MANIPULATION OF GUT MICROBIOTA AS A KEY TARGET TO INTERVENE ON THE ONSET AND PROGRESSION OF DIGESTIVE SYSTEM DISEASES

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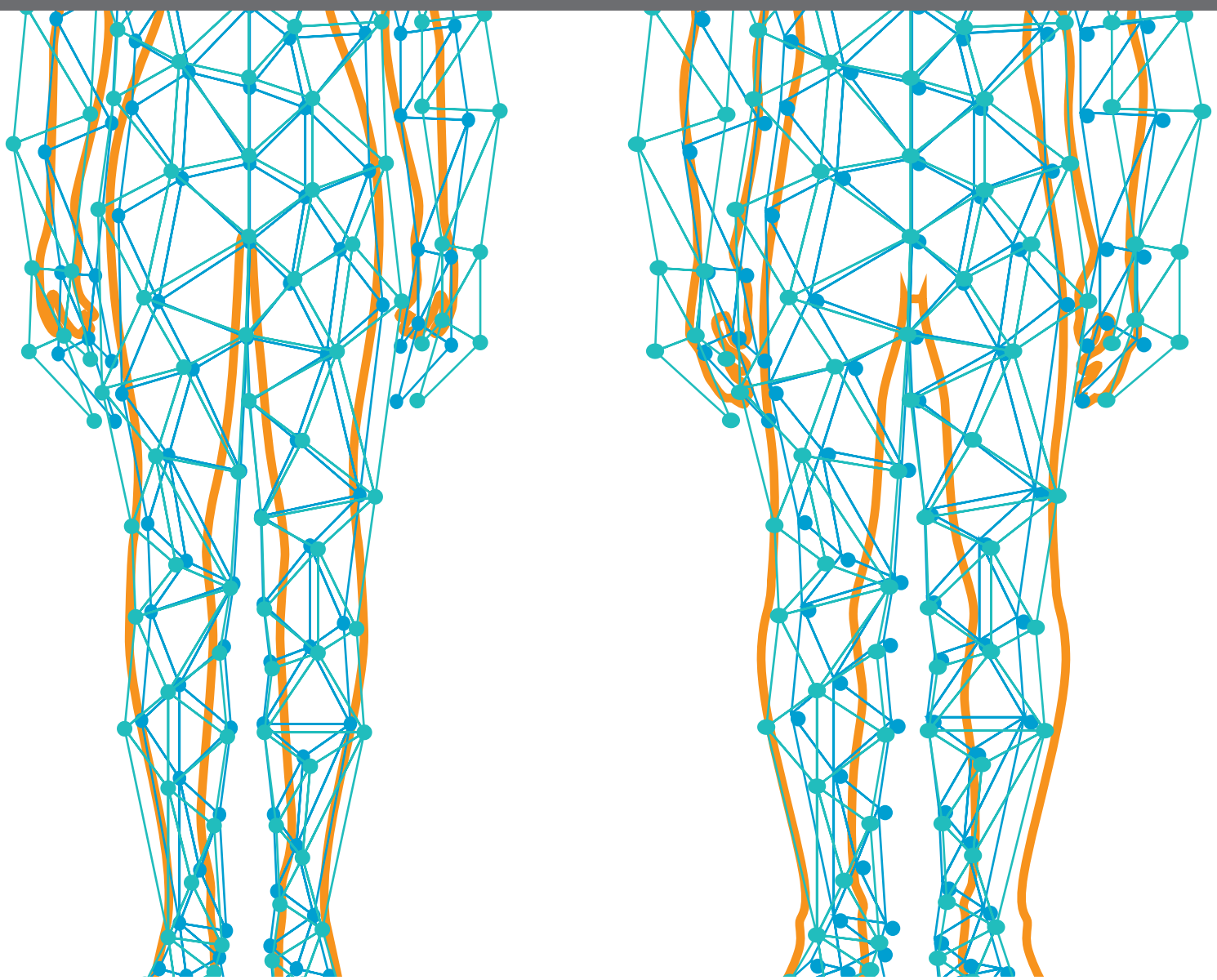

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MANIPULATION OF GUT MICROBIOTA AS A KEY TARGET TO INTERVENE ON THE ONSET AND PROGRESSION OF DIGESTIVE SYSTEM DISEASES

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Editorial: Manipulation of gut microbiota as a key target to intervene on the onset and progression of digestive system diseases

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Editorial on the Research Topic
Manipulation of gut microbiota as a key target to intervene on the onset and progression of digestive system diseases

It has been well acknowledged that the gut microbiota (GM), the collection mainly of bacteria that symbiotically inhabit the gut, acts as an "invisible organ" exerting profound impacts on the physiology and pathology of the gastrointestinal tract and beyond (1). In the specific context of intestinal and liver diseases, both endogenous and exogenous influences could disrupt the intestinal barrier, increasing intestinal permeability and contributing to the establishment and perpetuation of GM dysbiosis (2). Such a disturbance can result in the translocation of bacteria, bacterial-derived components (lipopolysaccharides, peptidoglycan, DNA, etc.), and bacterial metabolites from the "leaky gut" to the systemic circulation, with mechanistic implications in a variety of extra-intestinal diseases (2, 3). Therefore, researchers have proposed that gut microbes could be used as biomarkers for diagnosing diseases and predicting prognosis (4, 5). Besides, an increasing number of studies are exploring the therapeutic potential of GM manipulation. Current methods for regulating gut microecology include dietary intervention, probiotics, prebiotics, synbiotics, antibiotics, fecal microbiota transplantation (FMT), postbiotics and even genetically engineered bacteria, although for most of them there is no evidence of long-term efficacy and safety based on clinical trials. So far, dietary intervention has been successfully tested in metabolic-associated liver disorders, such as non-alcoholic fatty liver disease (NAFLD) (6). On the other

hand, probiotics and prebiotics are widely used as intestinal microecological regulators in clinical practice. Probiotics are effective in the treatment of ulcerative colitis (UC), *Clostridioides difficile* infection (CDI), NAFLD, cirrhosis, and its complication, hepatic encephalopathy (HE) (7–11). Regarding the clinical treatment of the latter, the non-absorbable antibiotic rifaximin has also been widely used due to its benefits in effectively preventing the recurrence of dominant HE (12, 13). In addition, rifaximin has been proven to improve liver enzymes and endotoxemia in NAFLD and non-alcoholic steatosis hepatitis (NASH) patients (14, 15). FMT as arguably the most direct means of manipulating GM has been reported as meaningful intervention in animal experiments and some clinical applications, mainly CDI, but also UC, irritable bowel syndrome (IBS), chronic hepatitis B, alcoholic liver disease (ALD), NAFLD, liver cirrhosis, and HE (16–19). However, large randomized controlled trials are still needed to validate its implication in terms of long-term efficacy and safety. Another emerging method that precisely targets GM is bacteria that are genetically engineered to deliver bioactive molecules or express certain functionalities. Based on their activity, they may improve bacterial colonization, immune regulation, toxin metabolism, and anti-pathogen colonization by targeting relevant genes (18).

In this scenario, this special issue appeared highly topical and attracted great attention. In particular, our Research Topic aimed to discuss whether and how modulating GM by different methods could become an integral part of the prevention and treatment of digestive system diseases. In this special collection, we published original research, providing novel insights into the possible causal relationships between GM and digestive system diseases, as well as reviews specifically focused on certain disorders, namely Crohn's disease (CD) and alcohol-associated liver disease.

As anticipated above, GM has a profound and sometimes crucial influence on the health of the gut and the entire human body. With reference to intestinal diseases, Rashed et al. reviewed GM dysbiosis in CD, and enumerated the different approaches available to modulate GM as a whole (meaning bacteria, archaea, fungi, and viruses), including antibiotics, probiotics, prebiotics, synbiotics, personalized diets, and FMT. In another review, Watanabe and Kamada focused on the pathophysiological mechanisms of CD with intestinal fibrosis, which is a critical determinant of prognosis. They summarized current knowledge on the link between intestinal fibrosis in CD and GM, and discussed some GM-dependent animal models that demonstrated causality. As for colorectal cancer (CRC), previous studies have shown differences in GM communities throughout the various stages of the disease; however, the drivers of GM imbalances and how they contribute to the onset of CRC remain to be determined. In this context, Shaw et al. found that the composition of the mucosa-associated microbiota is influenced by body mass index (BMI) status in CRC patients. In particular, having a BMI >25 kg/m^2 was associated with an overabundance of *Prevotella* and Fusobacteria, as well as an increase in trans-phylum relationships (i.e., co-occurrences and co-exclusions). Approaches to manipulate GM towards a healthier (eubiotic) profile can therefore hold great promise in treating digestive system diseases. Among them, FMT from healthy donors is one of the most effective strategies for replacing the GM of patients with various diseases. In this regard, Amorim et al. refined the gut decontamination protocols prior to FMT engraftment. Their results showed that 7 days of broad-spectrum antibiotic treatment followed by 3 weekly doses of FMT provide a simple, reliable, and cost-effective methodology for FMT in animal research.

Due to the close anatomical and functional interaction of the gut-liver axis, it is not surprising that GM also plays a crucial role in the onset and progression of liver disease. Interestingly, GM alterations may already be present at the early stage of liver damage. In particular, Sheng et al. found that GM structure and function in males with hyperuricemia and elevated levels of liver enzymes were significantly different from those in healthy individuals. Such alterations included the enrichment of pathways involved in the production of 5-aminoimidazole ribonucleotide, aromatic amino acids, and chorismate, and the depletion of pathways involved in the synthesis of beneficial metabolites, i.e., short-chain fatty acids, as well as producing taxa (e.g., *Roseburia*, *Ruminococcus* and *Butyricimonas* species). Changes in GM have also been observed in patients with alcohol-induced liver dysfunction. Jiao et al. identified an underrepresentation of *Faecalibacterium prausnitzii* and *Roseburia hominis*, as well as four metabolic pathways (associated with galacturonate and glucuronate catabolism, β-D-glucuronide degradation, D-galacturonate degradation, and mixed acid fermentation) in the drinking case group compared to the non-drinking case group. The changed bacterial species have been proven to be anti-inflammatory and protect liver function. Administering probiotics and/or changing dietary patterns to reverse GM dysbiosis may therefore improve liver function of alcohol drinkers. Recently, advances have also been made in elucidating the interaction between GM and alcohol-associated liver disease (ALD). Chen et al. reviewed the mechanisms by which gut bacteria and fungi contribute to the onset and development of ALD and proposed some effective treatments to restore GM, including probiotics, FMT, and specific bacteriophages.

Liver cirrhosis is the end-stage of many chronic liver diseases. GM dysbiosis is likely to occur gradually during its development but again, evidence of GM involvement and the usefulness of its manipulation is still scant. In this context, Triantos et al. discussed the risks of variceal bleeding in cirrhotic patients and found that anti-endotoxin antibody and TGF-β levels were significant predictors. Moreover, fatty acid-binding protein 2 (FABP2), a marker of enterocyte damage, was associated with 6-week mortality, suggesting that impaired intestinal barrier (potentially affected by GM) and subsequent

microbial translocation may be crucial for the prognosis of liver cirrhosis patients. Therefore, GM-modulating interventions may be promising strategies for preventing/treating liver diseases. Lu et al. combined GM profiling through shotgun metagenomics with targeted metabolomics to uncover the role of lactitol supplementation in regulating GM dysbiosis and metabolic dysregulation in cirrhotic patients. The relative abundance of some health-promoting lactic acid bacteria (i.e., lactobacilli and bifidobacteria) increased, while the proportions of the pathogen *Klebsiella pneumoniae* and associated antibiotic-resistant genes/virulence factors decreased after lactitol intervention. Finally, hepatocellular carcinoma (HCC) typically develops as a result of persistent and long-term chronic liver injures followed by progression to severe fibrosis and cirrhosis. The presence of microvascular invasion (MVI) is a critical indicator of long-term survival and tumor recurrence. Zhang et al. observed significant differentiation in GM composition and structure between the HCC-MVI group and HCC patients without vascular invasion. Based on key GM signatures, a non-invasive HCC-MVI microbial prediction model was constructed using 20 bacterial families with an area under the curve (AUC) value of 94.81%.

