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Original Article

Gut microbiome and metabolome signatures in liver cirrhosis-related complications

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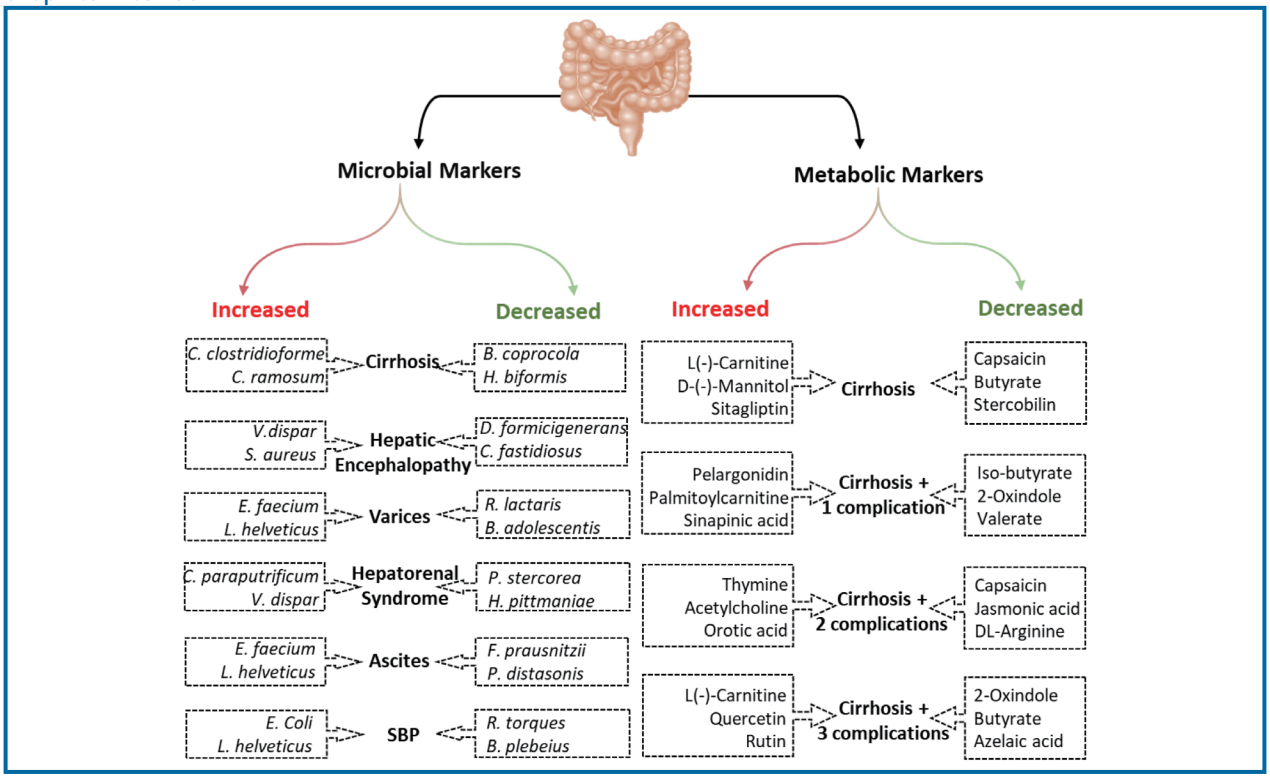
Graphical Abstract



Study Highlights

- Gut microbial dysbiosis intensifies significantly with liver cirrhosis progression which is marked by concurrent incremental changes in specific microbes and metabolites. Interestingly, cirrhosis-induced shift increases in gut microbes and metabolites are closely associated with cirrhosis-related clinical markers. This study magnifies the scope of cirrhosis biomarkers tailored to specific liver cirrhosis-associated complications. Additionally, this study highlights the relevance of decreased fecal microbial and metabolic markers in cirrhosis patients, which are closely related to clinical markers such as the MELD and CTP scores, and AST, ALT, bilirubin, and γ-GT levels. These findings enhance our understanding of the gut microbiome in cirrhosis and its linkage to associated microbial species and metabolites.

Background/Aims: Shifts in the gut microbiota and metabolites are interrelated with liver cirrhosis progression and complications. However, causal relationships have not been evaluated comprehensively. Here, we identified complication-dependent gut microbiota and metabolic signatures in patients with liver cirrhosis.

Methods: Microbiome taxonomic profiling was performed on 194 stool samples (52 controls and 142 cirrhosis patients) via V3-V4 16S rRNA sequencing. Next, 51 samples (17 controls and 34 cirrhosis patients) were selected for fecal metabolite profiling via gas chromatography mass spectrometry and liquid chromatography coupled to time-of-flight mass spectrometry. Correlation analyses were performed targeting the gut-microbiota, metabolites, clinical parameters, and presence of complications (varices, ascites, peritonitis, encephalopathy, hepatorenal syndrome, hepatocellular carcinoma, and deceased).

Results: *Veillonella* bacteria, *Ruminococcus gnavus*, and *Streptococcus pneumoniae* are cirrhosis-related microbiotas compared with control group. *Bacteroides ovatus*, *Clostridium symbiosum*, *Emergencia timonensis*, *Fusobacterium varium*, and *Hungatella*_uc were associated with complications in the cirrhosis group. The areas under the receiver operating characteristic curve (AUROCs) for the diagnosis of cirrhosis, encephalopathy, hepatorenal syndrome, and deceased were 0.863, 0.733, 0.71, and 0.69, respectively. The AUROCs of mixed microbial species for the diagnosis of cirrhosis and complication were 0.808 and 0.847, respectively. According to the metabolic profile, 5 increased fecal metabolites in patients with cirrhosis were biomarkers (AUROC >0.880) for the diagnosis of cirrhosis and complications. Clinical markers were significantly correlated with the gut microbiota and metabolites.

Conclusions: Cirrhosis-dependent gut microbiota and metabolites present unique signatures that can be used as noninvasive biomarkers for the diagnosis of cirrhosis and its complications. **(Clin Mol Hepatol 2024;30:845-862)**

Keywords: Microbiota; Metabolite; Biomarker; Cirrhosis; Complication

INTRODUCTION

The gut-liver axis exhibits a unique bidirectional relationship; therefore, dysbiosis in the gut microbiome has a profound impact on liver disease establishment and progression, especially in patients with liver cirrhosis. In dysbiosis, loss of diversity not only is defined by a relative increase in pathological species but also indicates the loss of bacteria that are beneficial for health, especially autochthonous species, which are important for stabilizing the ecological balance. Numerous clinical studies have revealed a robust and deep connection between gut dysbiosis and cirrhosis

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Abbreviations:

ACLF, Acute-on-Chronic Liver Failure; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUROC, Area Under the Receiver Operating Characteristic Curve; CTP, Child-Turcotte-Pugh; F/B ratio, firmicutes/bacteroidetes ratio; γ-GT, gamma-glutamyl transferase; HCC, hepatocellular carcinoma; HRS, hepatorenal syndrome; IFNγ, interferon gamma; IL, interleukin; INR, International Normalized Ratio; KW test, Kruskal-Wallis Test; LDA, Linear Discriminant Analysis; LEfSe, Linear Discriminant Analysis Effect Size; MELD, Model for End-Stage Liver Disease; NAFLD, nonalcoholic fatty liver disease; OUT, Operational Taxonomic Units; PCA, Principal Component Analysis; PT, Prothrombin Time; SBP, spontaneous bacterial peritonitis; VIP score, variable importance in projection score

progression from the asymptomatic compensated phase to the more severe decompensated phase.[1,2] The results of these studies indicated that pathogenic families such as *Staphylococcaceae*, *Enterobacteriaceae* and *Enterococcaceae* dominate the gut microenvironment and are linked to the severity of the disease, whereas the abundances of the autochthonous taxa *Ruminococcaceae*, *Lachnospiraceae*, and *Clostridiales XIV* decrease significantly. Moreover, the severity of cirrhosis is more strongly associated with dysbiosis at the species level, as shown by a recent study in which patients with decompensated cirrhosis had increased abundances of *Eubacterium*, *Faecalibacterium*, and *Ruminococcus* species in their gut microbiome, in contrast to *Peptostreptococcus* and *Enterococcus* species, which were more abundant in acute-on-chronic liver failure (ACLF) patients' gut microbiomes.[1] Gut dysbiosis is also associated with an altered metabolite profile, which is a compounding factor in liver diseases.[3,4] Specifically, gut microbial-derived metabolites exhibited a close association with ACLF.[5,6]

Considering these relationships between the liver-gut axis and the microbiome and metabolites, we hypothesized that metagenomics analysis at the species level and metabolite analysis would broaden our current understanding of the liver-gut axis in cirrhosis. Thus, we evaluated the differences between gut microbial biomarkers in healthy controls (HCs) and patients with cirrhosis and cirrhosis with complications such as varices, ascites, peritonitis, encephalopathy, HRS, HCC, and deceased. Furthermore, microbial and metabolite biomarkers correlated with cirrhotic clinical markers were identified to obtain detailed insights into complication-dependent bacterial species as biomarkers.

