Gut Pathogens

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Fecal carriage of multidrug-resistant organisms increases the risk of hepatic encephalopathy in patients with cirrhosis: insights from gut microbiota and metabolite features

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Abstract

Background The impact of the fecal multidrug-resistant organism (MDRO) carriage on the gut microbiota, metabolite alterations, and cirrhosis-related complications remains unclear.

Methods Eighty-eight patients with cirrhosis and 22 healthy volunteers were analyzed for plasma metabolites, fecal MDROs, and microbiota composition. The fecal bacterial and fungal composition was assessed using 16S ribosomal RNA and internal transcribed spacer sequencing, whereas plasma metabolomic analysis was evaluated via untargeted liquid chromatography-mass spectrometry. Predictors of cirrhosis-related outcomes, risk factors for MDRO carriage, and microbiota-metabolite correlations were analyzed.

Results Fecal MDRO carriage was detected in 33% of patients with cirrhosis. MDRO carriers had a higher risk of hepatic encephalopathy (HE) compared to non-carriers (20.7% vs. 3.2%, $p=0.008$). Patients carrying MDROs had higher plasma lipopolysaccharide (LPS) levels, and both elevated LPS and MDRO carriage independently predicted HE occurrence within 1 year. Compared with non-carriers, MDRO carriers had higher fecal bacterial and fungal burdens and exhibited different gut microbiota compositions, characterized by increased *Streptococcus salivarius* and enrichment of *Saccharomycetes* and *Candida albicans*. Thirty-one metabolites differed significantly among healthy controls, and patients with cirrhosis, with and without MDRO carriage. Six metabolites were significantly correlated with specific microbial taxa in MDRO carriers. Isoaustin, a fungal-derived metabolite, was significantly elevated in MDRO carriers with HE.

Conclusions Fecal MDRO carriage was associated with endotoxemia, altered gut microbiota, metabolic changes, and a higher risk of HE. It's worthy to monitor fecal MDRO colonization in cirrhosis.

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Keywords Multidrug resistant organisms, Cirrhosis-associated complications, Microbiota, Metabolite, Hepatic encephalopathy

Introduction

Patients with cirrhosis are highly susceptible to infections [1]. The widespread use of antibiotics has led to a rapid increase in multidrug-resistant organism (MDRO)-related infections for these patients [2]. Colonization of MDROs is an important clinical issue as it increases the risk of subsequent MDRO infections in such patients, contributing to poorer survival outcomes [3–5]. Therefore, strategies targeting MDRO colonization in cirrhosis are crucial in improving patient outcomes.

The intestinal tract serves as the main reservoir for MDROs [6], and the gut microbiome plays an important role in maintaining the gut barrier and protecting against resistant pathogens. Certain commensals, such as *Klebsiella oxytoca, Blautia producta*, and *Clostridium bolteae*, prevent the colonization of MDROs in animal models [7–10]. The successful decolonization of resistant pathogens through fecal microbiota transplantation (FMT) highlights the role of the gut microbiota in MDRO regulation [11, 12]. Furthermore, probiotics and prebiotics have emerged as potential strategies for MDRO decolonization through modulating the diversity of gut microbiota, although their effectiveness depends on factors such as dosage, probiotic strain, and the target pathogens [13]. Bacterial metabolism also influences antibiotic resistance by affecting energy production, cell envelope modifications, and biofilm formation [14]. Metabolic alterations, including defects in glucose and amino acid metabolism, have been observed in specific resistant bacteria [15]. Furthermore, supplementation of specific metabolites, such as mannitol or fructose, effectively increases bacterial susceptibility to antibiotics [16]. In addition, microbial metabolites, such as short-chain fatty acids, strengthen the integrity of the intestinal barrier, potentially protecting against MDRO colonization [17, 18].

The gut microbiota undergoes significant alterations with the progression of cirrhosis, accompanied by gut barrier dysfunction, systemic inflammation, and immune dysregulation, all of which compromise resistance to MDRO colonization [19, 20]. In hospitalized patients without cirrhosis, MDRO colonization has been associated with reduced microbial diversity and a change in microbiota composition, with higher abundances of the Enterobacteriaceae family and *Enterococcus* spp. in MDRO carriers, whereas the Bacteroidales order and *Lactobacillus* spp. are more abundant in non-carriers [21–23]. In cirrhosis, MDRO colonization increases the risks of MDRO-related infections, failure of spontaneous bacterial peritonitis (SBP) prophylaxis, lower transplant-free survival, and higher mortality [3, 4, 24–26]. However, the interaction between gut microbiota alteration, metabolite modulation, and MDRO colonization in patients with cirrhosis remains poorly understood. Furthermore, the long-term impact of fecal MDRO colonization on specific outcomes related to cirrhosis is unclear. Therefore, this study aimed to investigate the interactions between fecal MDROs, gut microbiota, and their associated metabolites, and their association with clinical outcomes in patients with cirrhosis.

Materials and methods

Participants

This prospective study was conducted at Taipei Veterans General Hospital between October 2018 and April 2022. Cirrhosis was diagnosed based on histological, clinical, biochemical, endoscopic, and imaging findings suggestive of cirrhosis in patients with chronic liver disease [27]. Patients with previous episodes of hepatic encephalopathy (HE) episode or active hepatocellular carcinoma status; with malignancies other than hepatocellular carcinoma; with human immunodeficiency virus infection or severe comorbidities, such as chronic renal failure, heart failure, or chronic obstructive pulmonary disease; and who received proton pump inhibitor, nonsteroidal anti-inflammatory drugs, antibiotics, or probiotics within 1 month were excluded. Twenty-two healthy adults without underlying systemic disease were enrolled as healthy controls. This study was approved by the Institutional Review Board of Taipei Veterans General Hospital (IRB No., 2017-09-013 C and 2019-08-013 A). Written informed consent was obtained from each participant.

Data collection

Demographic characteristics, laboratory data, and medical history were collected. Blood and stool samples were collected on the day of enrollment. Fresh stool samples were collected using the Commode Specimen Collection System (Thermo Fisher, MA USA). Stool samples from 18 hospitalized patients with cirrhosis were collected directly in the ward, whereas samples from the 70 outpatients with cirrhosis and 22 healthy participants were collected at home and transported to the laboratory within 2 h of collection. All stool samples were collected following standardized protocols as previously described

to minimize individual variability across sample handling [28]. On arrival at the laboratory, all stool samples were immediately processed and stored at − 80 °C until analysis. Patients with cirrhosis were followed for at least 1 year, or until death or liver transplant. During the follow-up period, clinical events were recorded, which included (1) complications of cirrhosis, such as SBP, overt HE [29], newly developed or worsening ascites, acute kidney injury, first or recurrent variceal bleeding; (2) newly diagnosed hepatocellular carcinoma; (3) bacterial infections; (4) death; and (5) liver transplant.

Detection and definition of MDROs

Stool samples were inoculated onto selective agar plates at 37 °C for 24 h to detect MDRO. The identification of MDRO was based on bacterial cultures and antimicrobial susceptibility testing. MDRO was defined as bacteria resistant to at least one agent in three or more antimicrobial categories, as previously described [30]. These included methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci, and multidrug-resistant Gram-negative bacteria, such as Enterobacterales, *Pseudomonas aeruginosa*, and *Acinetobacter* spp. Enterobacterales were classified as third-generation cephalosporin-resistant pathogens if they were not susceptible to one or more third-generation cephalosporins and multidrug-resistant Gram-negative bacteria were considered carbapenem-resistant pathogens if they exhibited resistance to carbapenem antibiotics. Bacterial isolates were identified using matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (bioMérieux SA, Marcy l'Etoile, France). The antibiotic susceptibility test was performed using the VITEK2 system (bioMérieux), with results interpreted according to the 2021 Clinical and Laboratory Standards Institute guidelines [31]. Healthy adults or patients with cirrhosis in which MDRO were detected in fecal specimens were considered carriers of MDRO. Patients with cirrhosis were then divided into the MDRO carrier and non-carrier groups for further analysis. The residual stool samples were placed in 10% glycerol vials and stored at − 80 °C to further assess the microbiome.

