetic acid tubes from all patients at 4 days after the initiation of detoxification treatment. Peripheral blood mononuclear cells were isolated using Ficoll density gradient centrifugation (GE Healthcare Life Sciences, Marlborough, MA, USA) for 30 min at 900 ***g***. Cells were counted under a microscope (100x), and viability was always >95%, as determined by Trypan Blue exclusion (Sigma-Aldrich, St. Louis, MO, USA).

2.7 | Immunophenotyping

Multicolour flow cytometry was used to identify a comprehensive panel of lymphocyte subsets. Peripheral blood mononuclear cells were washed with flow cytometer buffer (phosphate-buffered saline containing 1% foetal calf serum and 0.01% sodium azide) and treated with flow cytometry blocking solution for 20 minutes. The cells were stained for 30 minutes at 4°C with combinations of anti-CD3, anti-CD4, anti-CD8, anti-CD16 and anti-CD56 monoclonal antibodies. The fluorescent dyes used were fluorescein isothiocyanate, phycoerythrin and allophycocyanin; all antibodies were purchased from BD Biosciences (San Jose, CA, USA). At least 20,000 lymphocytes were identified by granularity and size using a FACS Canto II flow cytometer (BD Biosciences). FlowJo 7.2.5 software (Tree Star Inc, Ashland, OR, USA) was used to analyse the data.

2.8 | Bioinformatics analysis

Sequencing reads, quality trimming, demultiplexing and taxonomic assignments were performed using QIIME (version 1.9.0).[19] The diversity and richness of bacteria were evaluated using multiple estimates, including the level of operational taxonomic units (OTUs), as well as the Chao1, Shannon and Simpson indices. Principal coordinates analysis was used to explore the relationship between the AHE and HEV-ALF groups. The linear discriminant analysis effect size method was applied to the OTU table to determine the abundances of different bacterial taxa. Metagenomic profiling (STAMP) software was used for further analysis of output files.[20] The sequence data from this study were deposited in the sequence reading file of GenBank under accession number ERP 119119.

2.9 | Statistical analysis

Statistical analyses were performed with SPSS software (v. 18.0; SPSS Inc, Chicago, IL, USA) and Statistical Analysis of Metagenomic Profiles software. The time ROC package in R software was used to compare the time-dependent area under the receiver operating characteristic curve. Cox proportional hazards modelling was performed to identify factors associated with prognosis of patients with HEV-ALF. All data are expressed as means ± standard deviations. *P* values <.05 were considered statistically significant.

3 | RESULTS

3.1 | Collection of 16S data

We obtained 1 856 359 high-quality sequences from 24 faecal samples of enrolled participants, which constituted 95.1% of valid sequences among 1 952 007 total reads. The mean read length was 414 bp (range: 400-428 bp). The total number of unique sequences from the AHE and HEV-ALF groups was 6606; all phylotypes were represented. In particular, 2836 OTUs were obtained in the AHE group and 3770 OTUs were obtained in the HEV-ALF group. The coverage rate of all sequences in the two groups was near 99.8%, which indicated excellent sequencing depth.

3.2 | Overall structure of the faecal bacterial communities

We evaluated the ecological characteristics of faecal bacterial communities using a variety of indicators based on OTU level in the AHE and HEV-ALF groups (Table 1). Species richness refers to the number of bacterial species distributed by OTUs detected in the sample. Richness estimates were derived from the number of species observed by extrapolation using estimates such as abundance-based coverage estimator and Chao1 indices. Diversity estimates were derived from species richness. No significant differences were found in Shannon and Simpson indices between the AHE and HEV-ALF groups (both $P > .05$, Figure S1). Rarefaction analysis estimates showed that the species richness trend in the AHE group was lower than that in the HEV-ALF group (Figure S2). OTU analysis revealed a long tail in the rank abundance curves, indicating that the majority of OTUs were low in abundance (Figure S3).