In conclusion, the modulation of unbalanced GMs as an adjunct therapy for digestive system diseases is attracting increasing attention. Diet, probiotics, prebiotics, synbiotics, antibiotics, FMT, postbiotics and genetically engineered bacteria have been shown as potential treatment strategies for digestive and liver diseases. However, these treatments are still controversial in terms of efficacy, safety, and mechanisms of action, and must necessarily be confirmed in clinical trials with a large sample size. Nevertheless, it is still an interesting, challenging, and promising research field.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Lactitol Supplementation Modulates Intestinal Microbiome in Liver Cirrhotic Patients

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Background: Cirrhosis is a common chronic liver disease characterized by irreversible diffuse liver damage. Intestinal microbiome dysbiosis and metabolite dysfunction contribute to the development of cirrhosis. Lactitol (4-β-D-galactopyranosyl-D-glucitol) was previously reported to promote the growth of intestinal *Bifidobacteria*. However, the effect of lactitol on the intestinal microbiome and fecal short-chain fatty acids (SCFAs) and bile acids (BAs) and the interactions among these factors in cirrhotic patients pre- and post-lactitol treatment remain poorly understood.

Methods: Here, using shotgun metagenomics and targeted metabolomics methods.

Results: we found that health-promoting lactic acid bacteria, including *Bifidobacterium longum*, *B.pseudocatenulatum,* and *Lactobacillus salivarius,* were increased after lactitol intervention, and significant decrease of pathogen *Klebsiella pneumonia* and associated antibiotic resistant genes /virulence factors. Functionally, pathways including Pseudomonas aeruginosa biofilm formation, endotoxin biosynthesis, and horizontal transfer of pathogenic genes were decreased in cirrhotic patients after 4-week lactitol intervention compared with before treatment.

Conclusion: We identified lactitol-associated metagenomic changes, and provide insight into the understanding of the roles of lactitol in modulating gut microbiome in cirrhotic patients.

Keywords: cirrhosis, lactitol, microbiome, short-chain fatty acids, bile acids, shotgun metagenomics, targeted metabolomics

INTRODUCTION

Cirrhosis is a common chronic liver disease characterized by irreversible diffuse liver damage. It is an important risk factor for hepatocellular carcinoma and hepatic decompensation development. The early diagnosis and treatment of cirrhotic patients are essential to achieve the best outcomes. Antiviral drug treatments fail to effectively prevent the progression to liver cirrhosis or its associated complications, which suggests that hepatitis virus replication is not the only driving force of cirrhosis development in China (1, 2). The intestinal microbiota was demonstrated to be associated with the pathogenesis and progression of liver diseases, including alcoholic liver

disease (3), nonalcoholic fatty liver disease, non-alcoholic steatohepatitis (4), total parenteral nutrition/intestinal failure-related liver disease (5), and primary sclerosing cholangitis (PSC) (6). Moreover, there are direct interactions between metabolites produced by the gut microbiota and cirrhosis (7). In particular, the intestinal microbiota is a topic of high interest because an increasing number of researchers have focused their efforts on the potential roles of intestinal microbiota-targeted therapies in ameliorating the progression of liver disease (8, 9). Furthermore, it has been suggested that therapies targeting the intestinal microbiome (10) are pivotal for the treatment of liver fibrosis and cirrhosis.

Lactitol (4-beta-D-galactopyranosyl-D-glucitol), as a prebiotic, can be metabolized by intestinal bacteria to lactic acid and other natural acids. The efficacy of lactitol in the treatment of constipation and hepatic encephalopathy has been evaluated in various clinical trials (11–14). Lactitol supplementation can effectively increase the abundance of beneficial intestinal bacterial communities, such as *Bifidobacterium* and *Lactobacillus*. Moreover, lactitol decreased the levels of plasma endotoxin compared with other standard medical treatments, such as antibiotics (15). Although other alterations in the interactions between the microbiota and metabolites after the oral administration of lactitol have not been clearly and comprehensively elucidated, lactitol is widely used in the clinic treatment of portal-systemic encephalopathy to reduce liver injury through regulating the intestinal microbial community and decreasing gut-derived endotoxemia (12, 13). Therefore, it is important to understand the physiological mechanisms of the intestinal microbiome and metabolites associated with lactitol supplementation, and identify microbiome involved in benefiting patients and clinical outcomes.

In this study, we provided insight into benefits of lactitol to cirrhotic disease by profiling of intestinal microbiome and metabolites of cirrhotic patients before and after 4 weeks lactitol treatment. Compared with healthy individuals, we examined the correlation between the microbiome and metabolites and found shifts of the abundance of microbial antibiotic-resistant genes (ARGs), virulence factor genes (VFGs) in microbiome, and metabolites including short-chain fatty acids (SCFAs) and bile acids (BAs).

PATIENTS AND METHODS

Subjects and Study Design

Subjects with histologically confirmed liver cirrhosis and a body mass index (BMI) < 25.78 kg/m2 were classified as the lactitol group (LC). Clinical, metabolic biochemical, and epidemiological characteristics are described in **Supplementary Table 1**. This study was performed in accordance with the ethical guidelines of the 1975 Declaration of Helsinki for the participation of human subjects and approved by the Ethics Committee of the First Affiliated Hospital, School of Medicine, Zhejiang University (reference number: 2017HL668-1). Written informed consent and questionnaires were obtained from all study subjects at the time of enrolment. The inclusion and exclusion criteria were presented in **Supplementary File 1**. Finally, the study included 24 patients and 29 healthy controls (HCs). The details of these subjects are presented in **Table 1**. In the patient group, lactitol was administered orally at a dose of 5 g three times a day, and samples were collected after 4 weeks of treatment and subjected to intestinal microbiome metagenomics and metabolomics analyses.

Biological Specimen Collection and DNA Extraction

Well-formed stools were collected in sterile bags, refrigerated, and then taken directly to the lab. The samples were then divided into 200-mg aliquots, frozen rapidly in liquid nitrogen and stored at −80° until DNA extraction. Three blood samples were collected in tubes (two tubes without additives and one EDTA-containing tube) labeled with the subject's study number and delivered immediately to our clinical diagnostics laboratory for routine blood tests biochemical parameter measurements, and serum collection. Serum samples were divided into 100-μL aliquots and stored at −80°C until BA quantification. Samples (including stools and peripheral blood samples) were collected from cirrhotic patients at two time points: enrolment and 4 weeks after lactitol treatment (5 g, three times a day). HCs provided their samples only once at the time of enrollment.

Fecal DNA was extracted from the fecal pellets with a QIAGEN DNeasy PowerSoil Kit (ref. no.12888, Germany) in accordance with the manufacturer's instructions. The DNA samples were quantified with a Qubit dsDNA HS Assay Kit (Invitrogen, Q32854), normalized to 20 ng/L, and stored at −80°C until sequencing library preparation.

Metagenomic Sequencing and Analysis

For this assay, 10 L of DNA were fragmented by sonication to a size of 350 bp using an Ultrasonic 5-cell crusher (Covaris LE220R-plus, USA). An NEB Next® UltraTM DNA library Prep Kit for Illumina (NEB, USA) was used to generate the DNA sequencing library, and an attribute sequence index code was added to each fecal DNA sample. The library quality was assessed using the Qubit® 2.0 Fluorometer (Life Technologies, CA, USA) and Agilent Bioanalyzer 2100 system. Sequencing was performed on an Illumina Novaseq 6000 platform (Illumina) using NovaSeq 6000 Reagent Kits v1.5 (Illumina, USA) in accordance with the manufacturer's instructions. Finally, 150-bp paired-end reads were generated, and the raw sequencing data were submitted to the National Microbiology Data Center. The accession number is NMDC10017859.

Raw sequencing reads were quality-controlled with KneadData (version 0.6.1, https://bitbucket.org/biobakery/kneaddata). Briefly, low-quality bases were trimmed from the 3′ end of reads with Trimmomatic (version 0.36) (16), and then trimmed reads <50 nucleotides in length were discarded. Host (human) contaminant reads were identified and removed by mapping against the human genome (hg19) with Bowtie 2 (17). Quality-filtered reads were taxonomically profiled using MetaPhlAn2 (version 2.2.0)

(18) against the ChocoPhlAn database, and community-level functional metabolic pathways were identified using HUMAnN2 (19) against the UniRef90 protein reference database with default settings. The resulting taxonomic and pathway abundance files from all samples were joined and normalized to the relative abundance. Merged data were unstratified, and LEfSe (20) was used to identify differentially abundant features, as described above.

To explore the metagenomic information of the intestinal microbiota, all quality-filtered reads from samples were co-assembled using MEGAHIT (v.1.0.3) (21), and potential genes were predicted over the assembled contigs by Prokka (22). Genes with a length greater than or equal to 100 bp were retained. All predicted genes were combined and clustered using CD HIT (23) into a non-redundant gene set by setting the similarity threshold to 95%, and the relative abundance of unique genes was calculated using SALMON (24). Then, the predicted genes were annotated using eggNOG 4.5 (25), and Clusters of Orthologous Group (COG) (26) profiles were generated. Furthermore, all protein predictions were searched against the CARD database using resistance gene identifier software (27) and the VFDB database (28) using DIAMOND (29). Additional details are presented in **Supplementary File 1**.

BA Quantification

Samples (20 mg for each fecal sample and 50 μL for each blood sample) were extracted with 200 μL methanol after being ground with a ball mill, and the proteins were precipitated at −20°. The extracts were evaporated to dryness and reconstituted in 100 μL 50% methanol (V/V) for further LC-MS analysis using an LC-ESI-MS/MS system (UHPLC, ExionLC^{TM} AD; MS, Applied Biosystems 6500 Triple Quadrupole). Details were presented in **Supplementary File 1**. The standards, including cholic acid-d4, lithocholic acid-d4, glycolithocholic acid-d4, glycochenodeoxycholic acid-d4, taurocholic acid-d4, tauroursodeoxycholic acid-d5, glycodeoxycholic acid-d4, chenodeoxycholic acid-d4, deoxycholic acid-d4, and 2-chloro-L-phenylalanine, were purchased from Olchemim Ltd. (Olomouc, Czech Republic) and Sigma (St. Louis, MO, USA). A set of diluted standards (1000 μg/mL, 100 μg/mL, 10 μg/mL, 1 μg/mL, 0.1 μg/mL, and 0.01 μg/mL) was prepared with MeOH before analysis. BA concentrations were depicted as nanograms per gram (ng/g) of feces in fecal samples and nanograms per milliliter (ng/mL) of blood in blood samples. Data analysis was performed with MetWare (http://www.metware.cn/) based on the AB SciexQTRAP 6500 LC-MS/MS platform.

SCFA Quantification

Stool SCFAs were measured using a gas chromatography-mass spectrometry (GC-MS; Gas chromatograph (type 7890A, Agilent, USA) equipped with a mass selective detector (type 5975C, Agilent, USA). The measurement details were described in our previous study (30) and **Supplementary File 1**. The standards, including acetate-d3, propionate-d5, butyrate-d7, valerate-d9, isobutyric acid, 2-methylbutyric acid, and 3-methylbutyric acid (isovaleric acid), were purchased from Sigma (St. Louis, MO, USA). A set of diluted standards was prepared (1,000 μg/mL, 100 μg/mL, 10 μg/mL, 1 μg/mL, 0.1 μg/mL, and 0.01 μg/mL) with ethyl acetate before analysis. Data analysis was performed with ChemStation (Agilent, CA, USA, v E.02.02.1432) and Chroma TOF software (LECO, St. Joseph, MI, USA, v 4.34). SCFA concentrations in fecal samples were reported in milligrams per gram (mg/g) of feces.