Processing and analysis of stool bacterial and fungal genomic data

For each stool specimen, total genomic DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. The extracted total DNA was stored at − 80 °C for further PCR amplification. To analyze the bacterial composition, the hypervariable V3–V4 region of 16S ribosomal RNA (rRNA) genes was amplified by PCR using the 341 F (V3) and 805R (V4) primers designed for Illumina sequencing [32]. For the analysis of fungal composition, fungal-specific internal transcribed spacer (ITS) genes of distinct regions of ITS1 were amplified using the primers of ITS5-1737 F and ITS2-2043R.

Next-generation sequencing was performed using the Illumina MiSeq Desktop Sequencer following the standard protocol. Raw reads from the sequencing of 16S rRNA and fungal ITS genes were processed using QIIME 2 version 2024.5 and DADA2 plugin [33–35]. The 16S rRNA (V3–V4) and ITS1 sequencing were conducted with an average sequencing depth of 35,000 and 100,000 reads per sample, respectively. Rarefaction was performed at 20,000 reads per sample to normalize sequencing depth. To normalize the variations in sequence depth across samples, amplicon sequence variant abundance information was rarefied to the minimum sequence depth using the QIIME script (single_rarefaction.py) [36]. Further analyses of alpha and beta diversities were both performed using the normalized data. Relative abundance normalization was then used to analyze sequencing data. The taxonomic compositions of all samples were identified.

Bacterial and fungal DNA quantification

Bacterial and fungal DNA quantification was performed using real-time PCR. The total bacterial DNA was quantified by amplifying 16S rRNA genes using specific primers as previously described [37, 38], and the fungal DNA was quantified using the Femto Fungal DNA Quantification Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol [39].

Measurement of lipopolysaccharides

Plasma lipopolysaccharides (LPS) were measured using enzyme-linked immunosorbent assay kits (Cloud-Clone Corp, Katy, TX, USA) according to the manufacturer's instructions.

Liquid chromatography mass spectrometry-based metabolomics analysis

Plasma samples from all participants were immediately stored at −80 °C until analysis. An untargeted metabolomics approach was used. Each plasma sample (60 µL) was spiked with two internal standards (6 ppm lysine-^{13}C6, 1 ppm stearic acid-^{13}C18), selected to represent two distinct ends of the metabolite spectrum in the 9-min liquid chromatography gradient based on prior liquid chromatography mass spectrometry (LC–MS) data from our laboratory: lysine-^{13}C6 (hydrophilic) elutes at 0.87 ± 0.07 min, whereas stearic acid-^{13}C18 (hydrophobic) elutes at 3.85 ± 0.25 min. The samples were then deproteinized by adding 240 µL 100% methanol [40], followed by centrifugation at 4 °C and 13,000 *g* for 10 min. The supernatant

was vacuum-dried and reconstituted in 60 µL ultrapure water for subsequent LC–MS analysis.

LC–MS analysis was performed on a Waters Xevo G2-S Q-Tof tandem mass spectrometer coupled with an Acquity UPLC system (Waters). A BEH C18 column (2.1 × 100 mm 1.7 µm, Waters) was used, and maintained at 40 °C. The total run time was 9 min, at a flow rate of 0.3 mL/min. The gradient started at 1% mobile phase B (acetonitrile with 0.1% ammonium hydroxide) from 0 to 0.5 min, and then increased to 100% B from 0.5 to 4 min. The composition was held at 100% B for 1 min, followed by a return to the initial composition over 1 min. The final conditions were maintained for an additional 3 min. Mobile phase A was an aqueous solution containing 0.1% ammonium hydroxide. Leucine enkephalin ([M − H] − = 554.2615 m/z) was used for continuous mass calibration. MS^E data were acquired under both low and high collision energy conditions with a mass scan range of 50–1200 m/z. A pooled quality control sample was prepared by mixing plasma samples, aliquoting, and analyzing during each batch of LC–MS analysis to provide a basis for normalization. Following quality control-based normalization using the statTarget R package [41], the normalized metabolic feature levels from three measurements of each individual sample were averaged before statistical analysis.

The ionic features were tentatively identified by matching monoisotopic mass (m/z), fragmentation pattern, and retention time (if available) with the in-house library, Human Metabolome Database (http://www.hmdb.ca/), and the Human Microbial Metabolome Database (https://mimedb.org) [42]. Only [M −H] −adduction form was considered for compound identification. The MS1 and MS/MS mass tolerance were set to 20 ppm and 50 ppm, respectively.

Statistical analysis

Clinical data were expressed as the median (25 th to 75 th percentiles) or as counts, as appropriate. The chi-square or Fisher's exact test was used to analyze categorical variables. The normality of continuous variables was assessed using the Kolmogorov–Smirnov test. Because the continuous variables were not normally distributed, Mann–Whitney U test was applied to compare continuous variables between MDRO carriers and non-carriers in the cirrhotic population. Cox regression analysis was performed to identify predictors of clinical outcomes. A logistic regression model was used to identify risk factors for the fecal carriage of MDROs. The cutoff values of the Model for End-Stage Liver Disease (MELD) score and LPS levels were determined using Youden's index. Variables with $p < 0.1$ in the univariate analysis were included in the multivariable analysis. Statistical significance was defined as $p < 0.05$. The Kruskal–Wallis test was used to assess the differences in metabolites between healthy controls and patients with and without MDRO, and between healthy controls and patients with and without HE. Data were considered significant when $p < 0.05$. All statistical analyses were performed using IBM SPSS Statistics for Windows, Version 24.0 (IBM Corp. Armonk, NY, USA).

For gut bacterial and fungal analyses, the alpha diversity was evaluated using Faith's phylogenetic diversity (Faith's PD) and Shannon indices, with Kruskal–Wallis and Wilcoxon tests applied for statistical analysis. Beta diversity was evaluated using Bray–Curtis and Unweighted UniFrac distances and were visualized through principal coordinates analysis and nonmetric multidimensional scaling (NMDS). Results were tested for significance using the permutational multivariate analysis of variance test. Differences in diversity were further analyzed using the R vegan package. Quality filtering was performed using DADA2, and rarefaction curves confirmed sequencing saturation across all samples. To identify candidate taxa most likely to explain the differences between groups, linear discriminant analysis (LDA) effect size (LEfSe) analysis was performed with an α value = 0.05 (using Kruskal–Wallis and Wilcoxon tests) and an effect size threshold of 3 for bacteria and 2 for fungus on LDA [43, 44].

Correlations between bacteria/fungi and metabolic profiles were evaluated using Spearman's rank test. Statistical computations were considered significant when Spearman's rank correlation coefficient > 0.2 and $p < 0.05$. The values of the correlation tests were adjusted using the Benjamini–Hochberg method, and a false discovery rate-adjusted $p < 0.05$ was considered statistically significant for multiple comparisons.

Results

Fecal microbiological findings in healthy controls and patients with cirrhosis

Eighty-eight patients with cirrhosis and 22 healthy volunteers were enrolled. The median age and sex distributions were comparable across groups (Supplementary Table 1). The fecal MDRO colonization rate was higher in patients with cirrhosis than in the healthy controls (33% vs. 9.1%, $p = 0.026$) (Supplementary Table 2). Of the 29 patients with fecal MDRO carriage, six patients (30.7%) were colonized with ≥ 2 MDROs. The MDRO most isolated was third-generation cephalosporin-resistant *Escherichia coli* (58.6%), followed by vancomycin-resistant *Enterococcus* spp. (48.3%).