Beta diversity analysis was used to measure similarity between microbial communities by measuring the degree to which members or structures were shared between communities. Because of significant differences among patients, the two groups of faecal microbiota could not be divided into different clusters based on community composition using the unweighted uniFrac metric and weighted uniFrac metric; moreover, they could not be clearly divided by using principal coordinates analysis (Figure S4).

Clustering was performed by an analysis of bacterial richness based on the numbers of shared and unique OTUs between groups; these data were visualized using a Venn diagram, which was used to compare OTUs between the two groups (Figure S5).

3.3 | Altered microbiota compositions in AHE and HEV-ALF groups

Metastats was used to investigate correlations related to the altered faecal microbiota between the AHE and HEV-ALF groups. At the phylum level, *Proteobacteria* were significantly more abundant in the faecal microbiota of the HEV-ALF group than in the faecal microbiota of the AHE group. However, *Firmicutes* were more abundant in the AHE group than in the HEV-ALF group (Figure 1A). At the family level, the abundance of *Xanthomonadaceae* was higher in the HEV-ALF group than in the AHE group, while the abundances of *Lactobacillaceae* and *Streptococcaceae* were lower (Figure 1B). There were significant differences at the genus level between the AHE and HEV-ALF groups. Among the abundant genera, *Stenotrophomonas* was more prevalent in the HEV-ALF group, while the abundances of *Streptococcus*, *Subdoligranulum* and *Lactobacillus* were significantly higher in the AHE group (Figure 1C) (Table S1).

The metagenomics linear discriminant analysis effect size approach was also used to identify key phylogeny types that differed between the AHE and HEV-ALF groups. *Lactobacillaceae*, *Streptococcus* and *Firmicutes*, which were most abundant in the AHE group, and *Gammaproteobacteria* and *Xanthomonadceae*, which were most abundant in the HEV-ALF group, were the dominant phylotypes that contributed to differences regarding intestinal microbiota between the two groups (Figure 1D,E).

Receiver operating characteristic curves were generated for microbiota samples, and areas under the curve were calculated (Figure 1F,G). The cut-off values for *Lactobacillaceae* and *Gammaproteobacteria* could distinguish patients with HEV-ALF from those with AHE.

3.4 | Associations between faecal microbiota and clinical indicators

There were significant differences in INR and the severity of hepatic encephalopathy between the AHE and HEV-ALF groups ($P < .05$, Table 2). We further evaluated correlations among the

TABLE 1 Comparisons of richness and diversity of faecal microbiota in AHE and HEV-ALF groups

Group	No. of Reads	No. of OTUs	Good's (%)	Richness estimator				Diversity index	
				ACE	95% CI	Chao1	95% CI	Shannon	Simpson
AHE	921 398	2836	99.8	3581	3443 ~ 3718	3509	3367 ~ 3651	4.79442	0.91767
HEV-ALF	934 961	3770	99.7	5071	4943 ~ 5198	4877	4740 ~ 5014	4.07608	0.81867

Note: Parameters were calculated by QIIME software. Operational taxonomic units (OTUs) were defined at the 97% similarity level.
Abbreviation: ACE, abundance-based coverage estimator.

relative abundances of bacterial taxa (*Lactobacillaceae*, *Streptococcus*, *Firmicutes* and *Gammaproteobacteria*), the INR and the severity of hepatic encephalopathy; both the INR and hepatic encephalopathy are important indicators for diagnosis of HEV-ALF. Notably, the relative abundance of *Lactobacillaceae* was negatively correlated with both serum INR (0.74, $P = .0082$; Figure 2A) and severity of hepatic encephalopathy (0.82, $P = .0011$; Figure 2B); moreover, the relative abundance of *Gammaproteobacteria* was positively correlated with both serum INR (0.76, $P = .0066$; Figure 2C) and severity of hepatic encephalopathy (0.68, $P = .0015$; Figure 2D).

3.5 | Associations between faecal microbiota and Th lymphocytes

We evaluated the lymphocyte levels with anti-CD3, anti-CD4, anti-CD8, anti-CD16 and anti-CD56 antibodies in the AHE and HEV-ALF groups. No significant differences in cytotoxic T cells (CTL) and natural killer cell (NK) levels were observed between groups, but the level of T helper (Th) lymphocytes was significantly lower in the HEV-ALF group than in the AHE group ($P < .05$, Figure 3A).

