ORIGINAL ARTICLE

Distinct responsiveness to rifaximin in patients with hepatic encephalopathy depends on functional gut microbial species

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Abstract

Hepatic encephalopathy (HE) is the neuropsychiatric complication of liver cirrhosis (LC). The influence of gut microbiota on HE pathogenesis has been suggested but not precisely elucidated. Here, we investigate how the gut microbial profile changed in patients with HE to clarify the functional gut microbial species associated with HE. We focused on their responses to rifaximin (RFX), a nonabsorbable antibiotic used in HE therapy. Feces samples were collected from patients with decompensated LC (all HE), patients with compensated LC, and healthy controls, and fecal gut microbial profiles were compared using 16S ribosomal RNA gene amplicon and metagenomic sequencing. The linear discriminant analysis effect size was used to identify specific species. *Urease*-positive *Streptococcus salivarius*, which can produce ammonia, was identified as the most significantly abundant gut microbiota in the HE group, and its ability to elevate the levels of blood ammonia as well as brain glutamine was experimentally verified in mice. *Urease*-negative *Ruminococcus*

gnavus was also identified as a significantly abundant species in patients with RFX-nonresponsive HE after RFX administration. Interestingly, R. gnavus enhanced urease activity of recombinant urease itself, implying that R. gnavus could amplify ammonia production of surrounding urease-positive microbiota. Furthermore, the sensitivity of S. salivarius and R. gnavus to RFX depended on conjugated secondary bile acid levels, suggesting a therapeutic potential of the combined use of secondary bile acid levels with RFX for enhancing the efficacy of RFX. This study identified specific gut bacterial species abundant in patients with HE and verified their functions linked to HE pathophysiology. Targeting these bacteria could be a potentially effective strategy to treat HE.

INTRODUCTION

Hepatic encephalopathy (HE) is an end-stage clinical feature of liver cirrhosis (LC) that manifests as neuropsychiatric disorders. Ammonia is one of the important molecules exerting neurotoxic effects in HE. In LC, portosystemic shunting and impaired hepatic ammonia metabolism, which normally converts ammonia to urea through the ornithine cycle in hepatocytes, leads to increased serum ammonia levels. The neurotoxic effect of ammonia is attributed to its ability to cross the blood–brain barrier. When ammonia levels increase within the brain, glutamine synthetase in astrocytes rapidly synthesizes glutamine, consequently promoting astrocyte swelling, which potentially leads to the onset of neuropsychiatric disorders in patients with HE.[1]

HE can range from low grade (minimal HE) to high grade (overt HE) and even coma.[2] Although ammonia is considered to play a central role in HE by functioning as a neurotoxin, blood ammonia levels are not always correlated with the severity of HE, implying that other factors could influence HE severity. Recently, increasing evidence suggests that the gut microbiota play an important role in HE pathogenesis. Indeed, gut microbiota influence liver function through the translocation of microbial components and metabolites to the liver by absorption. Because the liver is abundantly exposed to gut-derived factors, enteric dysbiosis is directly associated with liver diseases, including LC.[3,4] For example, short-chain fatty acid levels in blood or feces are inversely correlated with the severity of HE.[5] Moreover, altered bile acid composition is associated with blood–brain barrier dysfunction.[6] Therefore, improving microbial composition and targeting microbial function have been explored for the treatment of HE. Recently, probiotics treatment and fecal microbiota transplantation have been effectively applied to increase beneficial microbiota.[7] Moreover, the nonabsorbable antibiotic rifaximin (RFX) has been recognized not only to decrease blood ammonia levels by directly targeting bacteria but also to improve gut barrier function and alleviate neuropsychiatric features of patients with HE.[8]

This study investigates how the gut microbial profile changes in patients with HE to clarify the functional gut microbial species associated with HE pathogenesis. To evaluate this, we used 16S ribosomal RNA (rRNA) gene amplicon and metagenomic sequencing analyses. For this purpose, we particularly focused on the responses to RFX in patients with HE. Our analysis has identified *Streptococcus salivarius* and *Ruminococcus gnavus* as unique functional bacterial species associated with HE. We also uncovered that conjugated secondary bile acid levels in patients could influence the RFX sensitivity of bacteria. Targeting these specific bacteria using approaches such as phage therapy[9,10] could provide novel therapeutic strategies to treat HE.

MATERIALS AND METHODS

Patients and controls

Ninety patients with LC were retrospectively recruited at Osaka City University Hospital (Osaka, Japan) from April 2017 to March 2020. LC was diagnosed according to international guidelines by the comprehensive evaluation of liver biopsy, endoscopic or radiologic imaging examination, clinical symptoms, physical signs, laboratory tests, and LC-associated complications. Any patient with an unclear LC diagnosis was excluded. Patients were divided into compensated (with no prior or current history of HE) and decompensated cirrhosis groups.[11] Patients in the decompensated cirrhosis group had medical histories of hyperammonemia (peripheral venous blood ammonia concentration ≥70 μg/dl) with objective neurologic features. Patients meeting any of the exclusion criteria, such as comorbidity or medical history of hematemesis, severe infection, alcohol withdrawal, primary psychiatric diseases, drug overdose, or electrolyte disturbances, were excluded.[12]

A total of 27 patients with compensated cirrhosis and 26 patients with decompensated cirrhosis were included in this study (Figure 1; Table 1). Fecal samples from patients were immediately stored by direct
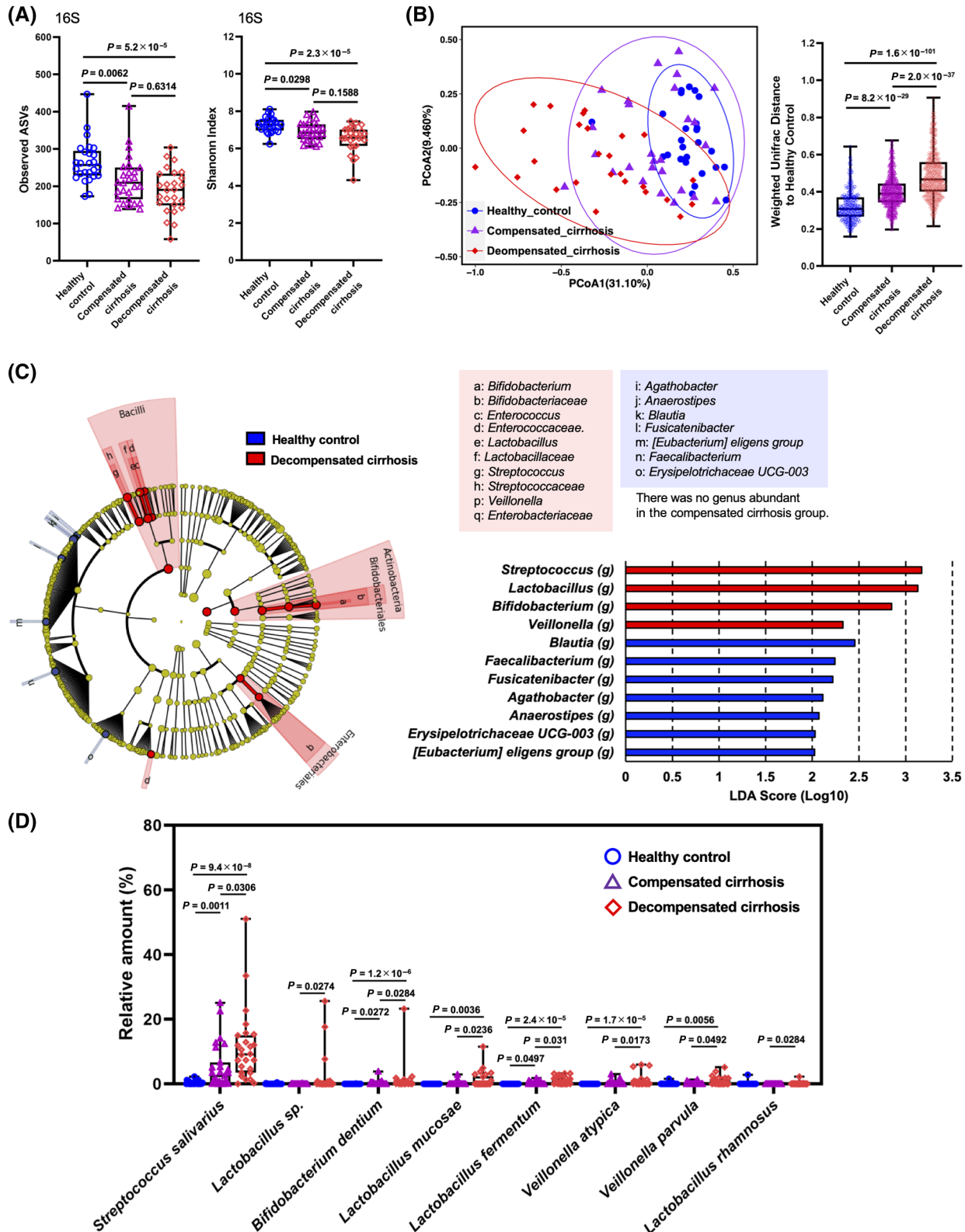
FIGURE 1 The difference in gut microbiota composition between patients with LC and HCs. Comparison of gut microbiota composition among patients with compensated or decompensated cirrhosis and HCs. (A) Alpha diversity was determined using the observed amplicon sequence variants and Shannon index. (B) Weighted UniFrac principal coordinate analysis of the gut microbiota harvested from the three groups. The weighted UniFrac distance to HCs was compared with each group. (C) Differences in gut microbiota composition at the genus level were determined through LDA effect size analysis among the three groups (LDA score >2) (D) Box plot shows relative abundance at the species level for *Streptococcus*, *Lactobacillus*, *Bifidobacterium*, and *Veillonella*. Species with significant differences among the three groups are arranged in descending order (Benjamini-Hochberg adjusted *p* value after Mann-Whitney U test). Data in (A,B) were analyzed using the Kruskal-Wallis test with Dunn's multiple comparisons. Data are shown as boxplots. Abbreviations: ASV, amplicon sequence variant; HC, healthy control; LC, liver cirrhosis; LDA, linear discriminant analysis; PCoA, principal coordinate analysis

