Gut microbiome and metabolome signatures in liver cirrhosis-related complications

Short title: Gut microbiota and metabolites in cirrhosis

Satya Priya Sharma1†, Haripriya Gupta1, Goo-Hyun Kwon1, Sang Yoon Lee1, Seol Hee Song1, Jeoung Su Kim1†, Jeong Ha Park1, Min Ju Kim1, Dong-Hoon Yang1, Hyunjoon Park1, Sung-Min Won1, Jin-Ju Jeong1, Ki-Kwang Oh1, Jung A Eom1, Kyeong Jin Lee1, Sang Jun Yoon1, Young Lim Ham2, Gwang Ho Baik1,3, Dong Joon Kim1,3, Ki Tae Suk1,3*,

* Corresponding author
† These authors equally contributed

Authors’ current affiliations and institutions participating in the study

1Institute for Liver and Digestive Diseases, Hallym University, Chuncheon, Republic of Korea 24253
2Department of Nursing Daewon University College Jecheon, Republic of Korea 27135
3Department of Internal Medicine, Hallym University College of Medicine, Chuncheon, Republic of Korea 24253

Corresponding author information

Ki Tae Suk, M.D., Ph.D.

Department of Internal Medicine, Hallym University Chuncheon Sacred Heart Hospital
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, *f 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
ABSTRACT

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.

Conclusion: Cirrhosis-dependent gut microbiota and metabolites present unique signatures that can be used as noninvasive biomarkers for the diagnosis of cirrhosis and its complications.

Key Words: Microbiota; Metabolite; Biomarker; Cirrhosis; Complication
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.
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 progression from the asymptomatic compensated phase to the more severe decompensated phase.\textsuperscript{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.\textsuperscript{1} Gut dysbiosis is also associated with an altered metabolite profile, which is a compounding factor in liver diseases.\textsuperscript{3,4} Specifically, gut microbial-derived metabolites exhibited a close association with ACLF.\textsuperscript{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 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.
METHODS

Study Design

The fecal samples from 52 healthy and 142 liver cirrhosis patients collected for fecal microbiome profiling at Hallym university hospitals, prospectively. Liver cirrhosis was defined with combination of blood, liver imaging, and pathological finding and patients were grouped based on complications. The detailed baseline characteristics are explained in supplementary table 1. 16S rRNA sequencing employed on 194 samples for complication based differential microbiome profiling. Subsequently, 17 healthy and 34 cirrhosis (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 (figure 1A). Detailed methods used for stool microbiota-metabolome analysis included in online supplementary files.

Statistical Analysis

The group associated difference between mean abundance in fecal microbiome and metabolite were estimated by Analysis of Variance (ANOVA) by using GraphPad Prism 8 (GraphPad Software, San Diego, USA). Biomarker ability thru 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 less 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), INR, Model for End-Stage Liver Disease (MELD), and Child-Turcotte-Pugh (CTP) score 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) (figure 1A). A compositional shift was observed at each hierarchical level, starting at the phylum level (figure 1, supplementary figure S1-S4); the abundance of *Bacteroidetes* decreased significantly within all complications (KW p<0.0001) except HCC, however *Actinobacteria* increased significantly (KW p=0.0.310) and F/B ratio (KW p=0.05253) insignificantly (figure 1B, supplementary figure S1A, B). Compared with those of HCs, significant decline in number of OTUs (KW p<0.0001) (figure 1C) and Shannon index (KW p<0.0001) but the total read count (KW p=0.8936) unchanged compared to HCs (supplementary figure S1 C, D). Conditions such as Varices, SBP, and mortality significantly changed numbers of OTUs between occurred and non-occurred (figure 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*
Linear discriminant analysis effect size (LEfSe) (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 (figure 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. gnarus 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 (figure 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, figure 1C), with unchanged total reads (supplementary figure 3A) compared to HCs. Likewise, alpha (Shannon index) and beta (UniFrac distance) indices were also significantly altered with complications (supplementary figure 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 figure 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 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 (figure 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 (figure 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 figure 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 was also assessed and Firmicutes eight species with AUROCs exceeding 0.70 were identified (supplementary figure 4B) including V. parvula (0.744), C. innocuum (0.740), C. ramosum (0.733), E. faecium (0.721), G. adiacens (0.719), V. dispar (0.716), S. pneumoniae (0.714), and R. gnavus (0.706), underlining 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 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 (figure 3B).

The complication group exhibited a greater variety of species than did to 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 0.729, (figure 3C).