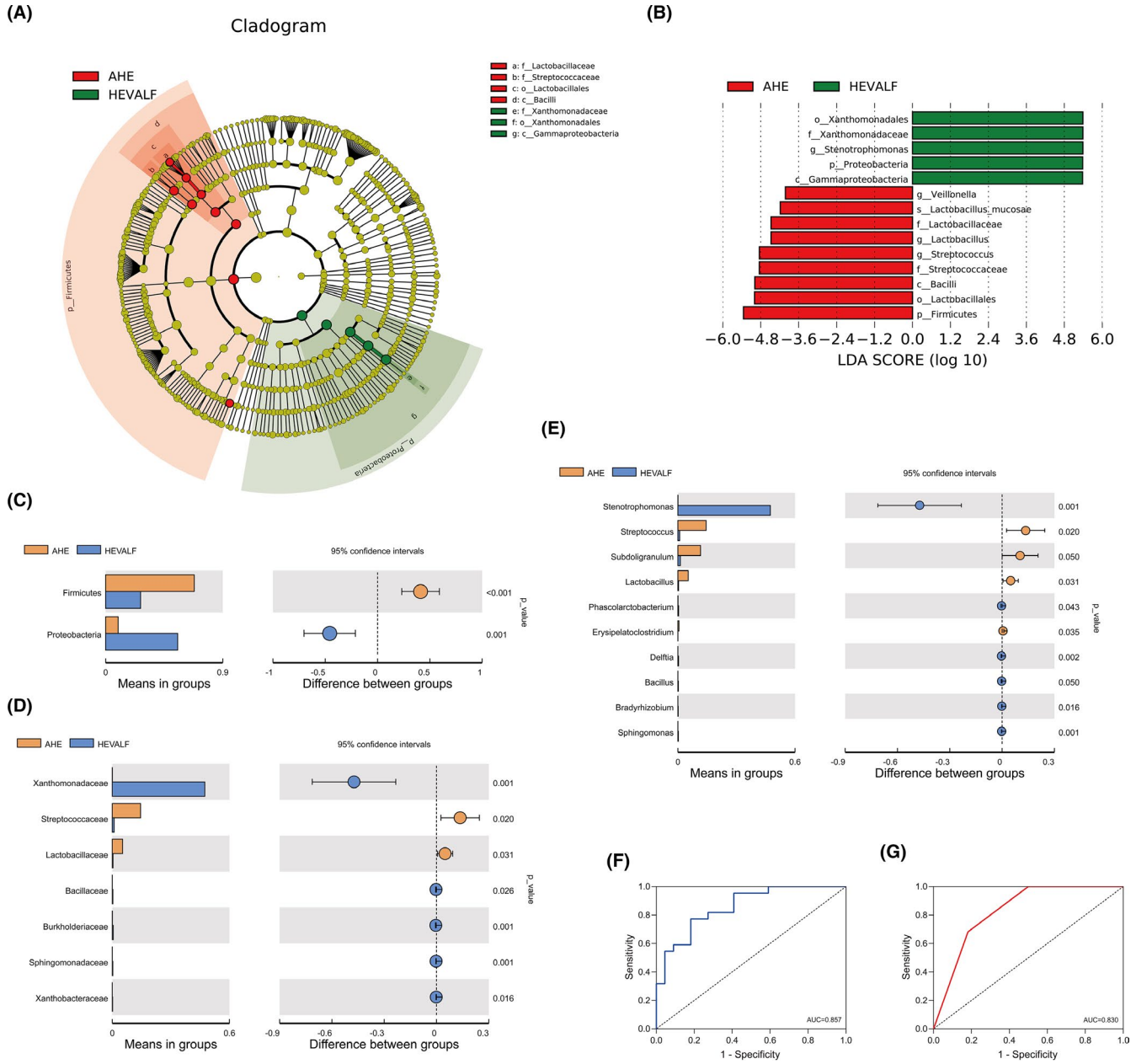

FIGURE 1 Taxonomic differences in faecal microbiota composition between AHE and HEV-ALF groups. Cladogram representing features of bacterial hierarchies determined using the linear discriminant analysis-based model (A). Linear discriminant analysis and effect size measurements identified the most differentially abundant taxa between the two groups (B). Comparisons of relative abundances at bacterial phylum (C), family (D) and genus (E) levels in samples from the two groups; receiver operating characteristic curves for *Lactobacillaceae* (F) and *Gammaproteobacteria* (G) were used to distinguish patients with HEV-ALF from those with AHE

TABLE 2 Characteristics of the enrolled patients

Variables	AHE group	HEV-ALF group	*P*
Clinical characteristics			
Age (y)	49.08 ± 15.49	56.00 ± 10.44	.213
Gender (F/M)	5/7	5/7	1.000
BMI	22.46 ± 2.29	23.10 ± 2.15	.496
PH	7.41 (7.31-7.46)	7.42 (7.32-7.47)	.549
HE	0 (0.00)	4 (33.33)	.039
Symptoms			
Fever	5 (41.67)	7 (58.33)	.389
Jaundice	8 (88.89)	12 (100.00)	.039
Abdominal pain	2 (16.67)	3 (25.00)	.536
Nausea/Vomit	8 (66.67)	9 (75.00)	.283
Laboratory parameters			
WBC (10^9/L)	6.25 (2.9-9.9)	7.28 (4.4-12.0)	.261
ALT (U/L)	963.25 (53-2330)	1224.83 (26-4638)	.564
AST (U/L)	581.17 (16-1877)	606.67 (50-2714)	.929
Variables	AHE group	HEV-ALF group	*P*
GGT (U/L)	318.00 (113-1067)	184.25 (68-326)	.102
TP (g/L)	65.93 ± 6.44	59.72 ± 8.97	.054
ALB (g/L)	37.74 ± 8.60	34.04 ± 5.39	.220
TBIL (umol/L)	92.33 ± 65.20	253.88 ± 152.71	.003
UREA (mmol/L)	4.15 (2.65-6.32)	5.20 (1.79-10.71)	.163
CR (umol/L)	70.83 (39-124)	68.08 (24-83)	.740