MATERIALS AND METHODS

Study design

The fecal samples from 52 healthy and 142 liver cirrhosis patients were collected for fecal microbiome profiling at Hallym University Hospitals, prospectively. Liver cirrhosis was defined with a combination of blood, liver imaging, and pathological findings and patients were grouped based on complications. The detailed baseline characteristics are explained in Supplementary Table 1. 16S rRNA sequencing was employed on 194 samples for complication based differential microbiome profiling. Subsequently, 17 healthy and 34 cirrhosis (Model for End-Stage Liver Disease [MELD] score >10) were randomly selected for fecal metabolites profiling with gas chromatography mass spectrometry and liquid chromatography coupled to time-of-flight mass spectrometry (Fig. 1A). Detailed methods used for stool microbiota-metabolome analysis were included in online supplementary files.

Statistical analysis

The group-associated difference between mean abundance in fecal microbiome and metabolite was estimated by Analysis of Variance (ANOVA) using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Biomarker ability through Receiver Operating Characteristic (ROC) curves and Spearman's rank correlation coefficient between microbiome and metabolite were evaluated by Origin Pro 2021.

RESULTS

Study population

In the cohort of 142 cirrhosis patients, 33% were females (n=46, age 61.0±13.4 years) with lower mortality rate compared to male (74%), where overall mortality rate was 24% (n=34, age 60.9±9.1 years). Patients were classified into six complication-based groups: only cirrhosis (n=10), cirrhosis with HCC (n=26), cirrhosis with varices (n=7), cirrhosis with ascites (n=26), cirrhosis with two complications (n=44), and cirrhosis with three or more complications (n=29). Etiologically, alcohol was the leading cause of cirrhosis (55.6%), followed by viral causes (30%), nonalcoholic causes (5.5%), and a combination of 2 etiologies (8.5%). Cirrhosis-related clinical markers such as AST, ALT, GGT, bilirubin, prothrombin time (PT), international normalized ratio (INR), MELD, and Child-Turcotte-Pugh (CTP) scores were significantly increased, whereas cholesterol, albumin, and platelet levels were significantly decreased in patients with cirrhosis compared to HCs (Supplementary Table 1).

Cirrhosis-related complication-dependent gut microbial variations

A multistep complication-dependent approach was utilized to evaluate shifts in the gut microbiome from healthy individuals to patients with cirrhosis and from patients with cirrhosis to patients with cirrhosis with complications (occurring and non-occurring) (Fig. 1A). A compositional shift was observed at each hierarchical level, starting at the phylum level (Fig. 1, Supplementary Figs. 1–4); the abundance of *Bacteroidetes* decreased significantly within all complications (Kruskal-Wallis test [KW] P<0.0001) except

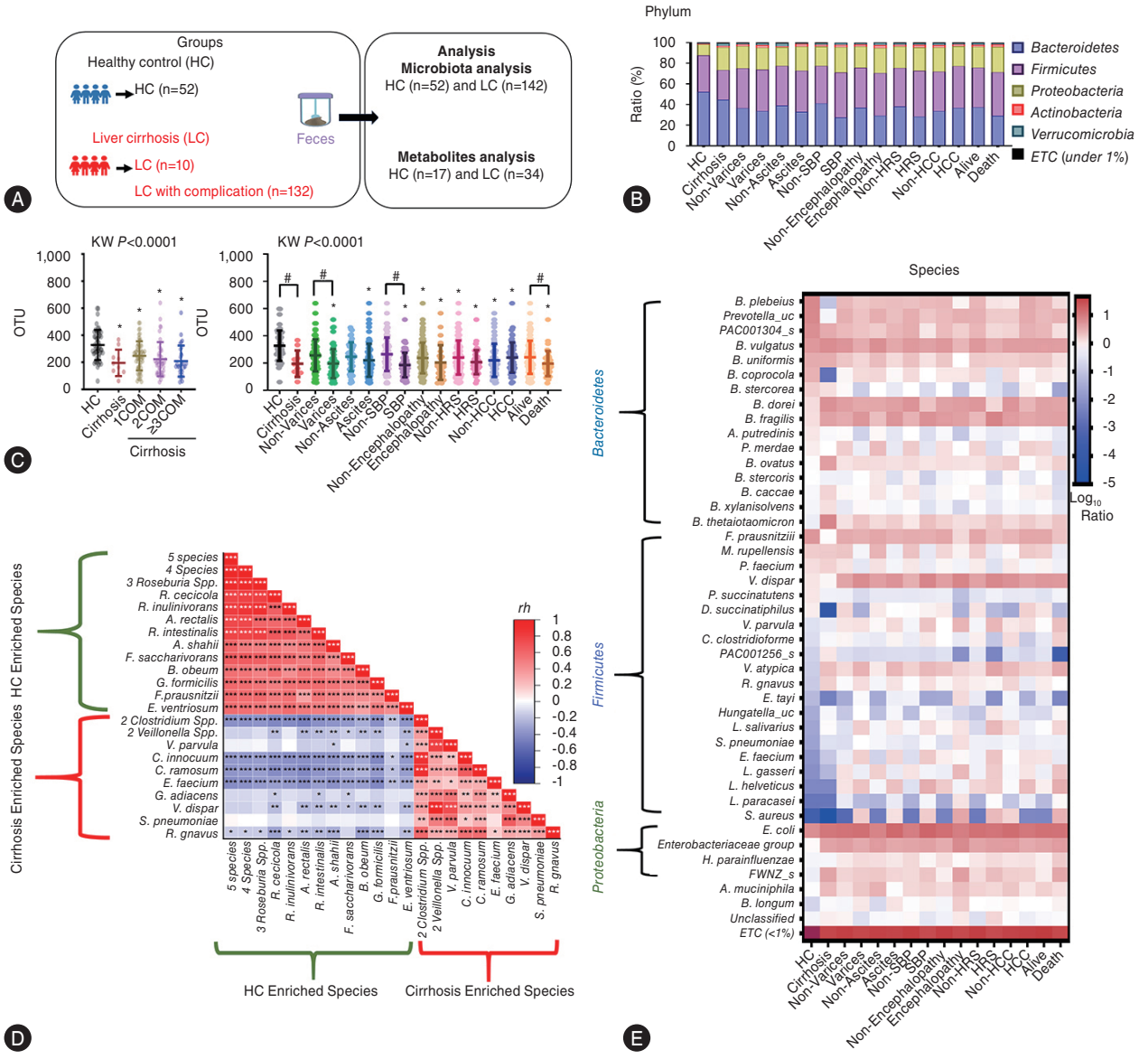

Figure 1. Complication dependent shift in fecal microbiome observed in cirrhosis patients. (A) Study work flow. (B) Relative diversity at phylum level between HC, cirrhosis, and, complication specific patients' groups. (C) Comparative OUTs observation in HC, cirrhosis and cirrhosis with compilations (in left), (in right) HC, cirrhosis and non-complication and complication specific groups. (D) Spearman correlation between cirrhosis depleted and cirrhosis enriched bacterial species. (E) Relative diversity at species level between HC, cirrhosis, and, complication specific patients' groups. OUT, Operational Taxonomic Units; HC, healthy control; SD, standard deviation. Data represented as mean±SD and statistical difference in mean between the groups measured by ANOVA using Kruskal–Wallis sum-rank test (KW) and represented by; *P<0.05, and difference between two groups measured by t-test using Mann–Whitney test and represented by $^{\#}P$<0.05.

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HCC, however *Actinobacteria* increased significantly (KW *P*=0.0.310) and *firmicutes*/*bacteroidetes* ratio (F/B ratio) (KW *P*=0.05253) insignificantly (Fig. 1B, Supplementary Fig. 1A, B). Compared with those of HCs, significant decline in number of OTUs (KW *P*<0.0001) (Fig. 1C) and Shannon index (KW *P*<0.0001) but the total read count (KW *P*=0.8936) unchanged compared to HCs (Supplementary Fig. 1C, D). Conditions such as varices, SBP, and mortality significantly changed numbers of operational taxonomic unit (OTU)s between occurred and non-occurred (Fig. 1C, right). At genus, *Veillonella* (KW *P*<0.0001), *Lactobacillus* (KW *P*<0.0001), *Enterococcus* (KW *P*<0.0001), and *Streptococcus* (KW *P*=0.0318) significantly increased in cirrhosis patients, contrasting to *Oscillibacter* (KW, *P*<0.0001), *Faecalibacterium* (KW, *P*<0.0001), and *Prevotella* (KW, *P*=0.0043) (Supplementary Fig. 2A, B)

Linear discriminant analysis effect size (LEfSe) (linear discriminant analysis [LDA] score>2 and *P*<0.05) was used to identify enriched and depleted species compared with HCs to determine the complication-dependent gut microbial signature. Later, Spearman rank correlation model confirmed a linear association between these biomarkers (Fig. 1D), with cirrhosis-enriched species inversely correlated to those enriched in HCs. In particular, *C. innocuum*, *C. ramosum*, and *E. faecium* exhibited strong negative correlations, while *V. dispar* and *R. gnavus* had moderate negative correlations. The abundances of the species *V. parvula* and *G. adiacens* were slightly negatively correlated, while S. *pneumoniae* showed almost no correlation. Complication-dependent microbial species also showed significant changes in the microbiome profile (Fig. 1E).