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 10.4%, and PC3 at 5% (figure 4A). Further complication based classification enhanced discrimination, with PC1 increasing to 30.9%, PC2 increasing to 10.6%, and PC3 increasing to 6.5% (figure 4B). This discrepancy pattern persisted across all cirrhosis patients and was particularly distinct when patients were grouped by complication number (supplementary figure S5-S7). A sum of 28 metabolites significantly differed between cirrhosis patients and HCs, with 19 increased and 9 decreased in cirrhosis patients (higher in HCs) (figure 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 (figure 4D; supplementary figure 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 figure S7). Complication-specific variations in fecal metabolites (figure 5A) altered metabolic pathways, as shown by the pathway enrichment ratio (figure 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 figure 8 C, D) were strongly negatively correlated (figure 5D, supplementary figure 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 (figure 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 above mentioned 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, 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 (figure 7, supplementary figure 10). In this correlation model, a total of 19 metabolites were included, 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, palmitoyl carnitine, L-threonic acid, and prolylleucine.

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 (figure 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 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 multi-group 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.\(^9\)

One of the vital outcomes of the current study is that depleted microbial species as biomarkers in patients with cirrhosis presented a greater AUROC 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.\textsuperscript{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.\textsuperscript{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\textsuperscript{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 (\textit{A. putredinis}, \textit{B. eggerthii}, and \textit{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, \textit{C. clostridioforme}, which is a prominent biomarker of liver function, showed a positive association with liver function; previously, the cirrhosis-related biomarker taxa, \textit{Hungatella}, showed a negative correlation with liver function.\textsuperscript{12} Cirrhosis-dependent depletion of bacterial species compared to the control showed greater biomarker ability, in which the depleted species \textit{B. coprocola} indicated a greater risk of hepatic encephalopathy when its fecal concentration increased\textsuperscript{15}, and \textit{H. bifformis} was also positively associated with fibrosis.\textsuperscript{16} The hepatic encephalopathy-associated gut microbial species \textit{W. confusa} is associated with promoting the development of fatty liver by increasing the circulatory ethanol concentration.\textsuperscript{17} Hepatic encephalopathy-dependently reduced microbial taxa, such as \textit{D. formicigenerans}, are related to improved ICI-dependent antitumor immunity\textsuperscript{18}, and \textit{B. cellulosilyticus} reduces hyperlipidemia and improves atherosclerosis\textsuperscript{19}. The HRS-associated gut microbial species \textit{C. paraputrificum} is responsible for producing gas-forming liver abscesses\textsuperscript{20} and ulcerative colitis.\textsuperscript{21} The second gut microbial species associated with HRS completion is \textit{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\textsuperscript{22}. On the other hand, gut microbial species depleted in the HRS group, such as \textit{R. faecis}, have been shown to alleviate fibrosis.\textsuperscript{3} \textit{R. planticola} has been identified as a gut microbial biomarker associated with deceased in liver cirrhosis patients and is reported to cause liver abscesses.\textsuperscript{23} The 3 gut microbial species recognized as being depleted in patients with fatal cirrhosis are \textit{A. muciniphila}\textsuperscript{24,25}, \textit{R. intestinalis}\textsuperscript{26,27}, and \textit{R. lactatiformans}\textsuperscript{28,29}, and they are known to have beneficial health effects, especially in patients with liver diseases.
We found significant differences in fecal metabolites between healthy controls and cirrhosis patients, similar to the findings of a previous study of blood. Overall, 5 metabolites with cirrhosis-dependent increases and decreases showed significantly high diagnostic ability, although the individual AUROCs was 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, and butyrate regulates the LKB1-AMPK-Insig signaling pathway to reduce hepatic injury in addition to other health-promoting effects. 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. However, its depletion in feces is related to autism therefore, further investigations are required to establish its strong relationship with cirrhosis. Among metabolite increased in cirrhosis, Indole-3-lactic acid is a gut microbe produced Indole intermediate metabolites that can regulate T-cell-controlled immunomodulation. 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 and N6,N6,N6-trimethyl-L-lysine is an intermediate of lysine degradation and is related to liver and cardiac diseases.

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, hemostatic markers, AST, conjugated bilirubin levels, GGT, and albumin 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 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.

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, choline metabolites (TMA and TMAO), bile acid, and byproducts 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. 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 a 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 are 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 with 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, multi-centric 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 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-point 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.
Acknowledgments

This research was supported by the Hallym University Research Fund, the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF-2020R1I1A3073530 and NRF-2020R1A6A1A03043026), the Korea Institute for Advancement of Technology (P0020622).

Author’s contributors

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.

Competing interests

All authors declare no conflicts of interest.

Data availability statement

Data are available upon reasonable request. Data generated or analyzed during the study are available from the corresponding author by request.

Supplemental material
This content has been supplied by the author(s). It has not been vetted by Clinical and Molecular Hepatology (CMH) and may not have been peer-reviewed. Any opinions or recommendations discussed are solely those of the author(s) and are not endorsed by CMH. CMH disclaims all liability and responsibility arising from any reliance placed on the content. Where the content includes any translated material, CMH does not warrant the accuracy and reliability of the translations (including but not limited to local regulations, clinical guidelines, terminology, drug names and drug dosages), and is not responsible for any error and/or omissions arising from translation and adaptation or otherwise.