INR	1.18 (0.88-1.66)	1.51 (0.95-2.47)	.033
AFP (ng/mL)	56.15 (1.20-392.50)	141.48 (2.40-703.40)	.248
PLT (10^9/L)	170.67 (49-336)	180.08 (86-288)	.755
TCH (mmol/L)	3.58 ± 1.28	2.96 ± 0.72	.160
CHE	5366.83 (1983-8649)	4073.58 (136-8188)	.172
GLU (mmol/L)	3.69 (2.98-5.99)	3.72 (2.97-5.56)	.525
IgM (+)	12 (100.00)	12 (100.00)	1.000
HEV RNA	6 (50.00)	7 (58.33)	.219

Abbreviations: AFP, alpha-fetoprotein; ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CR, creatinine; GGT, γ-glutamyl transpeptidase; GLU, glucose; HE, hepatic encephalopathy; INR, international normalized ratio; PH, degree of acid or alkali; PLT, platelets; TBIL, total bilirubin; TCH, total cholesterol; UREA, urea nitrogen; WBC, white blood cell.

Correlations were evaluated between the relative abundances of bacterial taxa (*Lactobacillaceae* and *Gammaproteobacteria*) and Th lymphocytes. The relative abundance of *Lactobacillaceae* was positively correlated with Th lymphocyte level, while that of *Gammaproteobacteria* was negatively correlated with Th lymphocyte level (both $P < .05$, Figure 3B,C).

To further explore correlations between faecal microbiota composition and Th lymphocytes in patients with HEV infections, the patients with AHE and patients with HEV-ALF were divided into two groups: the 'High Th lymphocytes' group, in which the percentage of Th ($CD3^+$ $CD4^+$) cells was ≥27.4%, and the 'Low Th lymphocytes' group, in which the percentage of Th ($CD3^+$ $CD4^+$) cells was <27.4%. We found that *Lactobacillaceae* was enriched in the High Th lymphocytes group (Figure 4A,B).