TABLE 1 Clinical characteristics of the enrolled participants

	Healthy control (n = 26)	Compensated cirrhosis (n = 27)	Decompensated cirrhosis (n = 26)	Kruskal-Wallis test p value	Mann-Whitney U test /Fisher's test HC versus CC	HC versus DC	CC versus DC
Demographics							
Age (years)	61.4 (50–79)	67.6 (54–81)	68.4 (52–85)	0.006	0.039	0.008	>0.99
Sex (M/F)	14/12	16/11	15/11	–	>0.99	>0.99	>0.99
Body mass index	22.1 (18.9–24.6)	24.5 (16.9–31.4)	25.8 (19.1–31.6)	<0.001	0.025	<0.001	0.49
Clinical							
Etiology (viral/nonviral)	–	26/1	12/14		–	–	<0.001
HBV/HCV/NASH/Alcohol/PBC/AIH (%)	–	0/96.3/3.7 /0/ 0 /0	11.5/34.6/26.9 /11.5/11.5/3.8				
HE grade (I/II)	–	–	20/6		–	–	–
Medication (+/−)							
BCAA	0/26	8/19	21/5	–	0.004	<0.001	<0.001
Synthetic disaccharides	0/26	4/23	17/9	–	0.111	<0.001	<0.001
Proton pump inhibitor or H_2 blocker	0/26	11/16	20/6	–	<0.001	<0.001	0.012
Biochemical examinations							
Hb (g/dl)	14.0 (11.9–15.9)	13.0 (8.0–16.5)	11.4 (7.0–14.9)	<0.001	0.150	<0.001	0.040
Platelet count (×10⁴/μl)	21.7 (15.6–33.9)	10.7 (3.5–20.2)	9.9 (3.0–21.4)	<0.001	<0.001	<0.001	>0.99
Prothrombin activity (%)	–	82.7 (53–101)	65.3 (26–94)	<0.001	–	–	<0.001
Blood ammonia concentration (μg/dl)	–	–	131.0 (78–293)	–	–	–	–
Albumin (g/dl)	4.2 (3.8–4.6)	3.8 (2.1–4.7)	2.8 (2.3–3.8)	<0.001	0.44	<0.001	<0.001
Total bilirubin (mg/dl)	0.7 (0.5–1.2)	1.0 (0.4–2.4)	1.9 (0.5–9.5)	<0.001	0.050	<0.001	0.015
Aspartate aminotransferase (U/L)	22.2 (16–34)	33.2 (14–67)	59.0 (23–270)	<0.001	<0.001	<0.001	0.032
Alanine aminotransferase (U/L)	21.0 (6.0–46)	25.8 (10–67)	36.1 (13–176)	0.019	0.43	0.014	0.49
γ-Glutamyl transpeptidase (U/L)	36.3 (10–114)	51.1 (12–253)	50.2 (9–208)	0.70	–	–	–
Creatinine (mg/dl)	0.8 (0.5–1.0)	0.8 (0.5–1.1)	0.9 (0.6–2.0)	0.100	–	–	–
MELD score	–	8.2 (6–12)	11.7 (6–19)	–	–	–	–
FIB-4 index	1.48 (0.66–2.26)	5.20 (1.91–19.43)	7.80 (2.54–20.96)	<0.001	<0.001	<0.001	0.080
ALBI score	−2.87 (−3.21 to −2.56)	−2.43 (−3.33 to −1.04)	−1.48 (−2.52 to −0.75)	<0.001	0.25	<0.001	<0.001

Note: Mean (range) or number.

Abbreviations: AIH, autoimmune hepatitis; ALBI, albumin–bilirubin score; BCAA, branched-chain amino acid; CC, compensated cirrhosis; DC, decompensated cirrhosis; FIB-4, fibrosis-4; Hb, hemoglobin; HBV, hepatitis B virus; HC, healthy control; HCV, hepatitis C virus; HE, hepatic encephalopathy; MELD, Model for End-Stage Liver Disease; NASH, nonalcoholic steatohepatitis; PBC, primary biliary cholangitis.

addition of a preservation solution in preparation for bacterial DNA extraction using a fecal sampling kit (TechnoSuruga Laboratory Co., Ltd, Shizuoka, Japan). Samples were stored at 4°C until 16S rRNA gene sequencing was performed. The primary fecal samples from these patients were collected before the use of antibiotics, such as RFX. Alteration of the gut microbial profiles of patients under long-term medication of conventionally used proton-pump inhibitors (PPIs) and lactulose was evaluated (Figure S1B,C). An additional 13 fecal samples were collected from the patients with decompensated cirrhosis after oral administration of RFX for 2–12 weeks. Patients whose plasma ammonia levels were reduced by RFX were defined as responders, and patients whose plasma ammonia levels showed no decrease were considered to be nonresponders. Sampling for healthy controls was performed at the medical check-up center (Department of Premier Preventive Medicine, Osaka City University Hospital, Osaka, Japan)[13] from January 2020 to June 2020.

A total of 73 healthy controls were enrolled in the study. Exclusion criteria were as follows: (a) age <50 years old[14]; (b) body mass index <18.5 kg/m^2 or >25 kg/m^2[15]; (c) history of liver disease and diabetes mellitus; (d) the use of antibiotics, including RFX and probiotics, within 2 weeks before sampling. Finally, 26 healthy controls were included. All metadata describing each sample are provided in Table S1. The ethics committee of the Graduate School of Medicine, Osaka City University (Osaka, Japan), approved the protocol (approval number: 3722). This study was conducted following Helsinki Declaration II, and written informed consent was obtained from all study participants.

Mouse experiments

Details of the animal experiments are described in the Supporting Materials. All animal experiments were approved by the Institutional Animal Care and Use Committees of Osaka City University (approval numbers: 17206, 18079). Other experimental procedures are also described in the Supporting Materials.

RESULTS

Bioinformatics analysis of the gut microbiota revealed the bacterial species, including *Streprococcus salivarius*, associated with HE

To determine how the gut microbial profile changes in patients with HE, fecal gut microbiota of 26 patients with decompensated cirrhosis (all with HE), 27 patients with compensated cirrhosis, and 26 healthy controls (Table 1; Table S1) were compared through 16S rRNA

gene sequencing. The gut microbial composition of the decompensated cirrhosis group displayed the lowest alpha diversity and taxonomic richness (as measured using the Shannon index) among the three groups (Figure 1A). Analysis of taxonomic richness based on the number of amplicon sequence variants (ASVs) at the genus level revealed that a reduction in the number of ASVs was consistent with the low diversity of the bacterial taxa in the decompensated cirrhosis group.

The differences in gut microbiota composition of individuals in the three groups were assessed through principal coordinate analysis of the weighted UniFrac distances, which assessed the community structure, and the abundance of ASVs was included in the analysis (Figure 1B). Because microbial diversity differed between the compensated and decompensated cirrhosis groups (Figure 1B), the gut microbiota composition in the fecal DNA of individuals from the three groups was further analyzed using 16S rRNA gene sequencing data at the genus level.