ORCID IDs

Satya Priya Sharma, https://orcid.org/0000-0001-5994-8179

Ki Tae Suk, https://orcid.org/0000-0002-9206-9245.
References


<table>
<thead>
<tr>
<th>Complication</th>
<th>Complication relating microbiota compared with control group</th>
<th>Complication relating microbiota compared with cirrhosis group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Varices</td>
<td>Veillonella dispar</td>
<td>Veillonella dispar</td>
</tr>
<tr>
<td></td>
<td>Anaerostipes hadrus</td>
<td>Lactobacillus fermentum</td>
</tr>
<tr>
<td></td>
<td>Clostridium innocuum</td>
<td>Lactobacillus paracasei</td>
</tr>
<tr>
<td></td>
<td>Dorea longicatena</td>
<td>Paraprevotella uc</td>
</tr>
<tr>
<td></td>
<td>Clostridium ramosum</td>
<td></td>
</tr>
<tr>
<td>Ascites</td>
<td>Escherichia coli</td>
<td>Veillonella dispar</td>
</tr>
<tr>
<td></td>
<td>Lactobacillus gasseri</td>
<td>Lactobacillus helveticus</td>
</tr>
<tr>
<td></td>
<td>Anaerostipes hadrus</td>
<td>Clostridium clostridioforme</td>
</tr>
<tr>
<td></td>
<td>Anaeroglobus geminatus</td>
<td>Lactobacillus fermentum</td>
</tr>
<tr>
<td></td>
<td>Clostridium ramosum</td>
<td>Holdemanella biformis</td>
</tr>
<tr>
<td>SBP</td>
<td>Escherichia coli</td>
<td>Veillonella dispar</td>
</tr>
<tr>
<td></td>
<td>Anaeroglobus geminatus</td>
<td>Clostridium clostridioforme</td>
</tr>
<tr>
<td></td>
<td>Clostridium ramosum</td>
<td>Lactobacillus fermentum</td>
</tr>
<tr>
<td></td>
<td>Fusobacterium nucleatum</td>
<td>Lactobacillus paracasei</td>
</tr>
<tr>
<td></td>
<td>Romboutsia timonensis</td>
<td>Flavonifractor plantii</td>
</tr>
<tr>
<td>Encephalopathy</td>
<td>Veillonella dispar</td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td></td>
<td>Veillonella atypica</td>
<td>Lactobacillus paracasei</td>
</tr>
<tr>
<td></td>
<td>Bacteroides stercolis</td>
<td>Enterococcus faecium</td>
</tr>
<tr>
<td></td>
<td>Megasphaera micronuciformis</td>
<td>Lactobacillus fermentum</td>
</tr>
<tr>
<td></td>
<td>Clostridium ramosum</td>
<td>Anaerotignum lactatfermentans</td>
</tr>
<tr>
<td>HRS</td>
<td>Anaerostipes hadrus</td>
<td>Veillonella dispar</td>
</tr>
<tr>
<td></td>
<td>Clostridium ramosum</td>
<td>Veillonella parvula</td>
</tr>
<tr>
<td></td>
<td>Dorea longicatena</td>
<td>Lactobacillus fermentum</td>
</tr>
<tr>
<td></td>
<td>Veillonella uc</td>
<td>Veillonella uc</td>
</tr>
<tr>
<td></td>
<td>Campylobacter gracilis</td>
<td>Coprococcus comes</td>
</tr>
<tr>
<td>HCC</td>
<td>Megamonas rupellensis</td>
<td>Prevotella uc</td>
</tr>
<tr>
<td></td>
<td>Roseburia inulinivorans</td>
<td>Alistipes putredinis</td>
</tr>
<tr>
<td></td>
<td>Anaerostipes hadrus</td>
<td>Bacteroides eggerthii</td>
</tr>
<tr>
<td></td>
<td>Gemmiger formicilis</td>
<td>Holdemanella biformis</td>
</tr>
<tr>
<td></td>
<td>Lachnospira pectinoschiza</td>
<td>Parabacteroides goldsteinii</td>
</tr>
<tr>
<td>Death</td>
<td>Enterobacteriaceae group</td>
<td>Veillonella dispar</td>
</tr>
<tr>
<td></td>
<td>Akkermansia muciniphila</td>
<td>Clostridium clostridioforme</td>
</tr>
<tr>
<td></td>
<td>Roseburia inulinivorans</td>
<td>Lactobacillus fermentum</td>
</tr>
<tr>
<td></td>
<td>Bifidobacterium dentium</td>
<td>Bifidobacterium dentium</td>
</tr>
<tr>
<td></td>
<td>Ruthenibacterium lactatiformans</td>
<td>Lactobacillus delbrueckii</td>
</tr>
<tr>
<td>Cirrhosis related microbiota compared with control group</td>
<td>Complication relating microbiota compared with cirrhosis group</td>
<td></td>
</tr>
<tr>
<td>--------------------------------------------------------</td>
<td>---------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Veillonella parvula</td>
<td>Bacteroides ovatus</td>
<td></td>
</tr>
<tr>
<td>Veillonella atypica</td>
<td>Clostridium symbiosum</td>
<td></td>
</tr>
<tr>
<td>Veillonella dispar</td>
<td>Emergencia timonensis</td>
<td></td>
</tr>
<tr>
<td>Ruminococcus gnavus</td>
<td>Fusobacterium varium</td>
<td></td>
</tr>
<tr>
<td>Streptococcus pneumoniae</td>
<td>Hungatella_uc</td>
<td></td>
</tr>
</tbody>
</table>
Figure legends