Characteristics and outcomes between MDRO carriers and non-carriers in patients with cirrhosis

Demographic characteristics were similar between MDRO carriers and non-carriers, including age, sex, cirrhosis etiology, comorbidities, and severity of liver disease (Table 1). However, MDRO carriers had higher plasma LPS levels (15.1 vs 10.4 ng/L, $p = 0.006$) and a higher admission rate within 30 days (34.5% vs 13.6%, $p = 0.002$). During a median follow-up of 16.4 months (range, 0.7–40.4 months), 36 patients (40.1%) developed complications associated with cirrhosis within 1 year (Table 2). HE occurred more frequently in MDRO carriers than in non-carriers (20.7 vs 3.4%, $p = 0.008$). However, other complications, including infectious events, did not differ significantly. Among infectious events, five patients developed SBP, whereas ten had non-SBP infections, including bacteremia (n = 5), intraabdominal infections (n = 2), aspiration pneumonia (n = 2), and urinary tract infection (n = 1). The positive culture rate for these infectious events was 53%, but none of the pathogens were MDROs.

Univariate Cox regression analysis showed that plasma LPS levels ≥ 14.9 ng/mL and fecal MDRO carriage were independent predictors for HE occurrence, which maintained their statistical significance on multivariable analysis, further supporting their association with HE occurrence (Table 3). Table 4 shows the risk factors associated with fecal MDRO colonization in patients with cirrhosis. In the univariate analysis, higher LPS levels (≥ 11.9 ng/mL) and prior admission in the last 30 days

Table 1 Baseline characteristics of MDRO carriers and non-carriers in patients with cirrhosis

Variables	MDRO− (n = 59)	MDRO + (n = 29)	*p*-value
Age, years	61.8 (54.4–65.4)	56.36 (46.9–64.9)	0.067
Male	45 (76.3)	23 (79.3)	0.749
Etiology of cirrhosis (%)			
Viral/alcohol/others	42 (71.2)/12 (20.3)/5 (8.5)	15 (51.7)/8 (27.6)/6 (20.7)	0.142
Laboratory			
White blood cell (1000/uL)	4.3 (3.2–5.3)	4.1 (3.0–5.5)	0.862
Platelet (1000/uL)	85.0 (58.0–120.0)	80.0 (46.0–117.5)	0.470
Sodium (mEq/L)	140 (138–142)	139 (136–141)	0.185
Creatinine (mg/dL)	0.8 (0.7–1.0)	0.8 (0.7–1.0)	0.535
Total bilirubin (mg/dL)	1.4 (1.0–2.2)	1.6 (0.9–3.7)	0.742
ALT (U/L)	27.0 (22.0–37.0)	32.0 (21.5–41.0)	0.260
Albumin (g/dL)	3.7 (3.3–4.1)	3.5 (3.1–4.0)	0.054
INR	1.3 (1.2–1.4)	1.3 (1.2–1.5)	0.131
Ammonia (ug/dL)	24.0 (17.8–29.9)	22.5 (17.7–30.7)	0.568
LPS (ng/mL)	10.4 (8.7–15.1)	15.1 (10.0–20.9)	0.006
Fecal Alb (g/dL)	4.7 (0.8–15.1)	3.4 (0.7–31.6)	0.759
Comorbidity			
Diabetes mellitus	17 (28.8)	8 (28.4)	0.904
Hypertension	8 (13.6)	2 (6.9)	0.355
Hepatocellular carcinoma	10 (16.9)	5 (17.2)	0.973
Presence of ascites	31 (52.5)	14 (48.3)	0.707
Presence of varices	53 (89.8)	25 (86.2)	0.615
Child–Pugh score	6 (5–8)	7 (5–8)	0.177
Child–Pugh class			
A/B/C	35 (59.3)/19 (32.2)/5 (8.5)	13 (44.8)/12 (41.4)/4 (13.8)	0.417
MELD score	10.5(9.0–13.4)	11.6(8.4–16.4)	0.221
Prior admission			
< 30 days	8 (13.6)	10 (34.5)	0.022
< 60 days	15 (25.4)	11 (37.9)	0.247
< 90 days	19 (32.2)	13 (44.8)	0.227

The data are expressed as median (25 th–75 th percentiles), or number (percent)

MDRO multidrug-resistant organism, *ALT* alanine aminotransferase, *INR* international normalized ratio, *LPS* lipopolysaccharide, *MELD* Model for End-stage Liver Disease

Table 2 Outcomes of MDRO carriers and non-carriers in patients with cirrhosis within one year of follow-up

Variables	MDRO− (n = 59)	MDRO+ (n = 29)	*p*-value
SBP	3 (5.1)	2 (6.9)	1.000
Infections other than SBP	6 (10.2)	4 (13.8)	0.615
Hepatic encephalopathy	2 (3.4)	6 (20.7)	0.008
Variceal bleeding	2 (3.4)	1 (3.4)	1.000
Acute kidney injury	13 (22.0)	5 (17.2)	0.600
Newly onset ascites	3 (5.1)	2 (6.9)	1.000
Newly developed HCC	4 (6.8)	2 (6.9)	1.000
Mortality	1 (1.7)	2 (6.9)	0.252
Liver transplant	1 (1.7)	0 (0)	1.000

The data are expressed as number (percent)

MDRO multidrug-resistant organism, *SBP* spontaneous bacterial peritonitis, *HCC* hepatocellular carcinoma

were significant risk factors for fecal MDRO colonization. However, in multivariable analysis, prior admission within 30 days did not predict MDRO colonization. Only LPS ≥ 11.9 ng/mL (odds ratio = 3.84; $p = 0.009$) remained an independent risk factor for MDRO colonization. Consistently, higher fecal bacterial DNA loads were observed in MDRO carriers compared with non-carriers and healthy adults (Supplementary Fig. 1 A).

Comparison of the fecal microbiota of healthy adults and patients with cirrhosis with and without fecal MDRO carriage

Compared to healthy adults, Proteobacteria was predominant in fecal samples of patients with cirrhosis (Fig. 1A). At the family level, Bacteroidaceae, Enterobacteriaceae, Lactobacillaceae and Streptococcaceae increased, whereas Lachnospiraceae and Ruminococcaceae decreased. The richness and evenness of fecal bacteria measured by the Faith's PD index and Shannon index were significantly reduced in patients with cirrhosis (Fig. 1B). In addition, the principal component analyses of the unweighted UniFrac distance and the Bray–Curtis distance showed a significant bacterial dissimilarity between these two groups (both $p = 0.001$; Fig. 1C).

The phylogenetic diversity of the fecal bacteria measured by Faith's PD index and Shannon index decreased significantly in patients with and without MDRO carriage when compared with healthy controls (Fig. 2A). No significant difference was observed between patients with and without MDROs; however, a trend toward a decrease in alpha diversity was observed in patients with cirrhosis carrying MDROs. According to unweighted UniFrac metrics, a significant dissimilarity in the fecal bacteria was observed both between healthy controls and patients with cirrhosis regardless of MDRO carriage ($p = 0.001$) and between patients with and without MDRO colonization ($p = 0.033$). However, despite overall differences across groups, MDRO-associated bacterial dissimilarity was not significant between MDRO carriers and non-carriers when measured using the Bray–Curtis distance ($p = 0.134$) (Fig. 2B). Furthermore, LEfSe analysis showed

Table 3 Cox regression for hepatic encephalopathy within one year in patients with cirrhosis

Variables	Univariate analysis			Multivariable analysis		
	HR	95%CI	*p*-value	HR	95%CI	*p*-value
Age	1.01	0.94–1.08	0.826			
Male sex	2.16	0.27–17.58	0.471			
Type 2 diabetes mellitus	0.34	0.04–2.78	0.316			
Albumin (< 3.5/≥ 3.5 g/dL)	1.81	0.45–7.23	0.403			
Sodium (< 135/≥ 135 mEq/L)	3.99	0.80–19.82	0.091	4.60	0.65–32.60	0.127
LPS (≥ 14.9/< 14.9 ng/mL)	14.97	1.84–121.83	0.011	11.01	1.24–97.50	0.031
Child–Pugh class						
A			Reference			Reference
B	3.29	0.60–17.94	0.170	1.12	0.19–6.71	0.903
C	6.59	0.93–46.82	0.060	1.71	0.18–15.82	0.639
MELD scores (≥ 10/< 10)	4.89	0.60–39.72	0.138			
History of HCC before enrollment	0.70	0.09–5.67	0.736			
MDRO carriage	6.68	1.35–33.13	0.020	5.47	1.02–29.31	0.047