Streptococcus was enriched in the microbial populations of the compensated and decompensated cirrhosis groups (Figure S1A). The linear discriminant analysis effect size (LEfSe) was analyzed for each pair of groups. When the linear discriminant analysis (LDA) score was set to >2 in the LEfSe analysis, significantly different genera were not detected between the healthy control and the compensated cirrhosis groups or between the compensated and decompensated cirrhosis groups. However, significantly different genera were identified between the healthy control and the decompensated cirrhosis groups (Figure 1C). In addition, *Streptococcus*, *Lactobacillus*, *Bifidobacterium*, and *Veillonella* were enriched in the decompensated cirrhosis group (LDA score >2). Interestingly, the LEfSe analysis of patients with LC who received long-term PPI treatment to reduce the risk of relapse of bleeding after treatment of varices (Figure S1B) indicated an abundance of *Streptococcus* in the feces of the PPI-treated group. This suggested that PPI-induced dysbiosis was potentially involved in the dominance of *Streptococcus* in the PPI-treated patients with LC, in accordance with published reports.[16–18] Similarly, the LEfSe analysis indicated that lactulose treatment, which is a standard therapy in patients with HE, also affects the abundance of *Lactobacillus* and *Bifidobacterium* (Figure S1C). Conversely, *Streptococcus, Lactobacillus,* and *Bifidobacterium* were not significantly abundant in the samples from patients with decompensated cirrhosis who did not receive PPI or lactulose treatment when compared with those from healthy controls or the compensated cirrhosis group following LEfSe analysis (Figure S1D,E).

Next, to identify the most abundant species in the decompensated cirrhosis group, we searched the aforementioned genera for the ASVs of specific species (Figure S2A–D). We found that *S. salivarius* was

the most abundant bacterial species in the decompensated cirrhosis group among the three groups (Figure 1D). The abundance of the other highly ranked bacterial species, including *Bifidobacterium longum* and *Lactobacillus salivarius,* was consistent with the species reported to be abundant in patients with LC (Figure S2A–D).[3] These results suggest that *S. salivarius* is a candidate bacterial species involved in HE pathophysiology.

Decompensated cirrhosis is associated with the abundance of *S. salivarius*

In this study, patients with decompensated cirrhosis (HE) who did not receive PPI treatment and patients with compensated cirrhosis receiving PPI treatment were included. We therefore further evaluated the abundance of *S. salivarius* in PPI-nonuser and PPI-user groups. Interestingly, in the PPI-nonuser group, *S. salivarius* abundance in patients with decompensated cirrhosis (HE) was significantly higher than that in patients with compensated cirrhosis (Figure S1F), suggesting that decompensated cirrhosis contributes to the abundance of *S. salivarius*. In contrast, *S. salivarius* abundance was similar between patients with decompensated and those with compensated cirrhosis who use PPIs (Figure S1F).

Model for End-Stage Liver Disease (MELD) scores in patients with compensated cirrhosis were lower than those of patients with decompensated cirrhosis (Figure S1G), reflecting the maintained liver function to metabolize ammonia in patients with compensated cirrhosis. These results suggest that the abundance of *S. salivarius* is associated with not only PPI administration but also cirrhosis pathophysiology, although the frequent administration of PPI in patients with LC is likely to increase *S. salivarius* abundance in PPI users. Intriguingly, there are reports showing reduced gastric acid output in patients with LC,[19,20] implying that *S. salivarius* in patients with LC might be more prone to transfer from the oral cavity to the intestines by passing through the weakened gastric acid barrier.

S. salivarius is the key gut microbial species involved in HE pathophysiology

S. salivarius, the most abundant species in the decompensated cirrhosis group, is well known to produce ammonia through urease by catalyzing urea hydrolysis.[21] However, a few *S. salivarius* strains do not harbor the complete *urease* operon despite having similar 16S rRNA gene sequences (Figure S3A). To confirm whether *S. salivarius* in patients is indeed capable of producing ammonia, we determined whether the relative abundance of *S. salivarius* is correlated with the DNA amount of *urease subunit alpha* (*UreC*), encoding the catalytic subunit alpha of urease, in fecal DNA samples in each individual from the three groups. Quantitative polymerase chain reaction (qPCR) analysis revealed a significantly higher amount of *UreC* DNA in patients with LC than in the healthy controls. The highest amount of *UreC* DNA was detected in the decompensated cirrhosis group (Figure 2A). Moreover, a significant positive correlation was noted between the relative abundance of *S. salivarius* and *UreC* DNA (Figure 2A,B). These results indicated that the dominance of *S. salivarius* species in the decompensated cirrhosis group reflects the function of *urease*-positive *S. salivarius* strains on hyperammonemia pathogenesis. Indeed, patients in the decompensated cirrhosis group had medical histories of hyperammonemia (peripheral venous blood ammonia concentration ≥70 µg/dl) with objective neurologic features (hereafter, patients with HE) (Table S1). In addition, we found that the abundance of *S. salivarius* in the fecal samples was significantly correlated with encephalopathy grade (Figure 2C,D), although it was not correlated with blood ammonia levels (Figure S2E,F).

Next, we investigated the biological effects of *urease*-positive *S. salivarius* on the pathophysiology of HE. To

FIGURE 2 Administration of urease-expressing *Streptococcus salivarius* induced hyperammonemia in mice. (A) Abundance of the *S. salivarius UreC* gene in human fecal DNA was assessed through qPCR. Data were analyzed using the Kruskal-Wallis test with Dunn's multiple comparisons. Data are shown as boxplots. (B) Correlation between the abundance of *S. salivarius* and *UreC* based on Spearman's rank correlation coefficient. (C) Abundance of *S. salivarius* in compensated cirrhosis (unimpaired) and HE grade I and II was analyzed using the Kruskal-Wallis test with Dunn's multiple comparisons. Data are shown as boxplots. (D) Correlation between the abundance of *S. salivarius* and HE grade based on Spearman's rank correlation coefficient. The compensated cirrhosis group was defined as the unimpaired group. (E) The *S. salivarius* strain (*urease*-expressing strain, NCTC 7366; a strain lacking *urease gene*, ATCC 25975) was examined for urease activity with or without urea. The abundance of bacteria was determined by measuring the culture medium using an absorptiometer at a wavelength of OD_{595}. Each bacterial species was used in 1 ml of culture medium adjusted to OD_{595} = 2.0 (Student t test, mean ± SD, n = 3/group; average value of 25975/urea+ = 1). (F) Schematic representation of the experimental design. Mice were administered water containing four antibiotic cocktails for 14 days during weeks 12–14 and 0.5 g/L metronidazole for 3 additional days. Mice were orally administered with the *urease*-expressing *S. salivarius* daily for 14 days, during weeks 14–16. Mice were then killed after week 16, and blood plasma and stool were collected. (G) Amounts of *S. salivarius* were determined by 16S rRNA qPCR in the two groups as shown (Mann-Whitney U test, mean ± SEM). (H,I) Concentration of blood plasma ammonia and brain glutamine from the two groups. Data indicate mean ± SEM, Student t test. Abbreviations: 4Abx, cocktail of four antibiotics; CCI_4, carbon tetrachloride; HE, hepatic encephalopathy; ip, intraperitoneal; MNZ, metronidazole; po, per os (orally); qPCR, quantitative polymerase chain reaction; rRNA, ribosomal RNA; SS, *S. salivarius*; *UreC*, urease subunit alpha

this end, the urease activities of *urease*-positive *S. salivarius* in the NCTC 7366 strain were examined *in vitro* using a described method.[22] Phenol red discoloration in the culture supernatant of the *S. salivarius* NCTC 7366 strain (resulting from ammonia production) was observed following urea supplementation (Figure 2E;