Statistical Analysis

All statistical analyses were performed using R software. Data are presented as the mean ± standard error of the mean (SEM). The Kruskal-Wallis sum test was used for comparisons between groups when appropriate. Bray-Curtis distance was calculated as the beta diversity measurement using the vegan package. The PERMANOVA test with the adonis function was used to calculate the community structure differences. Principal coordinate analysis was carried out to visualize the Bray-Curtis dissimilarity among samples for microbial species (Hellinger transformed). The different treatments were fitted as centroids onto the ordination plots (31). Different genes were identified using the DESeq2 package with the cutoff threshold of adjusted $P < 0.05$ and the absolute value of log2 fold change (log2 FC) > 1. KEGG enrichment analyses (http://www.genome.jp/kegg) were performed using different genes as the foreground genes and all genes as the background. Discriminatory metabolites between groups were determined by VIP ≥ 1 and fold change ≥ 2 or ≤ 0.5. VIP values were extracted from OPLS-DA results, which included score plots and permutation plots generated using R package MetaboAnalystR. The data were log-transformed (log2) and mean-centered before OPLS-DA. To avoid overfitting, a permutation test (200 permutations) was performed. Significantly enriched KEGG pathways were identified for the differentially expressed genes identified above with the FDR multiple testing-corrected $P < 0.05$. All figures were created using the ggplot2 and DEseq2 packages. $P < 0.05$ or BH-adjusted $P < 0.05$ was considered statistically significant.

RESULTS

Baseline Characteristics of Subjects

Table 1 presents the detailed characteristics of each group, including clinical, metabolic, biochemical, and histological profiles. The levels of albumin, globulin and platelet in subjects with liver cirrhosis were lower than in the HC group. All clinical parameters including MELD showed no difference between pre- and post-lactitol treatment groups. During lactitol therapy, none of the patients experienced abdominal pain, bloating, diarrhea and other uncomfortable clinical manifestations.

Compositional Alterations in the Gut Microbiome

Each sample was sequenced at an average 12G depth of data and contained 3,199,274 non-redundant ORFs. A total of 387 bacterial species were identified across all subjects

TABLE 1 | Subject characteristics.

Characteristics		Group HC	Group LC_pre	Group LC_post	P-value		
		N=29 (%)	N=24 (%)		LC_pre vs HC	LC_post vs HC	LC_ post vs LC_ pre
Gender	Female	8 (27.58)	7 (29.17)		0.840	0.840	–
	Male	21 (72.42)	17 (70.83)				
Age		50.77±6.76	51.58 ± 10.95		0.751	0.751	–
BMI (kg/m2)		21.58 ± 3.05	22.30 ± 2.74		0.370	0.370	–
ALT (5-40U/L)		25.23 ± 10.46	26.83 ± 12.23	31.08 ± 18.11	0.678	0.132	0.297
AST (8-40U/L)		24.97 ± 6.52	34.54 ± 14.55	34.83 ± 14.98	0.007	0.005	0.936
Albumin (35-55g/L)		45.34 ± 2.71	41.96 ± 4.68	43.24 ± 4.85	0.012	0.196	0.744
Globulin (20-35g/L)		26.27 ± 2.98	30.31 ± 5.43	29.98 ± 5.15	0.009	0.012	0.995
TB (0-21mg/dL)		11.37 ± 5.29	25.08 ± 14.70	24.87 ± 11.82	<0.001	<0.001	1.000
PLT(83-303*109/L)		233.83 ± 49.99	84.71 ± 56.69	85.08 ± 57.44	<0.001	<0.001	1.000
Crea(59-104mg/dL)		73.50 ± 11.96	69.54 ± 11.97	66.42 ± 11.31	0.232	0.034	0.370
INR		–	1.18 ± 0.14	1.17 ± 0.11	–	–	1.000
MELD		–	5.37 ± 0.34	5.34 ± 0.30	–	–	0.984

The data were depicted as Mean ± SD. Continuous variables with a normal distribution were assessed using two-tailed independent sample t tests, whereas the data that did not fit a normal distribution were assessed using a non-parametric Mann-Whitney test.

and classified into 122 genera (>99% in each sample; **Supplementary Materials 1A,B**) for subsequent analysis.

Although no significant differences were observed in α and β bacterial community diversities between patients and HCs or pre-/post-lactitol treatment groups (**Supplementary Figures 1A–F**; all $P > 0.05$), significant differences in abundances of bacterial taxa were found in the intestinal microbiota of the three groups. Detailed overviews of the dominant bacterial profiles in each group were illustrated at the genus and species level (**Supplementary Figures 2A,B**). Among these 20 dominant genera, *Bifidobacterium*, *Veillonella*, *Enterobacter*, *Sutterella*, *Haemophilus,* and *Aggregatibacter* were found to be enriched in LC-post group, whereas *Klebsiella* and *Pseudoflavonifractor* were enriched in the LC-pre group, and *Prevotella*, *Roseburia*, *Parvimonas*, *Butyrivibrio*, *Methanobrevibacter,* and Clostridiales XIII_incertae_sedis_noname were enriched in HCs (all LDA scores (log10) > 2 and $P < 0.05$, using the LefSe approach; **Figure 1A**). At the species level, *Adlercreutzia_equolifaciens* (lactitol-decreased) and *Veillonella_atypica* (lactitol-increased) were found to be further deepened the discrepancy in their abundance after 4 weeks of lactitol supplementation (**Figure 1B**, by Kruskal–Wallis test along with the Bonferroni correction); Furthermore, the effects of lactitol treatment on *Haemophilus_parainfluenzae*, *Ruminococcus_obeum,* and *Eubacterium_ventriosum* were minimal, and the shifts in the other species that were significantly discriminatory between the LC-pre vs. HC groups tended to be similar to the level of those in HCs, including three increased species (*Prevotella_copri*, *Streptococcus_australis,* and *Granulicatella*_unclassified) and two decreased species (*Klebsiella_pneumoniae* and *V. dispar*) (**Figure 1C**). Species that showed no difference in abundance between LC-pre and HC groups exhibited a significant difference in abundance between LC-post and HC groups, including five increased species (*Bacteroides_ovatus*, *Bifidobacterium_pseudocatenulatum*, *B. longum*, *Lactobacillus_salivarius,* and *Rothi_mucilaginosa* (**Figure 1D**) and three decreased species (*Peptostreptococcaceae*_noname_unclassified, *Roseburia_inulinivorans* and *Eubacterium_rectale* (**Figure 1E**). Additionally, we found some health-beneficial species enriched in the LC-post group (including *B. ovatus*, *B. pseudocatenulatum*, *B. longum*, *Lactobacillus_salivarius*, *L. fermentium*, and *L. oris*), while some opportunistic pathogen enriched in the LC-pre group (including *B. massiliensis*, *K. pneumoniae*, *R. onavus*, *R. torques* and *V. dispar*) (all LDA scores (log10) > 2 and $P < 0.05$; **Figure 1F**). Phylogenic trees based on single-nucleotide polymorphisms were constructed for these discriminatory species, and two distinct clusters were formed for most cirrhotic patients and HCs based on the genome of *K. pneumoniae* ($P < 0.001$, Fisher's exact test; **Figure 1G**). Interestingly, there was a significant shift in the genotypes of *K. pneumoniae* strains as 10 LC-post patient strains were clustered together with HC strains, suggesting that liver cirrhotic patients are colonized by different *K. pneumonia* strains compared with HCs, which were partially changed by lactitol treatment.

Lactitol Treatment Modulated Microbial Functional Genes

Metagenomic analysis revealed a total of 469 functional metabolic pathways from all fecal samples. A scatter plot based on principal coordinate analysis obtained from the Bray–Curtis distance matrix of functional metabolic pathways showed significant differences among LC-pre, LC-post, and HC groups ($P < 0.001$; **Figure 2A**). Subsequently, the differences in the top 50 abundant functional metabolic pathways were tested between LC-pre and HC groups and between LC-post and HC groups by the Wilcox test. Among the significantly differentially abundant biological processes between LC-pre and HC groups ($P < 0.05$), six pathways were upregulated

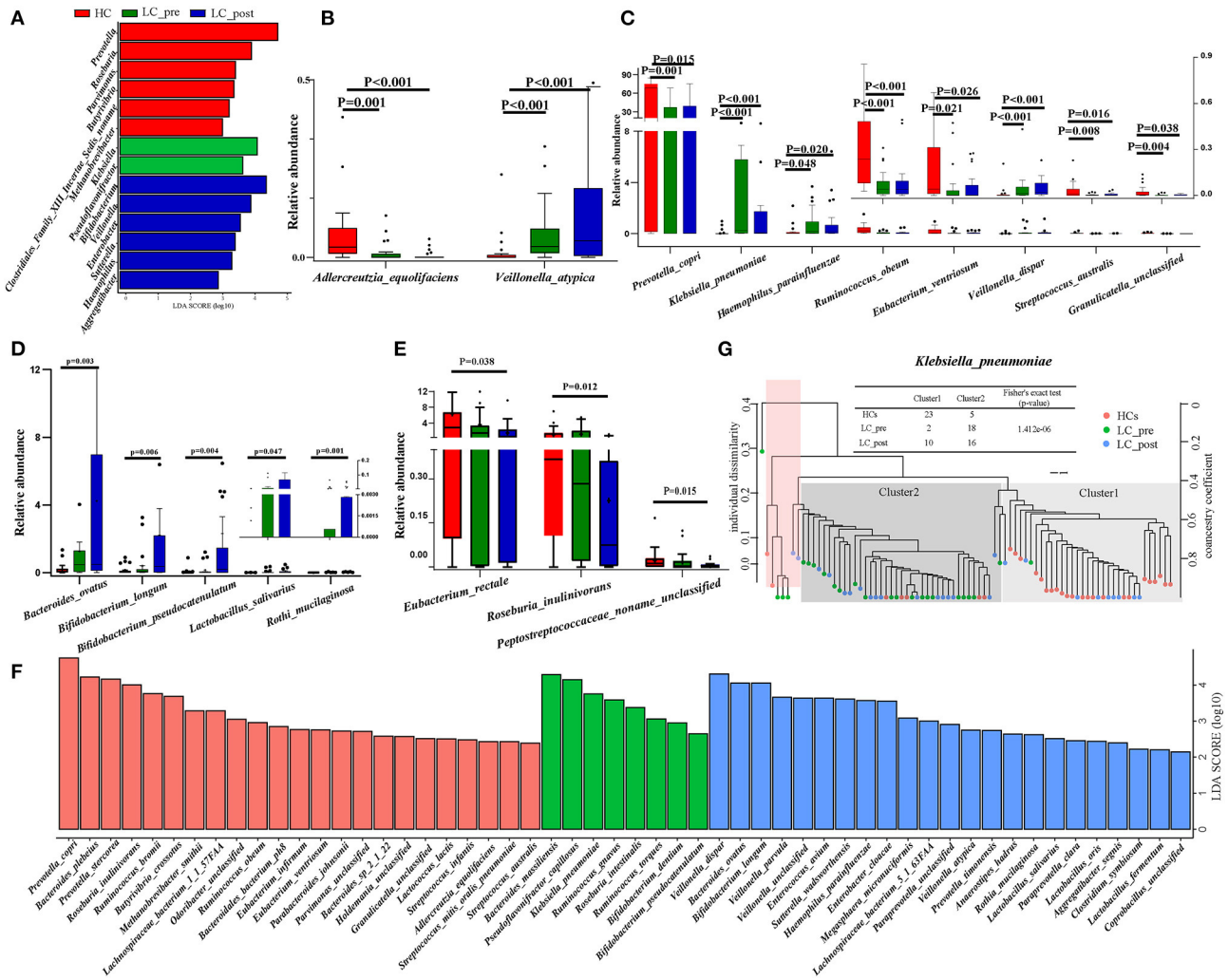

FIGURE 1 | Comparison of intestinal microbial communities in cirrhotic patients before (LC-pre, green) and after (LC-post, blue) lactitol intervention and healthy controls (HCs, red). **(A)** LEfSe and LDA based on divergent genera between the three groups, which identified the most differentially abundant genera between the three groups. LDA scores of red bars represented genera enriched in HCs, the green represented the LC-pre group, and the blue represented the LC-post group. **(B–E)** Statistical analysis of differentially abundant species by a non-parametric Kruskal–Wallis test with the Bonferroni correction between the three groups. The box plots indicated the median and 25th to 75th percentiles. **(F)** LEfSe and LDA based on divergent species between the three groups. **(G)** Strain-level analysis of *K. pneumoniae* based on SNPs using the fecal metagenome of subjects in the three groups. LEfSe, Linear discriminant analysis Effect Size; LDA, linear discriminant analysis.