HR hazard ratio, *CI* confidence interval, *LPS* lipopolysaccharide, *MELD* Model for End-stage Liver Disease, *HCC* hepatocellular carcinoma, *MDRO* multidrug-resistant organism

Table 4 Risk factors for fecal MDRO carriage in patients with cirrhosis

Variables	Univariate analysis			Multivariable analysis		
	OR	95%CI	*p*-value	OR	95%CI	*p*-value
Age	0.95	0.91–1.01	0.077	0.96	0.91–1.00	0.062
Male sex	1.19	0.41–3.51	0.749			
Type 2 diabetes mellitus	0.94	0.35–2.53	0.904			
Albumin (< 3.5/≥ 3.5 g/dL)	0.63	0.25–1.57	0.321			
Sodium (< 135/≥ 135 mEq/L)	1.25	0.28–5.62	0.775			
LPS (≥ 11.9/< 11.9 ng/ml)	4.02	1.56–10.40	0.004	3.84	1.40–10.49	0.009
Child–Pugh class						
A			Reference			
B	1.70	0.65–4.46	0.280			
C	2.15	0.50–9.28	0.303			
MELD scores (≥ 15/< 15)	2.30	0.84–6.29	0.106			
Prior admission within 30 days	3.36	1.15–9.77	0.026	1.04	0.38–2.85	0.945
Frequency of hospitalization in 3 months						
0			Reference			
1	1.29	0.46–3.62	0.625			
2–3	0.53	0.06–5.01	0.575			
History of HCC before enrollment	1.02	0.31–3.32	0.973			
Presence of ascites	0.84	0.35–2.05	0.707			

MDRO multidrug-resistant organism, *OR* odds ratio, *CI* confidence interval, *LPS* lipopolysaccharide, *MELD* Model for End-stage Liver Disease, *HCC* hepatocellular carcinoma

a prominent abundance of *Streptococcus salivarius* in MDRO carriers, whereas *Megamonas* genus was abundant in non-carriers (Fig. 2C).

Identification of the metabolic signature associated with hepatic encephalopathy in patients with fecal MDRO carriage

Total 4869 metabolic features were detected in plasma from patients with cirrhosis using untargeted metabolomic analysis, of which 1618 metabolites were putatively identified. Thirty-one metabolites differed significantly among healthy controls, and patients with and without MDRO carriage (Supplementary Table 3). The correlation analysis showed that abundance of six metabolites were significantly associated with dominant bacterial taxa in patients carrying MDROs (Fig. 3). The abundance of *Clostridioides difficile* was positively correlated with the abundance of isoaustin and 2,3-butanediol glucoside, both of which were significantly higher in MDRO carriers. Conversely, *C.difficile* was negatively correlated with DG (14:0/18:0/0:0), thelephoric acid, and 5-(3',4',5'-trihydroxyphenyl)-gamma-valerolactone, all of which were significantly reduced in MDRO carriers compared with non-carriers. Additionally, *Streptococcus salivarius* exhibited a negative correlation with PE-NMe2(24:0/20:3), which was negatively regulated in MDRO carriers compared with non-carriers. However, no definitive positive correlation was observed between *Streptococcus salivarius* and other metabolites. Among these six metabolites, only isoaustin was significantly elevated in MDRO carriers who developed HE, compared with MDRO carriers without HE and healthy controls (Fig. 4).

Differences in fungal characteristics in patients with cirrhosis with fecal MDRO carriage

Isoaustin is a fungal derived metabolite [45] and we observed that fecal samples from patients with cirrhosis carrying MDROs showed a notable increase in fungal DNA expression compared with healthy controls

(See figure on next page.)

Fig. 1 The composition and diversity of fecal microbiota in healthy subjects and patients with cirrhosis. **A** Stacked bar plots of phylogenetic composition of common bacterial taxa (> 0.1% abundance) at the phylum level and family level in fecal samples of healthy subjects and patients with cirrhosis. **B** Alpha diversity indices of fecal bacteria measured by Faith's PD index and Shannon index. **** $p < 0.0001$, *** $p < 0.001$. **C** Principal coordinate analysis of fecal microbiota by unweighted Unifrac distance metrics and Bray–Curtis distance. *Faith's PD* Faith's phylogenetic diversity, *NMDS* non-metric multidimensional scaling

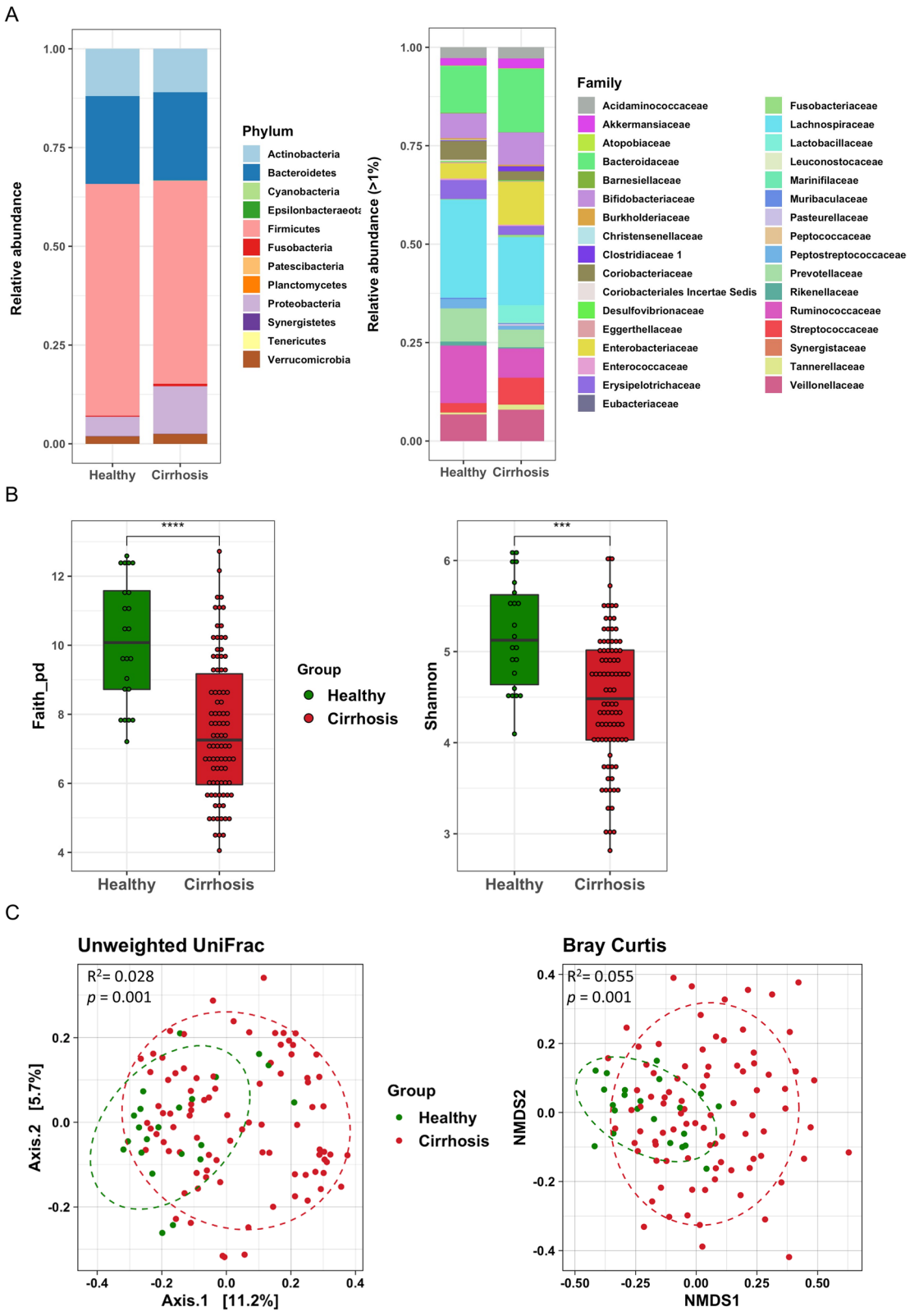

Fig. 1 (See legend on previous page.)

