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Alterations in Gut Microbiota and Metabolism in Cirrhotic Portal Hypertension: Implications for Disease Progression

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ABSTRACT

Background: Although gut microbiota has been implicated in various liver disorders, its relationship with cirrhotic portal hypertension (CPH) remains unclear.

Aims: To investigate the structural and functional alterations of gut microbiota in patients with CPH and the potential role of these alterations in the progression of CPH.

Methods: We collected faecal samples from 35 patients with CPH and 71 patients without CPH (controls) to conduct microbiome and metabolomic analyses. Gut microbes, faecal metabolites and their functional pathways associated with CPH were identified using multiple bioinformatics approaches. To understand the role of gut microbiota in the pathogenesis of CPH, we carried out faecal microbiota transplantation, CPH-characteristic bacterial transplantation and antibacterial experiments in mice.

Results: Microbial diversity was diminished, and gut microbial structures were altered in patients with CPH compared to the controls, primarily manifested as increased abundance of lipopolysaccharide-producing bacteria and decreased abundance of anti-inflammatory bacteria. This dysbiosis of gut microbiota was accompanied by changes in the faecal metabolome, particularly in arginine biosynthesis and nitric oxide production. Transplantation of gut microbiota from CPH patients, as well as the transplantation of CPH-associated bacteria *Veillonella nakazawae*, was found to exacerbate CPH progression in mice. Antibiotic treatment significantly alleviated the CPH progression induced by N-dimethylnitrosamine in mice.

Conclusions: Our study reveals that gut microbiota dysbiosis is implicated in CPH progression, potentially providing new avenues for microbiome-based treatment for CPH.

Qun Zhang, Jinghua Cui and Yixin Hou are co-first authors.

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1 | Introduction

Cirrhotic portal hypertension (CPH) is an important driving force behind the occurrence and development of various cirrhotic complications, including ascites, gastroesophageal variceal haemorrhage and hepatic encephalopathy, which are leading causes of death and liver transplantation worldwide [1]. The prime determinants of CPH are increased intrahepatic vascular resistance and expanded splanchnic blood flow [2]. Currently, the main treatments for CPH include etiological treatment, vasodilators, non-selective beta-blockers and transjugular intrahepatic portosystemic shunts [3]. Despite these interventions, the therapeutic outcomes and prognoses of patients with CPH remain unsatisfactory [4]. To develop more effective therapeutic strategies, it is critically important to identify the previously unrecognised mechanisms that drive CPH.

In the past few years, gut microbiota dysbiosis has been found to play a key role in the pathogenesis of numerous liver diseases, including hepatitis, cirrhosis, acute-on-chronic liver failure and hepatocellular carcinoma [5, 6]. In the liver, approximately two-thirds of blood supply comes from the portal vein, of which 75% comes from the mesenteric vein [7, 8]. Therefore, the liver is readily exposed to gut microbes and their products. The term 'gut-liver axis' was thus coined to emphasise the close anatomical and functional interplay between the two organs [5, 6]. A growing body of evidence supports the potential important role of the gut-liver axis in the pathogenesis of portal hypertension [7, 9]. García-Lezana et al. reported that restoring healthy gut flora could alleviate portal hypertension in a rat model of metabolic steatohepatitis [10]. Moghadamrad et al. found that the presence of intestinal mucosal lymphatic and blood vessels induced by bacterial colonisation could contribute to the development of portal hypertension [11]. However, most of these studies focused on experimental animals, and there have been few clinical studies on this subject.

In this study, we aimed to investigate the impact of the gut microbiota and its metabolism in patients with CPH. We performed integrative microbiome and metabolomic analyses of stool samples from patients with and without CPH, and identified CPH-associated microbial species, faecal metabolites and functional pathways. We further performed faecal microbiota transplantation (FMT), CPH-characteristic bacteria transplantation and antibacterial experiments to show that gut microbiota play an important role in CPH progression.

2 | Materials and Methods

2.1 | Ethics Statement

This study complied with the ethical guidelines of the Declaration of Helsinki. It was approved by the Ethical Review Committee of Beijing Ditan Hospital (Beijing, China) and registered in the Chinese Clinical Trial Registry, International Clinical Trials Registry Platform of the World Health Organization (ChiCTR2000040465). Prior to participating in the study, all participants provided written informed consent. In addition, all experiments with mice were approved by the Capital Medical University's Animal Ethics Committee (Beijing, China) (permit

number: 201901A009), and all mice received humane care in accordance with institutional guidelines.

2.2 | Patients and Animal Subjects

2.2.1 | Patients

From March 2020 to October 2023, we prospectively and consecutively recruited 35 patients with CPH and 71 individuals with non-cirrhotic portal hypertension (NC, serving as the control group) at Beijing Ditan Hospital of Capital Medical University. Notably, all participants were positive for the hepatitis B surface antigen. The clinical features of the two cohorts are summarised in Table 1. There were no significant differences between patients with CPH and NC in age, gender or body mass index. Participants were considered to have CPH if they were diagnosed with cirrhosis by radiological imaging and exhibited oesophageal/gastric varices by endoscopy [3, 12]. All subjects in the NC group were patients with chronic hepatitis B who were either on maintenance antiviral therapy or had newly initiated treatment, with confirmed absence of cirrhosis and no oesophageal/gastric varices on endoscopy. The absence of cirrhosis was confirmed by meeting all of the following criteria: (a) liver stiffness <9.0 kPa by elastography [13, 14]; (b) FIB-4 score <1.45 [15]; and (c) absence of cirrhosis on imaging [16, 17]. The exclusion criteria included: (a) patients aged >70 years or <20 years; (b) coexisting liver or organ malignancies; (c) chronic renal diseases, diabetes mellitus, hypertension or other metabolic disorders; (d) serious internal organ or infectious diseases; and (e) reception of medication containing any antibiotics, metformin, probiotics, proton pump inhibitors, steroids or other hormones, within 1 month before stool sampling. All patient information was obtained from the questionnaires and the hospital's electronic medical records.

2.2.2 | Mice

Healthy male ICR mice (weight: 18–22 g; age: 4–6 weeks) were purchased from Beijing Weitong Lihua Biotechnology Co. Ltd. The mice were adaptively fed for a week prior to the formal experiments. The mice were housed in standard specific-pathogen-free conditions (65% humidity, 23°C, 12 h/12 h light/dark) and had free access to food and water.

2.3 | Collection of Stool Samples

Fresh stool samples were collected from each participant at approximately the same time in the early morning and immediately delivered to the laboratory under cold conditions (4°C). Each sample was divided into five 200 mg portions under sterile conditions and frozen at –80°C until assayed.

2.4 | DNA Extraction, Amplification and Sequencing

Faecal genomic DNA extraction was done using the MagPure Soil DNA LQ Kit (Magen Biotechnology Co. Ltd., Guangzhou, China) according to the product instructions. The V3–V4 region

TABLE 1 | Baseline characteristics of the participants.

Characteristics	CPH (<i>n</i> = 35)	NC (<i>n</i> = 71)		<i>p</i> ^a
		VUS (<i>n</i> = 55)	VSS ^b (<i>n</i> = 16)	
Age (year)	49.0 (43.0–53.0)	34.0 (28.0–50.0)	50.0 (28.5–51.5)	0.121
Gender, male (%)	22 (62.9%)	31 (56.4%)	11 (68.8%)	0.714
Body mass index (kg/m ²)	21.9 (20.7–23.0)	22.7 (20.8–24.1)	22.7 (22.1–24.2)	0.055
ALT (U/L)	21.3 (16.2–31.4)	246.1 (75.8–365.0)	34.6 (30.3–38.7)	<0.001
AST (U/L)	28.8 (20.6–45.3)	98.7 (45.2–174.0)	22.7 (19.7–25.6)	0.007
ALP (U/L)	87.6 (55.2–109.3)	75.1 (66.4–93.4)	52.9 (46.8–59.5)	0.285
GGT (U/L)	29.8 (17.1–47.9)	80.6 (39.1–153.3)	20.2 (14.6–29.7)	0.007
TBil (mmol/L)	23.5 (15.7–35.6)	20.7 (16.2–26.8)	8.5 (7.7–9.4)	0.074
DBil (mmol/L)	10.3 (7.3–15.9)	8.6 (5.8–13.2)	3.5 (2.9–3.7)	0.019
ALB (g/L)	34.2 (31.7–39.6)	41.3 (37.8–46.9)	50.8 (48.9–54.5)	<0.001
WBC (×10 ⁹ /L)	2.4 (1.8–3.5)	6.0 (4.4–7.3)	5.7 (4.4–6.5)	<0.001
PLT (×10 ⁹ /L)	58.6 (32.7–96.4)	211.0 (180.0–265.0)	150.5 (200.5–204.0)	<0.001
PT (s)	14.8 (13.5–17.5)	12.6 (11.1–13.4)	11.1 (10.4–11.5)	<0.001
PTA (%)	67.0 (55.0–79.0)	82.0 (74.0–93.0)	101.5 (90.0–106.8)	0.002
INR	1.3 (1.2–1.6)	1.1 (1.0–1.2)	1.0 (1.0–1.3)	0.001
Spleen thickness (mm)	53.5 (44.3–60.3)	31.0 (27.0–40.0)	28.5 (27.0–31.5)	<0.001
Portal vein diameter (mm)	12.5 (11.0–15.0)	11.0 (10.0–12.0)	9.0 (8.0–10.0)	0.002
Spleen length (mm)	157.0 (140.2–177.3)	100.0 (100.0–121.0)	109.5 (105.3–113.8)	<0.001
Liver stiffness (Kpa)	18.5 (15.7–25.7)	6.2 (5.4–6.8)	5.3 (4.8–6.6)	<0.001
FIB-4 scores	6.3 (2.8–10.3)	0.8 (0.7–1.1)	0.8 (0.7–1.1)	<0.001
Child-Pugh scores	9.0 (8.0–10.0)	7.0 (7.0–7.0)	7.0 (6.0–7.0)	0.001
MELD scores	11.4 (9.4–13.6)	8.1 (7.3–11.0)	8.0 (6.6–9.0)	0.003

Abbreviations: ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CPH, cirrhotic portal hypertension; DBil, direct bilirubin; FIB-4, fibrosis-4 index; GGT, γ -glutamyl transferase; HCT, haematocrit; INR, international normalised ratio; MELD, Model for End-stage Liver Disease; NC, non-cirrhotic portal hypertension; NLR, neutrophil-lymphocyte ratio; PLT, platelet; PT, prothrombin time; PTA, prothrombin activity; TBil, total bilirubin; VSS, virologically suppressed subgroup; VUS, virologically unsuppressed subgroup; WBC, white blood cells.