(including guanosine ribonucleotide *de novo* biosynthesis, queuosine biosynthesis, methylerythritol phosphate pathway I, urate biosynthesis/inosine 5′-phosphate degradation, glycolysis III, and thiamin formation from pyrithiamine and oxythiamine) (**Figures 2B–G**), two showed minimal alterations (peptidoglycan biosynthesis III and superpathway of branched amino acid biosynthesis) (**Figures 2H,I**), and the alterations in the rest were similar to the levels observed in HCs (including superpathway of guanosine nucleotides de novo biosynthesis I, superpathway of guanosine nucleotides de novo biosynthesis II and purine ribonucleoside degradation after 4-week lactitol intervention) (**Figures 2J–L**). the differential abundance of the dominant KEGG Orthologs (KOs) among LC-post, LC-pre and HCs was observed using Heatmap of KOs analysis (**Figure 3A**). Further characterization of the functionalities of KOs in metagenomes of patients pre- actitol treatment by comparison with HCs, of note is that LPS biosynthesis, homologous recombination and mismatch repair (vital for horizontal transfer of pathogenic genes), and Pseudomonas aeruginosa biofilm formation (vital for translocation infection) were enriched in metagenomes of LC-pre (**Figure 3B**, **Supplementary Material 2A**), which is different from LC-post vs. HCs (**Figure 3C**, **Supplementary Material 2B**). Additionally, we also assigned the significant detection gene to the Cluster of Orthologous Groups (COG) database for microbialfunction analysis, and distribution and differences in relative abundances of COG categories were showed in

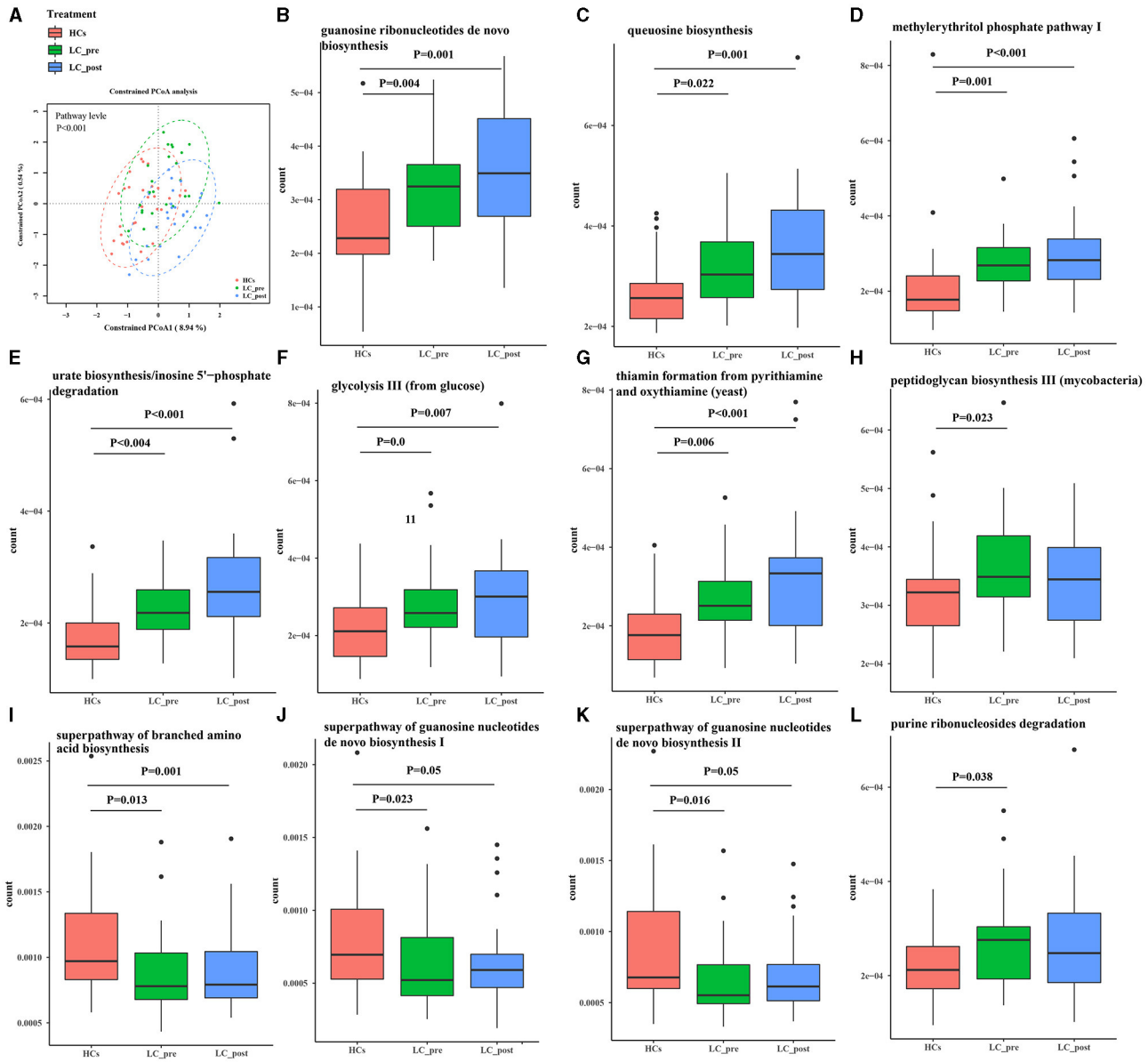

FIGURE 2 | Functional pathways of the gut microbiome in cirrhotic patients before (LC-pre, green) and after (LC-post, blue) lactitol intervention and healthy controls (HCs, red). The pathways were annotated by HUMAnN2. **(A)** Beta diversity analyses of the Bray–Curtis similarity index scores of microbial pathways between the three groups, and statistical significance was measured using adonis analysis. Ellipses show 95% confidence intervals, and the different colors of ellipses represent different groups. **(B–L)** Wilcox test of the top 50 microbial metabolic pathways between groups. Data were shown as box plots with the median and 25th to 75th percentiles. Benjamini–Hochberg correction was further applied to adjust derived *p*-values. Only pathways with p-values under a threshold of 0.05 were considered as significant.

Supplementary Figures 3A–R. Importantly, the count of COG categories of Defense mechanisms were increased significantly in LC-post when compared with LC-pre (**Supplementary Figure 3I**). We also found that the differentially expressed carbohydrate-active enzymes (CAZy) between the three groups mainly included glycoside hydrolases (GHs), glycosyltransferases (GTs), polysaccharide lyases (PLs), carbohydrate esterases (CEs), and carbohydrate-binding modules (CBMs) (**Supplementary Figures 4A–C**, Foldchange $>$ 2 and $P < 0.05$ after adjusting for the Bonferroni correction).

We also profiled particular genes related to antibiotic resistance and known pathogenic factors in the gut microbiome data by comparison with CARD, and VFDB databases, and 218 ARGs, and 1560 VFGs were derived (**Supplementary Materials 3A, B**). In terms of ARGs, matching against the CARD database revealed a significantly higher number of ARGs in both LC-pre and LC-post groups than the

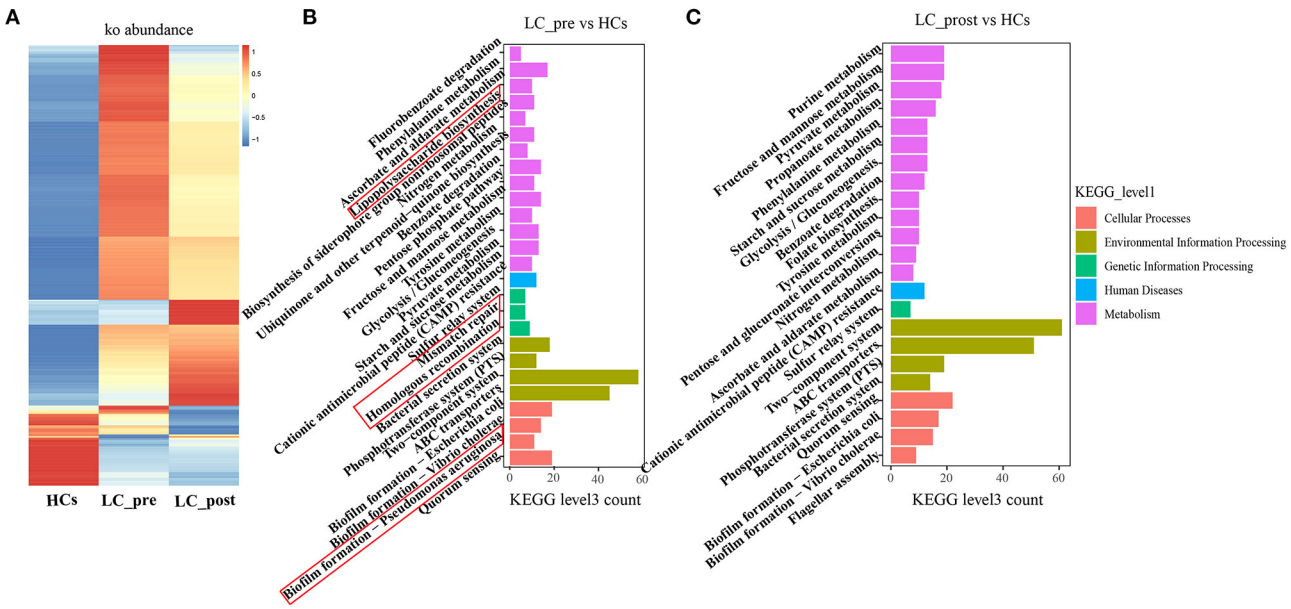

FIGURE 3 | KEGG pathway enrichment analyses. **(A)** Heatmap analysis shows the difference among the cirrhotic patients before (LC-pre) and after (LC-post) lactitol intervention and healthy controls (HCs). **(B)** the number of differential KEGG metabolism level 3 from LC-pre vs. HCs. **(C)** Abundance of differential KEGG metabolism level 3 from LC-post vs. HCs. KEGG, Kyoto Encyclopedia of Genes and Genomes.

HC group (**Figure 4A**). In contrast, mapping VFGs against the VFDB database showed a significant increase in the number of VFGs in patients compared with HCs, but the number of VFGs tended to be reduced in the LC-post group and was similar to that in the HCs (**Figure 4B**). Specifically, compared with HCs, patients in the LC-pre group were characterized by 32 (20% of total 160 ARGs) cirrhosis-enriched ARGs, 119 (7.6% of total 1560 VFGs) cirrhosis-enriched VFGs, 4 (2.5% of total 160 ARGs) cirrhosis-depleted ARGs, and 42 (2.7% of total 1560 VFGs) cirrhosis-depleted VFGs (**Figure 4C**, Fold change > 2 and adjusted $P < 0.01$). After 2 months of lactitol supplementation, we found 7 (4.3% of total 160 ARGs) lactitol-upregulated ARGs, 44 (2.8% of total 1560 VFGs) lactitol-upregulated VFGs, 15 (9.37% of total 160 ARGs) lactitol-downregulated ARGs, and 80 (5.13% of total 1560 VFGs) lactitol-downregulated VFGs in the microbiome of the LC-post group (**Figure 4D**, Fold change > 2 and adjusted $P < 0.01$). Among these lactitol-susceptibility ARGs, we found that 22% of ARGs belonged to *Klebsiella*, 19.6% to *Escherichia*, 16% to *Enterobacteriaceae,* and 8.3% to *Enterobacter*. Additionally, 17.3% of VFGs belonged to *Klebsiella*, 12.4% to *Escherichia*, 8.1% to *Salmonella,* and 5.9% to *Pseudomonas*. Network analysis between ARGs and bacterial abundances mainly generated three different covarying clusters: (1) *P. copri* (enriched in HCs) significantly positively correlated with tet37, (2) *En. cloacae* (enriched in LC-post) with acrA and MIR-3, and 3) *K. pneumonia* (enriched in LC-pre) with SHV-161, KpnH, oqxA, KpnG, acrA, FosA6, UhpT, and FosA5 (**Figure 4E**, $P < 0.05$). Additionally, network analysis between VFGs and bacterial abundances mainly generated six different covarying clusters, especially for the *K. pneumonia* (enriched in LC-pre) cluster, which was significantly positively associated with 16 virulence factors, such as AHA-1846, exeB, mrkA, and lefB, vagw/ecpD (**Figure 4F**, $P < 0.05$). Importantly, these ARGs and VFGs that were correlated to *K. pneumonia* were significantly decreased after the 4-week lactitol intervention.