Figure S3B,C), confirming that the *S. salivarius* NCTC 7366 strain was an active *urease*-expressing bacterial strain. Next, the carbon tetrachloride (CCl_4)-treated liver fibrosis models were evaluated. Mice were intraperitoneally injected with CCl_4 (diluted to 20% in olive oil) at a dose of 1.0 ml/kg body weight twice per week for
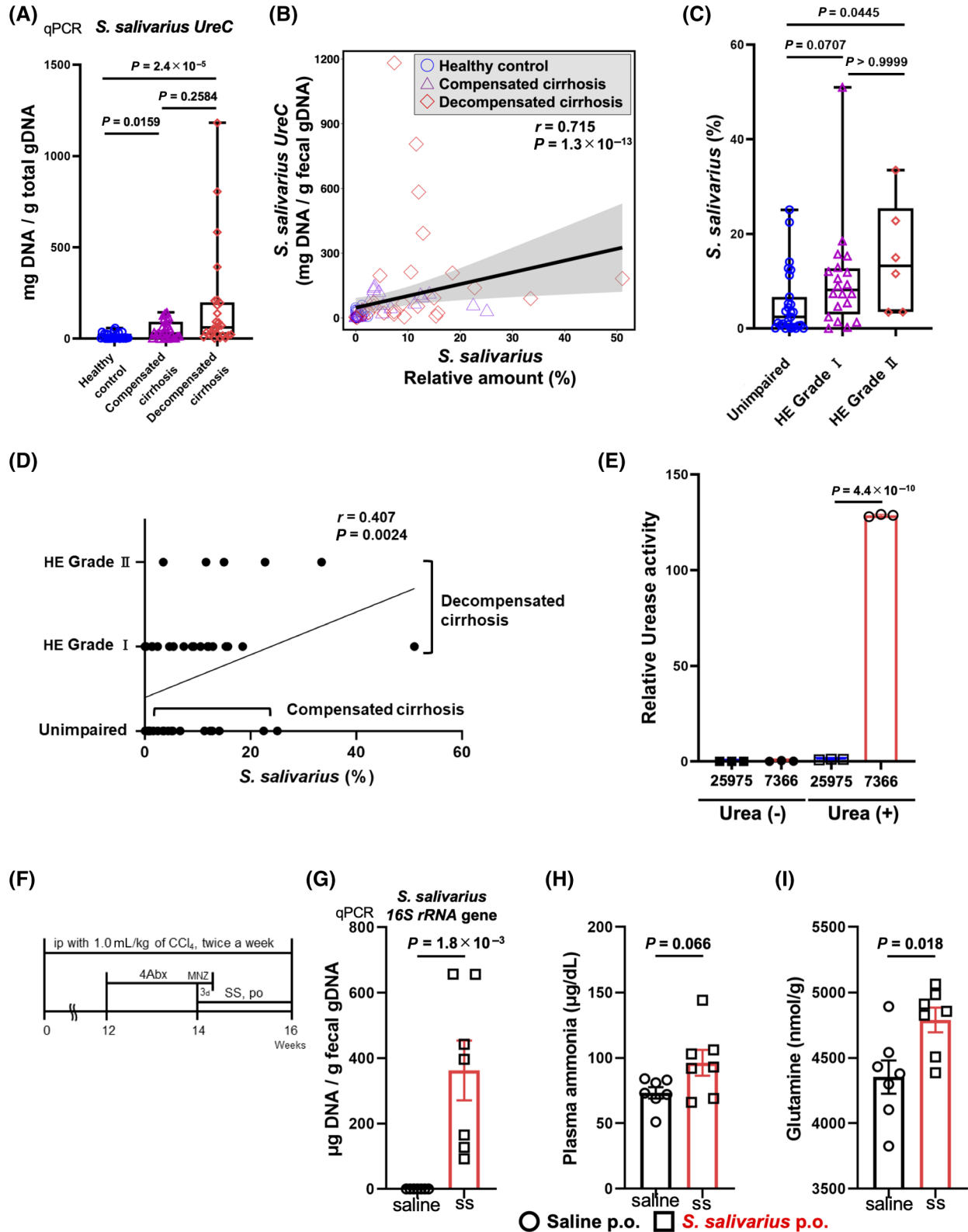
16 weeks to induce advanced hepatic fibrosis; control mice were injected only with olive oil (vehicle). Severe fibrosis was induced in the CCl$_4$-treated mouse liver as determined by sirius red staining (Figure S4A,B). Levels of plasma ammonia were significantly elevated in the CCl$_4$-induced hepatic fibrosis mouse model compared to the vehicle-treated mice, confirming that this mouse model mimics hyperammonemia in LC (Figure S4C). We further investigated whether hyperammonemia influenced the mouse brain metabolites associated with neuropsychiatric phenotypes in HE. Ammonia can cross the blood–brain barrier; astrocytic glutamine synthetase then converts ammonia and glutamate into glutamine, elevating osmolyte levels and increasing cerebral volume, which leads to brain dysfunction.[23] Therefore, to assess the increase in brain glutamine levels, the amino acid metabolites were quantified in the brains of the CCl$_4$-treated mice (Figure S4D,E). We investigated whether the *urease*-positive *S. salivarius* NCTC 7366 strain enhances hyperammonemia *in vivo* in the CCl$_4$-treated mouse model (Figure 2F). Bacterial colonization in the mice was confirmed through qPCR analysis of the amount of *S. salivarius* 16S rRNA gene DNA in the mice treated with *S. salivarius* (Figure 2G). Plasma ammonia levels were increased in the mice treated with the *urease*-positive *S. salivarius* NCTC 7366 strain compared to those treated with only the antibiotic cocktail (Figure 2H). Moreover, significantly higher glutamine levels were detected in the mouse brains treated with the *urease*-positive *S. salivarius* NCTC 7366 strain compared to those with the antibiotic cocktail, thus confirming the pathologic influence of hyperammonemia on the brain of the mouse model mimicking HE (Figure 2I). These results strongly indicated that *S. salivarius* could be a gut microbial species involved in HE pathophysiology.

RFX targets *S. salivarius* to improve hyperammonemia in patients with HE

RFX, a nonabsorbable oral antibiotic, has recently been used as an advanced therapy for HE to alter the gut microbial environment.[24] We particularly focused on the efficacy to RFX in patients with HE because its beneficial effects in HE, such as reducing ammonia levels, alleviating gut barrier dysfunction, and improving neuropsychiatric symptoms, have been suggested in many studies.[8] RFX reportedly alters the relative abundance of gut bacteria[8] and is effective against numerous strains, such as *Staphylococcus, Streptococcus, Enterococcus, Bacteroides*, and *Clostridium*.[25] Although RFX has been hypothesized to exert effects beyond those of an antibiotic, such as improving the intestinal barrier,[26] the precise target bacterial species of RFX in HE or the effect on the perturbations in the gut microbial community have not been completely elucidated.

RFX was first confirmed to effectively eliminate the *S. salivarius* NCTC 7366 strain *in vitro* (Figure 3A). Therefore, we next investigated whether the gut microbial composition of patients was altered after RFX administration. Patients with HE were separated into two groups, RFX responder and RFX nonresponder, according to changes in their plasma ammonia levels (Figure 3B). Responder patients comprised those patients whose plasma ammonia levels were reduced after RFX treatment, whereas nonresponder patients were those whose plasma ammonia levels showed no decrease after RFX treatment (Figure 3B). Furthermore, qPCR analysis of the total 16S rRNA gene in patient fecal DNA samples revealed no significant differences between samples before and after RFX administration (Figure 3C), indicating that RFX administration was unlikely to alter the total amount of fecal bacteria. However, the abundance of *S. salivarius* (Figure 3D) as well as the level of *S. salivarius UreC* were significantly reduced after RFX treatment in the responder group (Figure 3E), suggesting that RFX effectively targeted *UreC*-positive *S. salivarius* in the responder group.

Furthermore, the metagenomic analysis of the whole fecal microbial genome from the three responder and three nonresponder patients revealed significantly reduced abundance of *S. salivarius* and expression of the *urease* operon of *S. salivarius* after RFX administration (Figure 3F). These results indicated that RFX alters the gut microbial composition at the species level in patients with HE and particularly targets *S. salivarius* strains harboring the *urease* operon. We checked the abundance of *urease* operons from other genera excluding *S. salivarius* (Figure S5B) and found that several genera harboring the *urease* operon existed in both responder and nonresponder patients. Therefore, there is a possibility that species other than *S. salivarius* are associated, to some extent, with the HE phenotype. Nevertheless, *S. salivarius* may be the major influencer for the HE phenotype because it is the most abundant species in patients with decompensated cirrhosis, particularly in patients who respond to RFX (Figure 1D). Moreover, we noticed that *urease*-positive genera were abundant in patients who were nonresponders after RFX treatment (Figure S5). Therefore, these findings strongly indicate that *S. salivarius* is a key species associated with hyperammonemia-associated HE, particularly in patients who respond to RFX.