and patients without MDRO carriage (Supplementary Fig. 1B). To further investigate the influence of MDROs on fungal composition, we analyzed gut fungal profiles in healthy adults and patients with cirrhosis (Supplementary Fig. 2). Patients with cirrhosis, compared with healthy controls, showed a distinct change in fungal composition with an increased abundance of *Candida* at the genus level. While fungal alpha diversity measured by the Shannon index showed no significant difference between groups, Faith's PD index revealed significantly greater phylogenetic richness in patients with cirrhosis. In the beta diversity analysis, unweighted UniFrac distances indicated substantial dissimilarity in fungal communities between patients with cirrhosis and healthy controls ($p = 0.022$), reflecting fungal dysbiosis in cirrhosis. The LEfSe analysis corroborated these findings, highlighting an elevated abundance of *Candida* in patients with cirrhosis. Further analyses of the fungal composition within the cirrhotic group revealed distinct patterns between MDRO carriers and non-carriers (Fig. 5). The taxa bar plot also showed an increased abundance of *Candida, Pichia,* and *Saccharomyces* in MDRO carriers compared with non-carriers at the genus level (Fig. 5A). Alpha diversity comparisons showed no significant differences between MDRO carriers and non-carriers on Faith's PD and Shannon index (Fig. 5B). However, the beta diversity analysis using Bray–Curtis distances demonstrated significant differences in fungal communities between the two groups ($p = 0.017$; Fig. 5C). The LEfSe analysis further identified a predominant abundance of Saccharomycetes and *Candida albicans* specifically in MDRO carriers (Fig. 5D). Furthermore, we assessed the correlation between isoaustin levels and the dominant fungal profile in MDRO carriers. A positive correlation was observed between isoaustin levels and specific fungi, notably the genera *Stemphylium* and *Stemphylium vesicarium*, in MDRO carriers (Fig. 5E). This association suggests potential metabolic interactions unique to the MDRO-associated fungal profiles.

Discussion

In this study, fecal MDRO carriage in patients with cirrhosis was associated with a significantly higher risk of HE within 1 year. MDRO colonization was also associated with altered gut bacterial/fungal composition and specific metabolites, suggesting a connection between MDRO carriage and the increased risk of HE in patients with cirrhosis.

MDRO infections in cirrhosis vary widely by regions, with a higher prevalence reported in Asia, particularly India (73%), compared to 38% in Europe, less than 20% in the United States, and 34% globally [2]. However, geographic data on fecal MDRO colonization in cirrhosis remain limited. Despite this, studies have reported high MDRO colonization rates among patients with cirrhosis under different clinical conditions [3, 4, 25, 46] MDRO colonization was observed in the intestine of more than 40% of patient with decompensated cirrhosis [3]. In liver transplant candidates, MDRO colonization rates (from skin, oral and rectal samples) ranged from 20% at listing to 37% at transplantation [46]. Furthermore, MDRO colonization rates in critically ill patients with cirrhosis ranged from 33 to 73% [4, 24, 25]. Our study further supports these findings, showing a high fecal MDRO colonization rate (33%) in patients with cirrhosis under general conditions, which was significantly higher than that in healthy participants (9.1%). These results emphasize the importance of monitoring MDRO colonization in cirrhosis.

Although previous studies have linked MDRO colonization to an increased risk of subsequent MDRO infections and poorer survival [3, 4, 24, 25, 46], we did not observe a significant difference in overall infection or mortality rates between MDRO carriers and non-carriers. Instead, we observed a higher rate of overt HE in MDRO carriers. These discrepancies may be explained by differences in the severity of liver disease at enrollment. Prior studies have focused mainly on decompensated or critically ill patients [3, 4, 25, 46]. In contrast, our study enrolled patients with cirrhosis of all severities, half of them being Child–Pugh class A, and the majority (80%) being outpatients.

Our study also observed that higher plasma LPS levels and fecal MDRO carriage were significant predictors of the occurrence of HE within the first year of follow-up. Additionally, MDRO carriers exhibited higher fecal bacterial burdens than in non-carriers, suggesting gut bacterial overgrowth. As LPS is produced primarily

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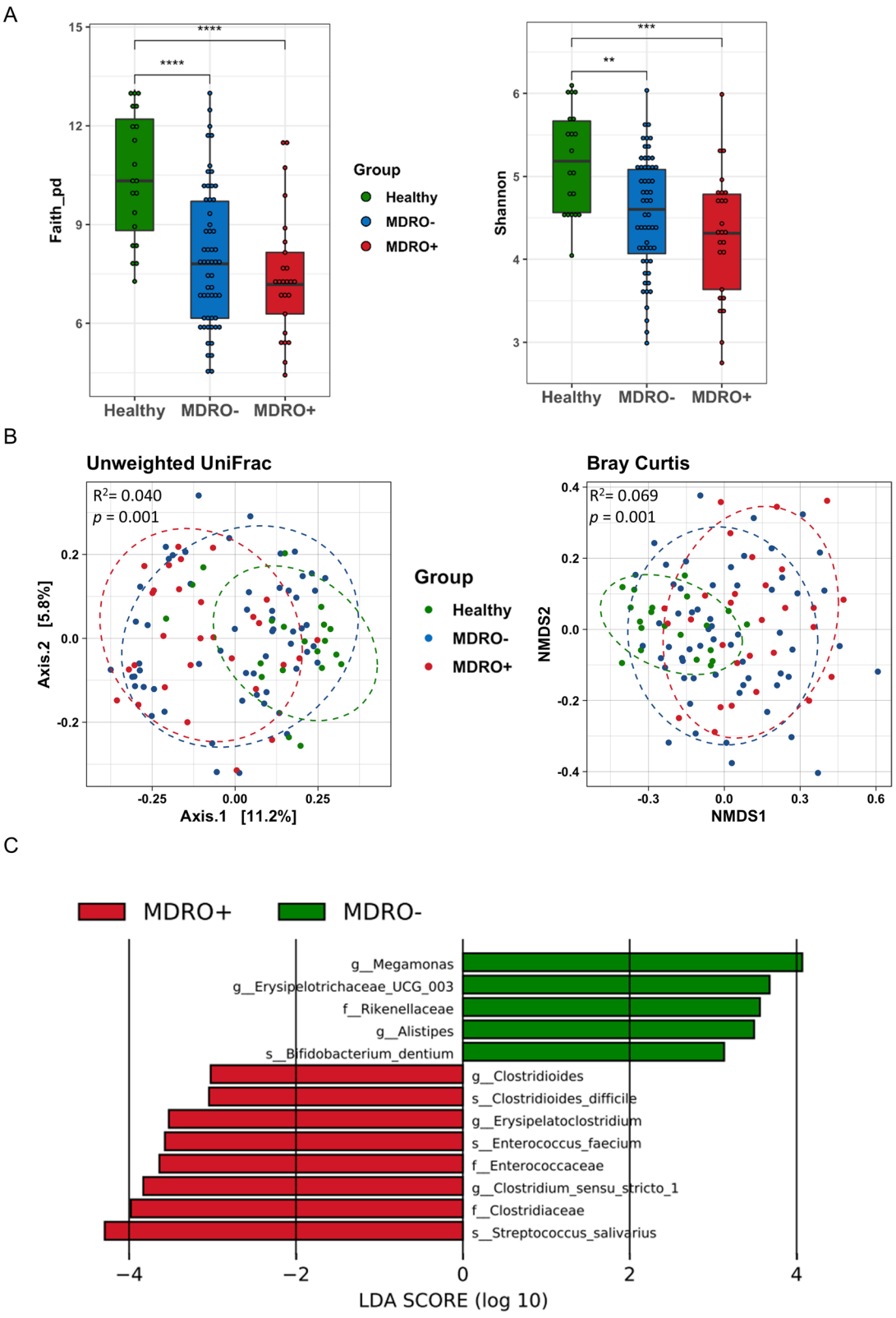
Fig. 2 The composition and diversity of fecal microbiota among healthy subjects, fecal MDRO– and MDRO + patients with cirrhosis. **A** Alpha diversity indices of fecal bacteria measured by Faith's PD index and Shannon index. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$. **B** Principal coordinate analysis of fecal microbiota by unweighted Unifrac distance metrics and Bray–Curtis distance. $p = 0.033$ by Unifrac distance metrics and $p = 0.134$ by Bray–Curtis distance between MDRO- and MDRO + patients. **C** Linear discriminant analysis (LDA) effect size (LEfSe) of differential abundance of bacterial taxa between fecal MDRO + and MDRO – patients with cirrhosis. MDRO, multidrug-resistant organism; NMDS, non-metric multidimensional scaling