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. 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.
Figure 2. Identification of cirrhosis and cirrhosis associated complication dependent fecal microbial biomarker. (A) LDA score for complication associated depleted and complication associated enriched bacterial species 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.
Figure 3. Assortment for differential fecal microbial biomarker between cirrhosis and cirrhosis associated complications. (A) LDA score for specific bacterial species depleted and enriched in complication compared to cirrhosis, selected microbial species presented significant difference (p<0.05) with cirrhosis measured by t-test using Mann-Whitney test and represented, (B) AUROC for specific bacterial species increased in cirrhosis compared to complications, (C) AUROC for specific bacterial species enriched in individual complication compared to cirrhosis.
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. The 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.
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 pathway in each groups based on enrichment ratio compare 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.
Figure 6. Correlation between gut microbial and fecal metabolic biomarker 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 to correlation is represented as *p<0.05, **p<0.01, ***p<0.001.
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.
Supplementary Materials and Methods
Participant inclusion and fecal collection

A hospital-based prospective cohort study was conducted between April 2017 and March 2022 at university hospitals to evaluate the microbial characteristics of cirrhosis patients (trial registration: NCT05786755 and NCT04339725, IRB No. 2016-134). A total of 194 subjects (52 controls and 142 cirrhosis patients) who were >40 years old were included in this study. This study involved patients with cirrhosis who had follow-up visits at the hepatology department of university hospitals.

Cirrhosis was diagnosed based on the presence of complications (varix, ascites, and encephalopathy), blood tests, imaging findings, fibroscan, or pathological liver results. Liver cancer was diagnosed by two or more imaging tests, such as computer tomography, magnetic resonance imaging, angiography, or contrast ultrasound. In addition, subjects taking drugs that affect the gut microbiota were excluded at enrollment. For the control group, we included healthy subjects who visited the center for a health check-up.

The cirrhosis with complications group (n=132) was classified into two individual complication groups, complications occurring and nonoccurring, where each complication was classified into one of six complication types: varices, ascites, SBP, encephalopathy, HRS, and HCC. Patients with HCC were in stage C and/or in stage D classified according to Barcelona Clinic Liver Cancer (BCLC) staging system for HCC. These patients were admitted in hospital for Transarterial chemoembolization (TACE). Additionally, we classified these patients as alive or dead to evaluate the mortality-dependent microbiome.

Baseline studies included family history, diet pattern, alcohol history, abdominal ultrasound and computed tomography scan, X-ray, electrocardiography, complete blood count, electrolytes, liver function test, viral markers, and Child–Pugh score. Blood analysis was performed using standard methodologies. Serum biochemical parameters included aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, bilirubin, alkaline phosphatase (ALP), gamma glutamyl transpeptidase (GGT), blood urea nitrogen, creatinine, international normalized ratio, α-fetoprotein, carcinoembryonic antigen, prothrombin
time, blood glucose, and total cholesterol. The levels of hepatitis A, B, and C and other virus markers were evaluated. Antinuclear antibody, antimitochondrial antibody, and antismooth muscle antibody tests were also performed.

This project followed the ethics of the 1975 Helsinki Declaration, as reflected by a prior approval by the institutional review board for human research in hospitals (2016-134). Informed consent was obtained from all participants. All authors had access to the study data and reviewed and approved the final manuscript.

**Stool sample and V3-V4 16S rRNA sequencing**

Sequencing was carried out according to the manufacturer’s instructions at CJ bioscience, Inc. (Seoul, Republic of Korea) with the Illumina MiSeq platform using reagent kit V3 in PE 250 bp mode. Human feces were stored at -20 °C as soon as the patient received 2-3 g of feces using the kit (stool paper and stool box) and moved to -80 °C within 1 day. Genomic DNA for metagenomic sequencing was extracted with a QIAamp stool kit (Qiagen, Hilden, Germany), and the library was prepared with a NEBNext Ultra II FS DNA Library Prep Kit for Illumina (New England BioLabs, Ipswich, MA, USA) according to the manufacturer’s directions. The quantification of libraries was checked using a Qubit dsDNA HS assay kit (Thermo Fisher Scientific, Waltham, MA, USA) and confirmed by quantitative polymerase chain reaction (qPCR) with a KAPA SYBR FAST qPCR Master Mix kit (Kapa Biosystems, Wilmington, MA, USA). The quality of the libraries was assessed on a Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA) using a DNA 12000 chip. All libraries were sequenced on the NovaSeq 6000 platform (Illumina, USA) with paired-end 150 bp reads.