^a*p* value refers to comparisons between CPH and NC groups.

^bVSS criteria: HBV DNA < 20 IU/mL and normal ALT (≤ 40 U/L). Values are expressed as medians (interquartile range) for quantitative variables and *n* (%) for qualitative variables. Quantitative data were compared using the Wilcoxon rank-sum test between both groups. Fisher's exact test compared qualitative data.

of the 16S rRNA in the extracted DNA (template) was amplified using the universal primers 343 F and 798 R. Amplicons were purified with AMPure XP beads (Beckman Coulter Co., USA), followed by another round of PCR amplification. After repurification, the final amplicon was quantified using the Qubit dsDNA assay kit (Thermo Fisher Scientific, USA). Detailed amplification and purification methods are described in the supplementary methods. Pooled equal amounts of purified amplicons were sequenced using Illumina NovaSeq 6000 (OE Biotech Co. Ltd., Shanghai, China) [18, 19].

2.5 | Bioinformatic Analysis of 16S rRNA Gene Sequencing Data

After sequencing, the raw data were processed with tag filtering, splicing, quality control and chimerism removal to obtain

high-quality reads for analysis. Using Vsearch software (version 2.4.2) [20], the high-quality valid tags were clustered to yield operational taxonomic units (OTUs) according to 97% similarity. Representative reads for each OTU were identified using quantitative insights into microbial ecology (QIIME) [21]. All representative reads were initially processed with the Ribosomal Database Project classifier for preliminary quality control and were subsequently annotated and classified against the SILVA database (v.123) [22, 23]. To account for variations in sampling across individuals, the relative abundance of each OTU and that of other taxonomic levels (from phylum to genus) were estimated for each sample. In the present study, bacterial diversity was determined from the relative abundance profiles of OTUs in individual samples and analyzed using Chao1 and Shannon indices, which were calculated according to QIIME [21]. Dissimilarity between community compositions was assessed using the Jaccard index (binary_Jaccard) and visualised with

OTU abundance-based principal coordinate analysis (PCoA). Bacterial taxonomic analyses and comparisons between the two groups at the phylum and genus levels were conducted using the Wilcoxon rank-sum test (significance threshold: $p < 0.05$). A random forest (RF) model was applied to these differentially expressed genera to rank their importance based on the mean decrease in Gini index [24, 25], and the top 10 bacterial genera were selected for subsequent analysis. The RF analysis was performed in Python 3.10.4 using the scikit-learn (v.1.2.2) and Matplotlib (v.3.7.1) packages. Associations of specific bacterial genera with phenotypic parameters were identified using Spearman correlation tests. Statistical analyses of the 16S rRNA sequencing data were performed using R version 3.5.3 [26].

2.6 | Untargeted Metabolomics Analysis

Untargeted metabolomic profiling of human faecal samples was performed by Metabo-Profile (Shanghai, China) using a gas chromatography time-of-flight mass spectrometry (GC-TOF/MS) system (Pegasus HT; Leco Corp., St. Joseph, MO, USA) with an Agilent 7890 B gas chromatograph [27]. Sample preparation, operating procedures and metabolite annotation are described in the supplementary methods. Considering that metabolites are often related due to their biological functions and metabolic pathways, we employed both univariate analysis (student's t -test) and multivariate analysis (orthogonal principal least-squares discriminant analysis, OPLS-DA) to identify differential metabolites between the CPH and NC groups [28, 29]. Metabolites were considered significant with the statistical cutoffs of $p < 0.05$ (student's t -test) and variable importance in the projection (VIP) > 1 (OPLS-DA). We further annotated the differential metabolites by matching them to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database using the pathway enrichment tool in MetaboAnalyst (<https://www.metaboanalyst.ca/>) [30]. Correlation analyses of metabolites with clinical parameters and specific bacterial genera were carried out using Spearman correlation tests.

2.7 | Isolation and Identification of *Veillonella nakazawae* and *Streptococcus anginosus*

Fresh stool samples were collected from patients with CPH and immediately transferred to an anaerobic environment. Each 2g of faecal samples was diluted in 20mL phosphate-buffered saline (PBS) and filtered with a 100 μ m filter. The filtrates were diluted from 1 to 10⁸ through 10-fold serial dilutions in PBS and spread onto Columbia blood plates to isolate target strains of intestinal microbiota. Plates were incubated in an anaerobic environment for ~48 h at 37°C maintained by a Whitley Jar gas-sing system. Candidate colonies were picked for DNA extraction and 16S rRNA sequencing. The obtained DNA sequences were subjected to BLAST analysis with the available databases at the National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov/>). Finally, *V. nakazawae* [31] and *S. anginosus* [32] were isolated and used in this study. Electron microscopy and matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry (Bruker Daltonics, Germany) were used to identify the two target strains. *V. nakazawae* was cultured anaerobically in Veillonella Medium Base

(VMB) (Product ID: PM22681, Shanghai HongSun Biotech Co. Ltd) supplemented with 40mL of 60% sodium lactate, at 37°C and 200 rpm for ~48 h. *S. anginosus* was cultured anaerobically in brain heart infusion (BHI) medium at 37°C and 200 rpm for ~33 h. The relevant growth curves for *V. nakazawae* and *S. anginosus* were also constructed.

2.8 | N-Dimethylnitrosamine-Induced CPH Mouse Model

A mouse model of CPH was constructed via intraperitoneal injection of 10 mg/kg N-dimethylnitrosamine (NDMN) once a day for three consecutive days per week for a total of 4 weeks according to the previously reported method [33].

2.9 | Antibiotic-Treated Mouse Model

Mice were treated with a mixture of broad-spectrum antibiotics comprising vancomycin (0.1g/L), ampicillin (0.2g/L), metronidazole (0.2g/L) and neomycin (0.2g/L) provided in the drinking water for 2 weeks [34]. We collected faeces immediately before and after antibiotic treatment and quantified the amount of culturable faecal bacteria on Columbia blood agar plates to confirm bacterial depletion. The viable bacterial count in mouse faeces was decreased by over 90%, suggesting the successful establishment of the antibiotic-treated animal model [35]. We also assessed changes in faecal microbiota before and after antibiotic treatment using alpha diversity (Shannon index), beta diversity (Bray-Curtis distances) and microbial community structure analysis.

2.10 | Faecal Microbiota Transplantation Experiment

Fresh stools were collected separately from four NC and four CPH patients, and 1.0g from each sample was mixed and suspended in 40mL PBS. The suspension was passed through 70 μ m filters and then centrifuged at 2000 rpm for 10 min. After removing the supernatant, the precipitates were resuspended in 2mL PBS. The resuspension was used for FMT via oral gavage [36]. Prior to gavage, the experimental mice were treated with a broad-spectrum antibiotic mixture for 2 weeks to construct the antibiotic-treated mouse model as described above and were randomly divided into three groups: NC-associated recipient mice (NC^{FMT}), CPH-associated recipient mice (CPH^{FMT}) and PBS-associated recipient mice (PBS^{FMT}) ($n=6$). The mice in the NC^{FMT} and CPH^{FMT} groups were then administered faecal suspensions (200 μ L per mouse) from NC or CPH patients, respectively, by gavage (once every other day) for 2 weeks, whereas mice of the PBS^{FMT} group received PBS at the same time points. To assess the effect of FMT on the gut microbiota of experimental mice, faecal samples were collected from NC^{FMT} and CPH^{FMT} groups before and after FMT for 16S rRNA sequencing. Subsequently, we established the CPH model in all mice by intraperitoneal injection of NDMN for 4 weeks. After the experimental period, liver hemodynamic indices, including portal vein diameter (PVD), portal vein velocity (PVV) and ascites depth (AD), were determined using a small animal ultrasound system (Esaote MyLab). In addition, serum samples were collected

for biochemical testing and amino acid targeted metabolomic analysis.

2.11 | *Veillonella nakazawae* Transplantation Experiment

Two groups of experimental mice ($n=6$) were treated with the broad-spectrum antibiotic mixture for 2 weeks as described in the antibiotic-treated mouse model above. One group of mice was then gavaged for 2 weeks (once every other day) with 2×10^8 colony-forming units (CFU)/200 μ L of *V. nakazawae* (CCUG 74597T) isolated from the faecal samples of CPH patients. The other group of mice was gavaged with PBS. Subsequently, all experimental mice were injected with NDMN to induce CPH, using the same method. Microbial transplantation was continued twice a week to enhance its effects on the experiments. Blood and tissue samples were collected for further analysis.

2.12 | Amino Acid Targeted Metabolomic Analysis

We conducted a quantitative analysis for amino acid metabolites in mouse serum samples using a Q300 Kit (Metabo-Profile, Shanghai, China) [37]. An ultra-performance liquid chromatography system coupled to a tandem mass spectrometry (UPLC-MS/MS) system (ACQUITY UPLC-Xevo TQ-S; Waters Corp., Milford, MA, USA) was used by Metabo-Profile Biotechnology (Shanghai, China) Co. Ltd. to quantify all the metabolites targeted in the present study. Detailed information regarding the measurements and analyses is provided in the [Supporting Information](#).

2.13 | Laboratory Measurements

Mouse blood samples were centrifuged at 3000 rpm for 15 min to separate serum. Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin (TBil) were determined using an automatic biochemical analyser (Skyla Vb1, CVM practice, Navarra, Spain). Serum nitric oxide (NO), inducible nitric oxide synthase (iNOS), interleukin [IL]-1 β , IL-6 and tumour necrosis factor alpha (TNF- α) were assayed using enzyme-linked immunosorbent assay (ELISA) kits (Jiancheng Biotech Ltd., Nanjing, China) according to the product instructions. The lipopolysaccharide (LPS) content was assayed using a Chromogenic Endotoxin quantitative kit (Xiamen Bioendo Technology Co. Ltd., Xiamen, China), following the kit instructions. Platelet counts were measured using a TEK-II Mini full-automatic animal blood analyser (Tecom Science, Jiangxi, China). The liver tissue pathology was evaluated using haematoxylin eosin (HE) staining. Additionally, the ultrastructure of the colon was observed by transmission electron microscopy (TEM). A detailed description of HE staining and TEM is provided in the [Supporting Information](#).