R. gnavus detected in nonresponders enhances urease activity

Plasma ammonia levels of patients who are nonresponders after RFX administration were highly sustained despite their decreased fecal *S. salivarius UreC* levels (Figure 3B,E). We noticed that *urease* operon-positive genera remained in patients who were
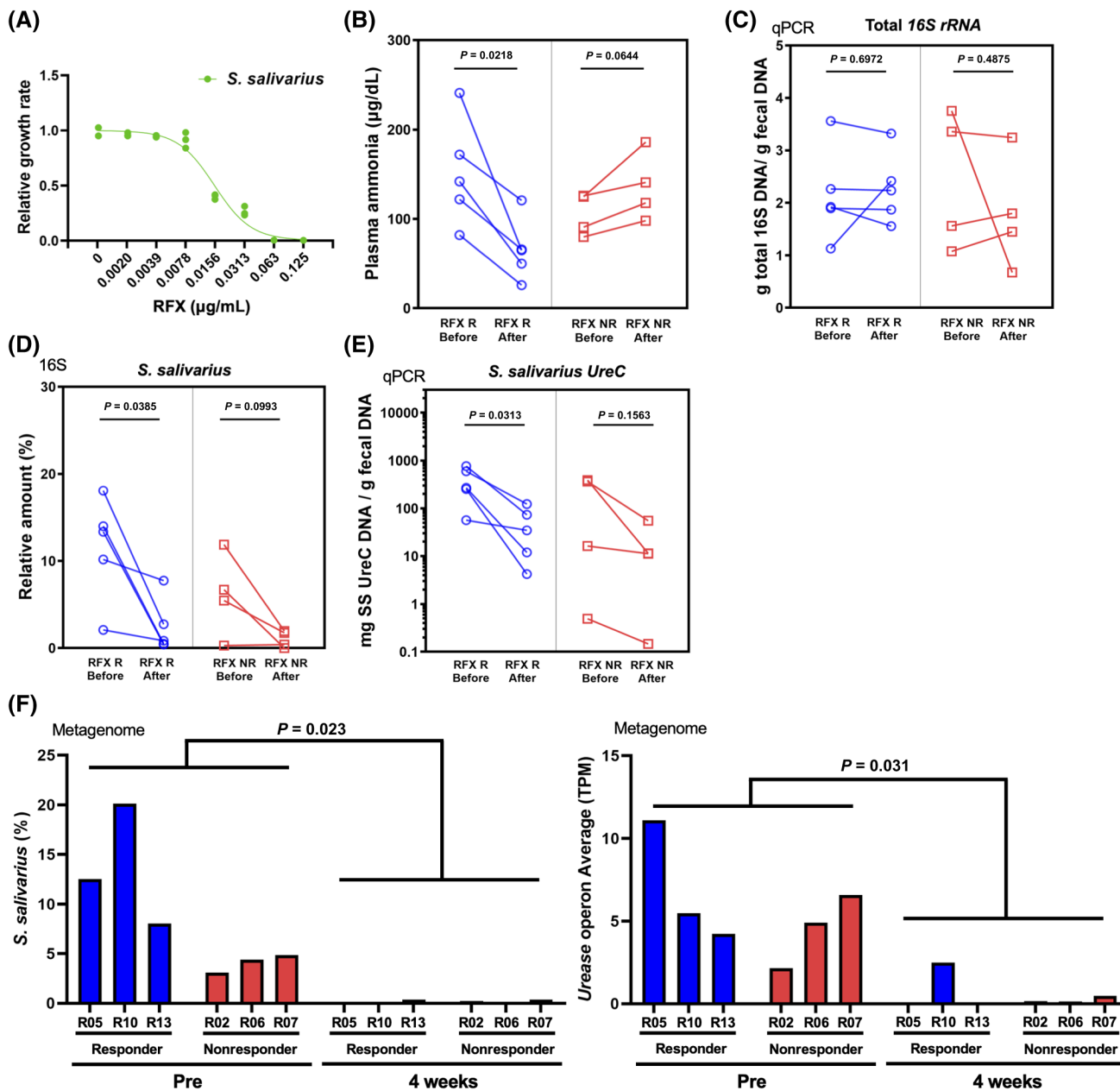
FIGURE 3 Rifaximin was effective for reducing *Streptococcus salivarius* in the responder group. (A) Growth of *S. salivarius* in the presence of RFX. (B–F) Patients with HE were divided into two groups, responder and nonresponder, based on changes in blood plasma ammonia levels before and after RFX administration. (B) Blood plasma ammonia levels, (C) total amount of fecal bacteria, (D) relative abundance of fecal *S. salivarius*, and (E) amount of fecal *S. salivarius UreC* gene transcripts of the two groups are shown. (F) Relative abundance of *S. salivarius* and the average amount of countable *urease* gene TPM from *S. salivarius* were determined by metagenomic sequencing analysis. Data in (B–F) were analyzed using the Wilcoxon signed-rank test. Abbreviations: HE, hepatic encephalopathy; NR, nonresponder; qPCR, quantitative polymerase chain reaction; R, responder; RFX, rifaximin; rRNA, ribosomal RNA; SS, *S. salivarius*; TPM, transcripts per million; *UreC*, urease subunit alpha

nonresponders after RFX treatment (Figure S5); however, despite the high plasma ammonia levels, the total amount of *urease* gene transcripts per million (TPM) in the nonresponders after RFX administration appeared to be less than those in patients who were responders before RFX administration (Figure 3F; Figure S5A,B). Because ammonia-producing gut bacteria markedly influence HE pathogenesis, we screened

for the bacterial species causing hyperammonemia in the nonresponder group by comparing the gut microbial profiles of the two groups before and 4 weeks after RFX administration. LEfSe analysis ranked *R. gnavus* as significantly abundant bacteria in the nonresponder group compared to the responder group at the genus (LDA score >4) and species levels (Figure 4A). 16S rRNA gene sequencing and relative abundance
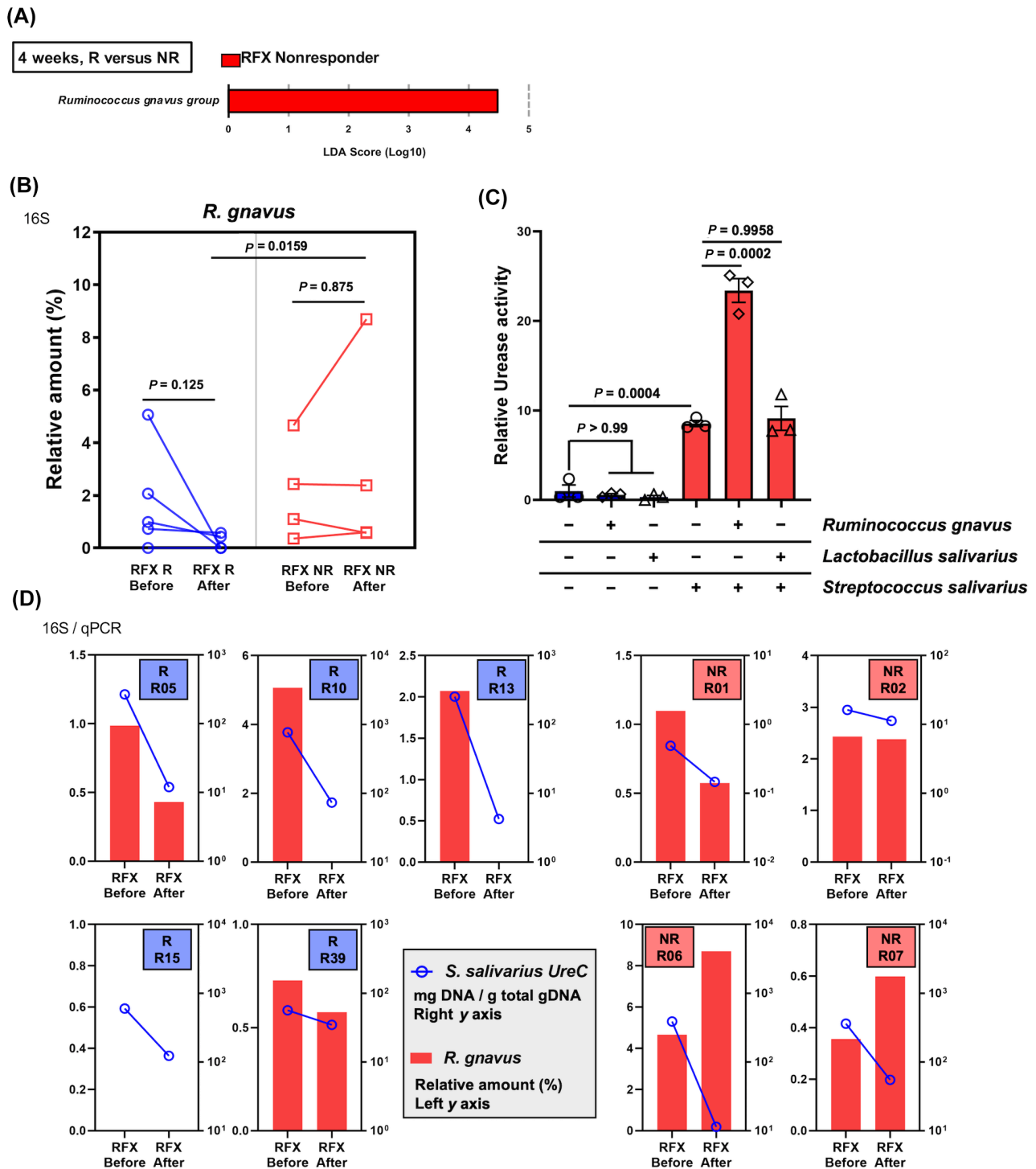
FIGURE 4 *Ruminococcus gnavus* was significantly abundant in the nonresponders and displayed enhanced urease activity. (A) Differences in gut microbiota composition at the genus level through linear discriminant analysis effect size analysis between the responders and nonresponders after RFX administration. (B) Relative abundance of *R. gnavus* in responders and nonresponders before and after RFX administration (Wilcoxon signed-rank test and Mann-Whitney U test). (C) *Streptococcus salivarius* strain (*urease*-expressing strain, NCTC 7366) with or without *R. gnavus* and *Lactobacillus salivarius* were tested for urease activity. We used 1 ml of culture medium adjusted to OD_{595} = 2.0, 2.0, and 0.2 (*R. gnavus*, *L. salivarius,* and *S. salivarius*, respectively) (n = 3/group; mean ± SD, one-way analysis of variance using Tukey's multiple-comparisons test; average value of *S. salivarius* only = 1). (D) Relative abundance of the indicated bacterial species (shown as percentage indicated in left vertical axis) and the amount of fecal *S. salivarius UreC* gene (right vertical axis as calculated in Figure 3E) in each individual responder (n = 5) and nonresponder (n = 4) before and after RFX administration. Abbreviations: LDA, linear discriminant analysis; NR, nonresponder; qPCR, quantitative polymerase chain reaction; R, responder; RFX, rifaximin; *UreC*, urease subunit alpha

analyses revealed that the abundance of *R. gnavus* in nonresponders was significantly higher than that in the responder group after RFX administration (Figure 4B), although there was no difference in the levels of *R. gnavus* between the two groups before RFX administration (Figure 4B). These results suggested that *R. gnavus* could be associated with hyperammonemia in patients who are nonresponders.