Altered Fecal Metabolites After Lactitol Consumption

To investigate the functional consequences and causes of microbiome shifts, we further performed metabolome analysis of SCFAs and BAs in fecal samples and BAs in serum. The overall metabolic signatures of LC-post patients were significantly different from those of HCs but similar to those of LC-pre patients (**Supplementary Figure 5**, $P < 0.01$). The fecal SCFA profiles of the LC-pre group displayed increases in pentanoic and acetic acids and decreases in butyric, propionic, 2-methylpropionic, and 2_methylbutyric acids compared with the HCs (**Figure 5A**). Lactitol treatment further reduced 2_methylpropionic, propionic acid, and butyric acid and fecal pentanoate but had a minimal effect on 2_methybutyric and acetic acid. Overall, co-occurrence analysis showed that discriminatory bacterial species were strongly correlated with the fecal SCFAs (**Figure 5B**). Within the fecal SCFA and bacteria correlation profile, differential bacterial species mainly generated two different covarying clusters: (1) a cluster of 2-methylpropionic acid, propionic acid, and butyric acid significantly positively associated with bacteria mainly enriched in HCs (except four species: one enriched in LC-post and three in LC-pre) and (2) acetic acid with species enriched in

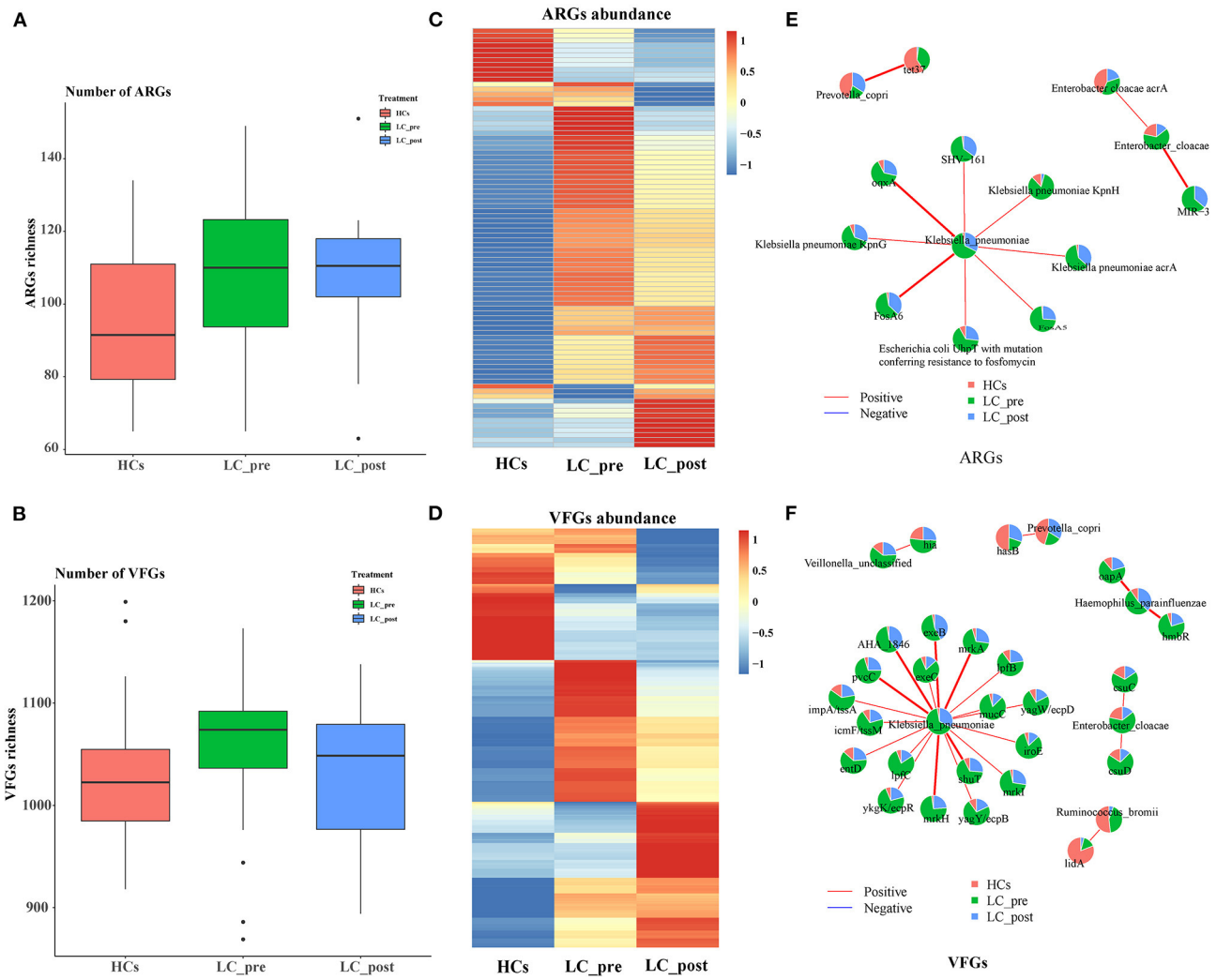

FIGURE 4 | Comparison of fecal ARGs and VFGs in subjects of cirrhotic patients before (LC-pre, green) and after (LC-post, blue) lactitol intervention and healthy controls (HCs, red), and correlation analysis between discriminatory genes and bacteria. **(A)** comparison of ARGs enrichment between the three groups. **(B)** Comparison of VFG enrichment between the three groups. **(C)** Heatmap of differential ARGs. **(D)** Heatmap of differential VFGs. **(E)** Correlation between ARGs and bacteria. **(F)** Correlation between VFGs and bacteria. Wilcoxon rank-sum test with a significance level of $P < 0.05$. ARGs, antibiotics resistance genes; VFGs, virulence factor genes.

the LC-post group (except for two species enriched in group LC-pre) (32).

We also observed similar trends for both discriminatory serum and fecal BA profiles in LC-pre vs. HC groups and LC-post vs. HC groups but different trends for the most discriminatory BAs in fecal and serum samples in cirrhotic patients compared with HCs (**Figure 6A**). Lactitol intervention reduced some BAs, especially F-TCA, F-TβMCA, and F-TCDCA (**Supplementary Material 4**, VIP>1 and log2FD>1 or <-1.), but without statistical significance ($P > 0.05$). We also explored the potential correlations between discriminatory bacterial species and fecal BAs among the three groups. No significant positive associations were observed between three slightly increased fecal BAs, including F-GCDCA, F-GCA, and F-CA, in lactitol intervention groups and discriminatory species enriched in the LC-post group (**Figures 6B,C**). Additionally, F-LCA, F-12_KLCA, F-GLCA, F-DCA, and F-GDCA were positively significantly associated with two species (belonging to *Paraprevotella*) enriched in the LC-post group and 11 species (mainly belonging to *Bacteroides* and *Prevotella*) enriched in HCs and negatively significantly associated with eight species (mainly belonging to *Veillonella*) enriched in the LC-pre group. We also found that F-CA, F-CDCA, F-UDCA, and their conjugated BAs were positively significantly correlated with five species (three belonging to *Streptococcus*, one to *Parvimonas*, and one to *Granulicatella*) enriched in HCs (**Figure 6C**).

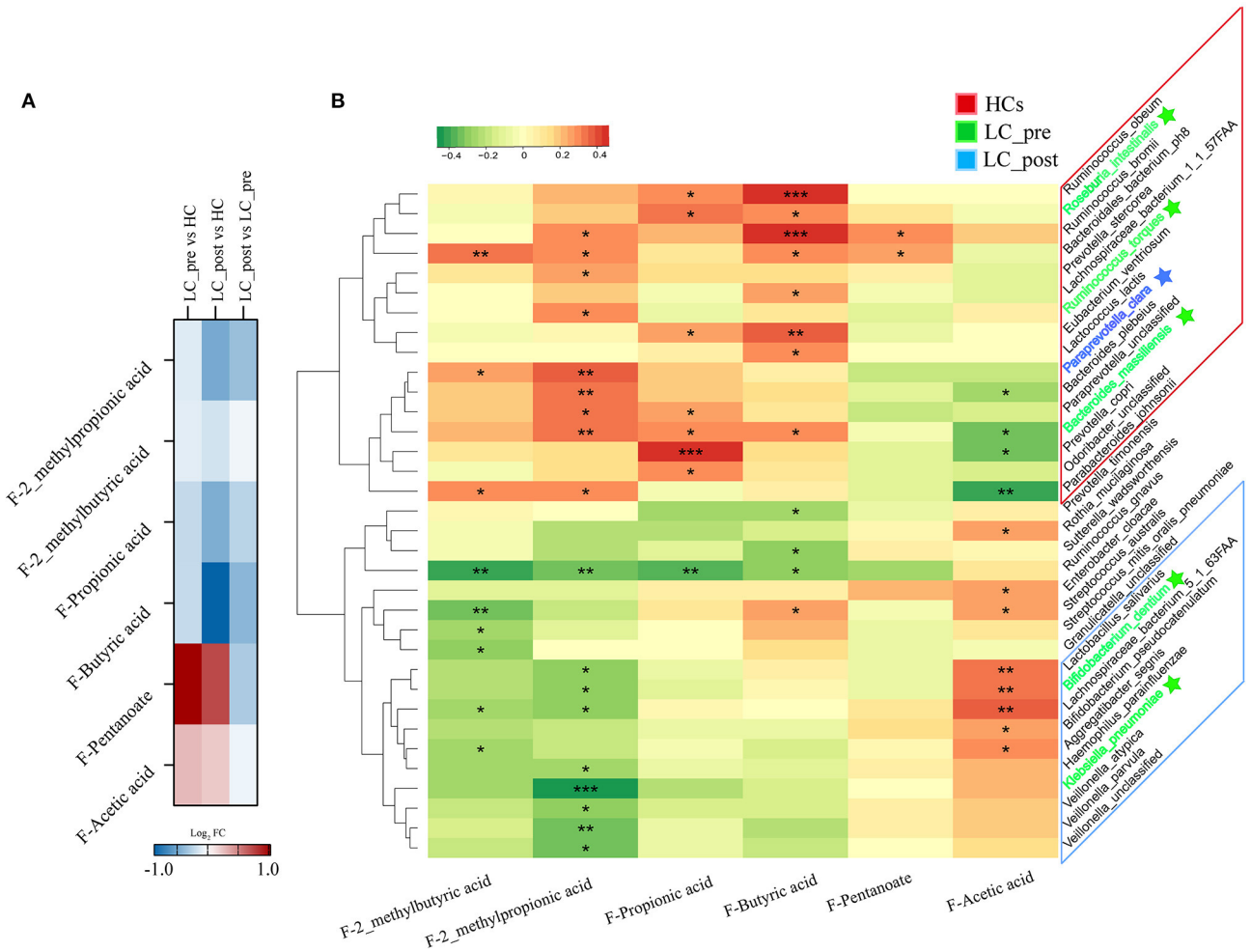

FIGURE 5 | Effect of lactitol on fecal SCFAs. **(A)** Heatmap of fecal SCFAs. **(B)** Correlation between fecal SCFAs and discriminatory species. SCFAs, short chain fatty acids; LC-pre (green), cirrhotic patients before lactitol intervention; LC-post (blue), cirrhotic patients before after lactitol intervention; HCs (red), healthy controls. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

DISCUSSION

In this study, we performed the analysis of intestinal microbiome and metabolome with respect to lactitol-related supplement in liver cirrhotic patients. We identified species-level bacteria and metabolites, such as SCFAs and BAs, that were associated with lactitol administration in cirrhotic patients. Although no significant differences were found in microbial diversity among the groups, significant differences in the functional genes of the intestinal microbiome were found, suggesting that the effects of lactitol on the intestinal microbiome presented as alterations in the functionality of the microbiome, rather than changes in the diversity of microbiota. Particularly, lactitol treatment resulted in a significant shift of *K. pneumoniae* at the strain level as observed that 40% of patients shift from cirrhotic patient-enriched strain clusters to HC-enriched strain clusters.