2.14 | Statistical Analysis

For clinical results, quantitative data were expressed as medians (interquartile range) and compared using the Wilcoxon

rank-sum test; qualitative variables were expressed as n (%) and compared using the Fisher's exact test. All the statistical analyses of the clinical data were performed in the Statistical Package for the Social Sciences (SPSS) version 22 (IBM Corp., USA). For animal experiments, all data were expressed as the mean $\pm$ standard deviation and compared using an unpaired Student's t -test in GraphPad Prism version 8.0.2 (GraphPad Software Inc., USA). Two-tailed $p < 0.05$ was regarded as a statistically significant difference. All correlations were analysed by the Pearson or nonparametric Spearman method according to the normal or nonnormal distribution of the data.

3 | Results

3.1 | Decreased Microbial Diversity and Altered Gut Microflora Structure in Patients With CPH

We analysed faecal samples from 35 patients with CPH and 71 individuals with NC using 16S rRNA sequencing and untargeted metabolomics (Figure S1). Alpha diversity was significantly decreased in patients with CPH compared with NC, according to the Chao 1 index ($p=0.036$; Figure 1A). The Shannon indices of the two groups showed a similar trend despite narrowly missing the statistical significance cutoff ($p=0.070$; Figure 1B). PCoA was used to evaluate the variation in microbial community composition. The results showed that the gut microflora structure differed significantly between patients with CPH and NC (Adonis test: $p=0.001$; Figure 1C), suggesting gut microbial dysbiosis in patients with CPH.

At the phylum level, the abundance of Bacteroidetes significantly decreased, whereas the abundance of Proteobacteria and the ratio of Firmicutes/Bacteroidetes significantly increased in patients with CPH compared to those in the NC group ($p < 0.05$; Figure 1D and Figure S2). At the genus level, we first assessed the microbial co-occurrence network among all genera in both the NC and CPH groups. We observed that the CPH group showed a greater number of statistically significant bacterial associations (Spearman correlation: $R > 0.5$ and $p < 0.05$) compared to the NC group (Figure 1E), suggesting tighter interactions among bacterial genera in CPH patients.

By Wilcoxon rank-sum test, 121 genera were found to be significantly different between the two groups ($p < 0.05$; Table S1). We built a 10-fold cross-validated RF model based on the 121 different bacterial genera to discriminate CPH from NC, achieving an area under the curve (AUC) of 0.86 (Figure S3). The RF model further identified the 10 most important bacterial genera (ranked by mean decrease in Gini index) associated with CPH (Figure 1F and Table S2). Among them, the abundances of *Flavonifractor*, *Veillonella*, *Klebsiella*, *Citrobacter*, *Clostridium* and *Streptococcus* were significantly increased in the CPH group, whereas the abundances of *Adlercreutzia*, *Alloprevotella*, *Parasutterella* and *Coprococcus_2* were significantly decreased (Figure 1G and Figure S4).

We further examined the correlations between the above 10 differentially expressed bacterial genera and several key clinical indicators. Most notably, we found that *Klebsiella*, *Veillonella* and *Streptococcus* significantly correlated with indicators of liver

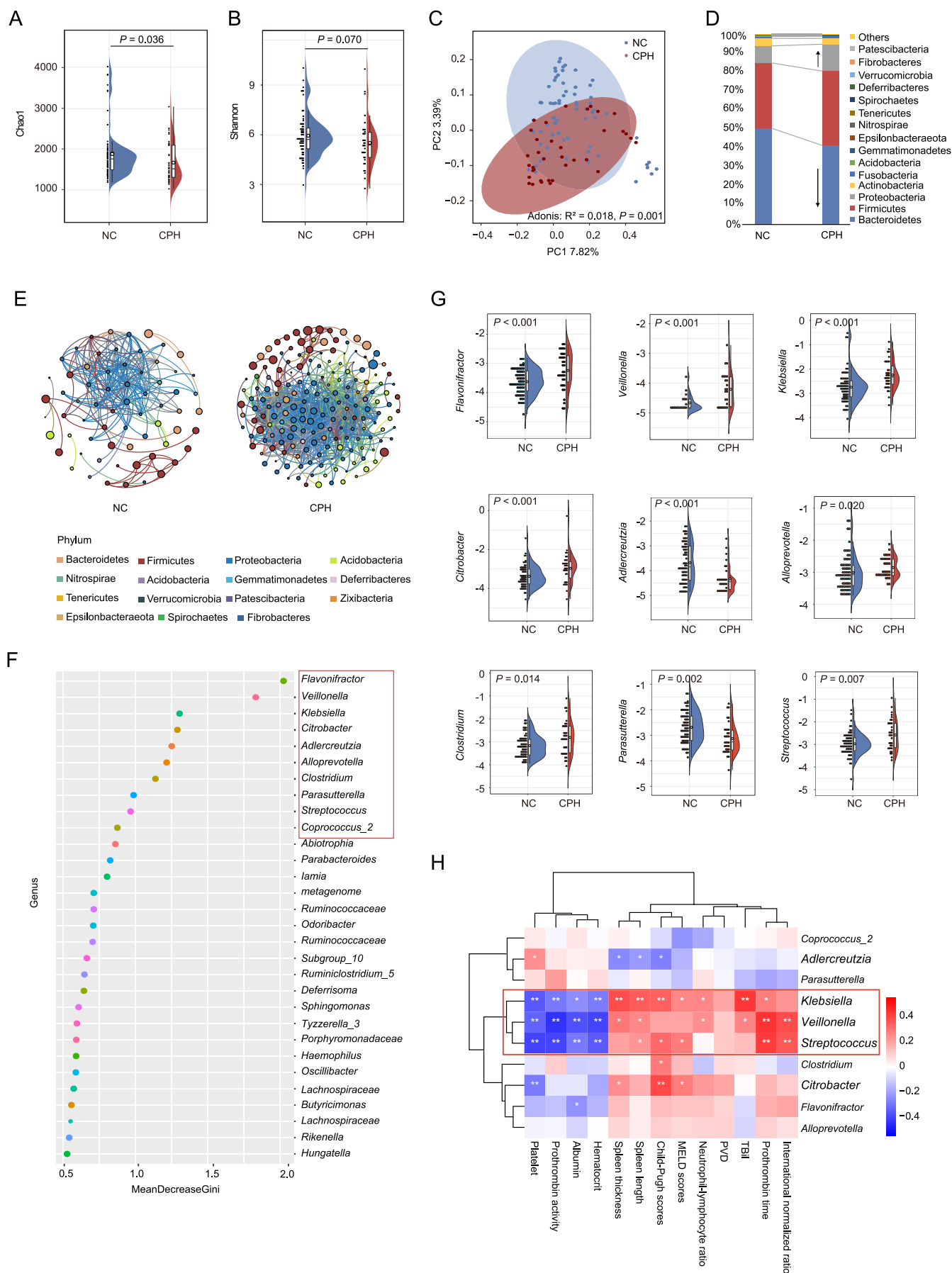


FIGURE 1 | Legend on next page.

FIGURE 1 | Alterations in gut microbial diversity and community structure. Differences in microbial diversity between CPH or NC, estimated using the Chao1 (A) and Shannon indices (B). (C) Beta diversity calculated by binary Jaccard using PCoA, depicting significant separation between overall composition of microbiota in faecal samples from patients with CPH or NC. Red and blue dots represent patients with and without CPH, respectively. (D) Stacked bar chart showing the proportion of components in top 10 bacterial phyla in each group. (E) Microbial co-occurrence network based on the Spearman correlation analysis ($R > 0.5$, $p < 0.05$) between all genera in patients with NC and CPH. Each node represents a genus, and the sizes of nodes represent the relative abundance of genera. The edges in red or blue represent the positive or negative correlation, respectively. (F) Random Forest featuring importance ranking for the 121 differentially expressed bacterial genera (Only the top 30 genera are shown). (G) Half violin plot of relative abundance for the top nine differentially expressed bacterial genera (log 10 transformed). (H) Heatmap of Spearman correlations between abundance of top 10 bacterial genera and clinical indices. Red and blue boxes represent positive and negative correlations, respectively (*, $p < 0.05$; **, $p < 0.01$). CPH, cirrhotic portal hypertension; MELD, Model for End-stage Liver Disease; NC, non-cirrhotic portal hypertension; PCoA, principal coordinate analysis; PVD, portal vein diameter; TBil, total bilirubin.

function (TBil, prothrombin time, international normalised ratio, Child-Pugh scores and Model for End-stage Liver Disease scores) and portal pressure (spleen thickness and spleen length), suggesting that the CPH phenotype may be associated with the alteration of these bacteria in the gut microbiome (Figure 1H and Table S3).

3.2 | Faecal Metabolomic Alterations in CPH

To further investigate the biological effects of gut microbiota on CPH, we analysed the metabolites in faecal samples using GC-TOF/MS. Principal least-squares discriminant analysis (PLS-DA) showed that the faecal metabolic profiles were significantly different between patients with CPH and NC (Figure 2A). By the combination of *t*-test and OPLS-DA, we identified 40 metabolites significantly altered between the two groups (Figure 2B–D and Tables S4–S6). Among these, 17 and 23 metabolites were enriched and depleted in the CPH patient group, respectively (Figure 2E and Figure S5). These results reflect an altered metabolomic environment in the guts of patients with CPH. KEGG pathway enrichment analysis showed that the significantly elevated metabolites in CPH mostly affected amino acid metabolic pathways, including alanine, aspartate and glutamate metabolism; D-glutamine and D-glutamate metabolism; nitrogen metabolism; arginine biosynthesis; and arginine and proline metabolism (Figure 2F and Table S7). These metabolic pathways interact with each other and ultimately affect the arginine biosynthesis pathway, which may promote NO biosynthesis (Figure 2G). These findings suggest that altered arginine biosynthesis pathways may play a role in the CPH phenotype.

To further assess whether the microbial and metabolic signatures associated with CPH were confounded by active hepatitis (present in 55/71 controls), we performed a subgroup analysis comparing the CPH group with the virologically suppressed subgroup (VSS; $n = 16$, defined as HBV DNA < 20 IU/mL and ALT ≤ 40 U/L) within the NC group. Albeit with reduced statistical power, we found that most differentially expressed microbial and metabolic features remained significant even in this ‘cleaner’ comparison (60% of original biomarkers retained significance at $p < 0.05$; Tables S8–S10). Crucially, key bacterial genera including *Veillonella*, *Streptococcus*, *Klebsiella*, *Adlercreutzia* and *Coprococcus_2* exhibited consistent directional changes

in the CPH group (Figure S6), and the arginine metabolism pathway remained significantly enriched among differential metabolites ($FDR < 0.05$; Figure S7 and Table S11). In addition, we performed a correlation analysis between serum ALT levels and the top 30 differentially expressed microbial genera and differential metabolites across all subjects. We found that 80% of CPH-associated biomarkers showed no significant correlation with ALT (e.g., *Veillonella*, $p = 0.320$; Figure S8 and Table S12). These findings suggest that the observed differences are primarily driven by portal hypertension rather than active hepatitis.