3.6 | Association between altered microbiota and mortality in HEV-ALF

Next, we explored whether an altered microbiota was associated with the prognosis of HEV-ALF. We found that *Lactobacillus mucosae* was enriched in surviving patients (Figure 4C,D). After adjustments for age, sex and MELD score in the Cox proportional hazards model, a high abundance of *L mucosae* was confirmed as a predictive factor for favourable prognosis in patients with HEV-ALF (Table 3).

4 | DISCUSSION

Several studies have been carried out to investigate the roles of altered human gut microbiota profiles in liver diseases.[21–23] However, there remains little knowledge regarding the roles of gut microbiota in patients with HEV infection, especially with respect to progression from AHE to HEV-ALF. To the best of our knowledge, the present study is the first to focus on the correlations between faecal microbiota and the occurrence and progression of HEV infection, with the aim of providing new insights into host-gut microbiota interactions in patients with HEV infection.

In this study, both the alpha diversity index (Shannon and Simpson) and the beta diversity index (principal coordinates analysis) did not demonstrate significant differences between the AHE and HEV-ALF groups; these indices were unexpectedly higher in patients with liver failure.[24,25] We speculate that this result may be related to the pathway of HEV infection. In addition, the diversity of faecal bacteria is influenced by several factors, including age, diet and antibiotics treatment.

In the present study, intestinal dysbiosis was characterized by significant taxonomic differences at the phylum level between the AHE and HEV-ALF groups. *Proteobacteria* were significantly more abundant in the HEV-ALF group, while the proportion of *Firmicutes* was markedly lower. Wang et al[26] reported that *Proteobacteria* tended to increase in patients with alcohol-induced chronic liver disease. Fernández et al[27] also reported that increased abundance of *Enterobacteraceae* was associated with decompensated cirrhosis and acute on chronic liver failure in Europe. Jasmohan et al[25] found that the incidence of acute on chronic liver failure was significantly reduced in patients with cirrhosis who exhibited high *Firmicutes* levels. The abundance of *Firmicutes* was previously found to be lower, while that of *Bacteroidetes* was higher, in patients with chronic hepatitis B infection, liver cirrhosis and hepatocellular carcinoma, compared with healthy controls.[28]

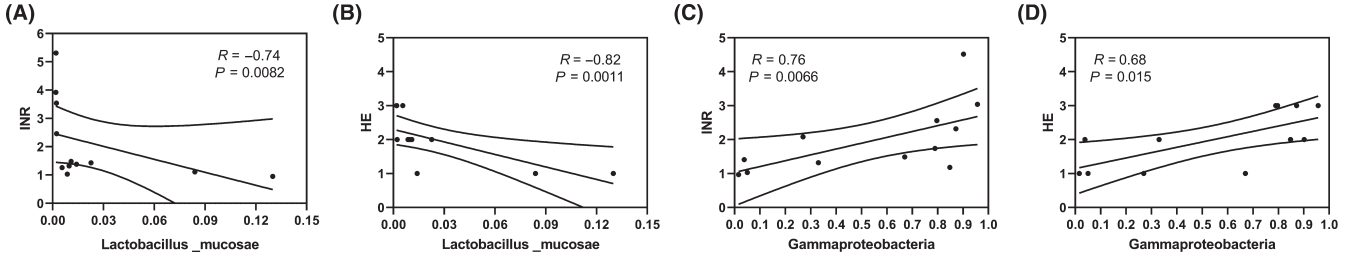

FIGURE 2 Correlations between relative abundances of *Lactobacillus mucosae* or *Gammaproteobacteria* with serum INR and hepatic encephalopathy. (A) The relative abundances of *Lactobacillus mucosae* with serum INR; (B) The relative abundances of *Lactobacillus mucosae* with HE; (C) The relative abundances of *Gammaproteobacteria* with serum INR; (D) The relative abundances of *Gammaproteobacteria* with HE; Spearman's rank correlation coefficient (R) and probability (P) were used to evaluate statistical significance. INR, international normalized ratio; HE, hepatic encephalopathy