Dysbiosis in gut microbiome composition is reported to correlate with cirrhosis severity (33), particularly associated with systemic inflammation and bacterial translocation (34). The changes observed in opportunistic pathogens in cirrhotic patients and HCs involve different strain *K. pneumonia,* which has been reported as cirrhosis associated species in previous studies (33, 35). We additionally found species enriched in cirrhotic patients, including *Eu. rectale*, *R. intestinalis*, *R. hominis*, *V. atypical*, *R. gnavus*, and *L. salivarius*, which were consistent with previous research (35). Our data then showed that 4 weeks of lactitol supplementation lead to decrease of cirrhosis-enriched species including pathogenic *K. pneumonia* (36) and *V. dispar* (37, 38), and increase of some health-beneficial species including *B. longum*, *B. pseudocatenulatum, L. salivarius* and *B. ovatus* (39). Previous studies have also demonstrated that lactitol increased the abundance of the first two species in the gut (11). Of particular note, *B. dentium*, an opportunistic pathogen enriched in cirrhotic

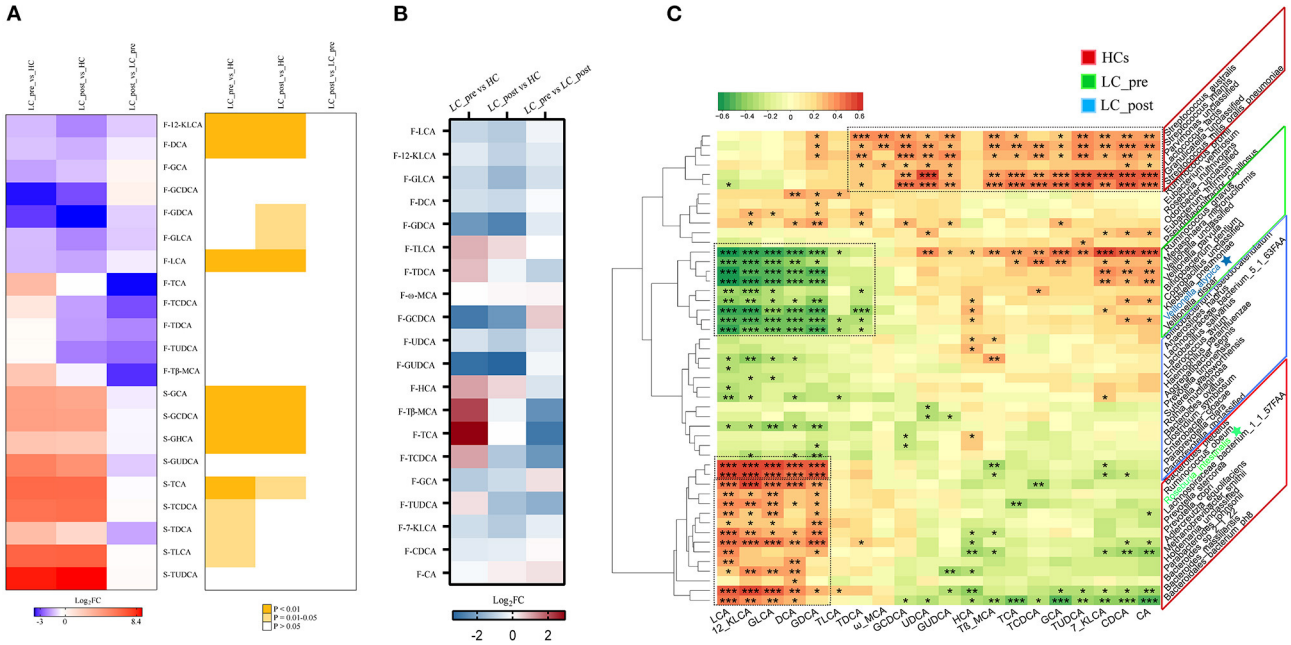

FIGURE 6 | Effect of lactitol on fecal and serum BAs. **(A)** Discriminatory BA levels (Foldchange > 2) and associated *P* values for LC-pre vs. HC, LC-post vs. HCs, and LC-pre vs. LC-post. **(B)** Heatmap of fecal BAs. **(C)** Correlation between fecal BAs and discriminatory species. BAs, bile acids; LC-pre (green), cirrhotic patients before lactitol intervention; LC-post (blue), cirrhotic patients before after lactitol intervention; HCs (red), healthy controls. Benjamini–Hochberg correction was further applied to adjust derived *p*-values. Only pathways with *p*-values under a threshold of 0.05 were considered as significant. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

patients (35), was also found to be enriched in the patients in this study, and reduced in abundance after lactitol supplementation.

We discovered that the lactitol supplement-modulated pathways include pathways associated with metabolic and liver function improvements, such as increased folate biosynthesis, propanoate metabolism, purine metabolism, pentose/glucuronate interconversions etc., and decreased lipopolysaccharide (LPS) and biofilm, endotoxin biosynthesis. Particularly, the alterations of functional pathways after 2-month lactitol supplement became more similar to that of HC. Therefore, we conclude that lactitol intervention could improve outcomes in cirrhosis-related intestinal microbiome dysbiosis, extending the observations from the previous study (40). Shifts in the abundance of these bacteria were correlated with alterations in functional pathways and some pathogenic genes, including ARGs and VFGs. For example, eight key ARGs and 18 key VFGs was identified to be associated with *K. pneumonia*, and decreased significantly after lactitol sumplemention. We postulate that the strain-level shift in *K. pneumoniae* contributed to these marked alterations in ARGs and VFGs as a result of lactitol treatment. Intestinal VFGs were suggested to influence host immune homeostasis (41) and be associated with disease severity (42). Of note, we found that alterations in the composition of lactitol-treated VFGs were correlated with the strain-level changes in *K.pneumonia*, which accounted for shifts in the highest proportion of differentiated VFGs in the gut microbiome of patients in LC-pre vs. LC-post groups. Lower VFG levels in *K. pneumonia* suggested weak invasiveness and reduced pathogenicity of the pathogen in lactitol-treated patients. Considering the probiotic properties of *B. longum* and *B. pseudocatenulatum*, a high abundance of both species in the LC-post group could also indicate an improvement in gut dysbiosis and therapeutic outcomes for hepatic cirrhosis.

SCFAs, particularly butyrate, have roles in the stimulation of tight junction and mucous production (43), reduction in systemic inflammation (44), and mediation of intestinal hormones (45). Although lactitol promoted the growth of *Bifidobacteria*, we did not find increase of fecal SCFAs in liver cirrhotic patients, which was reported in lactitol-treated healthy adults in the study by Finney and his colleagues (46). In fact, lactitol treatment further decreased the concentrations of intestinal SCFAs, except for 2-methylbutyric acid and acetate with no significant changes. The phenomena can potentially be explained by the fact that most species positively related to fecal 2-methylbutyric acid, methylpropionic acid, propionic acid, and butyric acid were enriched in the microbiome of HCs. Cirrhosis is always accompanied by a decreased conversion of primary to secondary fecal BAs (47). BA metabolism was found to be associated with BA biosynthesis in the liver and biotransformation by intestinal microbiota (48). Our results indicated that most of discriminatory secondary BAs in feces tended to be decreased in the LC-pre and LC-post groups compared with HCs. In contrast, the alterations in discriminatory serum BAs showed opposite trends in both patient groups vs. HCs. Together, these results suggested

the presence of hepatocytosis and enterohepatic circulatory deficiency in cirrhotic patients and were consistent with the higher total serum BA levels in both patient groups compared with HCs measured by clinical biochemical tests. Higher BA (including DCA, LCA, CDCA, and TCDCA) exposure may lead to cytotoxicity (49) and have cancer-promoting effects (50). However, both the clinical biochemical tests and BA mass spectrometry results showed that lactitol treatment had a minimal influence on serum BAs. Interestingly, lactitol treatment clearly reduced fecal BAs, especially some taurine conjugates, including F-TCA, F-TDCA, F-TCDCA, F-TUDCA, and F-TβMCA, although without statistical significance. Other previous studies of probiotic supplementation in humans also reported no metabolic changes between pre- and post-probiotic intervention (51). There is undoubtedly a correlation between intestinal microbiome dysbiosis and metabolic dysregulations (52). The abundance and diversity of bacteria that perform the various BA transformations vary widely. We found that bacteria enriched in HCs were classified into two clusters according to their relationships with fecal BAs: 13 species for LCA, KLCA, GLCA, DCA, and GDCA and 6 for the rest of the fecal BAs. However, those enriched in the LC-post group did not show clear or significant correlations with fecal BAs, except for two species belonging to *Paraprevotella*. Microbiota alterations affect host signaling but not necessarily BA synthesis (53). The phenomenon can be explained to a certain extent by the minor fluctuations in fecal BAs after a 4-week lactitol intervention. BA homeostasis in the circulation pool is necessary for normal health (48). For example, LCA and DCA are takeda G protein-coupled receptor (TGR5) agonists, and TGR5 activation in colonic L cells is involved in several metabolic activities, including energy homeostasis, thermogenesis, insulin signaling inflammation, and BA uptake (54). Restoration of the intestinal BA pool increases colonic RORγ+ regulatory T cell levels and ameliorates host susceptibility to inflammation (55). In the current study, the impact of lactitol on the microbiome was evident, and the lactitol-induced minor fluctuations in BAs theoretically influenced the course of cirrhotic disease states. Whether the effects of these changes on the disease are beneficial deserves further follow-up investigations to monitor disease progression with a larger number of patients and a multi-center cohort.

To our knowledge, this is the first study to combine microbiome with targeted metabolome analyses to uncover the role of lactitol supplementation in regulating the intestinal microbiome and metabolic dysbiosis in cirrhotic patients, and our findings extended the results of previous clinical trials for lactitol. Significant alterations were found in the microbiome profiles, including bacterial communities, microbiota-associated pathways, ABGs, and VFGs, in patients pre- and post-lactitol treatment, but we only identified minor lactitol-induced changes in BA and SCFA metabolites. However, one limitation of the current study was that it is only a single-center study performed in a small cohort. Therefore, it is necessary to perform future large-scale, multicenter, randomized, and placebo-controlled trials and experiments with a lactitol-treated cirrhotic rat model to further validate the lactitol-induced alterations are associated with improvements in disease status and the prevention of cirrhosis progression.

CONCLUSION

The results of the present study indicated that supplement of lactitol was associated with changes in bacterial abundances at the strain level were correlated with altered functions of the microbiome. Our results extended the observations from previous clinical trials of lactitol by providing evidence on lactitol-induced improvements in the microbiome of hepatic cirrhotic patients based on fecal metabolic pathways, ARG and VFG pools. Given the increasing importance of microbial resistance to antibiotics, fecal ARGs and VFGs may become the next research topic of high interest in probiotic or prebiotic clinical trials because epigenetic signatures have not yet been shown to predict the therapeutic results of microecological modulators. Lactitol-induced improvements in clinical outcomes require further follow-up investigations on to verify the clinical relevance of microbiome alterations.