3.3 | Associations Between Disease-Related Microbiota and Metabolites

We used Procrustes analysis to assess the overall correlations between the metabolomic and microbial datasets. Not surprisingly, we found a close association between faecal microbes and metabolites ($p = 0.001$; Figure 3A), likely reflecting that the metabolites were mainly derived from the gut microbiome activities. We then evaluated the associations between the differentially expressed bacterial genera and metabolites using the Spearman method (Table S13). Figure 3B shows the complex network of relationships between the key discriminatory microbial genera and metabolites. On the one hand, changes in the gut microbiota may affect host metabolism. We observed that CPH-enriched bacterial genera correlated positively with CPH-enriched metabolites. As mentioned above, elevated metabolites in CPH may affect host arginine biosynthesis pathways (Figure 2G). On the other hand, changes in the metabolic environment may provide energy for the growth of certain gut bacteria. Previous studies suggest that L-arginine is the sole substrate for NO synthesis via nitric oxide synthase and that the NO produced is rapidly oxidised to nitrate [38]. Certain gut bacteria express nitrate reductase (NIR), which reduces the nitrate produced by the host to nitrite (Figure 3C) [38, 39]. Here, we found that nitrate and nitrite levels in stool samples from patients with CPH were significantly higher than those from patients with NC ($p < 0.001$; Figure 3D,E), indicating that more bacteria capable of expressing NIR are present in the gut of CPH patients. To further support this idea, we isolated two target strains, *V. nakazawae* and *S. anginosus*, from CPH faecal samples (Figure 3F–H). We found that NIR activity in *V. nakazawae* and *S. anginosus* gradually increased along with the increasing nitrate content in the medium (Figure 3I,J).

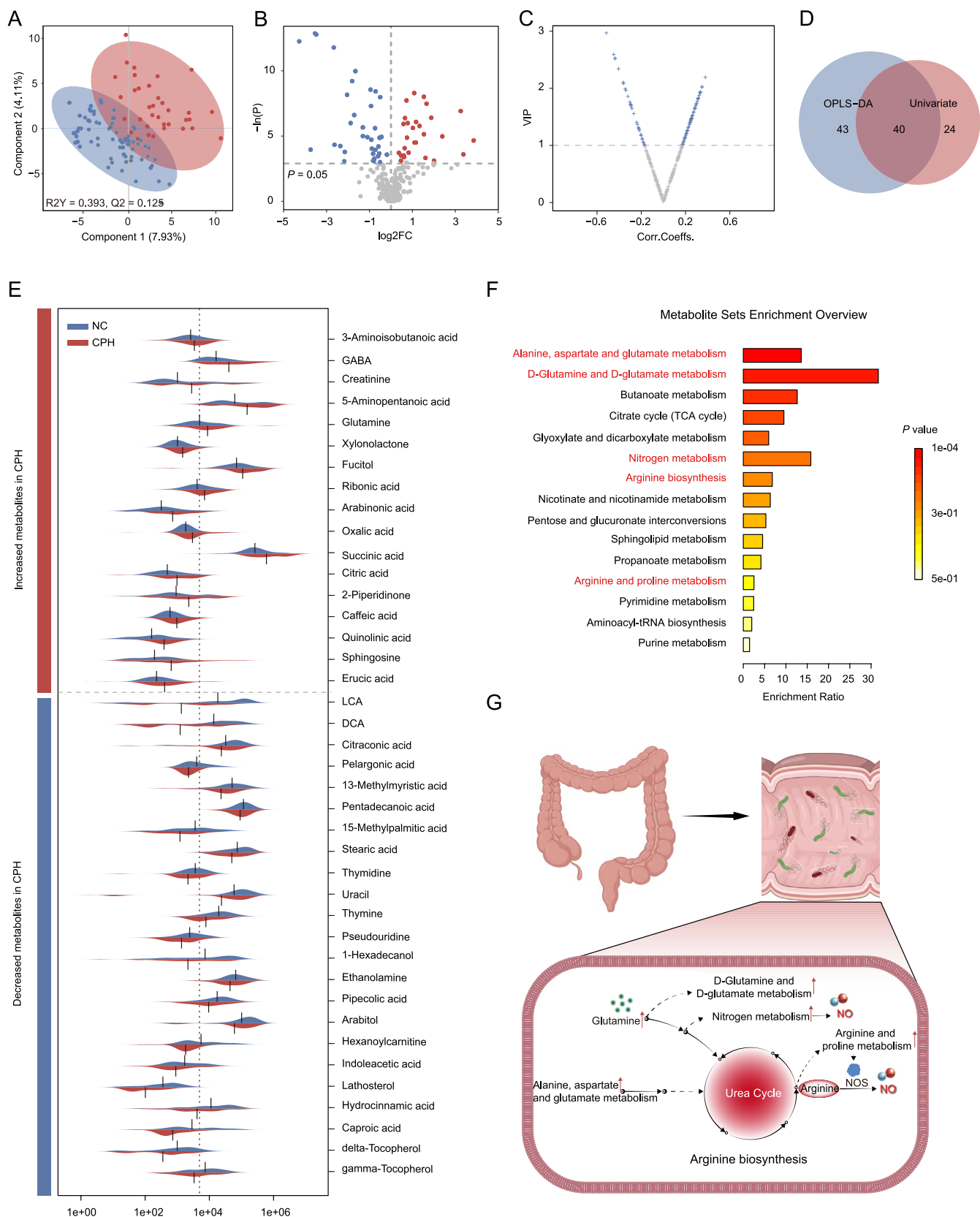


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These data suggest that *V. nakazawae* and *S. anginosus* can express NIR and therefore convert nitrate to nitrite, giving these pathogens a growth advantage in an anaerobic environment

[39]. Thus, the growth rates of these two bacterial strains were also significantly higher in the nitrate-containing medium (Figure 3K,L).

FIGURE 2 | Alterations of the faecal metabolome in patients with CPH. (A) PLS-DA 2D score plot for NC (blue) and CPH (red) samples. (B) Metabolite changes in patients with CPH vs. NC, determined using univariate analyses. Red and blue points represent increased and decreased metabolites, respectively. (C) Vplot showing the differentially expressed metabolite profiles, selected from the OPLS-DA model. (D) Venn plot of differentially expressed metabolites from multidimensional and univariate statistics. (E) Bean plot for the abundance of the 40 differentially expressed metabolites in samples from patients with CPH or NC. (F) Pathway enrichment and statistical significance of the differentially expressed metabolites that were significantly enriched in patients with CPH. (G) Interactions among five amino acid-related metabolic pathways. Upward-pointing arrows (↑) in the figure indicate that these pathways are upregulated in CPH. CPH, cirrhotic portal hypertension; DCA, deoxycholic acid; FC, fold change; GABA, γ -aminobutyric acid; LCA, lithocholic acid; NC, non-cirrhotic portal hypertension; NO, nitric oxide; NOS, nitric oxide synthases; OPLS-DA, Orthogonal Partial Least Squares-Based Discriminant Analysis; PLS-DA, Partial Least Squares-Based Discriminant Analysis; VIP, variable importance in projection.

3.4 | CPH-Associated Gut Microbiota Facilitate Portal Hypertension Progression in Mice

To assess the role of intestinal microbiota in CPH progression, we carried out FMT experiments in mice. As shown in Figure 4A, gut bacteria from patients with CPH or NC were transplanted into antibiotic-treated mice. We first analyzed changes in alpha diversity and beta diversity before and after transplantation in recipient mice that received CPH or NC flora. The results indicated that the alpha diversity in recipient mice was significantly increased after FMT (Shannon index: $p < 0.05$; Figure 4B,C), and PCoA based on Bray–Curtis distances revealed significant differences in microbial community structure before and after FMT ($p < 0.05$; Figure 4D,E). Further, we assessed whether the gut microbiota dysbiosis between CPH and NC patients could be reflected by FMT in mice. After FMT, alpha diversity was significantly reduced in CPH^{FMT} compared to that of NC^{FMT}, according to the Chao 1 ($p = 0.008$; Figure 4F) and Shannon indices ($p = 0.012$; Figure 4G). PCoA analysis showed a clear deviation in the structure of the gut microbiota between the two groups following FMT, and both groups significantly differed from their respective pre-FMT states ($p = 0.001$; Figure 4H). The CPH^{FMT} mice demonstrated an increased relative abundance of Proteobacteria compared to NC^{FMT} mice ($p = 0.025$; Figure 4I). Furthermore, although not all differences were statistically significant, the trends in the fluctuating abundances between the 10 different genera were consistent with those of our cohort study (Figure S9).

Next, we evaluated the effect of FMT on portal pressure-related indicators. Without an external stimulus, the CPH^{FMT} group exhibited higher levels of PVD and AD but lower levels of PVV, by comparison to the levels measured in the NC^{FMT} and PBS^{FMT} groups ($p < 0.05$; Figure 4J). In addition, compared to the NC^{FMT} and PBS^{FMT} groups, the spleen weight in the CPH^{FMT} group was significantly increased, whereas the platelet count was significantly decreased ($p < 0.05$; Figure 4K,L). These results suggest that the CPH-associated gut microbiota can facilitate the progression of CPH.