Microbial diversity among the four cirrhosis groups, namely, cirrhosis, cirrhosis with complications (1 complication [COM], 2 COM, and ≥3 COM), showed a marked decline in OTUs (*P*<0.0001, Fig. 1C), with unchanged total reads (Supplementary Fig. 3A) compared to HCs. Likewise, alpha (Shannon index) and beta (UniFrac distance) indices were also significantly altered with complications (Supplementary Fig. 3B, C).

Gut microbial signatures in patients with cirrhosis and complications

The top five bacterial species with increased and decreased abundances in each cirrhosis group compared to HCs based on greater significance in LDA were identified (Table 1 and Fig. 2A). A comprehensive list of complication specific species is provided in supplementary tables S2 and S3. Unique complication-specific microbial species whose abundance decreased or increased could be used as differential biomarkers for the early detection of cirrhosis and its complications. Therefore, the area under the receiver operating characteristic curves (AUROCs) was used to identify noninvasive differential biomarkers among disease-associated increased and decreased fecal microbial species. Among the decreased microbial species associated with cirrhosis and associated complications, the following AUROCs were detected: only cirrhosis (*B. coprocola* and *H. biformis*) 0.892, varices (*B. adolescentis* and *R. lactaris*) 0.732, ascites (*B. adolescentis* and *P. distasonis*) 0.669, SBP (*R. torques*) 0.657, encephalopathy (*D. formicigenerans* and *C. fastidiosus*) 0.855, HRS (*H. pittmaniae* and *R. faecis*) 0.707, HCC (*B. stercorirosoris*, *G. formicilis*, and *M. rupellensis*) 0.711, and mortality (*A. muciniphila*, *R. intestinalis*, and *R. lactatiformans*) 0.726 (Fig. 2B). The enriched microbial species associated with only cirrhosis (*C. clostridioforme*, *Hungatella_uc*, *C. ramosum*, and *F. plautii*) had an AUROC of 0.863, those associated with encephalopathy (*P. buccae* and *W. confusa*) had an AUROC of 0.733, those associated with HRS (*C. paraputrificum* and *S. salivarius*) had an AUROC of 0.709, and those associated with mortality (*R. planticola* and *Enterobacteriaceae* group) had an AUROC of 0.685 (Fig. 2C).

In addition, common bacterial species across cirrhosis groups were also identified for use as biomarkers for cirrhosis. After calculating the AUROCs for bacterial species enriched in HCs (depleted in cirrhosis), 10 species were found to have AUROCs >0.75 (Supplementary Fig. 4A). Species with high AUROCs including *R. cacicola* (0.797), *R. intestinalis* (0.792), *A. rectalis* (0.787), *R. inulinivorans* (0.780), and *A. shahii* (0.779), exhibited combined AUROCs of up to 0.826, whereas *Roseburia* species collectively achieved an AUROC of 0.803. The remaining five species *F. saccharivorans* (0.771), *B. obeum* (0.762), *G. formicilis* (0.758), *F. prausnitzii* (0.753), and *E. ventriosum* (0.751) also showed promising AUROCs values. Conversely, the AUROCs for cirrhosis enriched bacterial species were also assessed and *Firmicutes* eight species with AUROCs exceeding 0.70 were identified (Supplementary Fig. 4B) including *V. parvula* (0.744), *C. innocuum* (0.740), *C. ramo-*

sum (0.733), *E. faecium* (0.721), *G. adiacens* (0.719), *V. dispar (0.716)*, *S. pneumoniae* (0.714), and *R. gnavus* (0.706), underscoring their potential as noninvasive differential biomarkers for cirrhosis.

Differential gut microbial biomarkers between patients with cirrhosis and patients with cirrhosis-related complications

We extended the search for explicit microbial biomarkers that can differentiate between cirrhosis and associated

Table 1. Cirrhosis and complication-related microbiotas

Complication	Complication-related microbiota compared with control group	Complication-related microbiota compared with cirrhosis group
Varices	*Veillonella dispar* *Anaerostipes hadrus* *Clostridium innocuum* *Dorea longicatena* *Clostridium ramosum*	*Veillonella dispar* *Lactobacillus fermentum* *Lactobacillus paracasei* *Paraprevotella_uc*
Ascites	*Escherichia coli* *Lactobacillus gasseri* *Anaerostipes hadrus* *Anaeroglobus geminatus* *Clostridium ramosum*	*Veillonella dispar* *Lactobacillus helveticus* *Clostridium clostridioforme* *Lactobacillus fermentum* *Holdemanella biformis*
SBP	*Escherichia coli* *Anaeroglobus geminatus* *Clostridium ramosum* *Fusobacterium nucleatum* *Romboutsia timonensis*	*Veillonella dispar* *Clostridium clostridioforme* *Lactobacillus fermentum* *Lactobacillus paracasei* *Flavonifractor plautii*
Encephalopathy	*Veillonella dispar* *Veillonella atypica* *Bacteroides stercoris* *Megasphaera micronuciformis* *Clostridium ramosum*	*Staphylococcus aureus* *Lactobacillus paracasei* *Enterococcus faecium* *Lactobacillus fermentum* *Anaerotignum lactatifermentans*
HRS	*Anaerostipes hadrus* *Clostridium ramosum* *Dorea longicatena* *Veillonella_uc* *Campylobacter gracilis*	*Veillonella dispar* *Veillonella parvula* *Lactobacillus fermentum* *Veillonella_uc* *Coprococcus comes*
HCC	*Megamonas rupellensis* *Roseburia inulinivorans* *Anaerostipes hadrus* *Gemmiger formicilis* *Lachnospira pectinoschiza*	*Prevotella_uc* *Alistipes putredinis* *Bacteroides eggerthii* *Holdemanella biformis* *Parabacteroides goldsteinii*
Death	*Enterobacteriaceae group* *Akkermansia muciniphila* *Roseburia inulinivorans* *Bifidobacterium dentium* *Ruthenibacterium lactatiformans*	*Veillonella dispar* *Clostridium clostridioforme* *Lactobacillus fermentum* *Bifidobacterium dentium* *Lactobacillus delbrueckii*
Cirrhosis-related microbiota compared with control group		**Complication-related microbiota compared with cirrhosis group**
Veillonella parvula *Veillonella atypica* *Veillonella dispar* *Ruminococcus gnavus* *Streptococcus pneumoniae*		*Bacteroides ovatus* *Clostridium symbiosum* *Emergencia timonensis* *Fusobacterium varium* *Hungatella_uc*

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complications. We identified species that were more prevalent in the cirrhosis group rather than in the complication group, as detailed in Supplementary Table 4. The top 5 species for each complication are displayed in Figure 3A (left panel). Nine bacterial species, notably depleted in the complications groups, showed high AUROCs: *C. clostridioforme*, *B. ovatus*, *Hungatella_uc*, *C. symbiosum*, *F. plautii*, *A. lactatifermentans*, *F. varium*, *C. comes*, and *C. asparagiforme*. The AUROCs of three of these species exceeded 0.7, with a combined AUROC of 0.807. Including four additional species increased the combined AUROC to 0.788 (Fig. 3B).