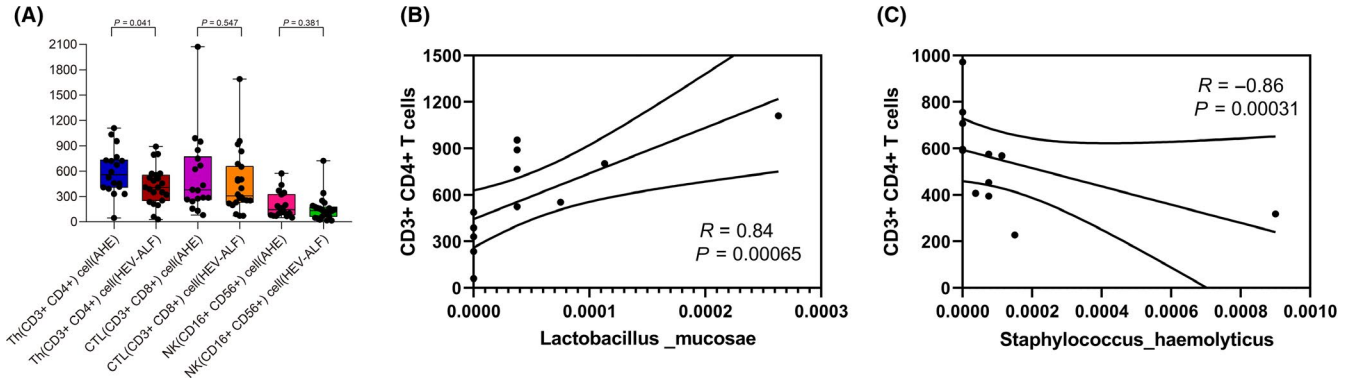

FIGURE 3 Correlations between relative abundances of *Lactobacillus mucosae* or *Gammaproteobacteria* and Th lymphocytes. (A) Comparisons of Th, CTL and NK lymphocyte levels in AHE and HEV-ALF groups; relative abundances of *L mucosae* (B), *Gammaproteobacteria* (C), and Th lymphocytes. Spearman's rank correlation coefficient (R) and probability (P) were used to evaluate statistical significance

The abundance of the *Xanthomonadceae* families was increased, while the abundances of *Lactobacillaceae* and *Streptococcaceae* were reduced, in the HEV-ALF group vs. the AHE group. Lactobacilli play important roles in the maintenance of health in humans and higher animals. Bajaj et al[29] reported that increased levels of *Lachnospiraceae* could effectively improve hepatic encephalopathy. Gu et al[30] also reported that *Lactobacillus rhamnosus* granules could restore the balance of microbiota and improve chronic alcoholic liver injury in a dose-dependent manner. Inoue et al[31] found that hepatitis C virus-infected people had lower bacterial diversity, fewer *Clostridium* species, and more *Streptococcus* and *Lactobacillus* species, compared with healthy people. Bajaj et al[32] showed that *L rhamnosus GG* is safe and well-tolerated in patients with cirrhosis, and reported that its use was associated with reductions in endotoxemia and dysbiosis.

There were also differences between the HEV-ALF and AHE groups at the genus level. *Stenotrophomonas* was significantly more abundant in the HEV-ALF group, while the proportions of three other genera (*Streptococcus*, *Subdoligranulum* and *Lactobacillus*) were significantly less abundant. We observed that the levels of *Lactobacillaceae* and *Gammaproteobacteria* in faecal microbiota samples could be used to distinguish patients with HEV-ALF from those with AHE (area under the curve = 0.857 and area under the curve = 0.830, respectively, Figure 1F,G).