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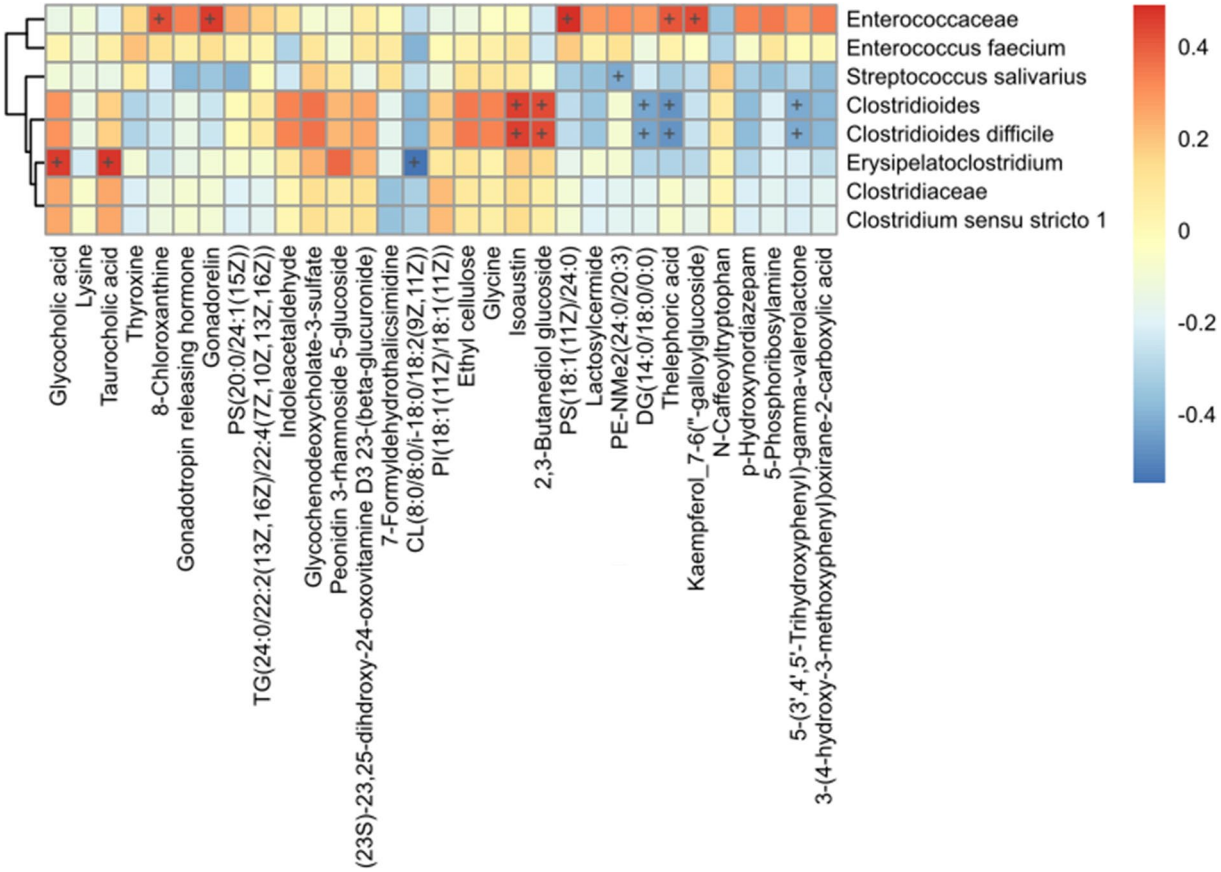

Fig. 3 Heatmap of the correlation analysis between plasma metabolites and the dominant bacterial taxa in MDRO + patients. MDRO, multidrug-resistant organism. *PS* phosphatidylserine, *TG* tracylglycerol, *CL* cardiolipin, *PE-NMe2* N,N-dimethyl phosphatidylethanolamine, *PI* phosphatidylinositol, *DG* diacylglycerol

by Gram-negative bacteria, this overgrowth may contribute to elevated systemic LPS levels and enhanced endotoxemia in MDRO carriers. Experimental studies have shown that LPS induces gut barrier dysfunction by reducing the expression of tight junction proteins and inducing mitochondrial dysfunction of the gut mucosa [47, 48]. Furthermore, chronic endotoxemia triggers the release of inflammatory cytokines (e.g., interleukin-6, interleukin-1β, and tumor necrosis factor-α), leading to systemic inflammation, which further contributes to dysfunction of the blood–brain barrier and neuroinflammation [49]. Gut microbiota alterations have also been implicated in the pathogenesis of HE in cirrhosis [50]. Bajaj et al. reported that patients with overt HE had an increased abundance of potentially pathogenic bacteria, such as *Enterococcus* and *Burkholderia,* in the colonic mucosa [51]. Zhang et al. found that *S. salivarius* was more abundant in patients with minimal HE than in those without and was positively correlated with ammonia levels [52]. Consistently, our study found that the composition of fecal bacteria differed between patients with and without MDRO carriage, with a prominent abundance of *S. salivarius* in MDRO carriers, whereas the *Megamonas* genus was abundant in non-carriers. *S. salivarius* belongs to the urease-producing bacteria, which could increase ammonia production, a key driver of HE. An overgrowth of *S. salivarius* in the MDRO carriers can exacerbate ammonia accumulation, worsening neurotoxicity and increasing the risk of HE. Though gnotobiotic models may help to clarify their roles, gnotobiotic rat models with cirrhotic portal hypertension and HE have not yet been developed, making it challenging to further evaluate the role of *S. salivarius* in HE pathogenesis.