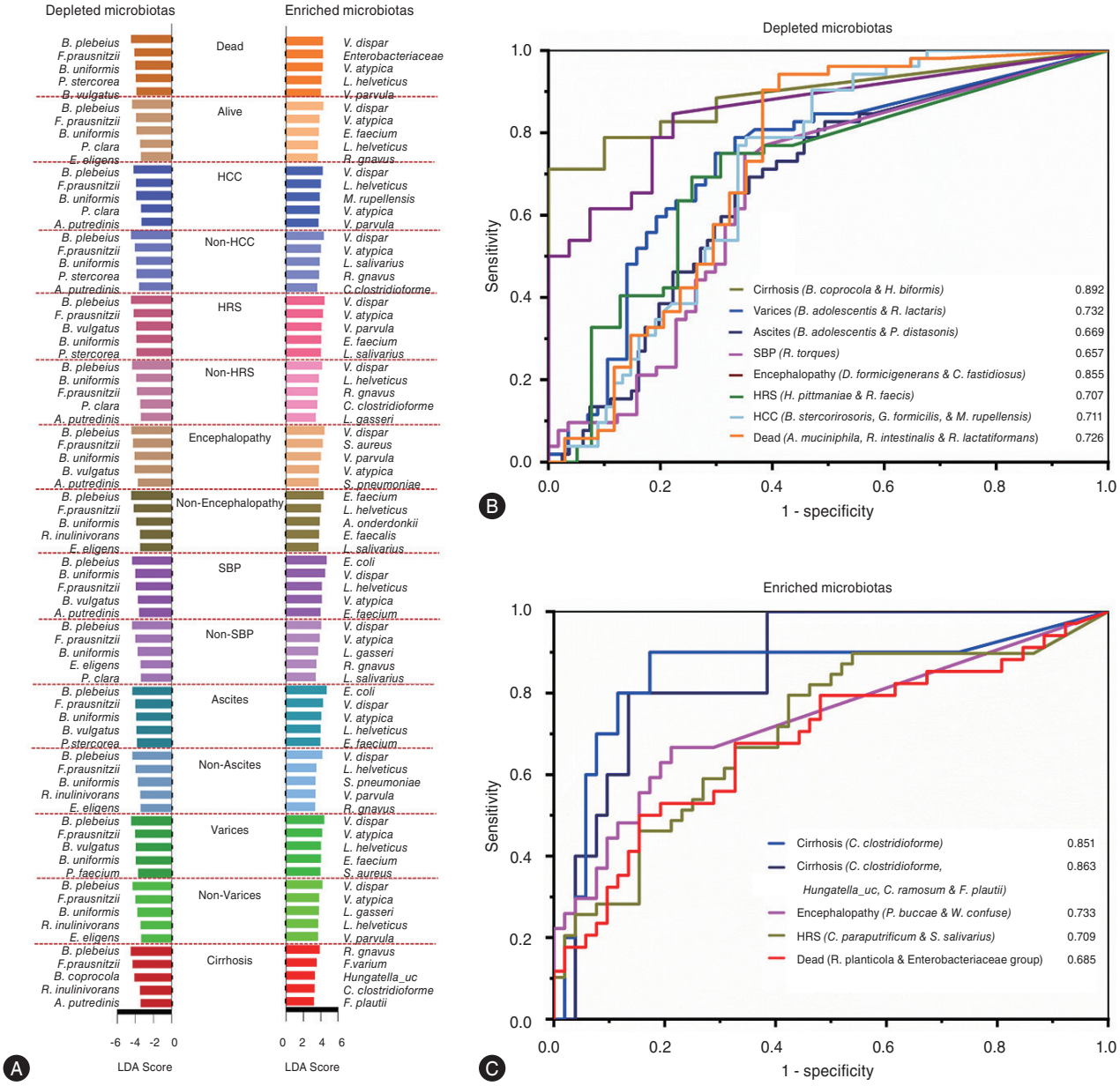

Figure 2. Identification of cirrhosis and cirrhosis-associated complication dependent fecal microbial biomarker. (A) Enriched and depleted microbiotas based on LDA score for complication associated depleted and complication associated enriched bacterial species that compared to HC, selected microbial species presented significant difference (P<0.05) with HC measured by t-test using Mann–Whitney test and represented. (B) AUROC for complications-dependent depleted bacterial species compared to HC. (C) AUROC for complications-dependent enriched bacterial species compared to HC. LDA, Linear Discriminant Analysis; HC, healthy control; AUROC, Area Under the Receiver Operating Characteristic Curve.

The complication group exhibited a greater variety of species than did the cirrhosis group (Supplementary Table 5), the top 5 most enriched complication-specific species are presented in Figure 3A (right panel). Notably, four species (*B. coprocola*, *B. coprophilus*, *A. finegoldii*, *P. goldsteinii*) showed AUROCs of up to 0.847. In cirrhosis with encephalopathy, the AUROCs of *E. faecium* and *S. aureus* reached 0.783. In HCC, the AUROCs of *A. putredinis*, *B. eggerthii*, and *Prevotella_uc* reached 0.716 In HRS *V. parvula* had an AUROC of 0.738, and in deceased patients, *B. dentium* had an AUROC of 0.729 (Fig. 3C).

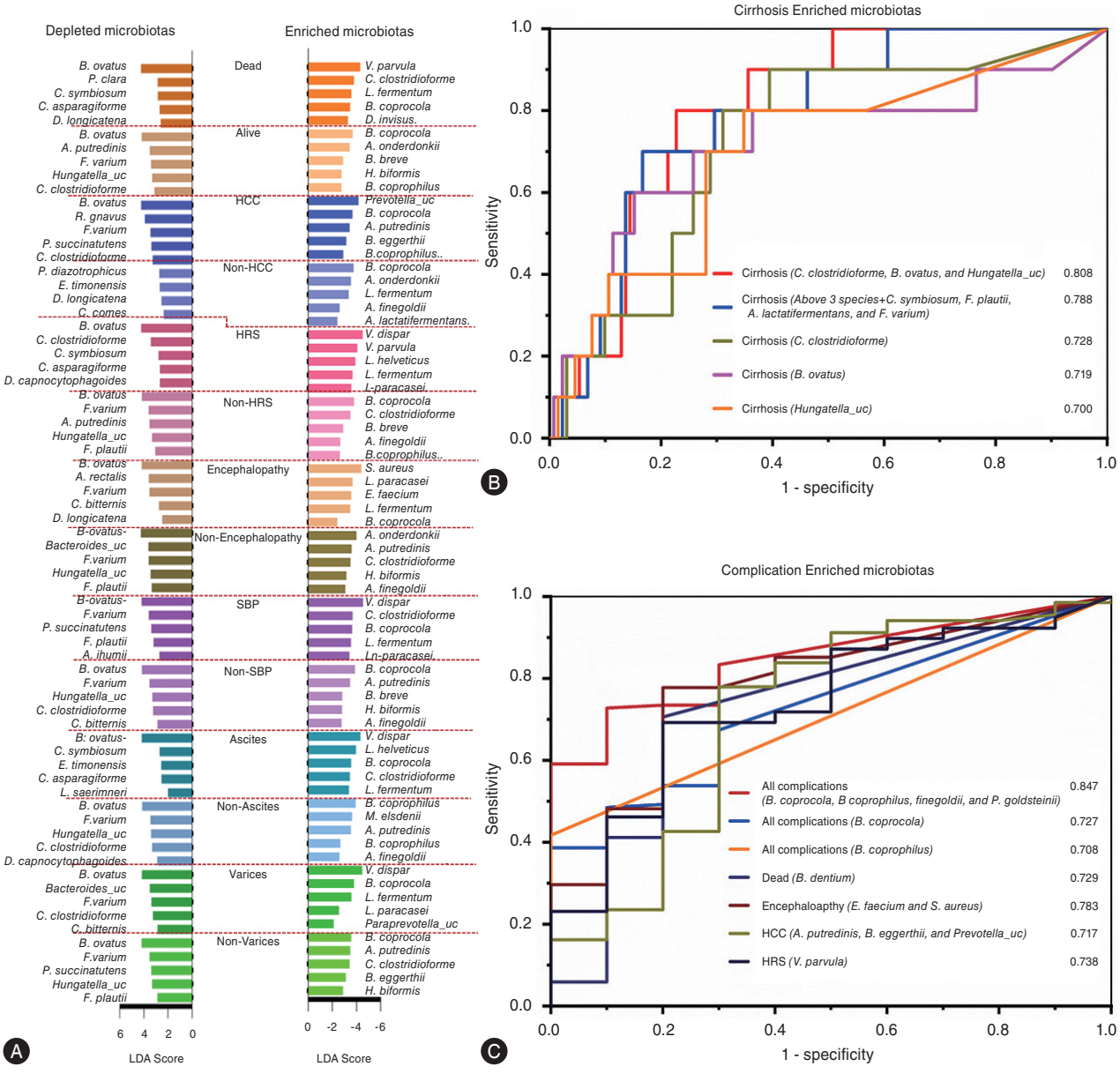

Figure 3. Assortment for differential fecal microbial biomarker between cirrhosis and cirrhosis-associated complications. (A) Depleted and enriched microbiotas based on LDA score for complication compared to cirrhosis, selected microbial species that presented significant difference (P<0.05) with cirrhosis measured by t-test using Mann–Whitney test and represented. (B) AUROC for species that bacterial species increased in cirrhosis compared to complications. (C) AUROC for specific bacterial species enriched in individual complication compared to cirrhosis. LDA, Linear Discriminant Analysis; AUROC, Area Under the Receiver Operating Characteristic Curve.