The analysis was performed following our previous reference. In brief, DNA was extracted with a QIAamp stool kit, and amplification of the V3-V4 region of the bacterial 16S rRNA gene was conducted using barcoded fusion primers. The forward fusion primer contained the p5 adapter, i5 index, and gene-specific primer 341F (5′-AATGATACGGCGACCACCGAGATCTACAC-XXXXXXXXTCGTCGCGCACGCAGTGTGTATAAGAGACAGCAG-GAGACACGCGCCGCGGCGCAGCAGCAG-3′; underlining
indicates the target region primer and X indicates the barcode region), and the reverse fusion primer contained the p7 adapter, i7 index, and gene-specific primer 805R (5′-CAAGCAGAGACGGGATACGAGATXXXXXXXXGTCTCGTGCGCTCAGAGATGCTGATAAGACAG-GACTACHVGGGTATCTAATCC-3′), which included sequencing adapters and dual-index barcodes of the Nextera XT kit (Illumina, San Diego, CA, USA). The amplification was performed in the C1000 touch thermal cycler PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with the following conditions: initial denaturation of 3 min at 95 °C; followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s and final extension at 72 °C for 5 min. Each amplified PCR product was confirmed with 1% agarose gel electrophoresis and visualized on a Gel Doc XR+ imaging system (Bio-Rad Laboratories, Inc., USA). The amplified products were purified and size-selected by Agencourt AMPure XP beads (Beckman Coulter, Chaska, MN, USA). The library was constructed with pooled PCR products, and the quality of the library was assessed on a Bioanalyzer 2100 (Agilent, USA) using a DNA 12000 chip and quantified by qPCR with a KAPA SYBR FAST qPCR Master Mix kit (Kapa Biosystems, USA).

Metabolite extraction

Thawed feces sample was added into 1.2mL ice-cold extraction solvent (methanol: acetonitrile: water, 3:3:2, v/v/v) and homogenized by sonication. Homogenized fecal solution were centrifuged at 13200 rpm for 5 min at 4 °C and supernatant was collected. Collected supernatant then dried by speed vacuum concentrator (SCANVAC, Korea). The concentrated dry fecal extract then derivatized with 40 mg/ml methoxyamine hydrochloride (Sigma-Aldrich, USA) in pyridine (Thermo, USA) and incubated at 30 °C for 90 min. The derivatized feces mixed with fatty acid methyl esters (FAME) and N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA+ 1% TMCS; Thermo USA) and reacted for 60 minutes at 800
rpm at 37 °C. The mixture of FAME included C8, C9, C10, C12, C14, C16, C18, C20, C22, C24, C26, C28, and C30.³

**Metabolic profiling**

The metabolomic profiles derived from human stool samples were acquired using two liquid chromatography–mass spectrometry (LC-MS) methods.⁴ Fecal samples were thawed at 4 °C and mixed with 1.1 ml of cold extraction solvent I (acetonitrile:water = 1:1, v/v). Those mixtures were vortexed for 1 min and sonicated for 5 min under ice and then centrifuged at 13,200 rpm for 5 min at 4 °C. Each supernatant (500 μl) was transferred into a new 2 ml tube (solution I) for the SCFA analysis (Method 1).⁵ The rest of the supernatant was mixed with 600 μl of cold extraction solvent II (acetonitrile:methanol = 1:3, v/v). For the second extraction step, the mixtures were vortexed for 1 min and centrifuged at 13,200 rpm for 5 min at 4 °C. The supernatants (500 μl) were aliquoted and transferred to new 1.5-ml tubes for LC–Orbitrap MS (Method 2). The aliquots were concentrated to complete dryness using a speed vacuum concentrator (SCANVAC, Korea).⁶

**Method 1: SCFAs analysis using LC-Orbitrap MS.**

The supernatant (40 μl) of solution I (acetonitrile:water = 1:1, v/v) was mixed with 20 μl of 200 mM 3-nitrophenylhydrazine-hydrochloride (HCL) in acetonitrile (70%) and 20 μl of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCL (120 mM) dissolved in a 6% pyridine solution. The mixture was incubated for 30 min at 40 °C and diluted with 1.92 ml of 70% of acetonitrile.⁵