While fungal dysbiosis has been described in cirrhosis [53], the specific link between MDROs and gut fungi has not been explored. In our study, patients with cirrhosis, particularly MDRO carriers, exhibited higher gut fungal burdens, with enrichment of Saccharomycetes and *C. albicans*. Beyond microbiota, gut-associated metabolites play a crucial role in gut-liver-brain interactions, contributing to HE development [54–57]. Our study found that isoaustin, a fungal metabolite primarily produced

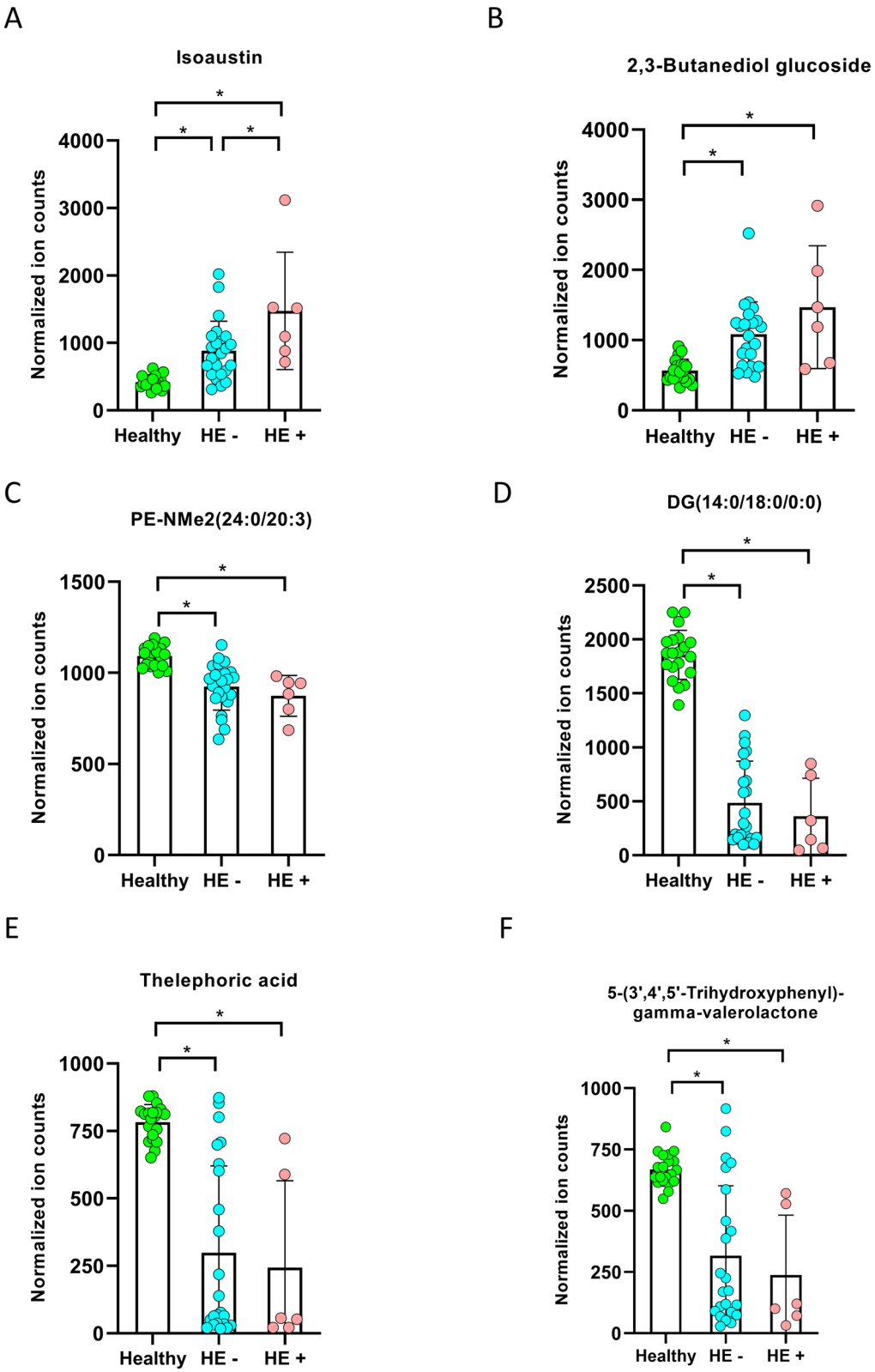

Fig. 4 Abundance of six metabolites linked to specific bacteria taxa in healthy subjects and MDRO + patients with (HE +) and without hepatic encephalopathy (HE–). *MDRO* multidrug-resistant organism, *HE* hepatic encephalopathy, *PE-NMe2* N,N-dimethyl phosphatidylethanolamine, *DG* diacylglycerol. * $p < 0.05$

by *Penicillium* spp. [45], significantly elevated in MDRO carriers with HE and was positively correlated with the abundance of *C. difficile*. Although the direct relationship between isoaustin and *C. difficile* remains unclear, previous studies have reported a higher abundance of *Penicillium* in *C. difficile* infections, supporting their potential link [58]. Furthermore, 10 out of the 11 dominant fungi in MDRO carriers were positively correlated with isoaustin levels, suggesting bacterial–fungal–metabolite interactions that may enhance isoaustin production. Liu et al. found a positive correlation between isoaustin levels and brain injury in infants, suggesting a role of isoaustin in gut-brain axis disturbances [59]. Bacteria and fungi interact bidirectionally in the gut to maintain microbiota balance [60]. Mucosa-associated fungi have been reported to promote gut homeostasis, strengthen intestinal barrier functions, and protect against bacterial infection [61]. However, some fungal metabolites with antimicrobial properties regulate bacterial growth, with penicillin being a well-known example [62]. On the other way, a reduction in short-chain fatty acids produced by bacteria has been shown to facilitate *C. albicans* growth and colonization [63]. In this study, the association of isoaustin levels with overgrowth of several fungi in MDRO patients implicated the relationship of isoaustin with fungal dysbiosis. Because bacteria and fungi interact bidirectionally in the gut to maintain microbiota balance, the isoaustin-related fungal alternation may contribute to bacterial dysbiosis, which may contribute to HE.

Although this study focused on six metabolites significantly correlated with dominant bacterial species in MDRO carriers, other metabolites potentially involved in the pathogenesis of HE may have been underexplored. For example, bile acid dysregulation has been implicated in HE, contributing to neuroinflammation and increased blood–brain barrier permeability [64]. Glycocholic acid and taurocholic acid, both elevated in MDRO carriers, have been found to be elevated in the cerebrospinal fluid in patients with HE [65]. Additionally, a mouse model of HE showed an increase in total bile acid content in brain tissue, with specific alterations in taurocholic acid isomers [66]. These findings suggest that bile acid dysregulation may also contribute to the increased risk of HE in MDRO carriers.

Taken together, altered gut bacterial/fungal composition with endotoxemia-driven systemic inflammation, ammonia-producing bacterial overgrowth, and altered gut metabolites may predispose MDRO carriers to ammonia accumulation and neuroinflammation, increasing the risk of HE.

While further studies are needed to elucidate the precise mechanisms, these findings suggest that targeting gut dysbiosis and reducing MDRO colonization may be a potential strategy for preventing HE in cirrhosis. Several approaches have been proposed for MDRO decolonization, including FMT, which has shown promise in restoring gut microbiota balance and reducing MDRO carriage [11, 12]. Additionally, FMT has demonstrated therapeutic efficacy in treating HE in patients with cirrhosis, further supporting the role of gut microbiota in HE pathogenesis [67]. However, data on its long-term safety and effectiveness in cirrhosis remain limited, underscoring the need for further research. Probiotics and prebiotics are also being explored as potential strategies for modulating gut microbiota and enhancing colonization resistance against MDROs. Certain probiotic strains, such as *Lactobacillus* spp., *Bifidobacterium* spp., and *Saccharomyces boulardii*, have been shown to inhibit MDRO colonization in small clinical studies, though their efficacy in cirrhosis requires further validation. Prebiotics promote the growth of beneficial bacteria and may help restore microbial balance, but their role in MDRO decolonization remains unclear due to insufficient human data [13].