Our untargeted metabolomics analysis of the clinical samples suggested that amino acid metabolism was altered in the CPH group (Figure 2F). To explore the potential mechanisms by which CPH-associated gut microbiota could exacerbate portal hypertension, we investigated amino acid metabolites in the serum of mice from the two FMT groups at week 4 using a UPLC-MS/MS-based targeted metabolomics method. PCoA revealed a clear separation of the serum metabolic profiles of the CPH^{FMT} and NC^{FMT} groups (Figure S10), suggesting the involvement of the intestinal

microflora in host metabolism. After filtering the metabolites using the intersection of univariate and multivariate statistical analyses, 11 amino acids were found to be significantly different between the two groups (Figure S11). The KEGG pathway analysis of the altered metabolites showed that changes in the arginine metabolism pathway were the most pronounced (Figure 4M and Table S14). By extracting the relative concentrations of arginine-related metabolites along the pathway, we found that arginine was not significantly elevated in the CPH^{FMT} group compared to the NC^{FMT} group, and even tended to decrease ($p = 0.423$; Figure 4N). The global arginine bioavailability ratio (GABR), which reflects steady-state arginine and is calculated as arginine/(ornithine + citrulline) [40], was significantly reduced in the CPH^{FMT} group ($p = 0.041$; Figure 4O), suggesting that arginine is involved in downstream catabolism. Consistent with this finding, blood citrulline and NO levels were also significantly increased in the CPH^{FMT} group following FMT ($p < 0.001$ and $p = 0.004$, respectively; Figure 4P,Q). Taken together, these observations suggest that CPH-associated bacteria may enhance the host arginine metabolism, resulting in subsequent NO facilitation accompanied by accelerated CPH progression in CPH^{FMT} mice.

3.5 | *Veillonella nakazawae* Promote Portal Hypertension Progression in Mice

We next explored if the CPH phenotype produced by FMT could be recapitulated by transplanting specific bacterial species associated with CPH. Among the differential species associated with CPH, we selected *Veillonella* because of the following reasons: (1) it was ranked as one of the top differential species between CPH and NC by RF analysis (ranked second; Figure 1F); (2) it had strong associations with liver function and CPH clinical indications (Figure 1H); and (3) it had strong associations with CPH altered metabolites (Figure 3B). In this experiment, we used the strains *V. nakazawae* isolated from the faecal samples of CPH patients (Figure 5A). First, we confirmed the successful colonisation of *V. nakazawae* using droplet digital PCR (Figure 5B). Liver function indicators, including ALT, AST and TBil, were significantly elevated in the *V. nakazawae* murine group relative to those in the reference PBS group ($p < 0.05$; Figure 5C–E). Compared to the PBS group, the *V. nakazawae* group showed significantly increased spleen weight and decreased platelet count ($p < 0.05$; Figure 5F,G). In addition, PVD and AD were significantly increased in the *V. nakazawae* group as compared to the control condition, whereas the PVV was significantly decreased at week 4 ($p < 0.05$; Figure 5H). This indicates that an increase in the abundances of *V. nakazawae* promoted CPH progression.

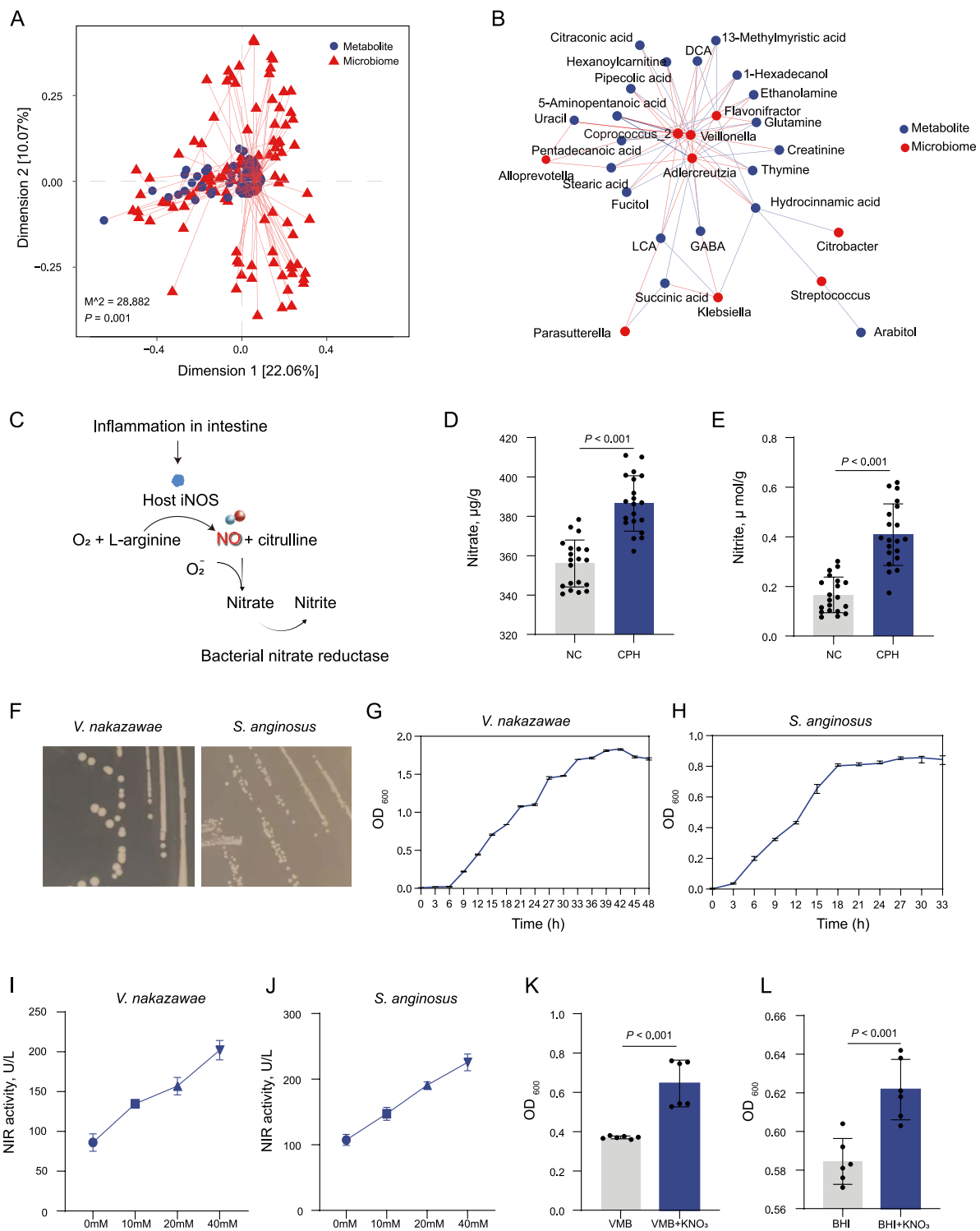


FIGURE 3 | Correlation between differential bacterial genera and metabolites. (A) Procrustes analysis of overall correlations between gut microbial profiles and faecal metabolic profiles among all participants. (B) Integrative network shows significant correlations between differentially expressed microbes and metabolites (Spearman tests: $P < 0.05$). Microbiomes and metabolites are represented by red and blue nodes, respectively. Positive (red) and negative (blue) correlations are shown as lines linking nodes. (C) Metabolic pathways of L-arginine-NO. (D) The amount of nitrate present in stool samples of patients with NC ($n = 20$) or CPH ($n = 20$). (E) The amount of nitrite present in stool samples of patients with NC ($n = 20$) or CPH ($n = 20$). (F) Images of typical colonies of *V. nakazawae* and *S. anginosus* isolated from patients with CPH. (G, H) Growth curves of *V. nakazawae* (G) and *S. anginosus* (H). (I, J) NIR activity of *V. nakazawae* (I) and *S. anginosus* (J) in media containing different concentrations of nitrate. (K, L) Growth curves of *V. nakazawae* (K) and *S. anginosus* (L) in media with and without KNO_3 . BHI, brain heart infusion; CPH, cirrhotic portal hypertension; DCA, deoxycholic acid; GABA, γ -aminobutyric acid; iNOS, inducible nitric oxide synthases; LCA, lithocholic acid; NC, non-cirrhotic portal hypertension; NIR, nitrate reductase; NO, nitric oxide; *S. anginosus*, *Streptococcus anginosus*; *V. nakazawae*, *Veillonella nakazawae*; VMB, Veillonella Medium Base.

We further examined the effects of *V. nakazawae* inoculation on the liver and colon. Mice in the PBS group exhibited normal liver structure without deterioration or inflammatory cell infiltration. Conversely, evidence of inflammatory cell infiltration and swollen hepatocytes were detected in the livers of *V. nakazawae*-infected mice (Figure 5I). Increased paracellular permeability and ultrastructural abnormalities of adherens junctions were observed in mice treated with *V. nakazawae* (Figure 5J). In addition, we found that serum inflammatory cytokines, such as IL-1 β , IL-6 and TNF- α were significantly higher in *V. nakazawae* group than those in the PBS group ($p < 0.05$; Figure 5K–M). Serum endotoxin, a LPS, was also significantly elevated in the *V. nakazawae* group ($p = 0.007$; Figure 5N) and was positively correlated with the levels of serum inflammatory cytokines, such as IL-1 β , IL-6 and TNF- α (Figure S12A–C). The NO levels and iNOS activity were also significantly elevated after *V. nakazawae* inoculation ($p < 0.05$; Figure 5O,P). These results showed that the overabundance of *V. nakazawae* may enhance the liver inflammatory responses and cause vasoactive substance release.

3.6 | Antibiotic Intervention Alleviates Portal Hypertension Progression in Cirrhotic Mice

To assess if suppression of CPH-associated bacteria could impact the progression of CPH, we designed a set of antibiotic intervention experiments using a CPH mouse model (Figure 6A). First, we analysed the differences in gut microbiota composition between normal mice and CPH model mice. The PCoA analysis showed significant differences in the gut microflora structure between the normal and model groups (Adonis test: $p = 0.002$; Figure 6B). At the phylum level, the Bacteroidetes abundance was significantly lower, whereas the Firmicutes/Bacteroidetes ratio was significantly higher in the model versus the normal group ($p < 0.05$; Figure 6C,D). This finding was consistent with the results of our population-based cohort study. At the genus level, the bacterial genera expressed differentially between the CPH model and normal mice, although different from the study cohort results (Figure S13), which also reflected changes in the gut flora structure caused by CPH induction.

In the antimicrobial mouse group (a 2-week antibiotic intervention prior to inducing CPH), the number of bacteria on Columbia blood agar plates after antibiotic treatment was significantly reduced compared with that before antibiotic treatment at the same multiple dilutions ($7.5 \pm 0.6 \times 10^5$ vs. $8.2 \pm 0.3 \times 10^8$ CFU/g; Figure 6E), indicating the success of antibiotics in inhibiting gut bacteria. Furthermore, alpha diversity analysis showed a significant decrease in microbial richness, with the Shannon index significantly decreased after antibiotic treatment compared to pre-treatment levels ($p < 0.001$; Figure 6F). PCoA based on Bray-Curtis distance revealed a significant difference in community structure before and after antibiotic treatment ($p = 0.003$; Figure 6G). The relative abundances of dominant phyla Bacteroidetes and Firmicutes showed significant decreases after antibiotic treatment compared to pre-treatment levels (Figure 6H). Together, these results validate the effectiveness of our antibiotic treatment protocol in eliminating gut microbiota.