DATA AVAILABILITY STATEMENT

The raw sequencing data have been deposited in the National Microbiology Data Center with the accession number is NMDC10017859. Further information and requests for resources and reagents should be directed to and will be fulfilled by the corresponding author, Zhongwen Wu (wuzhongwen@zju.edu.cn).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the First Affiliated Hospital, School of Medicine, Zhejiang University (reference number: 2017HL668-1). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZW and LL designed the study. HL, XP, YY, HZ, XZ, XL, CZ, LL, and ZW enrolled the subjects, collected clinical samples, and performed the experiments. LC, HL, ZW, and JW performed the analysis. HL and JW wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.762930/full#supplementary-material

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Structural and Functional Alterations of Gut Microbiota in Males With Hyperuricemia and High Levels of Liver Enzymes

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Objective: To investigate the correlation between the structure and function alterations of gut microbiota and biochemical indicators in males with hyperuricemia (HUA) and high levels of liver enzymes, in order to provide new evidences and therapeutic targets for the clinical diagnosis and treatment of HUA.

Methods: A total of 69 patients with HUA (HUA group) and 118 healthy controls were enrolled in this study. Their age, height, waist circumference, weight, and pressure were measured. The clinical parameters such as fasting plasma glucose (FBG), aspartate aminotransferase (AST), alanine aminotransferase (ALT), serum uric acid (SUA), serum creatinine (Scr), total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL), high-density lipoprotein (HDL), white blood cell (WBC), platelet (PLT), and absolute value of neutrophils (NEUT) were examined. We used whole-genome shotgun sequencing technology and HUMAnN2 MetaCyc pathway database to detect the composition and pathways of the gut microbiota. The main statistical methods were student's *t* test, chi-square tests, and Wilcoxon rank sum test. The correlations among bacterial diversity, microbial pathways, and biochemical indicators were evaluated by the R function "cor.test" with spearman method.

Results: The gut bacterial diversity in HUA group reduced significantly and the community of the microbiota was of significant difference between the two groups. The pathways that can produce 5-aminoimidazole ribonucleotide (PWY-6122, PWY-6277, and PWY-6121), aromatic amino acids, and chorismate (COMPLETE-ARO-PWY, ARO-PWY, and PWY-6163) were enriched in the HUA group; while the pathways that can produce short-chain fatty acids (SCFAs, such as CENTFERM-PWY and PWY-6590) and the gut microbiotas that can produce SCFAs (*Roseburia hominis, Odoribacter splanchnicus, Ruminococcus callidus, Lachnospiraceae bacterium 3_1_46FAA, Bacteroides uniformis, Butyricimonas synergistica*) and equol (*Adlercreutzia equolifaciens*) were enriched in healthy controls.

Conclusion: The structure and function of the gut microbiota in males with HUA and high levels of liver enzymes have altered apparently. In-depth study of related mechanisms may provide new ideas for the treatment of HUA.

Keywords: uric acid, liver enzymes, short-chain fatty acids, purine, aromatic amino acids, gut microbiota, equol

INTRODUCTION

Hyperuricemia (HUA) is a group of heterogeneous chronic metabolic pathological conditions caused by purine metabolism disorder and/or uric acid excretion disorder. The excessive production and/or decreased excretion of uric acid level caused by any cause can lead to the occurrence of HUA. HUA is not only the direct cause of gout, but closely related to the onset and progression of many diseases such as diabetes, metabolic syndrome, chronic kidney disease, cerebrovascular-related diseases, and hyperlipidemia. Studies have confirmed that uric acid level is an independent risk factor leading to the progression of cardiovascular-related diseases such as hypertension and coronary heart disease, and it has an important impact on its prognosis (1). The latest research data show that the prevalence of HUA in the Chinese population has risen from less than 1.5% in the early 1980s to as high as 13% (2), and it is still rising and the incidence is showing a younger trend. Therefore, it is of great significance to study in depth the pathogenesis and further explore effective treatment measures of HUA.

Healthy people excrete uric acid in two ways mainly, of which 70% is excreted through the kidney, and the remaining 30% is excreted through the intestine (3). Intestinal microbes are a group of microorganisms designated to be planted in the intestinal tract, which have a symbiotic relationship with the human host and have a variety of important physiological functions such as participation in digestion, metabolism, promotion of nutrient absorption, synthesis of trace elements, and regulation of immune function. They are of great significance for the maintenance of our health (4). Researches in recent years have shown that changes in intestinal microbiota are not only related to diabetes, obesity, hypertension, and other diseases, but closely related to the occurrence of HUA. Studies have shown that changes in the gut microbiota and HUA promote each other and jointly aggravate the disease progression. For example, the intestinal microbiota can participate not only in the catabolism of purine and uric acid through the regulation of *Escherichia coli* and *Lactobacillus* (5–7), but also in the other two ways to affect the excretion of uric acid such as the production of short-chain fatty acids (SCFAs) (8–10) and changes in the number and distribution of uric acid transporters (11, 12) in the body. Besides, purine metabolism increases the production of uric acid, which can induce intestinal oxidative stress and then further promotes the infiltration of inflammatory cells and causes chronic inflammation in the intestinal tract. However, there are not many studies on the gut microbiota as an entry point to investigate HUA, and most of them are animal experiments. In view of the fact that most patients with HUA are asymptomatic and the intestinal microecology can change correspondingly in other disease states, it is more important and suitable to select the asymptomatic people who take physical examination as the research subjects to explore the correlation between HUA and gut microbiota. This study intends to explore the relationship among the serum uric acid (SUA) level, liver enzymes, and the detection of intestinal microbes in the physical examination population, and to provide more evidence support for finding the relationship among the serum uric acid, liver enzymes, and intestinal microecology.

Abbreviations: WC, waist circumference; BMI, body mass index; FPG, fasting plasma glucose; DBP, diastolic blood pressure; SBP, systolic blood pressure; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, glutamyl transpeptidase; TBIL, total bilirubin; IBIL, indirect bilirubin; SUA, serum uric acid; Scr, serum creatinine; TC, total cholesterol; TG, triglyceride; LDL, low-density lipoprotein; HDL, high-density lipoprotein; WBC, white blood cell; PLT, platelet; NEUT, absolute value of neutrophil; NLR, neutrophil to lymphocyte ratio; SCFAs, short-chain fatty acids.

MATERIALS AND METHODS

Study Design

Totally, 89 persons and 118 participants were randomly enrolled in HUA group and healthy controls from the adult population who underwent the physical examination in the Department of health management center, the First Affiliated Hospital of Zhengzhou University, namely HUA group and healthy control group (healthy controls). According to the multidisciplinary expert consensus on the diagnosis and treatment of HUA-related diseases in China, the diagnostic criteria of HUA was defined as the fasting plasma uric acid value >420 μmol/L(7 mg/dL) on different days, excluding concomitant diabetes, tumors and hematological diseases. Exclusion criteria: persons with clear gastrointestinal diseases or severe diseases of cardiovascular, respiratory, kidney, and other systems; pregnant and lactating woman; minors under 18 years old; antibiotics and microbiota regulators, yogurt, gastrointestinal motility drugs, and other preparations that may affect the intestinal microbiota had been used in the past 2 months. According to the test, 89 people had HUA, including 2 females, 11 persons used antibiotics and yogurt preparations recently, and 7 people were of high blood sugar or used hypoglycemic drugs or antihypertensive drugs. After excluding the above population, 69 people were enrolled in HUA group (**Figure 1A**). The research protocol was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (2018-KY-56 and 2018-KY-90), and all of the study subjects signed an informed consent form.

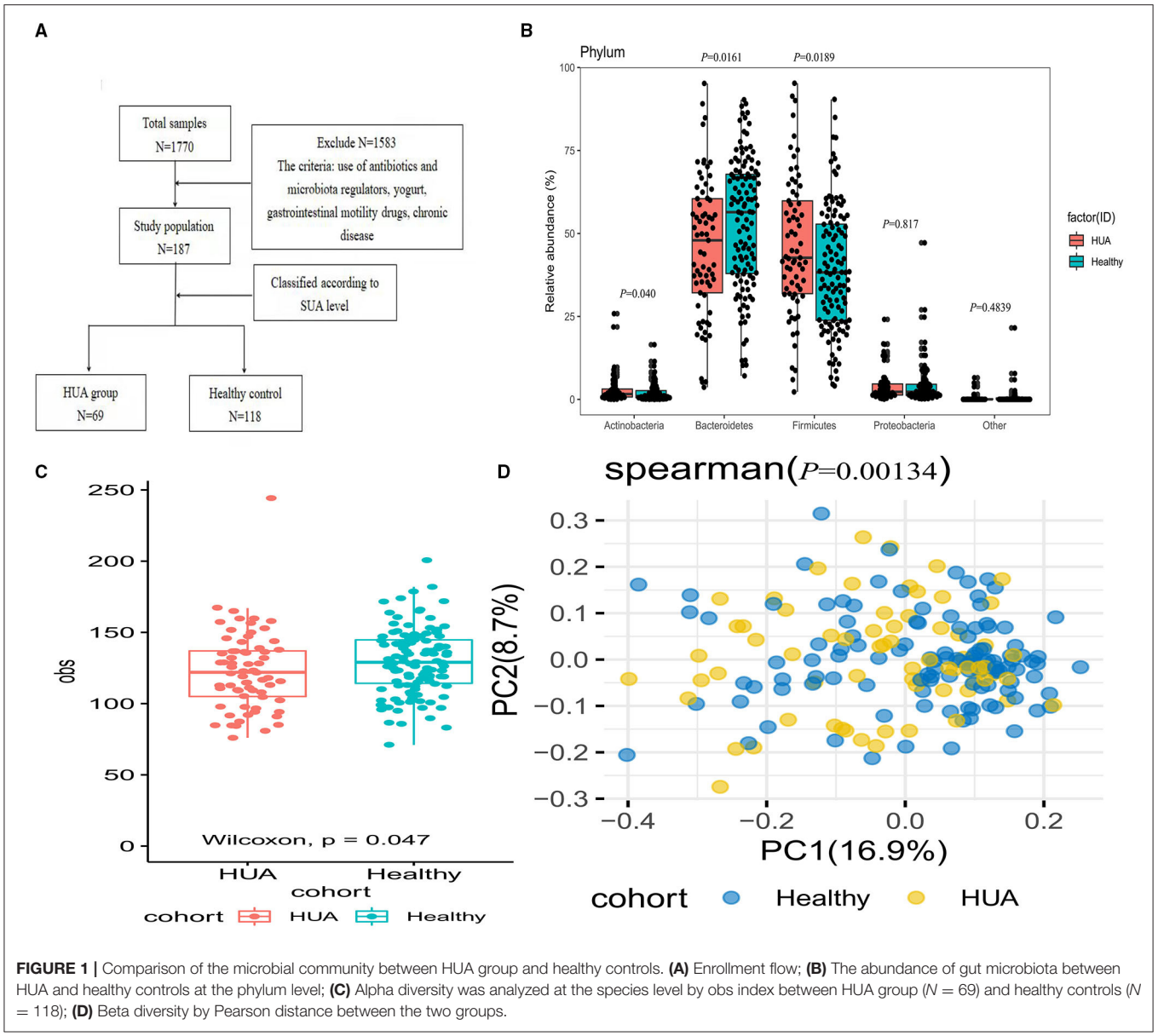

FIGURE 1 | Comparison of the microbial community between HUA group and healthy controls. **(A)** Enrollment flow; **(B)** The abundance of gut microbiota between HUA and healthy controls at the phylum level; **(C)** Alpha diversity was analyzed at the species level by obs index between HUA group (*N* = 69) and healthy controls (*N* = 118); **(D)** Beta diversity by Pearson distance between the two groups.