Next, we assessed the urease activity of *R. gnavus*; however, we obtained negative results (Figure 4C). In general, many bacterial species, including *R. gnavus*, harbor a series of genes encoding enzymes critical for nitrogen metabolism and ammonia assimilation,[27] implying that *R. gnavus* could use ammonia as a nitrogen source by receiving it from the other ammonia-producing bacteria. We hypothesized that *R. gnavus* influences coexisting ammonia-producing bacteria and conducted a urease activity assay by using the recombinant urease cultured in the presence or absence of *R. gnavus*. Interestingly, the urease activities of the *S. salivarius* NCTC 7366 strain and the recombinant urease were strongly enhanced in the presence of *R. gnavus* but not in its absence or in the presence of *Lactobacillus salivarius, Blautia producta,* or *Bacteroides uniformis* found in patients with HE (Figure 4C; Figure S6C–F). These results provide the first prospective evidence that *R. gnavus* can enhance surrounding urease activity, which could contribute to ammonia production of the coexisting *urease*-positive bacteria. Because *R. gnavus* was clearly associated with the enhancement of urease activity, we next investigated how the abundance of *R. gnavus* was altered in each patient with HE before and after RFX administration. The relative abundance of *R. gnavus* was strongly reduced in many patients who were responders after RFX administration (Figure 4B). In contrast, the abundance of *R. gnavus* was highly sustained or even increased after RFX administration in patients who were nonresponders (Figure 4D), despite the high sensitivity of this bacterium to RFX (Figure 5A).

We further tried to identify the distinct bacterial species remaining after RFX treatment by using more patients regardless of the time point of feces collection (samples collected at 2–12 weeks after RFX treatment were included). *Clostridium nexile* (shown as *Tyzzerella 4*) was additionally ranked by LEfSe analysis (Figure S6A,B). Intriguingly, similar to *R. gnavus, C. nexile* also enhanced recombinant urease activity up to more than 70-fold (Figure S6E), which was maintained following RFX treatment in some of the patients who were nonresponders (Figure S6G). Based on these results, we propose that *R. gnavus* and *C. nexile* are likely associated with hyperammonemia in patients with HE who are nonresponsive to RFX, through enhancing the urease activities of coexisting *urease*-positive gut bacteria, although the underlying mechanism remains to be elucidated.

Conjugated secondary bile acids increase the sensitivity of *R. gnavus* to RFX

Next, we questioned how the difference in RFX sensitivity was created in the patients who were nonresponders compared to those who were responders, particularly regarding abundantly sustained *R. gnavus* despite its innate sensitivity to RFX (Figure 5A). To resolve this discrepancy, we hypothesized that a certain factor in the nonresponders lowers the antibiotic efficacy of RFX. Using patient plasma samples before RFX administration, 101 plasma metabolites were detected through capillary electrophoresis time-of-flight mass spectrometry-based metabolome analysis, and five metabolite levels were found to be significantly different between the two groups (Figure S7A). Of these, the levels of β-alanine, valine, leucine, and isoleucine were significantly lower in the responder group than in the nonresponder group (Figure S7B). Although plasma ammonia levels and Child–Pugh scores calculated from the plasma biochemical data showed no difference in the reserved liver function between the two groups (Table S1), β-alanine and the branched-chain amino acid levels in the plasma were reduced in the responder group before RFX administration, which could suggest muscle atrophy linked to impaired ammonia detoxication in the livers of patients who were responders.[28] This muscle atrophy could also cause impaired glucose metabolism in muscles, thus increasing the metabolic production of ketone bodies, such as 2-hydroxybutyrate (2HB). Indeed, the plasma 2HB levels of patients who were responders were significantly correlated with the abundance of *S. salivarius* (Figure S7B,C). However, these findings may not be directly associated with RFX sensitivity.

Furthermore, plasma bile acid levels were assessed because secondary bile acids, metabolites peculiar to the gut microbiota, have been reported to influence the effect of antibiotics.[29] We determined the plasma bile acid levels of cholic acid (CA), deoxycholic acid (DCA), glycodeoxycholic acid (GDCA), taurodeoxycholic acid (TDCA), and others by using liquid chromatography–tandem mass spectrometry (Figure 5B) and compared them between the two groups before RFX administration. Interestingly, plasma levels of the conjugated secondary bile acids, including GDCA and TDCA, were profoundly and significantly lower in the nonresponder group than in responder group before RFX administration (Figure 5C). Moreover, a series of plasma bile acid levels (shown in the heatmap) indicated that the levels of secondary bile acids, including DCA and lithocholic acid and their conjugated forms, were lower in the plasma of the nonresponders (Figure 5B). Metagenomic analyses confirmed much lower amounts of the *bile acid-inducible (bai)* operon—gene clusters that synthesize the 7-α-dehydroxylated forms of primary bile acids—in
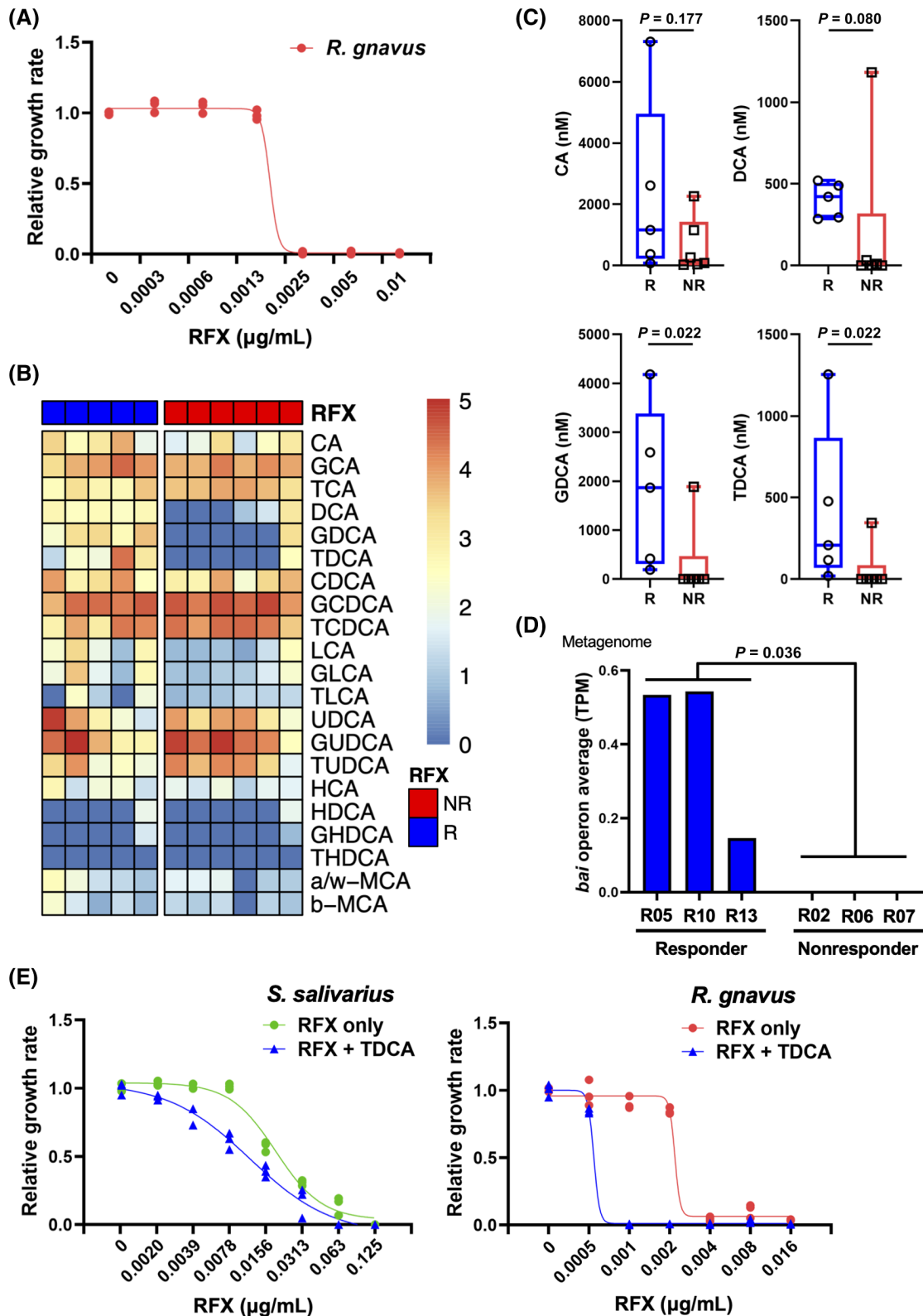
FIGURE 5 Comparison of bile acid levels of patients who were responders and nonresponders and the effect of secondary bile acids on RFX sensitivity to bacteria. (A) Growth of *Ruminococcus gnavus* in the presence of RFX. (B) Heatmap shows plasma bile acid concentrations in the responders and nonresponders before RFX administration. Each value uses a logarithmic scale. (C) Concentrations of CA, DCA, TDCA, and GDCA (Mann-Whitney U test, box plot). (D) Average amount of countable bai operon TPM was determined using metagenomic sequencing analysis (Mann-Whitney U test). (E) Growth of *Streptococcus salivarius* and *R. gnavus* in the presence of RFX in each medium and TDCA (1mM and 5 mM, respectively). Abbreviations: a/w, α/ω ; b, β; *bai*, bile acid-inducible; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; G-, glyco-; HCA, hyocholic acid; HDCA, hyodeoxycholic acid; LCA, lithocholic acid; MCA, muricholic acid; NR, nonresponder; R, responder; RFX, rifaximin; T-, tauro-; TPM, transcripts per million; UDCA, ursodeoxycholic acid