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Cirrhosis-related metabolic biomarkers

To determine differential fecal metabolic biomarkers between cirrhosis and cirrhosis with complications, metabolic data gathered from 34 cirrhosis patients (MELD score>10) including, patients with cirrhosis, cirrhosis with 1 COM (with HCC, varices, ascites), cirrhosis with 2 COM, and cirrhosis with ≥3 COM, were analyzed and compared with data gathered from HCs. A total of 104 fecal metabolites (Supplementary Table 6) were identified and distinct metabolite profiles in the cirrhosis groups were observed utilizing principal component analysis (PCA) with PC1 at 24.6%, PC2 at

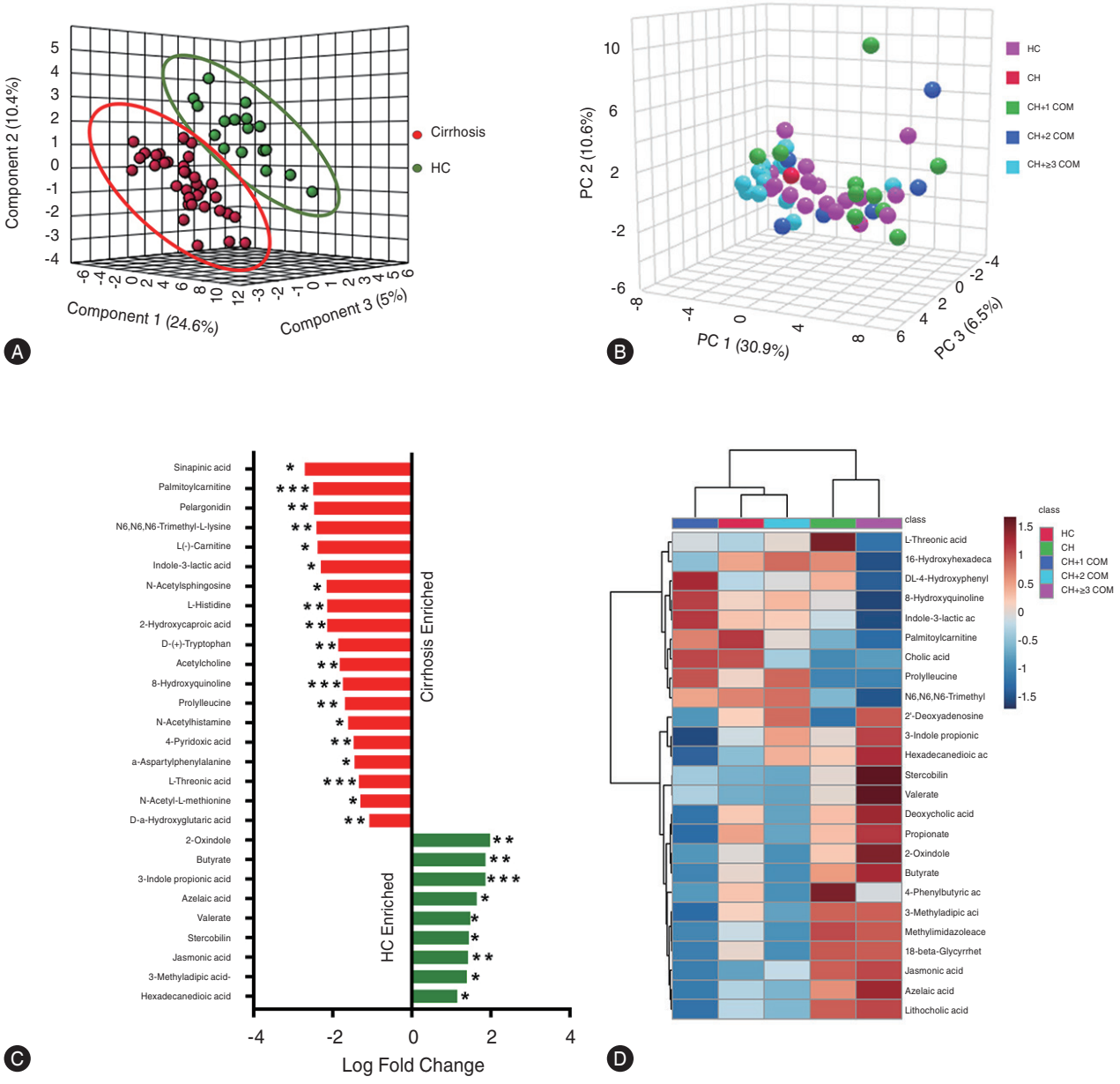

Figure 4. Cirrhosis altered fecal metabolite profiling. (A) Principal component analysis of fecal metabolites between HC and cirrhosis, and (B) principal component analysis of fecal metabolites between HC, cirrhosis and cirrhosis-associated complication patients. (C) Log fold change in metabolites in cirrhosis enriched and HC enriched. (D) Difference between HC, cirrhosis, and cirrhosis-associated complication groups based on top 25 variable fecal metabolites. HC, healthy control. The mean difference between two groups was measured by t-test using Mann–Whitney test and represented by *P<0.05, **P<0.01, ***P<0.001.

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10.4%, and PC3 at 5% (Fig. 4A). Further complication-based classification enhanced discrimination, with PC1 increasing to 30.9%, PC2 increasing to 10.6%, and PC3 increasing to 6.5% (Fig. 4B). This discrepancy pattern persisted across all cirrhosis patients and was particularly distinct when patients were grouped by complication number (Supplementary Figs. 5–7). A sum of 28 metabolites significantly differed between cirrhosis patients and HCs, with 19 increased and 9 decreased in cirrhosis patients (higher in HCs) (Fig. 4C). The top 25 differentially estimated fecal metabolites and the top 15 variables according to the variable projection (VIP) score clarified the distinctions between HCs, patients with cirrhosis, and patients with cirrhosis with complications (Fig. 4D; Supplementary Fig. 7). According to the VIP score, seven metabolites, stercobilin, lithocholic acid, butyrate, 3-Indole propionic acid, 2-oxindole, Indole-3-lactic acid, and palmitoylcarnitine, were consistently dysregulated across all groups compared to HCs (Supplementary Fig. 7). Complication-specific variations in fecal metabolites (Fig. 5A) altered metabolic pathways, as shown by the pathway enrichment ratio (Fig. 5C).

According to the microbial biomarker analysis, seven metabolites, including L (-)-carnitine (0.980), gluconic acid (0.901), cholic acid (0.882), N-acetylsphingosine (0.862), hesperetin (0.843), D-(-)-quinic acid (0.843), and 4-pyridoxic acid (0.824), exhibited increased levels only in cirrhosis patients. The differential biomarkers in non-HCC, encephalopathy, and deceased patients were acetylcholine (0.827), N-acetyl-L-phenylalanine (0.840), and alpha-aspartylphenylalanine (0.824), respectively. Conversely, six metabolites were notably depleted in cirrhosis patients: N-acetyl-L-tyrosine (0.961), DL-stachydrine (0.941), taurocholic acid (0.843), acetate (0.804), piperine (0.804), and urocanic acid (0.80392). Three metabolites, isobutyrate (0.814), isovalerate (0.814), and 3-methyladipic acid (0.807), are specifically related to cirrhosis with encephalopathy.

Furthermore, the analysis of cirrhosis-dependent metabolite biomarkers revealed that those metabolites whose levels were decreased in cirrhosis patients (in the top 5 B) had greater individual AUROCs than those whose levels were increased in cirrhosis patients (in the bottom 5 B). Conversely, combining the cirrhosis enriched metabolites presented higher AUROC (0.894) than the decreased metabolite (0.880). The top five decreased metabolites in cirrhosis patients included 3-Indole propionic acid (0.868), butyrate (0.851), jasmonic acid (0.820), azelaic acid (0.809), and stercobilin (0.802), while the most increased metabolites in cirrhosis patients were Indole-3-lactic acid (0.792), palmitoylcarnitine (0.790), N6,N6,N6-trimethyl-L-lysine (0.790), 8-hydroxyquinoline (0.778), and L-threonic acid (0.759). The enriched metabolites in HCs and cirrhotic patients (Supplementary Fig. 8C, D) were strongly negatively correlated (Fig. 5D, Supplementary Fig. 9), indicating their potential as differential biomarkers.

Correlations of gut microbial and metabolic biomarkers with cirrhosis-associated clinical markers

Twenty-three microbial species and 19 metabolites were previously shown to be correlated with 16 clinical parameters using a Spearman correlation model (Fig. 6) indicating their potential as noninvasive biomarkers. The bacterial species that were more abundant in HCs were significantly negatively correlated, and the bacterial species that were more abundant in patients with cirrhosis were significantly directly correlated with MELD and CTP scores, prothrombin time, PT/INR, GGT, AST, ALT, total bilirubin, conjugated bilirubin, and the conjugated/unconjugated bilirubin ratio. Additionally, a large number of bacterial species in HCs showed a significant positive correlation with serum ALB concentration, platelet count, and cholesterol level, and a larger number of bacterial species in patients with cirrhosis showed a significant negative correlation with those same parameters.

The combination of the 5 most prevalent species exhibited the most significant correlations with the abovementioned markers (negative and positive correlation). HC-enriched individual gut microbial markers species (*R. cecicola*, *A. rectalis*, *R. intestinalis, F. saccharivorans,* and *F. prausnitzii*) showed the most significant correlations with clinical markers associated with cirrhosis. In contrast, the most significant correlations with clinical markers were detected for 2 *Veillonella* spp., *V. parvula,* and *V. dispar,* followed by *2 Clostridium* spp., *C. innocuum*, and *E. faecium*, which are gut microbial markers associated with cirrhosis.