The diluted derivatives were chromatographically separated with a 150 × 2.1 mm ultra-high performance LC ethylene bridged hybrid (UPLC BEH) 1.7 μm C18 column (Waters, Milford, MA, USA) equipped with a 5.0 mm × 2.1 mm UPLC BEH 1.7 μm C18 VanGuard pre-column (Waters, Milford, MA, USA) controlled by an Ultimate-3000 UPLC system (Thermo Fisher Scientific, Waltham, MA, USA). The mobile phase
was composed of 0.01% formic acid in water (buffer A, v/v) and 0.01% formic acid in acetonitrile (buffer B, v/v) with a flow rate of 0.350 ml/min. The gradient for LC elution was programmed as follows: equilibration in 15% buffer B for 2 min, 15%–55% buffer B gradient over 9 min, 100% buffer B held for 1 min, and re-equilibration in 15% buffer B for 3 min. The injection volume was 2 μl for both the MS1 and MS/MS analyses. Mass spectra were acquired using a Q-Exactive Plus Orbitrap (Thermo Fisher Scientific, Waltham) equipped with an electrospray ionization interface (HESI-II) in negative ionization mode, and the system was controlled using Xcalibur 4.0 and Q-Exactive Tune software. Raw data were processed by Tracefinder software (v.4.0, Thermo Fisher Scientific, San José, CA, USA). The mass tolerance for precursor ions and retention time tolerance were set to 5 ppm and 0.5 min, respectively.  

Method 2: Secondary metabolites profiling of LC-Orbitrap MS.  
The dried extracts were reconstituted with 50 μl of 70% acetonitrile for the LC–Orbitrap MS analysis. Chromatographic separation was carried out using an Ultmate-3000 UPLC system (Thermo Fisher Scientific, Waltham) and a 150 × 2.1 mm UPLC BEH 1.7 μm C18 column (Waters, Milford, MA, USA) equipped with a 5.0 mm × 2.1 mm UPLC BEH 1.7 μm C18 VanGuard pre-column (Waters, Milford, MA, USA). The mobile phase consisted of buffer A (0.1% formic acid in water) and buffer B (0.1% formic acid in 100% acetonitrile). The flow rate was set to 0.350 ml/min, and the following gradient was programmed: equilibration in 3% buffer B for 1 min, 3%–100% buffer B gradient over 9 min, 100% buffer B held for 1 min, and re-equilibration in 3% buffer B for 3 min.  
The MS analysis was performed on a Q-Exactive plus Orbitrap (Thermo Fisher Scientific, Waltham) with ionization polarity-switching mode. A full MS scan was conducted on the metabolites (70–1000 m/z) with a resolution of 70,000 full widths at half maximum (FWHM), an automatic gain control (AGC) target of 1e6 ions, and a maximum injection time (IT) of 100 ms. A data-dependent MS/MS analysis was performed on pooled samples in each ionization mode. The data-dependent MS/MS settings were as follows: MS1 ions: Top5; resolution: 17,500; AGC target: 1e5; maximum IT: 50 ms; isolation window: 1.0
m/z; normalized collision energy (NCE): 30; intensity threshold: 2e3 ions; apex trigger: 3–6 s; dynamic exclusion: 5 s. The m/z values and retention times for six bile acids were added to the inclusion list for the application of a higher collision energy (NCE = 70).4

Data acquisition and pre-processing were conducted with Xcalibur software (Thermo Fisher Scientific, San José). The obtained RAW data files were processed using Compound Discoverer software (v.3.1, Thermo Fisher Scientific, San José). The data processing was done following a workflow procedure: Select spectra, Align Retention times, Detect Unknown Compounds, Group Unknown Compounds, Fill Gaps, and Search mzCloud. The mass tolerance of MS1 on every node was set at 5 ppm. The Align Retention Time node was set to 1 min to Maximum shift. Compound identification was done against mzCloud with criteria of 10 ppm (MS2 mass tolerance) and 70% of the assignment threshold.4 For excluding non-natural compounds (e.g., drugs, medicines), we changed compound class setting in general setting of Search mzCloud node.

**Bioinformatic Analysis**

Microbial Taxonomic profiling was performed on EzBioCloud Apps (CJ bioscience Inc., Republic of Korea) for each sample, and comparative Microbial Taxonomic analysis was done by EzBioCloud Apps. OTUs assignment at species level for each reads was conducted with CJ bioscience’s 16S rRNA database (DB ver. PKSSU4.0).7 The 97% sequence similarity cutoff was used for OTU determination in UNCLUST and CHDIT.8 The various alpha-diversity (Chao1, ACE, Jackknife, Simpson, Shannon, NPShannon) and Beta-diversity (PCoA and UPGMA) were calculated individually and grouped vise by using MTP analyzer. The 16S rRNA sequences data for each sample included in this study was deposited in the ChunLab’s EzBioCloud.