MDROs pose a significant challenge in cirrhotic patients, where immune dysfunction and frequent healthcare exposure increase susceptibility to colonization and infections related to MDROs, especially from *Klebsiella pneumoniae*, *Escherichia coli, Acinetobacter baumannii*, and *Pseudomonas aeruginosa*. These pathogens often share resistance genes like bla_{NDM}, bla_{KPC}, and *mcr* on mobile elements, facilitating rapid spread in healthcare settings and the community [68–73]. The combination of antimicrobial resistance and hypervirulence complicates treatment for infections in cirrhosis with high mortality rates. Although new therapies such as cefiderocol and ceftazidime-avibactam offer promising solutions [74, 75], emerging resistance remains a growing concern. Genomic surveillance has uncovered complex

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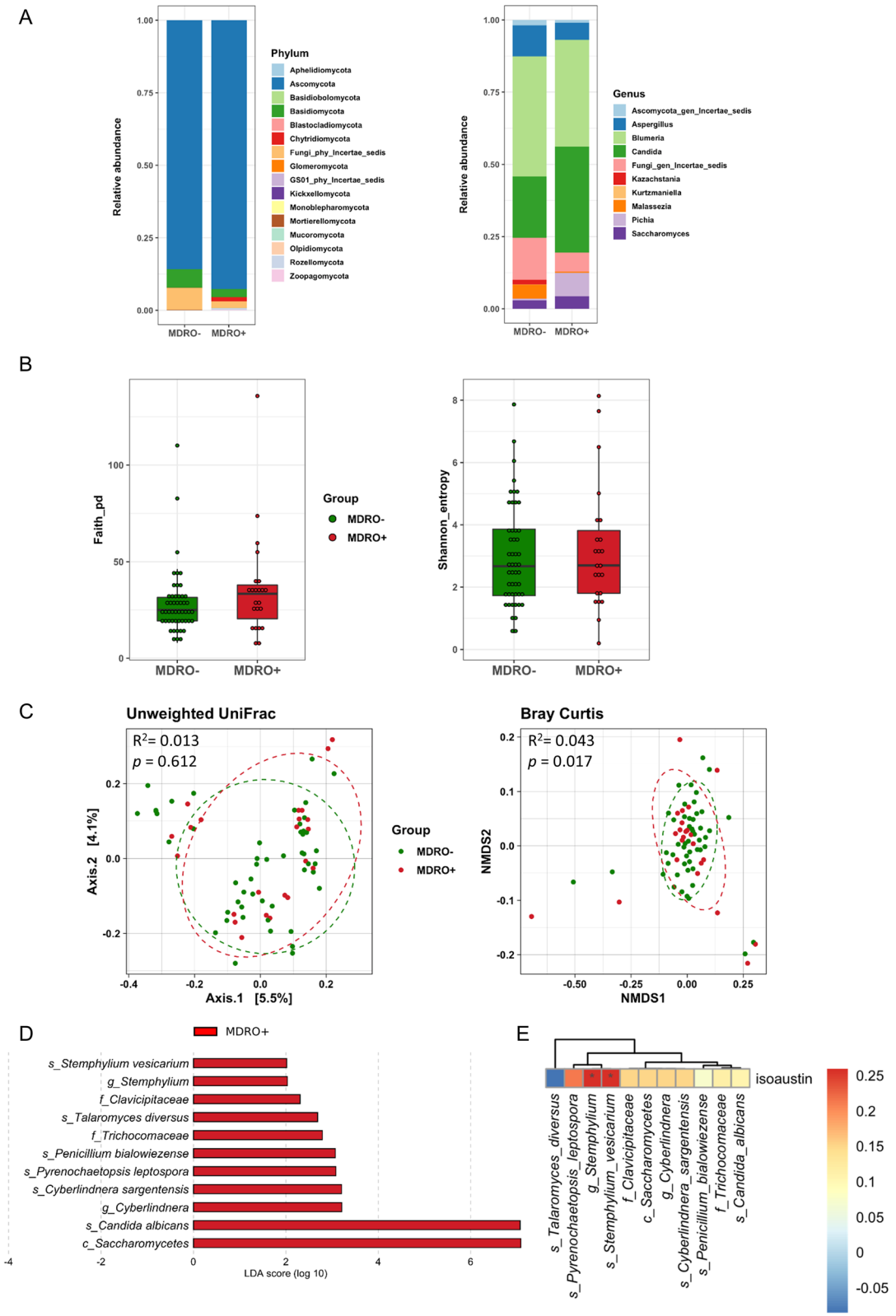
Fig. 5 The fecal fungal composition and diversity between fecal MDRO- and MDRO + patients. **A** Stacked bar charts representing relative abundance of fungal composition (> 0.1% abundance) at phylum and genus level. Only the top 10 enriched fugal taxa are shown at the genus level. **B** Alpha diversity indices of fecal fungi measured by Faith's PD index and Shannon index. **C** Principal coordinate analysis of fecal fungi by unweighted Unifrac distance metrics and Bray–Curtis distance. **D** Linear discriminant analysis (LDA) effect size (LEfSe) of differential abundance of fugal taxa. **E** Heatmap of the correlation analysis between dominant fugal taxa in MDRO + patients and isoaustin. MDRO, multidrug-resistant organism; Faith's PD, Faith's phylogenetic diversity; NMDS, non-metric multidimensional scaling



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transmission networks, reinforcing the need for precision diagnostics, antimicrobial stewardship, and coordinated global efforts. Our findings highlight the potential value of surveillance of fecal MDROs in high-risk patients and provide insights that may aid in establishing effective decolonization strategies to mitigate HE risk in cirrhosis.

This study had some limitations. First, as a single-center study with small patient numbers in Taiwan, the prevalence of MDROs and gut microbiota compositions can vary across regions due to differences in antibiotic practices and population genetics. The results may not be universally applicable across all geographic regions. Further larger, multicenter studies are warranted to validate our study findings. Second, although prior hospitalization or antibiotic exposure could potentially influence gut microbiota composition, the primary objective of this study was to investigate the interplay between gut microbiota, MDRO colonization, and cirrhosis-related outcomes. To minimize confounding effects, we excluded patients using antibiotics within 1 month prior to enrollment. Third, due to the observational design, we were unable to assess minimal HE, limiting our ability to evaluate microbial and metabolic influences in this subset of patients. Additionally, variability in sample handling by participants may cause some pre-analytical biases. To minimize individual variability across sample handling, all stool samples were collected following standardized protocols. Moreover, microbiota and metabolomic data were collected only at enrollment, hindering a time-course analysis of their fluctuations and association with HE development. Furthermore, many of the untargeted metabolites remained undiscovered, and only named metabolites were analyzed. While untargeted metabolomics provides broad metabolite coverage, it has inherent limitations in confidently identifying and quantifying all detected metabolites. For instance, isoaustin was found to correlate with HE development in MDRO carriers; however, its biological role and underlying mechanisms remain unclear. The unavailability of isoaustin in Taiwan has hindered our ability to perform mass spectroscopy or nuclear magnetic resonance spectroscopy for its precise identification and quantification, as well as to investigate bacterial-fungal-metabolite interactions and conduct experimental studies to elucidate its mechanistic role.

In conclusion, our study suggested that fecal MDRO carriage was associated with endotoxemia, gut microbiota alterations, and distinct metabolic changes, which may contribute to a higher risk of HE in patients with cirrhosis. These findings underscore the potential value of monitoring MDRO colonization in cirrhosis to improve patient outcomes. However, owing to the observational nature of the study, further research is anticipated to confirm these associations and elucidate the underlying mechanisms.

Abbreviations

MDRO	Multidrug-resistant organism
HE	Hepatic encephalopathy
LPS	Lipopolysaccharides
FMT	Fecal microbiota transplantation
SBP	Spontaneous bacterial peritonitis
ITS	Internal transcribed spacer
LC-MS	Liquid chromatography mass spectrometry
MELD	Model for End-Stage Liver Disease
Faith's PD	Faith's phylogenetic diversity
LEfSe	Linear discriminant analysis effect size

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Author contributions
PSW and KCL conceived and designed the study. All authors collected, analyzed, and interpreted the data. PSW and KCL drafted the manuscript. KCL, MCH, and BS provided critical revision during the drafting of the manuscript. All the authors read and approved the final manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
This study was approved by the Institutional Review Board of Taipei Veterans General Hospital (IRB No., 2017-09-013 C and 2019-08-013 A). Written informed consent was obtained from each participant.

Competing interests
The authors declare no competing interests.

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