The metabolic markers presented similar trends, with metabolites enriched in HCs patients and decreased in cirrhosis patients exhibiting significant negative relationships with MELD and CTP scores, prothrombin time, PT/INR,

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GGT, AST, ALT, total bilirubin, conjugated bilirubin, and the conjugated/unconjugated bilirubin ratio, and significant positive relationships with albumin, platelet count, and cholesterol. In contrast, the cirrhosis-enriched metabolites exhibited the opposite trend (Fig. 7, Supplementary Fig. 10). In this correlation model, a total of 19 metabolites were in-

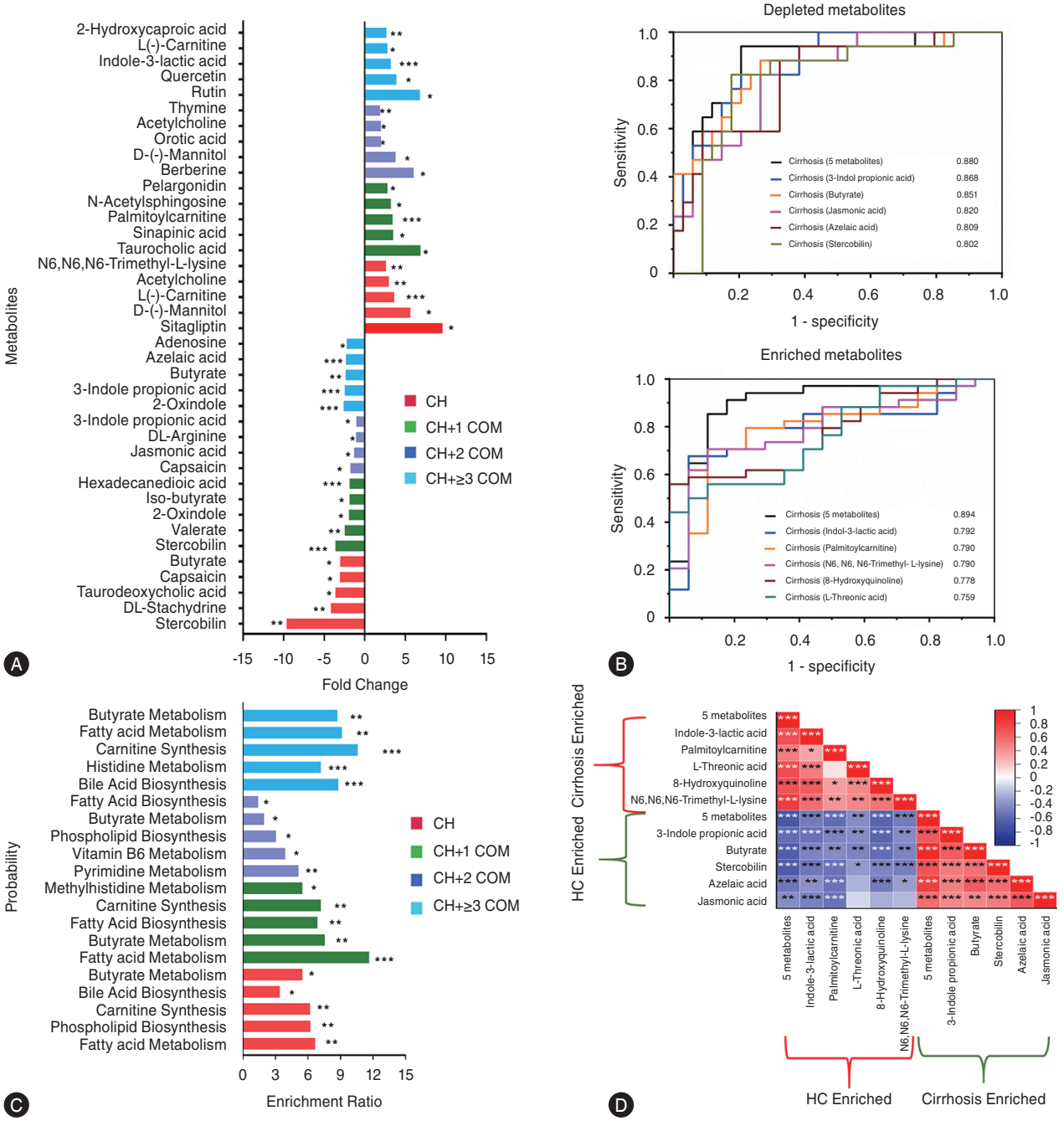

Figure 5. Cirrhosis-associated differential fecal metabolic biomarker identification. (A) 5 most significantly changed metabolite from each group compared to HC. (B) AUROC of cirrhosis depleted metabolites (top), AUROC of cirrhosis enriched metabolites (bottom). (C) Top 5 variable metabolic pathways in each group based on enrichment ratio compared to HC, mean difference between two groups measured by t-test using Mann-Whitney test and represented by *P<0.05, **P<0.01, ***P<0.001. (D) Spearman correlation analysis between cirrhosis depleted and enriched metabolites, and significance in correlation is represented as *P<0.05, **P<0.01, ***P<0.001. HC, healthy controls; AUROC, Area Under the Receiver Operating Characteristic Curve.

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cluded, with 8 in the HC-enriched group and 11 in the cirrhosis-enriched group. Among the HC-enriched metabolites, 3-Indole propionic acid, jasmonic acid, butyrate, azelaic acid, and hexadecanedioic acid were significantly highly correlated with clinical markers. However, among cirrhosis-related metabolites, N6,N6,N6-trimethyl-L-lysine, D-(+)-tryptophan, and 8-hydroxyquinoline were the most significantly correlated metabolites, followed by 3-Indole-3-lactic acid, palmitoylcarnitine, L-threonic acid, and prolyl-leucine.

We also established a correlation model between gut microbial and fecal metabolic biomarkers considering their strong and significant correlation with clinical markers. Gut microbial species were extracted from the fecal metabolites of patients and identified, and Spearman correlation models were used to evaluate the correlations (Fig. 7).

A strong negative correlation between HC-enriched microbial markers and cirrhosis-enriched metabolic markers and a strong positive correlation between HC-enriched microbial markers and HC-enriched metabolic markers were detected in the analysis. A significantly strong positive correlation was observed between butyrate, azelaic acid, and hexadecanedioic acid with all HC-enriched microbial markers. The abundances of the species *R. inulinivorans*, *R. intestinalis*, and *F. saccharivorans* presented significant strong negative correlations with most of the cirrhosis-enriched metabolic markers except acetylcholine. In contrast, cirrhosis-enriched microbial markers were weakly correlated with cirrhosis-enriched and HC-enriched metabolic markers. Whereas, cirrhosis-enriched microbial markers were poorly positively correlated with cirrhosis-enriched metabolic markers and poorly negatively correlated with HC-enriched metabolic markers. Species such as *C. innocuum, V. dispar*, and *S. pneumoniae* showed the most significant negative correlations with the highest number of HC-enriched metabolites, whereas *C. ramosum* showed