The patterns of T-cell levels in the development from AHE into HEV-ALF remained unclear. We compared Th, CTL and NK lymphocyte levels between the AHE and HEV-ALF groups. Only Th lymphocyte levels were significantly lower in the HEV-ALF group than in the AHE group. Pal et al[33] reported the existence of a Th2-cell bias in pregnant women with AHE, with a reduction in the production of Th1 cytokines and an increase in the production of Th2 cytokines. Cao et al[34] reported that hepatitis E virus infection of immunocompromised pigs reduced the serum levels of Th1 cytokines (interleukin [IL]-2 and IL-12) and Th2 cytokines (IL-4 and IL-10), especially during the acute phase of infection. Furthermore, the relative abundance of *Lactobacillaceae* was positively correlated with the level of Th lymphocytes, while there was a negative relationship between the relative abundance of *Gammaproteobacteria* and the level of Th lymphocytes. We also observed that *Lactobacillaceae* was enriched in the High Th lymphocytes group, which further confirmed that lactobacilli can induce human peripheral blood mononuclear cells to elicit a Th-type cellular immune response. Thus, we propose that lactobacilli may inhibit the production of Th cells, thereby impacting the inhibition and killing of HEV by T cells. Overall, more discussion is required on the speculation of the results.

As might be expected, HEV-ALF was associated with INR and the severity of hepatic encephalopathy. Our study showed that the relative abundance of *Lactobacillaceae* was negatively correlated