the nonresponder group (Figure 5D). To investigate the effects of the conjugated secondary bile acids on RFX sensitivity of the bacteria, *R. gnavus* was cultured with TDCA and RFX (Figure 5E). Although both *R. gnavus* and *S. salivarius* were sensitive to TDCA and RFX, their sensitivity was greatly enhanced in the presence of TDCA (Figure 5E), suggesting that very low levels of secondary bile acids in patients who were nonresponders strongly attenuated RFX sensitivity of *R. gnavus*. Interestingly, the majority of patients who were RFX nonresponders who showed extremely low levels of secondary bile acid in blood included patients with hepatitis C virus (HCV)-associated cirrhosis (Figure S7D). These results could explain why *R. gnavus* was retained in the nonresponder group after RFX administration, although other mechanisms could also be involved. These results suggest the therapeutic potential of conjugated secondary bile acids, including TDCA, in increasing RFX sensitivity of *R. gnavus* in patients with HE.

DISCUSSION

In this study, we investigated gut microbiota associated with HE pathophysiology through a series of multiomics analyses on gut microbiome and plasma metabolome in patients with HE. We particularly focused on the response to RFX in patients with HE because RFX targets gut microbiota and because of its beneficial effects for HE, such as reducing ammonia levels, alleviating gut barrier dysfunction, and improving neuropsychiatric symptoms.[8] As a result, we identified HE-associated bacterial species that produce ammonia and those that amplify urease activity, confirming that ammonia-related gut microbiota are important for HE pathogenesis.

First, we identified and experimentally verified *S. salivarius* as a specific gut microbial species strongly associated with HE pathophysiology. To do this, we performed gut microbial profiling analysis, using fecal DNA samples of patients with LC and of corresponding healthy individuals. Although the number of patients was limited and each patient's background was different, the common abundance of *S. salivarius* among patients with LC and those with HE was consistent with published reports on gut microbiota profiling in patients with LC.[3,16,30] *S. salivarius* reportedly inhabits both the oral cavity and the intestines.[31] The abundance of intestinal *S. salivarius* was detected in patients receiving long-term treatment with PPIs (Table S1). However, our analysis of PPI nonusers suggests that decompensated cirrhosis pathophysiology could be associated with the abundance of intestinal *S. salivarius* (Figure S1F; Figure 2D). This is possibly due to reduced gastric acid output in patients with LC,[19,20] suggesting that, similar to PPI-induced dysbiosis,[16–18] *S. salivarius* in

patients with LC is prone to transfer from the oral cavity to the intestines through a weakened gastric acid barrier.

Interestingly, many strains of *S. salivarius* harbor the *urease* operon encoding the ammonia-producing enzyme. Moreover, our samples displayed a significantly positive correlation between the 16S rRNA gene DNA levels of *S. salivarius* and the *urease* operon levels in fecal DNA samples obtained from patients with LC. Although several studies have reported that *S. salivarius* is abundant in patients with LC,[16,30] limited studies have experimentally verified the contribution of *S. salivarius* to HE. Using the advanced fibrosis model induced by peritoneal CCl_4 injection in mice, administration of the *urease*-positive *S. salivarius* strain significantly induced the HE phenotype by elevation of blood ammonia levels and brain glutamine levels, leading to brain dysfunction in mice. This suggests that *S. salivarius* is strongly associated with HE pathogenesis in patients with decompensated cirrhosis.

RFX is a nonabsorbable antibiotic approved for treating several diseases, including inflammatory bowel syndrome, functional bowel disorders, and HE.[32,33] Owing to its low systemic rate of absorption (only 0.4% of the orally administered dose), RFX exerts few side effects and minimal bacterial resistance without altering the overall composition of the gut microbiota.[8] In our study, all the patients who were RFX responders and half of those who were RFX nonresponders, despite relatively high levels of plasma ammonia in these patients, showed improved neuropsychiatric symptoms, implying that RFX could play a distinct role other than reducing ammonia levels. Because numerous favorable effects of RFX have been reported, further studies have not yet been performed on the gut microbiota of patients who are RFX nonresponders, who have been a focus of this study. Although plasma ammonia levels and Child-Pugh scores showed no difference in the reserved liver function between patients who were responders and those who were nonresponders (Table S1), all patients who were RFX nonresponders had hepatitis viral LC (mainly HCV-associated LC) while patients who were RFX responders tended to exhibit nonviral LC, such as nonalcoholic steatohepatitis (Figure S7D). These differences in disease background may potentially influence higher secondary bile acid levels in the plasma of RFX responders.[34] The *urease* gene of other genera excluding *S. salivarius* (Figure S5A,B) existed both in responders and nonresponders. Therefore, there is a possibility that these genera are associated with the HE phenotype to some extent, although *S. salivarius* (the most abundant) could be the major influencer for HE phenotype in patients with decompensated cirrhosis.

On assessing the gut microbiota of the RFX nonresponders, *R. gnavus* was ranked as a significantly abundant species in the nonresponders after RFX administration. Despite harboring no *urease* operon, *R.*

gnavus elevated coexisting urease activity, consistent with the sustained high ammonia levels in patients who were RFX nonresponders. Interestingly, *L. salivarius* did not have this function of elevating urease activity (Figure 4C; Figure S6E). Our analysis also revealed that lactulose administration greatly increased the amount of *L. salivarius* in the intestines (Figures S1C and S2C). Therefore, replacing gut microbiota might help reduce total gut microbial urease activity.

The sensitivity of *R. gnavus* to RFX markedly depended on the conjugated secondary bile acid concentration, thus partly explaining why *R. gnavus* was detected as a dominant species among patients who were nonresponders; these patients had plasma secondary bile acid levels that were much lower than those of patients who were responders. Furthermore, these data are concurrent with the metagenomic analyses performed using patient fecal DNA, particularly on the *urease* and *bai* operons (Figures 3F and 5D).

HE is a syndrome causing neuropsychiatric disorders among patients due to LC-associated liver dysfunction and concomitant dysbiosis in the intestines. Through multiomics analysis, we confirmed that bacterial species associated with ammonia production were important in HE pathogenesis. As a future perspective, precision medicine for each patient with HE is needed to reduce excess ammonia production from its exact source, possibly by targeting specific ammonia-producing gut bacteria using lytic bacteriophages (i.e., phage therapy).[9,10] This study also focused on the RFX responses in patients with HE. Intriguingly, *urease*-negative *R. gnavus,* which was retained in patients with HE who were RFX nonresponders, was discovered as a novel amplifier of coexisting urease activity. Furthermore, this study showed that the sensitivity of *R. gnavus* to RFX markedly depends on the concentration of conjugated secondary bile acids, including TDCA, suggesting the therapeutic potential for the combined use of TDCA and RFX to increase the sensitivity of patients with HE to RFX. We showed the importance of identifying the specific bacteria associated with HE. In addition, although blood ammonia levels are generally thought to be unstable,[35,36] future studies might be able to specify a new biomarker of HE through detailed characterization of specific gut microbiota, such as *S. salivarius*, identified here in patients with HE.

ETHICS STATEMENT

The ethics committee of the Graduate School of Medicine, Osaka City University (Osaka, Japan), approved the protocol (approval number: 3722). The study was conducted following Helsinki Declaration II, and written informed consent was obtained from all study participants. All animal experiments were approved by the Institutional Animal Care and Use Committees of Osaka City University (approval numbers: 17206, 18079).