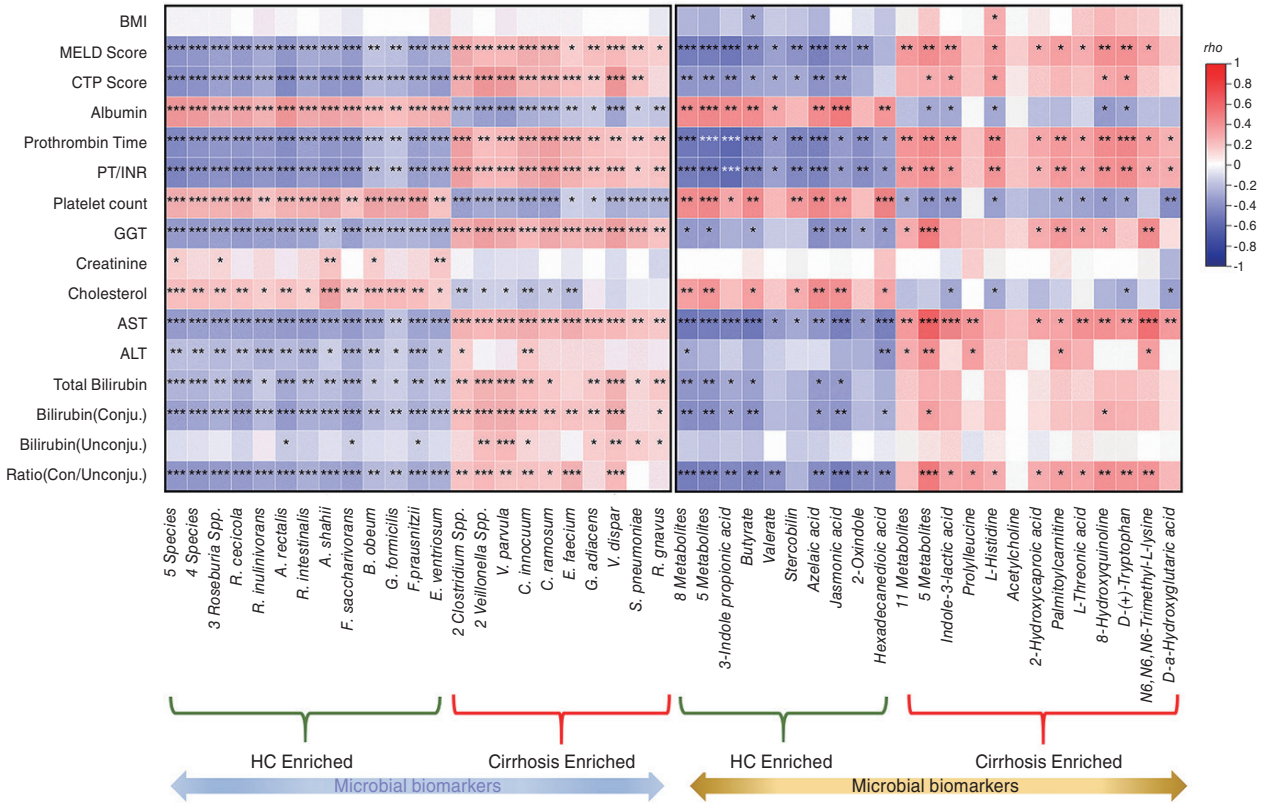

Figure 6. Correlation between gut microbial and fecal metabolic biomarkers and cirrhosis-associated clinical markers. Right panel showed correlation between gut microbial biomarker and cirrhosis-associated clinical markers and left side panel presented correlation between fecal metabolic biomarker and cirrhosis-associated clinical markers; significance in the correlation is represented as **P*<0.05, ***P*<0.01, ****P*<0.001.

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the highest correlation with cirrhosis-enriched metabolites.

DISCUSSION

Studies have shown that gut microbial biomarkers can differ between patients with compensated and decompensated cirrhosis[1,7,8] and may be useful in predicting disease progression and the risk of complications.[1,9] We systematically analyzed and compared the fecal microbial diversity at the species level in patients with cirrhosis and decompensated cirrhosis with complications and established a substantial relationship with well-known clinical markers in the present study. To make this study more inclusive of liver cirrhosis-related complications, we performed multigroup gut microbial analysis based on the occurrence and nonoccurrence of complications. Initially, in this analysis, we observed similarities in cirrhosis-dependent microbial abundances at various taxonomic levels with those of previously published studies, such as increased *Veillonella*, *Lactobacillus*, *Enterococcus*, and *Streptococcus* at the genus level and depleted *Bacteroidetes* (phylum), *Prevotella,* and *Faecalibacterium* (genus), and the similarities of results to those of previous studies validated our findings.[9]

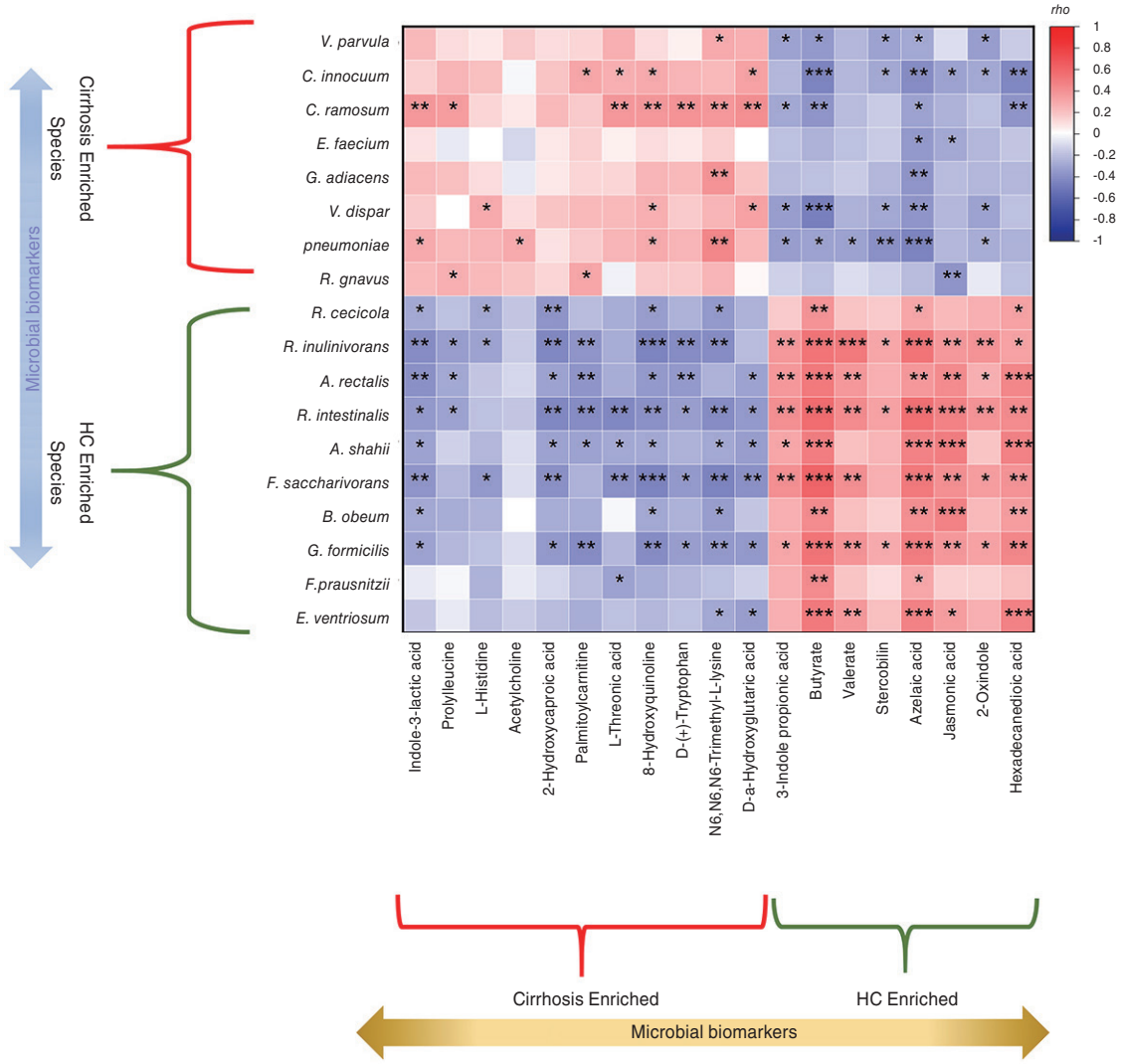

Figure 7. Correlation between cirrhosis-associated gut microbial and fecal metabolic biomarker. Correlation between gut microbial and metabolic biomarkers enriched in cirrhosis and HC, significance in correlation is represented as **P*<0.05, ***P*<0.01, ****P*<0.001. HC, healthy control.

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One of the vital outcomes of the current study is that depleted microbial species as biomarkers in patients with cirrhosis presented a greater AUROCs than increased microbial biomarkers in patients with cirrhosis when compared to HCs. The combination of the top 5 species had the highest AUROCs, that combination included 3 *Roseburia* spp., which are known autochthonous taxa and are considered next-generation probiotics that produce various beneficial health effects.[10,11] This analysis also revealed several new bacterial species that were depleted in cirrhosis patients and presented the greatest negative correlation with species increased in cirrhosis patients, especially *Clostridium spp.,* which also had the highest AUROC in cirrhosis patients. These depleted species are strict anaerobes and are responsible for producing short-chain fatty acids, particularly butyrate, which makes depletion of these species more important. Additionally, complications specifically decreased and increased bacterial species, also presented reasonably good biomarker ability, particularly in cirrhosis and encephalopathy conditions, and could have future utility.

Another unique finding of this study is the identification of complication-specific bacterial species that can serve as robust prognostic markers of cirrhosis progression from compensation to decompensation. We identified 3 gut bacterial species (*C. clostridioforme B. ovatus*, and *Hungatella_uc*) that were significantly more abundant in patients with cirrhosis than in patients with cirrhosis with complications and exhibited a high cumulative AUROC. All 3 of these species are obligate anaerobes, and 2 belong to *Firmicutes* (*C. clostridioforme*, and *Hungatella uc*). The species *C. clostridioforme* is related to liver diseases and is well known for its ethanol production.[12] In addition, 4 bacterial species (*B. coprocola, B. coprophilus, A. finegoldii, and P. goldsteinii*) were increased significantly in the cirrhosis with complications group and cumulatively showed the greatest diagnostic ability. Therefore, the ratios of the 3 species increased in non-complicated cirrhosis (*C. clostridioforme*, *B. ovatus*, and *Hungatella_uc*) and the 4 bacterial species increased in cirrhosis with complications (*B. coprocola, B. coprophilus, A. finegoldii,* and *P. goldsteinii*) could be good prognostic biomarkers for cirrhosis progression from compensated to decompensated cirrhosis.