Following antibiotic treatment, the mice were treated by intraperitoneal injection of NDMN to induce CPH. At week 4, PVD

and AD were significantly reduced in the antibacterial group compared to the CPH model group, whereas PVV was significantly increased ($p < 0.05$; Figure 6I). At the same time, compared to the CPH model group, the antibiotic-treated group showed a significant decrease in spleen weight and a significant increase in platelet counts ($p < 0.05$; Figure 6J,K). There was no significant difference in hemodynamic changes between the antimicrobial and the normal groups ($p > 0.05$; Figure 6I–K). Thus, the elimination of gut bacteria delayed the progression of CPH. In addition, the antibiotic intervention reduced LPS ($p = 0.002$; Figure 6L) and the levels of cytokines, including IL-1 β , IL-6 and TNF- α ($p < 0.05$; Figure 6M–O). The iNOS and NO levels were also significantly reduced after antibiotic treatment ($p < 0.05$; Figure 6P,Q). Thus, antibiotic treatment can significantly reduce the inflammatory response and the release of NO by eliminating intestinal bacteria.

4 | Discussion

Our present findings suggest that the gut microbiota of patients with CPH is dysbiotic. Altered gut microbiota play an important role in the progression of CPH, affecting host arginine metabolism to promote arginine biosynthesis, whereas the pro-inflammatory effects of certain gut bacterial species may induce iNOS expression, which collectively acts on the arginine biosynthesis pathway to promote NO synthesis, facilitating the progression of CPH.

To our knowledge, this is the first study to directly characterise the gut microbiota and metabolic profiles of patients with CPH. We observed lower faecal microbial diversity in patients with CPH than in those without CPH. Decreased microbial diversity is often associated with chronic illnesses and is thought to be a primary type of intestinal dysbiosis [41, 42]. Under circumstances of decreased microbial diversity, we observed increased microbial interactions, which may be due to multiple effects, such as: (1) the absence of key microbial communities leads to the reorganisation of competition/cooperation relationships among the remaining microbial communities, (2) low abundance microbial communities maintain community stability through metabolic interactions, (3) environmental perturbations, such as host inflammation, drive microbes to form highly connected networks to resist external pressures. Together, these processes contribute to the observed paradox of reduced taxonomic diversity coupled with heightened network complexity in the dysbiotic state. This pattern aligns with the ‘high fragility-high connectivity’ paradigm [43, 44]: when an ecosystem (here referring to microbial communities) is in a critical state, its internal interaction network temporarily increases complexity, but this structure is more susceptible to disruption and collapse. The coexistence of low diversity but high connectivity may represent a biomarker for microbial community instability in CPH, offering novel insights into disease-associated dysbiosis.

In addition, the expansion of Proteobacteria and increased Firmicutes/Bacteroidetes ratio are common markers of gut dysbiosis or unstable microbiota structure [45, 46]. Proteobacteria abundance and the Firmicutes/Bacteroidetes ratio were significantly increased in patients with CPH compared to NC, consistent with a dysbiotic gut microbiota state in CPH. Gut-derived

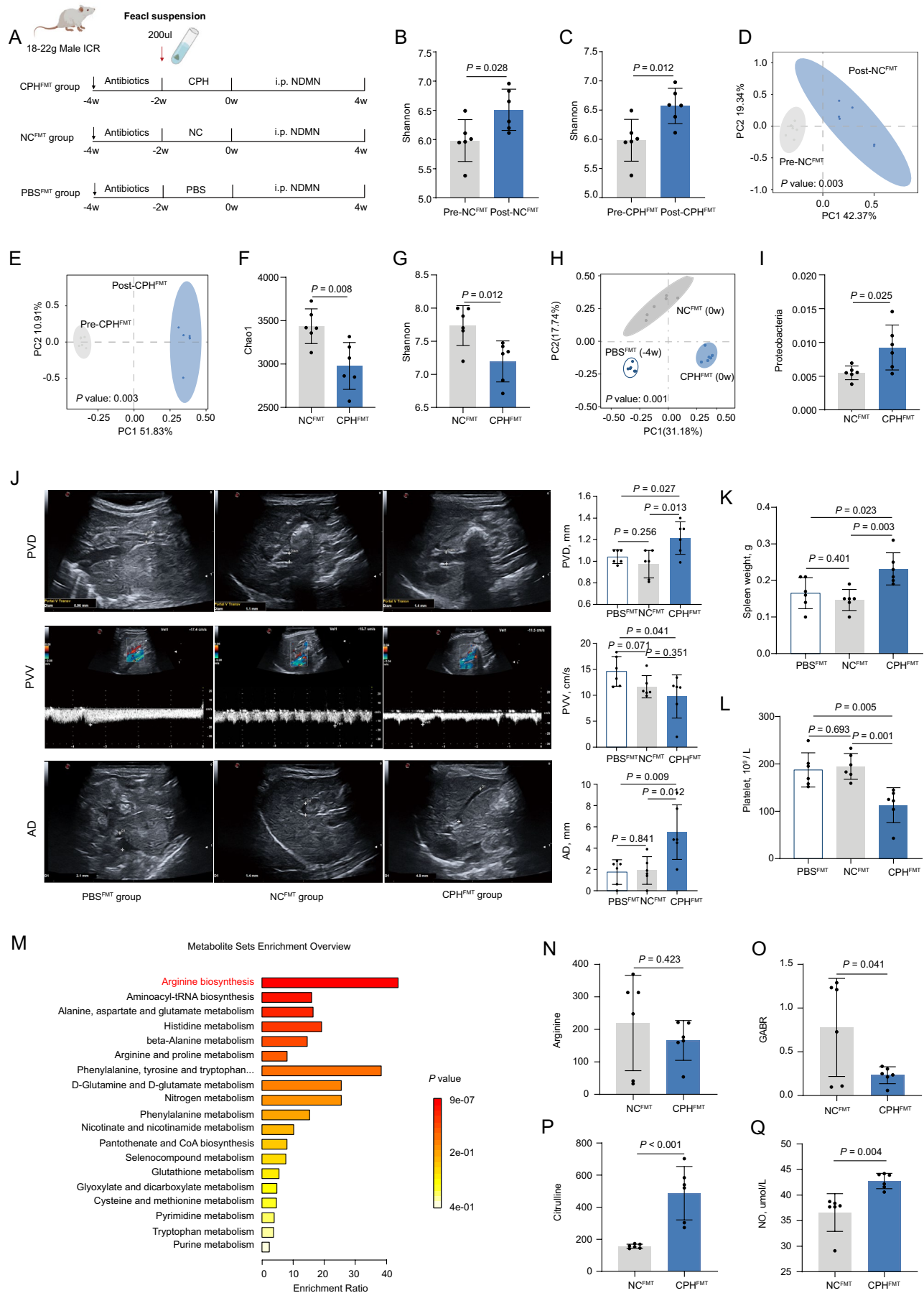


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FIGURE 4 | FMT experiment with CPH or NC faecal extracts. (A) Schematic of the FMT experiment conducted on mice using faecal extracts from patients with NC (NC^{FMT}) or CPH (CPH^{FMT}). Differences in microbial alpha diversity before and after transplantation were observed in recipient mice transplanted with NC flora (B) and CPH flora (C). PCoA analysis revealed significant differences in community structure before and after FMT in recipient mice transplanted with NC flora (D) and CPH flora (E). Differences in microbial diversity between mice in CPH^{FMT} and NC^{FMT} groups, estimated using the Chao1 (F) and Shannon (G) indices. (H) PCoA analysis of the microbiota depicting significant changes in intestinal flora composition before (−4w) and after (0w) FMT, as well as between the NC^{FMT} and CPH^{FMT} groups. (I) Relative abundance of Proteobacteria in the faecal samples of NC^{FMT} and CPH^{FMT} groups. (J) Ultrasound imaging and comparative analysis of PVD, PVV and AD between PBS^{FMT}, NC^{FMT} and CPH^{FMT} groups (image resolution: 860 × 808 pixels). The comparison of spleen weight (K) and platelet count (L) was performed among the three groups of mice. (M) Pathway enrichment and statistical significance of differentially expressed metabolites. The concentrations of arginine (N), GABR [defined as arginine/(ornithine + citrulline)] (O), citrulline (P) and NO (Q). AD, ascites depth; CPH, cirrhotic portal hypertension; CPH^{FMT}, CPH-associated recipient mice; FMT, faecal microbiota transplantation; GABR, Global arginine bioavailability ratio; NC, non-cirrhotic portal hypertension; NC^{FMT}, NC-associated recipient mice; NDMN, N-dimethylnitrosamine; NO, nitric oxide; PBS, phosphate-buffered saline; PBS^{FMT}, PBS-associated recipient mice; PCoA, Principal coordinate analysis; PVD, portal vein diameter; PVV, portal vein velocity.

endotoxins are involved in the progression of several liver diseases [47–50]. Herein, we detected an overabundance of gut bacterial genera involved in LPS biosynthesis, such as *Veillonella*, *Klebsiella* and *Streptococcus* in patients with CPH. LPS plays a key role in the initiation of various pathophysiological cascades [51]. Elevated LPS levels activate the nuclear translocation of nuclear factor- κ B, the release of proinflammatory cytokines (IL-1 β , IL-6 and TNF- α) and cause hepatic inflammatory and oxidative damage [52]. These might also be the main reason for increases in the three genera that correlated with more serious clinical indices. We also found that the decreased relative abundance of *Adlercreutzia* and *Coprococcus* was an additional essential characteristic of the intestinal microbiota in patients with CPH. *Coprococcus* is a major bacterial genus that produces butyrate and is an important anti-inflammatory commensal bacterium [53]. Butyrate, as the primary energy source of the intestinal mucosa, is critical for preventing inflammation and preserving intestinal barrier integrity [54]. Butyrate can modulate the function of intestinal macrophages and downregulate LPS-induced proinflammatory mediators, such as IL-12, IL-6 and NO [55]. *Adlercreutzia* was also confirmed to be an anti-inflammatory commensal bacterium and had decreased abundance in gut microbiota of patients with metabolic liver disease [56]. In short, the increase in bacteria that produce LPS and the decrease in anti-inflammatory bacteria in patients with CPH jointly contributed to exacerbate chronic inflammation in CPH. In addition, inflammatory cytokines and LPS upregulate iNOS, which augments the release of NO, a powerful vasodilator, thereby promoting the development of CPH [52]. Colonisation experiments with *V. nakazawae* further confirmed the contribution of intestinal bacteria in the pro-inflammatory response and the release of vasoactive substances. Therefore, the gut microbiome is a potential target for CPH prevention.