**Statistical Analysis**
Linear Discriminant Analysis Effect Size (LEfSe) analysis was implemented by using EzBioCloud Apps\(^9\) to estimate the group-based taxonomical differentiation. Firstly, ANOVA by Kruskal-Wallis sum-rank test and Mann-Whitney test compare rank test were used to distinguish significant differential abundance at each taxonomical level between the groups and later linear discriminant analysis (LDA) used to determine the effect size. The Dunn’s post-hoc test performed in ANOVA by Kruskal-Wallis sum-rank test to minimize the statistical errors occurred due to multiple comparisons. In the search of cirrhosis related and diseased dependent gut microbial biomarkers compared to HC, significant LDA score (LDA score>2 and \(p<0.05\)) were used to select bacterial species (increased and decreased in abundance) from each group. At first, species were having only number id and have no certain classification at species level were eliminated from the biomarker’s selection process. Afterward, complication-based significantly differential microbial catalog were prepared compared to control and compared to cirrhosis, individually. By using this complication specific differential microbial catalog complication specific microbial species were selected for biomarker ability based on operating characteristic curves (receiving operational curve, ROC). The Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was used to predict the 16S rRNA gene based microbial functional analysis.\(^10\) Quantitative analysis and graphs were made by GraphPad Prism 8 software and spearman’s rank correlation coefficient and operating characteristic curves (receiving operational curve, ROC) were constructed and area under curve (AUC) was calculated to assess the diagnostic performance of the model with the Origin Pro 2021.

The MetaboAnalyst 5.0 software (https://www.metaboanalyst.ca/) was used for metabolite’s quantitative analysis.\(^11\) The data distribution was measured by PCA and supervised particle least squares discriminant analysis (PLS-DA) and orthogonal PLS-DA (OPLS-DA) model were used for group-based differentiation. Along with this, score plot fit goodness (\(R^2\)) and goodness of prediction (\(Q^2\)) and for the score plot prediction, aligned and filtered list of metabolites were used. The Metabolomics Pathway Analysis (MetPa) tool was used for pathway analysis.
Supplementary Table 1. Patients’ characteristics

<table>
<thead>
<tr>
<th>Variables</th>
<th>HC (n=52)</th>
<th>Cirrhosis (n=10)</th>
<th>Cirrhosis with 1 COM</th>
<th>Cirrhosis with 2 COM</th>
<th>Cirrhosis with 3 COM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Mean [SD])</td>
<td>(n=26)</td>
<td>(n=7)</td>
<td>(n=26)</td>
<td>(n=44)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>60.9 (8.9)</td>
<td>60.7 (12.7)</td>
<td>61.6 (9.9)</td>
<td>48.4 (15.8)*</td>
<td>55.9 (8.5)</td>
</tr>
<tr>
<td>Female (n, %)</td>
<td>26 (50)</td>
<td>5 (50)</td>
<td>9 (35)</td>
<td>4 (57)</td>
<td>7 (27)</td>
</tr>
<tr>
<td>Mortality (n)</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.2 (3.7)</td>
<td>25.3 (2.9)</td>
<td>23.5 (3.4)</td>
<td>24.8 (1.1)</td>
<td>22.8 (3.5)</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>22 (4)</td>
<td>56 (18)*</td>
<td>85 (139)*</td>
<td>155 (104)*</td>
<td>113 (119)*</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>18 (7)</td>
<td>73 (76)*</td>
<td>59 (95)*</td>
<td>72 (41)*</td>
<td>56 (62)*</td>
</tr>
<tr>
<td>Cr (mg/dL)</td>
<td>0.9 (0.2)</td>
<td>0.8 (0.1)</td>
<td>0.8 (0.1)</td>
<td>0.8 (0.1)</td>
<td>1.4 (1.5)*</td>
</tr>
<tr>
<td>Chol (mg/dL)</td>
<td>172 (39)</td>
<td>142 (25)*</td>
<td>147 (32)*</td>
<td>123 (24)*</td>
<td>138 (52)*</td>
</tr>
<tr>
<td>γ-GT (IU/L)</td>
<td>25 (17)</td>
<td>157 (150)*</td>
<td>187 (231)*</td>
<td>138 (50)*</td>
<td>400 (441)*</td>
</tr>
<tr>
<td>Alb (g/dL)</td>
<td>4.3 (0.3)</td>
<td>4.0 (0.3)*</td>
<td>4.0 (0.5)*</td>
<td>3.9 (0.3)*</td>
<td>3.0 (0.4)*</td>
</tr>
<tr>
<td>T. bil</td>
<td>0.9 (0.5)</td>
<td>1.3 (0.7)*</td>
<td>2.0 (6.3)</td>
<td>4.2 (3.1)*</td>
<td>3.7 (3.8)*</td>
</tr>
<tr>
<td>D. bil</td>
<td>0.3 (0.4)</td>
<td>0.5 (0.3)</td>
<td>1.2 (4.7)</td>
<td>3.1 (2.6)*</td>
<td>2.2 (2.6)*</td>
</tr>
<tr>
<td>MELD</td>
<td>7.7 (1.7)</td>
<td>10.1 (1.5)*</td>
<td>9.6 (4.0)*</td>
<td>11.0 (1.7)*</td>
<td>15 (6.0)*</td>
</tr>
<tr>
<td>CTP</td>
<td>5.1 (0.3)</td>
<td>5.4 (0.7)</td>
<td>5.4 (0.9)*</td>
<td>6.6 (0.8)*</td>
<td>8.1 (1.7)*</td>
</tr>
<tr>
<td>PT (sec)</td>
<td>11.5 (0.6)</td>
<td>13.4 (0.8)*</td>
<td>13.6 (5.4)*</td>
<td>13.3 (0.4)*</td>
<td>22.4 (19.2)*</td>
</tr>
<tr>
<td>INR</td>
<td>1.0 (0.1)</td>
<td>1.2 (0.1)*</td>
<td>1.2 (0.4)*</td>
<td>1.2 (0.1)*</td>
<td>1.4 (0.4)*</td>
</tr>
<tr>
<td>Plt (10³ μL)</td>
<td>217 (49)</td>
<td>82 (47)*</td>
<td>144 (52)*</td>
<td>190 (65)</td>
<td>114 (72)*</td>
</tr>
<tr>
<td>Etiology</td>
<td>5</td>
<td>7</td>
<td>7</td>
<td>24</td>
<td>31</td>
</tr>
<tr>
<td>----------</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>Alcoholic</td>
<td>3</td>
<td>16</td>
<td>0</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>Viral</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