In addition, increased abundances of *E. faecium* and *S. aureus* could be early predictors of hepatic encephalopathy in cirrhosis patients. Both of these species are known to play critical roles in liver diseases,[13,14] thus, monitoring the abundance of these species is critical, especially for determining the prognosis of hepatic encephalopathy. Additionally, a constant increase in 3 species (*A. putredinis*, *B. eggerthii*, and *Prevotella_uc*) can be a predictor of end-stage liver disease, particularly HCC.

We identified 4 bacterial species as promising differential biomarkers between healthy individuals and patients with cirrhosis alone. Among the 4 bacterial species identified in this study, *C. clostridioforme*, which is a prominent biomarker of liver function, showed a positive association with liver function; previously, the cirrhosis-related biomarker taxa, *Hungatella,* showed a negative correlation with liver function.[12] Cirrhosis-dependent depletion of bacterial species compared to the control showed greater biomarker ability, in which the depleted species *B. coprocola* indicated a greater risk of hepatic encephalopathy when its fecal concentration increased,[15] and *H. biformis was* also positively associated with fibrosis.[16] The hepatic encephalopathy-associated gut microbial species *W. confusa* is associated with promoting the development of fatty liver by increasing the circulatory ethanol concentration.[17] Hepatic encephalopathy-dependently reduced microbial taxa, such as *D. formicigenerans,* are related to improved ICI-dependent antitumor immunity,[18] and *B. cellulosilyticus* reduces hyperlipidemia and improves atherosclerosis.[19] The HRS-associated gut microbial species *C. paraputrificum* is responsible for producing gas-forming liver abscesses[20] and ulcerative colitis.[21] The second gut microbial species associated with HRS completion is *S. salivarius,* whose higher abundance in the gut is correlated with a high accumulation of ammonia in hepatic encephalopathy patients and with SBP in patients who underwent liver transplantation.[22] On the other hand, gut microbial species depleted in the HRS group, such as *R. faecis*, have been shown to alleviate fibrosis.[3] *R. planticola* has been identified as a gut microbial biomarker associated with decease in liver cirrhosis patients and is reported to cause liver abscesses.[23] The 3 gut microbial species recognized as being depleted in patients with fatal cirrhosis are *A. muciniphila,*[24,25] *R. intestinalis,*[26,27] and *R. lactatiformans,*[28,29] and they are known to have beneficial health effects, especially in patients with liver diseases.

We found significant differences in fecal metabolites be-

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tween healthy controls and cirrhosis patients, similar to the findings of a previous study of blood.[30] Overall, 5 metabolites with cirrhosis-dependent increases and decreases showed significantly high diagnostic ability, although the individual AUROCs were high for cirrhosis-dependent decreased metabolites. The 3-Indole propionic acid protects against liver injury by inhibiting NF-κB signaling, boosting the production of proinflammatory cytokines, and hindering hepatic fibrosis by suppressing the activation of hepatic stellate cells,[31] and butyrate regulates the LKB1-AMPK-Insig signaling pathway to reduce hepatic injury[32] in addition to other health-promoting effects.[33,34] Another decreased metabolite, stercobilin, is a fecal pigment that is metabolized by gut bacteria, and increases in this metabolite in feces and plasma are related to inflammation.[35] However, its depletion in feces is related to autism;[36] therefore, further investigations are required to establish its strong relationship with cirrhosis. Among metabolites increased in cirrhosis, Indole-3-lactic acid is a gut microbe produced Indole intermediate metabolites that can regulate T-cell-controlled immunomodulation.[37,38] Thus, increased excretion of Indole-3-lactic acid in feces can be related to decreased T-cell-regulated immunomodulation and linked to cirrhosis progression. The other 2 metabolites, palmitoylcarnitine, are intermediate metabolites of fatty acid metabolism and are correlated with liver diseases[39,40] and N6,N6,N6-trimethyl-L-lysine is an intermediate of lysine degradation and is related to liver and cardiac diseases.[30]

The significant finding of this study is the correlation between hallmark clinical biomarkers of cirrhosis and the identified excretory microbial and metabolic biomarkers. Compared with metabolic biomarkers, clinical biomarkers and microbial markers exhibited superior correlations, particularly with MELD score,[41] hemostatic markers,[42] AST,[43] conjugated bilirubin levels,[44] GGT,[45] and albumin,[46] which are considered the most valuable prognostic tools for determining the severity of compensated to decompensated cirrhosis. This remarkable correlation with hallmark clinical biomarkers of cirrhosis and notably high AUROCs values make these noninvasive microbial biomarkers excellent substitutes for biomarkers that require invasive clinical tests. Furthermore, metabolic markers that are reduced in patients with cirrhosis are strongly correlated with clinical markers, whereas metabolic markers are increased in patients with cirrhosis. Hence, metabolic biomarkers can complement microbial biomarkers, and both provide a good experimental framework for discovering the pathophysiology of decomposition in cirrhosis.

Moreover, the associations of decreased microbial and metabolic markers with cirrhosis were greater than those of cirrhosis-enriched microbial and metabolic markers. These findings suggest that cirrhosis-dependent decreases in gut microbial species are more relevant than cirrhosis-enriched species. Five species (*R. cacicola, R. intestinalis, R. inulinivorans, F. prausnitzii,* and *E. ventriosum*) are known butyrate-producing bacteria[33] and are strongly negatively correlated with butyrate. Species such as *R. intestinalis* and *F. prausnitzii* are known for their ability to repair the gut barrier, ameliorate inflammation through increased production of anti-inflammatory cytokines (IL10 and IL22), suppress pro-inflammatory cytokines (IL17 and IFNγ), and improve energy metabolism.[26,47]

Empirical data from the scientific literature suggest that the gut microbiome influences various immunological, metabolic and molecular pathways through microbe-associated molecular patterns (MAMPs) and pathogen-associated molecular patterns (PAMPs). It exerts its effects through its metabolites such as: short-chain fatty acids (SCFAs), Indole and tryptophan metabolism-associated metabolites,[48] choline metabolites (TMA and TMAO), bile acid, and by-products of fermentation. Thus, gut microbiome alterations are crucial because they are connected with metabolic shifts that lead to altered physiological pathways that either deteriorate or improve liver health, accordingly changing the clinical and immunological markers related to the liver.[49,50] Consequently, increased liver cirrhosis severity in the gut is decisive, and this correlation strengthens the gut microbiome and cirrhosis progression and encourages the use of noninvasive microbial biomarkers as robust prognostic tools in patients with decompensated cirrhosis. This study also demonstrated the significance of cirrhosis-dependent decreases in the gut microbiome, which can be directly linked to metabolic profiles and can ameliorate the pathophysiological pathways involved in cirrhosis progression. Thus, the combination of these noninvasive microbial biomarkers for the early detection of decompensation in patients with cirrhosis and the use of new generations of probiotics to limit the progression of the disease could improve mortality related to ACLF.

Along with substantial positive outcomes, this trial also

has several limitations. Further investigation through future preclinical and clinical trials is necessary to improve our understanding of the role of gut microbes and metabolites as biomarkers in liver cirrhosis and associated complications. However, this current clinical trial has successfully identified and established correlations between several fecal microbial and metabolite biomarkers and clinical markers, nonetheless, the pathophysiological connections within these intra-correlations need extensive exploration. Therefore, preclinical and clinical trials are essential for validating associations between these markers and for the establishment of robust pathophysiological mechanisms related to the progression of liver cirrhosis severity. Despite having adequate patient numbers to identify the biomarkers, validation of these biomarkers is vital to ensure their accuracy, reliability, and clinical utility. Therefore, multicentric larger population-based clinical trials are required to determine the clinical relevance of these identified microbial and metabolic biomarkers corresponding to the progression of liver cirrhosis severity.

Since this was a cross-sectional observational trial, temporal variation in the gut microbial ecology and fecal metabolic profile as liver cirrhosis progresses is a major concern. These temporal variations are pivotal indicators of the progression of liver cirrhosis and its complications: thus, it is essential to measure these differences in the fecal microbiome and metabolites. To address these time-associated variabilities, a longitudinal clinical trial with specific and varied time-points for fecal samples collections following the liver cirrhosis advancement is required for cirrhosis progression-associated fecal microbiome and metabolite biomarker selection. This longitudinal clinical trial could possibly identify the liver cirrhosis progression associated fecal microbial and metabolite biomarkers.

Authors' contribution

Guarantor: The corresponding author (K.T.S.) has full access to all the data used in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Conception and design, administrative support, manuscript writing: S.P.S. Financial support: K.T.S. Collection and assembly of data: all authors. Data analysis and interpretation: all authors. Final approval of manuscript, accountable for all aspects of the work: all authors.

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Conflicts of Interest

All authors declare no conflicts of interest.

SUPPLEMENTARY MATERIAL

Supplementary material is available at Clinical and Molecular Hepatology website (http://www.e-cmh.org).

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