In the population cohort, our metabolomic analysis uncovered 15 metabolic pathways changed in the gut of CPH patients, especially amino acid metabolism. Amino acid homeostasis is essential to human health and diseases [57–59]. Previous studies have demonstrated that alterations in the gut microbiota could influence intestinal amino acid profiles through several mechanisms [60, 61]. For instance, colonisation of the gut microbiota can alter intestinal permeability, thereby affecting the transport and absorption of free amino acids in the gastrointestinal tract [62, 63]. Colonisation of intestinal bacteria could affect protein digestion enzymes like trypsin in the intestine [64]. Additionally,

gut microbes may directly utilise or metabolise amino acids within the gut or synthesise and supply amino acids to the host [65, 66]. Furthermore, these altered amino acid metabolic pathways interact with each other and eventually influence arginine biosynthesis pathways. The FMT experiment further confirmed the effect of CPH-associated intestinal flora on the arginine metabolism of the host. Arginine metabolism is the primary biochemical pathway for NO production [67, 68]. Although arginine was not significantly elevated in the CPH^{FMT} group compared to the NC^{FMT} group, the levels of its catabolite citrulline were significantly increased in the CPH^{FMT} group. The GABR, which reflects the steady-state arginine [40], was also decreased significantly in the CPH^{FMT} group, suggesting that arginine was involved in downstream catabolism. Consistent with this observation, the levels of NO were significantly increased in the CPH^{FMT} group following FMT.

In recent years, gut microbiota-targeted therapeutic strategies have been increasingly used in clinical practice, including intestinal flora transplantation, probiotic intervention and targeted drugs for specific intestinal flora [69–73]. A previous study showed that the transplantation of a healthy microbiota into metabolic steatohepatitis-afflicted rats yielded substantial reductions in portal pressure [10]. Consistent with this, we found that CPH mice transplanted with normal intestinal microbiota had significantly decreased portal pressure. We further showed that antibiotic treatment could suppress potential pathogenic bacterial species and alleviate the CPH phenotype in the CPH mouse model. These results provide new ideas for the clinical treatment of CPH.

The present study identified changes in the intestinal microbiota of patients with CPH and revealed key metabolic pathways that might promote CPH development, whereas also characterising specific microbiota markers. However, our work has several limitations. First, this was a single center study with limited sample sizes, and future multicenter studies with larger cohort sizes are required to corroborate our findings. In addition, while our control group included patients with active hepatitis B, subgroup analysis confirmed that the key microbial and metabolic differences still persist when comparing the CPH group with the virologically suppressed NC subgroup, strengthening their association with portal hypertension. Nevertheless, future studies employing entirely healthy cohorts could further validate these findings. Second, since liver biopsy and hepatic venous pressure gradient (HVPG)

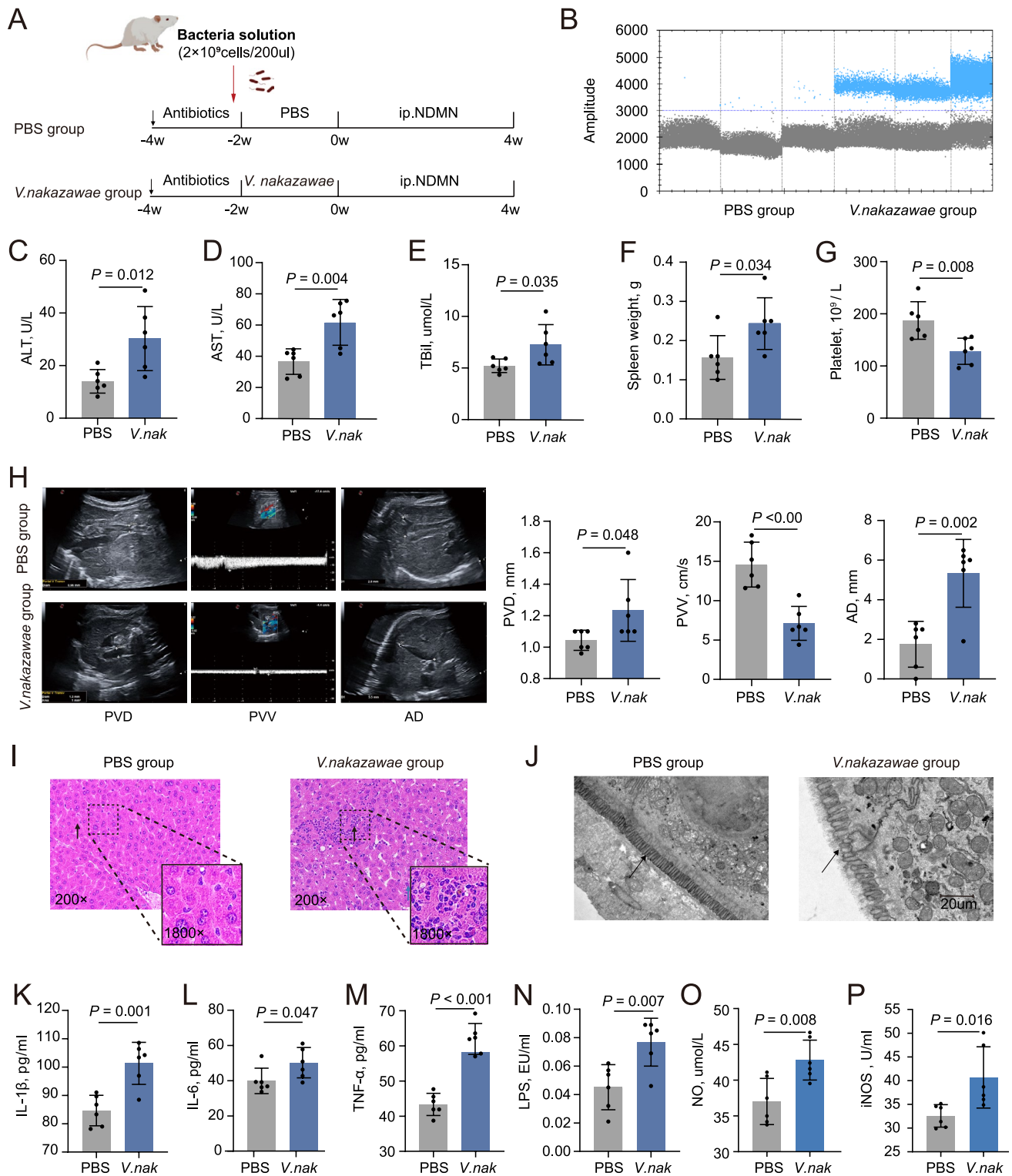


FIGURE 5 | Characteristic bacteria transplantation experiment using *Veillonella nakazawae*. (A) Schematic of the characteristic bacterial transplantation experiment. The groups used were PBS, *V. nakazawae*-infected mice. (B) Colonisation test of *V. nakazawae* in the faecal samples of mice administered intragastrically with PBS or *V. nakazawae* using ddPCR. Three mice were used for each group. Positive amplifications were detected in the *V. nakazawae* group. (C–E) Concentration of ALT, AST and TBil. The comparison of spleen weight (F) and platelet count (G) was performed between the PBS and *V. nakazawae* groups. (H) Ultrasound imaging and comparative analysis of PVD, PVV and AD (image resolution: 860 × 808 pixels). (I) Liver HE staining. (J) Electron micrographs of the colon from all mice groups. Concentrations of the serum pro-inflammatory cytokines IL-1β (K), IL-6 (L), TNF-α (M), LPS (N), NO (O) and iNOS (P). AD, ascites depth; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ddPCR, droplet digital PCR; HE, haematoxylin eosin; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NDMN, N-dimethylnitrosamine; NO, nitric oxide; PBS, phosphate-buffered saline; PVD, portal vein diameter; PVV, portal vein velocity; TBil, total bilirubin; TNF-α, tumour necrosis factor alpha; *V. nakazawae*, *Veillonella nakazawae*.