AUTHOR CONTRIBUTIONS

Conceptualization: Norifumi Kawada and Naoko Ohtani. Investigation: Yoshimi Yukawa-Muto, Tomonori Kamiya, Hiroshi Mori, Akiyoshi Hirayama, Shinji Fukuda, Ikuya Sato, and Naoko Ohtani. Formal analysis: Yoshimi Yukawa-Muto, Tomonori Kamiya, and Yusuke Konishi. Resources: Yoshimi Yukawa-Muto and Hideki Fujii. Data curation: Tomonori Kamiya, Hiroshi Mori, and Atsushi Toyoda. Writing the original draft: Yoshimi Yukawa-Muto, Tomonori Kamiya, and Naoko Ohtani. Review and editing: Norifumi Kawada and Naoko Ohtani. Supervision: Tomonori Kamiya, Shinji Fukuda, and Naoko Ohtani. Project Administration: Naoko Ohtani. Funding Acquisition: Tomonori Kamiya, Hideki Fujii, Atsushi Toyoda, Eiji Hara, Shinji Fukuda, Norifumi Kawada, and Naoko Ohtani.

DATA AVAILABILITY STATEMENT

All data relevant to the study are included in the article or materials. All amplicon and metagenome sequence data are available from the DDBJ Sequence Read Archive under BioProject ID PRJDB10484 (BioSample accession: SAMD00276658-SAMD00276750).

REFERENCES

1. Liu J, Lkhagva E, Chung HJ, Kim HJ, Hong ST. The pharmabiotic approach to treat hyperammonemia. Nutrients. 2018;10:140.
2. Bajaj JS. Hepatic encephalopathy: classification and treatment. J Hepatol. 2018;68:838–9.

3. Qin N, Yang F, Li A, Prifti E, Chen Y, Shao LI, et al. Alterations of the human gut microbiome in liver cirrhosis. Nature. 2014;513:59–64.

4. Seki E, De Minicis S, Osterreicher CH, Kluwe J, Osawa Y, Brenner DA, et al. TLR4 enhances TGF-beta signaling and hepatic fibrosis. Nat Med. 2007;13:1324–32.

5. Bloom PP, Tapper EB, Young VB, Lok AS. Microbiome therapeutics for hepatic encephalopathy. J Hepatol. 2021;75:1452–64.

6. DeMorrow S. Bile acids in hepatic encephalopathy. J Clin Exp Hepatol. 2019;9:117–24.

7. Bajaj JS, Fagan A, Gavis EA, Kassam Z, Sikaroodi M, Gillevet PM. Long-term outcomes of fecal microbiota transplantation in patients with cirrhosis. Gastroenterology. 2019;156:1921–3 e3.

8. Caraceni P, Vargas V, Solà E, Alessandria C, Wit K, Trebicka J, et al.; Liverhope Consortium. The use of rifaximin in patients with cirrhosis. Hepatology. 2021;74:1660–73.

9. Gordillo Altamirano FL, Barr JJ. Phage therapy in the postantibiotic era. Clin Microbiol Rev. 2019;32;e00066–18.

10. Duan YI, Llorente C, Lang S, Brandl K, Chu H, Jiang LU, et al. Bacteriophage targeting of gut bacterium attenuates alcoholic liver disease. Nature. 2019;575:505–11.

11. Bajaj JS, Sikaroodi M, Shamsaddini A, Henseler Z, Santiago-Rodriguez T, Acharya C, et al. Interaction of bacterial metagenome and virome in patients with cirrhosis and hepatic encephalopathy. Gut. 2021;70:1162–73.

12. Rose CF, Amodio P, Bajaj JS, Dhiman RK, Montagnese S, Taylor-Robinson SD, et al. Hepatic encephalopathy: novel insights into classification, pathophysiology and therapy. J Hepatol. 2020;73:1526–47.

13. Yoshida S, Kurajoh M, Fukumoto S, Murase T, Nakamura T, Yoshida H, et al. Association of plasma xanthine oxidoreductase activity with blood pressure affected by oxidative stress level: MedCity21 health examination registry. Sci Rep. 2020;10:4437.

14. Odamaki T, Kato K, Sugahara H, Hashikura N, Takahashi S, Xiao J-Z, et al. Age-related changes in gut microbiota composition from newborn to centenarian: a cross-sectional study. BMC Microbiol. 2016;16:90.

15. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature. 2006;444:1027–31.

16. Inoue T, Nakayama J, Moriya K, Kawaratani H, Momoda R, Ito K, et al. Gut dysbiosis associated with hepatitis C virus infection. Clin Infect Dis. 2018;67:869–77.

17. Tsai C-F, Chen M-H, Wang Y-P, Chu C-J, Huang Y-H, Lin H-C, et al. Proton pump inhibitors increase risk for hepatic encephalopathy in patients with cirrhosis in a population study. Gastroenterology. 2017;152:134–41.

18. Horvath A, Rainer F, Bashir M, Leber B, Schmerboeck B, Klymiuk I, et al. Biomarkers for oralization during long-term proton pump inhibitor therapy predict survival in cirrhosis. Sci Rep. 2019;9:12000.

19. Scobie BA, Summerskill WH. Reduced gastric acid output in cirrhosis: quantitation and relationships. Gut. 1964;5:422–8.

20. Lodato F, Azzaroli F, Girolamo MD, Feletti V, Cecinato P, Lisotti A, et al. Proton pump inhibitors in cirrhosis: tradition or evidence based practice? World J Gastroenterol. 2008;14:2980–5.

21. Chen YY, Weaver CA, Burne RA. Dual functions of Streptococcus salivarius urease. J Bacteriol. 2000;182:4667–9.

22. Young GM, Amid D, Miller VL. A bifunctional urease enhances survival of pathogenic Yersinia enterocolitica and Morganella morganii at low pH. J Bacteriol. 1996;178:6487–95.

23. Wijdicks EF. Hepatic encephalopathy. N Engl J Med. 2016;375:1660–70.

24. Bass NM, Mullen KD, Sanyal A, Poordad F, Neff G, Leevy CB, et al. Rifaximin treatment in hepatic encephalopathy. N Engl J Med. 2010;362:1071–81.

25. Hoover WW, Gerlach EH, Hoban DJ, Eliopoulos GM, Pfaller MA, Jones RN. Antimicrobial activity and spectrum of rifaximin, a new topical rifamycin derivative. Diagn Microbiol Infect Dis. 1993;16:111–8.

26. Kaji K, Takaya H, Saikawa S, Furukawa M, Sato S, Kawaratani H, et al. Rifaximin ameliorates hepatic encephalopathy and endotoxemia without affecting the gut microbiome diversity. World J Gastroenterol. 2017;23:8355–66.

27. Kim JN, Henriksen ED, Cann IK, Mackie RI. Nitrogen utilization and metabolism in Ruminococcus albus 8. Appl Environ Microbiol. 2014;80:3095–102.

28. Dasarathy S, Merli M. Sarcopenia from mechanism to diagnosis and treatment in liver disease. J Hepatol. 2016;65:1232–44.

29. Kang JD, Myers CJ, Harris SC, Kakiyama G, Lee IK, Yun BS, et al. Bile acid 7alpha-dehydroxylating gut bacteria secrete antibiotics that inhibit Clostridium difficile: role of secondary bile acids. Cell Chem Biol. 2019;26:27–34.e4.

30. Zhang Z, Zhai H, Geng J, Yu R, Ren H, Fan H, et al. Large-scale survey of gut microbiota associated with MHE via 16S rRNA-based pyrosequencing. Am J Gastroenterol. 2013;108:1601–11.

31. Kaci G, Goudercourt D, Dennin V, Pot B, Doré J, Ehrlich SD, et al. Anti-inflammatory properties of Streptococcus salivarius, a commensal bacterium of the oral cavity and digestive tract. Appl Environ Microbiol. 2014;80:928–34.

32. Simren M, Tack J. New treatments and therapeutic targets for IBS and other functional bowel disorders. Nat Rev Gastroenterol Hepatol. 2018;15:589–605.

33. Calanni F, Renzulli C, Barbanti M, Viscomi GC. Rifaximin: beyond the traditional antibiotic activity. J Antibiot (Tokyo). 2014;67:667–70.

34. Jiao NA, Baker SS, Chapa-Rodriguez A, Liu W, Nugent CA, Tsompana M, et al. Suppressed hepatic bile acid signalling despite elevated production of primary and secondary bile acids in NAFLD. Gut. 2018;67:1881–91.

35. Nicolao F, Efrati C, Masini A, Merli M, Attili AF, Riggio O. Role of determination of partial pressure of ammonia in cirrhotic patients with and without hepatic encephalopathy. J Hepatol. 2003;38:441–6.

36. Ong JP, Aggarwal A, Krieger D, Easley KA, Karafa MT, Van Lente F, et al. Correlation between ammonia levels and the severity of hepatic encephalopathy. Am J Med. 2003;114:188–93.

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