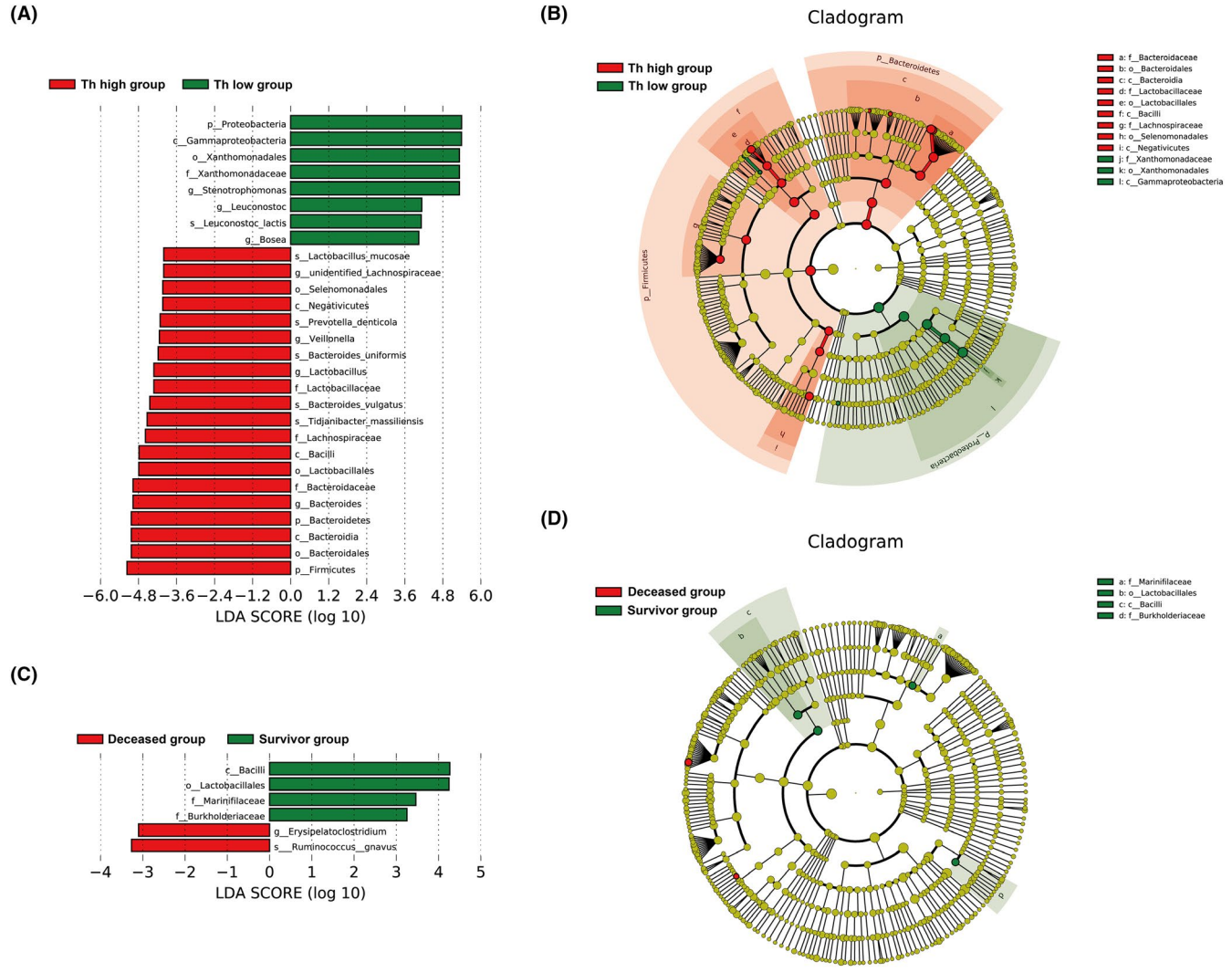

FIGURE 4 Comparisons of taxonomic abundance between High Th lymphocytes and Low Th lymphocytes groups (A, B) or between survivors and deceased patients (C, D) based on linear discriminant analysis score and the linear discriminant analysis effect size method. Histograms and cladograms represent differentially abundant taxa with linear discriminant analysis score > 4.0

TABLE 3 Risk factors associated with 28-day mortality in patients with HEV-ALF

	Univariate analysis			Multivariate analysis		
Variables	HR	95% CI	*P* value	HR	95% CI	*P* value
Age	0.990	0.985-1.048	.321	1.005	0.970-1.041	.798
Sex	1.254	0.371-4.245	.716	1.233	0.328-4.639	.756
Relative abundance of Lactobacillus _mucosae	0.286	0.103-0.681	.034	0.242	0.079-0.704	.045
MELD	1.390	1.042-1.855	0.025	1.338	0.804-2.226	.032

Note: MELD score (range, 6-40) is calculated as follows: 9.6*loge [creatinine (mg/dL)]+3.8*loge [bilirubin (mg/dL)]+11.2*loge (INR)+6.43*(aetiology: 0 if cholestatic or alcoholic, 1 otherwise).

with both serum INR and the severity of hepatic encephalopathy, while the relative abundance of *Gammaproteobacteria* was positively correlated with both serum INR and the severity of hepatic encephalopathy. Intriguingly, *L mucosae* was found to be associated with a favourable prognosis for patients with HEV-ALF, as this species was enriched in surviving patients compared with deceased patients.

This study had several limitations. First, the sample sizes for the AHE and HEV-ALF groups were small, which may have affected the reliability of the results. Second, this study lacks a control group with ALF without HEV infectious, which could rule out the supposition that the increase in Stenotrophomonas is typically considered a nosocomial infectious agent. Third, Th-type

cell-related cytokines were not analysed, which might have provided further information regarding potential underlying mechanisms for the observed effects with respect to *Lactobacillaceae* and Th lymphocytes.

5 | CONCLUSIONS

In summary, the results of this study add to the findings of previous reports regarding the roles of faecal microbiota in patients with liver disease. Specifically, the present results demonstrate that microbial populations are associated with the occurrence, development and exacerbation of HEV infection. Future studies should focus on whether *Lactobacillaceae* and *Gammaproteobacteria* can be serve as indicators for exacerbation of HEV infection, as well as whether *Lactobacillaceae* can influence the inhibitory effect of Th cells on HEV by interfering with the Th cell immune response, thus causing exacerbation of HEV. Further research of this nature may elucidate the temporal and causal relationships between faecal microbiota and the occurrence, development and exacerbation of HEV infection.

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

JW and FH contributed to the study concept and design, conducted the literature search and wrote the manuscript; ZL and YM contributed to the data analysis and made the tables and figures; SL, JL and JF contributed to the collection of patients' samples and medical information; JY, XJ and WW contributed to the acquisition and analysis of data; HC and LL contributed to the study concept, obtained funding and critically revised the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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