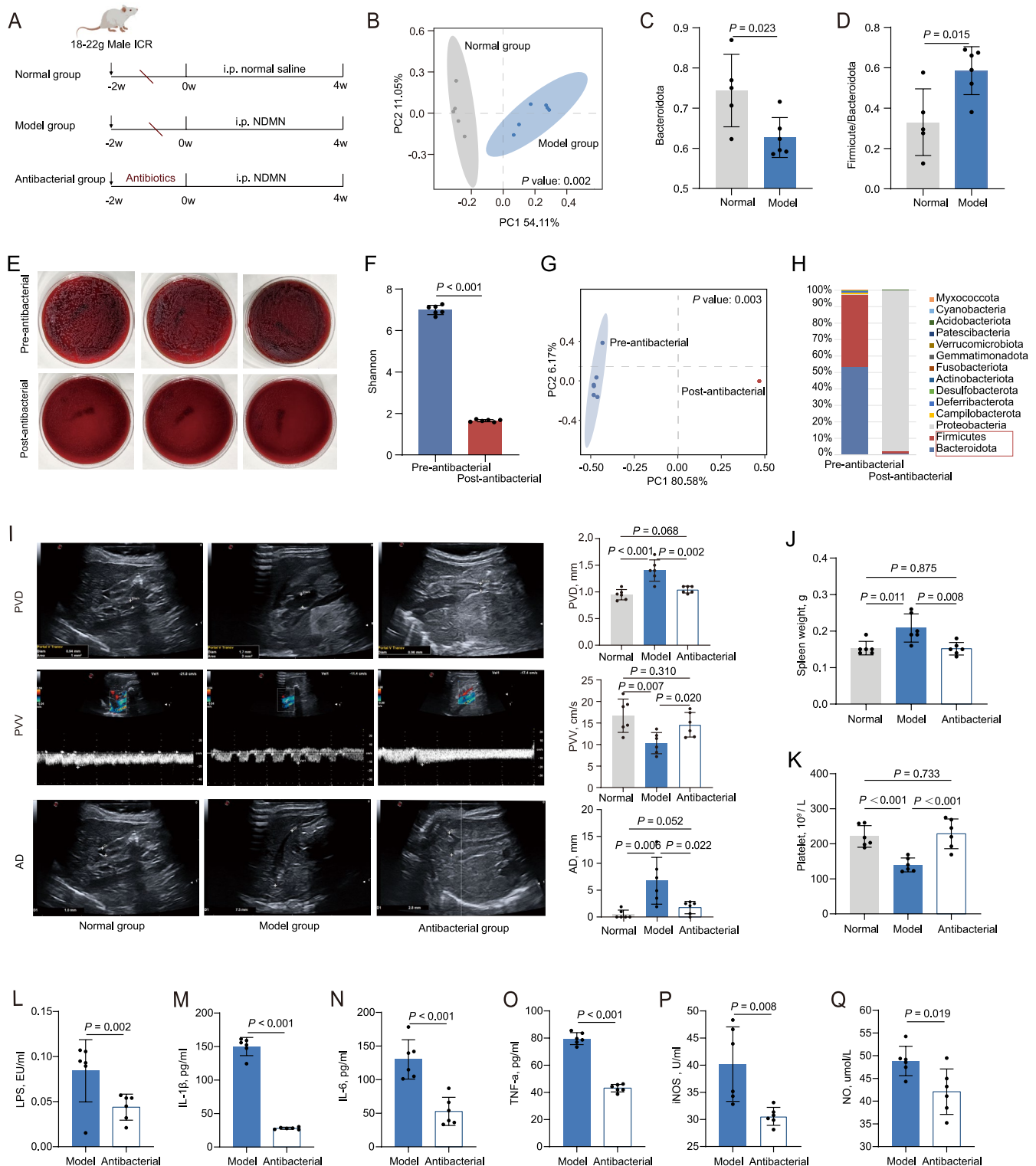


FIGURE 6 | Antibiotic intervention experiment in murine models. (A) Schematic of the antibiotic intervention experiment. (B) PCoA analysis of the microbiota between the normal and model mice groups. Bacteroidetes (C) and Firmicutes/Bacteroidetes ratio (D) in the faecal samples of the normal and model mice groups. (E) Comparison, at the same dilution, of bacteria on Columbia blood agar plates before and after antibiotic treatment. Analysis of alpha diversity (F), beta diversity (G) and microbial community structure at phylum level (H) was performed before and after antibiotic treatment. (I) Ultrasound imaging and comparative analysis of PVD, PVV and AD in normal, model and antibacterial groups (image resolution: 860×808 pixels). The comparisons of spleen weight (J) and platelet count (K) were performed among the normal, model and antibiotic-treated groups. (L) The serum LPS level in mice. The concentrations of serum pro-inflammatory cytokine IL-1 β (M), IL-6 (N) and TNF- α (O) in model and antibacterial mice. The concentrations of iNOS (P) and NO (Q) in model and antibacterial mice. AD, ascites depth; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NDMN, N-dimethylnitrosamine; NO, nitric oxide; PCoA, Principal coordinate analysis; PVD, portal vein diameter; PVV, portal vein velocity; TNF- α , tumour necrosis factor alpha.

measurement were not performed, there may be some subclinical or early-stage cirrhosis as well as early portal hypertension in our control group. Nevertheless, our approach maximises diagnostic accuracy while minimising invasiveness. Accordingly, future studies could incorporate liver biopsy and HVPG measurements to further validate these findings. Third, we sequenced 16S rRNA amplicons rather than using sequence-based metagenomics, which limited data interpretation at the species level and subsequent functional analyses. Although we isolated *V. nakazawae* for downstream analysis, other species might have been underrepresented due to cultivation biases. Consequently, further exploration of other *Veillonella* species would be valuable, and we will employ targeted metagenomics in future work to comprehensively evaluate their roles. Lastly, potential confounders, such as environmental factors, dietary intake and medications were not considered in our analysis due to the limited sample size.

In summary, we uncovered direct evidence that gut microbiome dysbiosis played a significant role in CPH pathogenesis. We characterised CPH altered metabolic pathways, particularly arginine biosynthesis, which likely played mediating roles in regulating inflammatory responses and blood pressure. Our study provided novel insights into the pathogenesis of CPH and potentially opens new avenues for developing novel microbiome-based therapeutic strategies for CPH.

Author Contributions

Qun Zhang: data curation, formal analysis, visualization, writing – original draft, methodology, investigation, supervision, writing – review and editing, software, validation, resources. **Jinghua Cui:** formal analysis, visualization, writing – original draft, methodology, supervision, writing – review and editing, software, validation. **Yixin Hou:** data curation, formal analysis, writing – original draft, methodology, investigation, writing – review and editing, software, validation. **Lining Guo:** supervision, writing – review and editing. **Hai Li:** supervision, writing – review and editing. **Guiqin Zhou:** data curation, resources. **Xiaojing Wang:** data curation, resources. **Bingbing Zhu:** data curation, validation. **Ke Shi:** data curation, validation, investigation. **Yi Zhang:** data curation, validation, investigation. **Yufei Bi:** data curation, validation. **Yanqiu Li:** data curation, validation. **Lina Sun:** data curation. **Ying Feng:** funding acquisition, conceptualization, methodology, supervision, project administration. **Jing Yuan:** conceptualization, methodology, supervision, project administration. **Xianbo Wang:** conceptualization, methodology, project administration, supervision, funding acquisition.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The 16S sequencing data of this study has been deposited in <https://ngdc.cncb.ac.cn>, under the accession number HRA002597. The metabolomics data is available in the MetaboLights database (www.ebi.ac.uk/metabolights/MTBLS10727).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** apt70392-sup-0001-supinfo.doc. **Figure S1:** The study design and flow diagram for this population-based cohort. CPH, cirrhotic portal hypertension; GC-TOF/MS, gas chromatography-time-of-flight mass spectrometry; NC, non-cirrhotic portal hypertension. **Figure S2:** Firmicutes/Bacteroidetes ratio in the faecal samples of patients with NC or CPH. CPH, cirrhotic portal hypertension; NC, non-cirrhotic portal hypertension. **Figure S3:** ROC curve and their corresponding AUC based on the cross-validation of the RF model employing differentially expressed genera. AUC, area under the curve; RF, random forest; ROC, receiver operating characteristic. **Figure S4:** Half violin plot of relative abundance of *Coprococcus_2* (log10-transformed) in the faecal samples of patients with NC or CPH. CPH, cirrhotic portal hypertension; NC, non-cirrhotic portal hypertension. **Figure S5:** Boxplots for the abundance of the 40 differentially expressed metabolites in samples from patients with CPH or NC. CPH, cirrhotic portal hypertension; DCA, deoxycholic acid; GABA, γ -aminobutyric acid; LCA, lithocholic acid; NC, non-cirrhotic portal hypertension. **Figure S6:** Half-violin plots of the log 10-transformed relative abundance of *Veillonella*, *Streptococcus*, *Klebsiella*, *Adlercreutzia* and *Coprococcus_2* in the CPH group versus the VSS. CPH, cirrhotic portal hypertension; VSS, virologically suppressed subgroup. **Figure S7:** Pathway enrichment analysis of differential metabolites between the CPH group and the virologically suppressed subgroup. CPH, cirrhotic portal hypertension. **Figure S8:** Spearman correlation analysis between serum ALT levels and (A) the top 30 differentially expressed microbial genera and (B) the 40 differential metabolites. ALT, alanine aminotransferase; DCA, deoxycholic acid; GABA, γ -aminobutyric acid; LCA, lithocholic acid. **Figure S9:** Average relative abundance of the top 10 differentially expressed bacterial genera in CPH^{FMT} and NC^{FMT} groups. Abscissa, mean relative abundance of specific genera. Grey and blue bars indicate mice in the NC^{FMT} and CPH^{FMT} group respectively; bars represent individual bacterial genera. Abbreviations: CPH, cirrhotic portal hypertension; CPH^{FMT}, CPH-associated recipient mice; FMT, faecal microbiota transplantation; NC, non-cirrhotic portal hypertension; NC^{FMT}, NC-associated recipient mice. **Figure S10:** 3D PCoA plots in faecal samples of NC^{FMT} and CPH^{FMT} groups. CPH, cirrhotic portal hypertension; CPH^{FMT}, CPH-associated recipient mice; FMT, faecal microbiota transplantation; NC, non-cirrhotic portal hypertension; NC^{FMT}, NC-associated recipient mice. **Figure S11:** Heatmap of differentially expressed metabolites in the NC^{FMT} and CPH^{FMT} mice groups. CPH, cirrhotic portal hypertension; CPH^{FMT}, CPH-associated recipient mice; FMT, faecal microbiota transplantation; NC, non-cirrhotic portal hypertension; NC^{FMT}, NC-associated recipient mice. **Figure S12:** Spearman's correlation analysis between LPS and inflammatory cytokines. Spearman's correlation analysis between LPS and IL-1 β (A), IL-6 (B) and TNF- α (C). IL, interleukin; LPS, lipopolysaccharide; TNF- α , tumour necrosis factor alpha. **Figure S13:** Heatmap of differentially expressed bacterial genera between normal and model groups. **Table S1:** List of 121 different genera determined by the Wilcoxon rank-sum test between patients with CPH and NC. **Table S2:** Random forest analysis ranking the 121 different genera according to importance. **Table S3:** Spearman correlation analysis of 13 clinical characteristics and 10 bacterial genera. **Table S4:** *p* value, basic information and FC of differentially expressed metabolites. **Table S5:** VIP and correlation coefficient (Corr. Coeffs) data of metabolites. **Table S6:** Statistics of 40 differentially expressed metabolites between patients with CPH and NC. **Table S7:** Pathway enrichment and

statistical significance of differentially expressed metabolites enriched in patients with CPH. **Table S8:** Differential microbial genera: CPH versus virologically suppressed NC subgroup. **Table S9:** Differential metabolites: CPH versus virologically suppressed NC subgroup (univariate analysis). **Table S10:** Differential metabolites: CPH versus virologically suppressed NC subgroup (multivariate analysis). **Table S11:** Pathway enrichment analysis from differential metabolites between CPH and virologically suppressed NC subgroup. **Table S12:** Spearman correlation of serum ALT with top 30 differential genera and 40 differential metabolites. **Table S13:** Spearman's correlation *p* values between 40 distinctly expressed metabolites and 10 differentially expressed bacterial genera. **Table S14:** Pathway enrichment and statistical significance of the differential metabolites between CPH^{FMT} and NC^{FMT} groups.