COM, complication; n, number; HC, healthy control; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; Cr, creatinine; Chol, cholesterol; GGT, gamma glutamyl transpeptidase; T, total; bil, bilirubin; MELD, Model for End-stage Liver Disease; CTP, Child-Turcotte-Pugh; PT, prothrombin time; INR, International Normalized Ratio; Plt, platelet.

The overall statistical difference between healthy control and the diseased groups measured by ANOVA; *p<0.05, and individual difference measured by t-test using Mann-Whitney test compare rank test #p<0.05
Supplementary Figure 1. Complication associated compositional changes in fecal microbiome. (A) significantly changed phylum in HC, cirrhosis and non-complication and complication specific groups, (B) Firmicutes and Bacteroidetes (F/B) log ratio, (C) Alpha diversity by Shannon indexing, (D) Total reads count. The overall statistical difference between HC and the diseased groups measured by ANOVA using Kruskal-Wallis sum-rank test (KW); *p<0.05 **p<0.01, ***p<0.001, and individual difference measured by t-test using Mann-Whitney test compare rank test #p<0.05, ##p<0.01, ###p<0.001.
Supplementary Figure 2. Variation at genus level in HC, cirrhosis and cirrhosis associated complication occurring and non-occurring groups. (A) Genus abundance ratio increased in cirrhosis and cirrhosis associated complication compared to HC, (B) Genus abundance ratio decreased in cirrhosis and cirrhosis associated complication compared to HC. The overall statistical difference between HC and the diseased groups measured by ANOVA using Kruskal-Wallis sum-rank test (KW); *p<0.05, **p<0.01, ***p<0.001,
Supplementary Figure 3. Cirrhosis and cirrhosis associated complications altered the compositional diversity parameters. (A) Total reads Count, (B) Alpha diversity by Shannon indexing, (C) Principal Component Analysis.
Supplementary Figure 4. Cirrhosis dependent biomarkers identification at species level compared to HC. (A) AUROC for complication depleted bacterial species, (B) AUROC for enriched bacterial species.
Supplementary Figure 5. Differential fecal metabolite profiling in cirrhosis and HC. Variations in the fecal metabolite profile in cirrhosis were observed compared to a healthy control group. These changes were assessed using a heat-map representation, including 104 distinct metabolites.
Supplementary Figure 6. Correlation between the fecal metabolites. Correlation between all identified metabolite in feces showed intra-metabolite positive and negative correlation. Highly correlated metabolite are represented in the red outlined panel.
Supplementary Figure 7. Difference between the HC, cirrhosis and cirrhosis associated complication groups based on variable importance in projection (VIP) score of fecal metabolites. Fifteen highest VIP score presenting fecal metabolites showed diversity between the groups.
Supplementary Figure 8. Distinctive cirrhosis related fecal metabolic marker profile compared with HC. (A) Most altered pathways based on pathway enrichment ratio analyzed by using KEGG pathway, (B) Variable importance in projection (VIP) score of fecal metabolites, Log fold change in metabolites (C) Depleted in cirrhosis, (D) Enriched cirrhosis. individual difference measured by mean difference between
Supplementary Figure 9. Cirrhosis associated depleted and enriched fecal metabolite showed high valued inter-correlation. Significant negative correlation observed between cirrhosis depleted and enriched metabolites analyzed by using spearman correlation analysis, and significant in correlation is represented as *p<0.05.
Supplementary Figure 10. Correlation between most variable fecal metabolites and cirrhosis associated clinical markers. Significant spearman correlation was observed between top 25 variable fecal metabolites in HC, cirrhosis, and cirrhosis associated complication and clinical markers, and significant in correlation is represented as *p<0.05.
References