General Information and Related Biochemical Indicators

The height, weight, waist circumference (Computerized body scale, SK-X80), and blood pressure (OMRON Medical automatic electronic blood pressure monitor, HBP-9021) were measured by designated staff after all subjects are enrolled in the group. Each indicator was measured for three times and the average value was taken, and then the body mass index (BMI) is calculated according to the following formula: BMI = weight Kg/ (height m)^2. Every study subject was required to fast and no water for 8–12 h and got 5 mL of cubital venous blood on an empty stomach in the morning. All the test tubes were placed in the specimen box and sent to the clinical central laboratory within 20 min by the logistics trolley transported by the hospital's special specimens after the blood was drawn. UniCel DxI 800 Immunoassay System from Beckman Coulter was used to test the blood samples. The following laboratory tests were collected such as fasting plasma glucose (FPG), aspartate aminotransferase (AST), alanine aminotransferase (ALT), glutamyl transpeptidase (GGT), total bilirubin (TBIL), indirect bilirubin (IBIL), SUA, serum creatinine (Scr), total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL), high-density lipoprotein (HDL), white blood cell (WBC), platelet (PLT), and absolute value of neutrophils (NEUT).

Collection of Stool Samples

Approximately 1 g of fresh stool specimen was collected from each study subject, placed the microbial sample preservation tube at −20°C, and transferred to −80°C for cryopreservation within

30 min. All blood and stool samples were collected before 10 o'clock on the day.

Statistical Analysis

R program (version 4.0.5) was used for the statistical analyses. Before analyzing the differences of the gut microbiota, we removed species with low expression levels and occurrence rates (positivity rates <10%). The laboratory test, demography, bacterial species, and pathways were analyzed by standardized statistical test methods. The main statistical methods were student's *t* test, chi-square tests, and Wilcoxon rank sum test. The continuous variables were expressed as mean ± SD ($\bar{x}\pm$ s) and classified variables were represented by counts. Normal test and homogeneity test were applied to analyze the difference between groups, and $P \geq 0.05$ was selected as the normal and homogeneity variances. Then, we used parametric test (*t* test) or non-parametric test (rank sum test) and $P < 0.05$ was regarded as statistically significant. The correlations among bacterial diversity, microbial pathways, and biochemical indicators were evaluated by the R function "cor.test" with Spearman method. The "ADE4" package in R program was applied to perform the principal coordinate analysis (PCoA) and the "vegan" package was used to calculate observed species number (obs) and Spearman index for each sample.

DNA Extraction, Shotgun Metagenomic Sequencing, and Reads Quantity Control

We extracted DNA from 1770 stool samples according to the operating instructions of the MagPure Stool DNA KF kit. All samples were tested using DNA nanospheres (DNB) based on DNA libraries construction and probe-anchored synthesis technology (cPAS) (MGI2000, MGI, Shenzhen, China) based on 100 bp paired-end reads of shotgun metagenomic sequencing. The overall accuracy (OA $\geq$ 0.8) control strategy were used to make sure the quality control (QC) of raw sequencing reads to filter out low-quality reads (13, 14). SOAPaligner/soap2 was applied to filter out human reads and hg19 was selected as the standard point of the high-quality reads (identity $\geq$ 0.9) (RRID:SCR_005503).

Microbiota Composition and Function Profiling

The taxonomic annotation and quantification on the base of MetaPhlAn2 with default settings were performed (15), and the generating gut microbial profiling of all levels such as bacteria, archaea, viruses, and eukaryotes were included. The HMP Unified Metabolic Analysis Network 2 (HUMAnN2) and National Center for Biotechnology Information (NCBI) (nlm.nih.gov) database (2014 Edition) were applied to annotate the nonredundant gene set and the functional genes into Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway, and then the corresponding metabolic pathways were generated (13, 14).

RESULTS

Clinical Characteristics of Subjects

A total of 69 patients with HUA (HUA group) and 118 healthy participants (healthy controls) were included in this cross-sectional cohort study. The level of SUA in the HUA group was significantly higher than that of the control group, accompanied by WC, BMI, AST, ALT, GGT, Scr, and TG were all higher than the control group, while HDL was lower than the control group. The differences were statistically significant (all $P < 0.05$) (**Table 1**).

Community Diversity in HUA Group and Healthy Controls

As shown in **Figure 1B**, four main abundant phylas dominated in the gut microbiota were discovered at the phylum level and there were significant differences in the phylum of *Bacteroidetes*, *Firmicutes,* and *Actinobacteria* between the two groups (**Supplementary Table 1**). Alpha and beta diversity was used for the evaluation of the community diversity at the species level. In **Figure 1C**, alpha diversity by obs was significantly different between HUA group and healthy controls (Wilcoxon rank sum test; $P < 0.05$). We then investigated the community variance among participants based on beta diversity by Pearson distance. Comparisons of the Pearson distance between the two groups showed significant differences in the first and second principal components (Wilcoxon rank sum test; $P < 0.05$) (**Figure 1D**).

Analysis of the Composition of Gut Microbiota Between HUA and Healthy Controls and the Correlation Among the Gut Microbiomes With a Panel of Clinical Characteristics

Analysis of the Composition of Gut Microbiota Between HUA and Healthy Controls

Totally 75 different species were identified between HUA group and healthy controls at the species level (Wilcoxon rank sum test; $P < 0.05$) (**Supplementary Table 2**), and 51 species were of significant difference after removing the low occurrence rate and low abundance species ($P < 0.05$; **Figure 2A**). In **Figure 2A,** we can see 37 species were enriched in the healthy controls and 14 species were enriched in the HUA group. From the view of species-level analysis, three high-abundance species (i.e., *Ruminococcus gnavus* and *torques* and *Lachnospiraceae bacterium 1_4_56FAA*) belonging to the *Firmicutes* were enriched in HUA group; and 12 species (i.e., *Alistipes finegoldii, senegalensis* and *shahii; Bacteroides caccae, faecis, intestinalis, ovatus, plebeius, uniformis* and *xylanisolvens, Odoribacter splanchnicus* and *Parabacteroides distasonis*) belonging to the *Bacteroidetes*; *five* species (i.e., Roseburia hominis, *Ruminococcus callidus* and *lactaris, Lachnospiraceae bacterium 7_1_58FAA,* and *Coprococcus_sp_ART55_1*) belonging to the *Firmicutes*; *three* species (i.e., *Adlercreutzia equolifaciens; Butyricimonas synergistica,* and *Akkermansia muciniphila*) belonging to other were enriched in healthy controls (**Figure 2B**).

TABLE 1 | The major demographic and serum features in males of HUA group and healthy controls.

Feature	HUA ($n = 69$)	Healthy ($n = 118$)	*P*
Age (year)	41.217 ± 10.345	43.805 ± 11.144	0.111
WC (cm)	92.622 ± 7.320	87.451 ± 8.352	<0.001***
BMI (Kg/m^2)	26.951 ± 2.880	24.595 ± 3.120	<0.001***
FPG (mmol/L)	5.194 ± 0.535	5.092 ± 0.464	0.190
DBP (mmHg)	81.319 ± 9.118	78.715 ± 9.929	0.070
SBP (mmHg)	129.493 ± 13.142	126.963 ± 14.468	0.223
AST (U/L)	24.841 ± 10.084	22.102 ± 5.641	0.041*
ALT (U/L)	33.942 ± 24.315	23.949 ± 9.718	0.002**
GGT (U/L)	47.565 ± 37.338	29.415 ± 23.757	<0.001***
TBIL (μmol/L)	13.126 ± 7.503	13.331 ± 7.422	0.857
IBIL (μmol/L)	8.03 ± 5.303	8.153 ± 5.699	0.883
SUA (μmol/L)	470.652 ± 48.647	330.212 ± 50.939	<0.001***
Scr (μmol/L)	80.681 ± 10.830	75.297 ± 9.111	<0.001***
TC (mmol/L)	4.935 ± 0.876	4.724 ± 0.902	0.119
TG (mmol/L)	2.311 ± 1.431	1.381 ± 0.756	<0.001***
LDL (mmol/L)	3.056 ± 0.778	2.94 ± 0.790	0.329
HDL (mmol/L)	1.204 ± 0.227	1.408 ± 0.346	<0.001***
WBC (10^9/L)	6.443 ± 1.267	6.051 ± 1.406	0.051
PLT (10^9/L)	230.899 ± 49.458	221.398 ± 48.270	0.203
NEUT (10^9/L)	3.699 ± 1.089	3.477 ± 1.023	0.169
NLR	1.847 ± 0.722	1.829 ± 0.619	0.858

*WC, waist circumference; BMI, body mass index; FPG, fasting plasma glucose; DBP, diastolic blood pressure; SBP, systolic blood pressure; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, glutamyl transpeptidase; TBIL, total bilirubin; IBIL, indirect bilirubin; SUA, serum uric acid; Scr, serum creatinine; TC, total cholesterol; TG, triglyceride; LDL, low-density lipoprotein; HDL, high-density lipoprotein; WBC, white blood cell; PLT, platelet; NEUT, absolute value of neutrophil; NLR, Neutrophil to lymphocyte ratio. *, **, and *** denote P < 0.05, 0.01, and 0.001 with statistical significance compared with healthy controls, respectively.*

Correlation Among the Gut Microbiomes With a Panel of Clinical Characteristics

Spearman's correlation analysis was applied to explore the association between the species abundances and clinical characteristics in this article. Totally 187 samples from HUA subjects and healthy controls were analyzed and 187 associations with original P values < 0.05 were found out. In **Figure 2C**, we could see that except HDL, TBIL, and IBIL, most of the gut microbiomes enriched in HUA group had significant positive correlations with the clinical characteristics, while those microbiomes enriched in the healthy controls were highly negatively correlated with these clinical indexes (**Supplementary Table 3**). Among the clinical parameters, SUA and TG had the largest number of correlations with bacterial species ($n = 26$ and $n = 28$ respectively, $P < 0.05$), followed by GGT ($n = 21$, $P < 0.05$), WC ($n = 19$, $P < 0.05$), BMI ($n = 15$, $P < 0.05$), TC ($n = 14$, $P < 0.05$), ALT ($n = 13$, $P < 0.05$), LDL, and HDL (both $n = 10$, $P < 0.05$). Besides, *Roseburia hominis* had the most frequent correlations with clinical parameters among the 51 bacterial species ($n = 10$, $P < 0.05$), followed by *Odoribacter splanchnicus* and *Adlerreutzia equolifaciens* (both $n = 8$, $P < 0.05$), *Butyricimonas synergistica, Bacteroides intestinalis, Bacteroides faecis, Alistipes senegalensis, Alistipes fonegoldii, Lachnospiraceae bacterium 1_4_56FAA,* and *Ruminococcus gnavus* (all $n = 7$, $P < 0.05$), *Lachnospiraceae bacterium 3_1_46FAA* ($n = 6$, $P < 0.05$), among the above bacterial species, two were enriched in HUA group (*Ruminococcus gnavus* and *Lachnospiraceae bacterium 1_4_56FAA*).

Functional Changes Brought About by the Microbiome in HUA Group and Healthy Controls

The Functional Differences From the Comparison of Different Participants

For each sample, MetaCyc pathway database by HUMAnN2 was further used to build the functional profiles with 494 pathways. Totally 41 pathways were identified in the comparison of MetaCyc pathway abundance between HUA group and healthy controls (Wilcoxon rank sum test; $P < 0.05$), and 28 pathways were of significant difference after removing the ones that were in low abundance and occurrence (**Supplementary Table 4**, **Figure 3**). Among the 28 pathways, 12 were enriched in healthy controls and 16 were enriched in HUA group. Within the 12 pathways in healthy controls, two were involved in the way of fermentation to SCFAs (CENTFERM-PWY and PWY-6590);one was related to the biosynthesis of GDP mannose (PWY-7323); five were related to the biosynthesis of gluconeogenesis (GLUCONEO-PWY and PWY66-399), pyrimidine deoxyribonucleotides (pwy-7198 and pwy-7210),

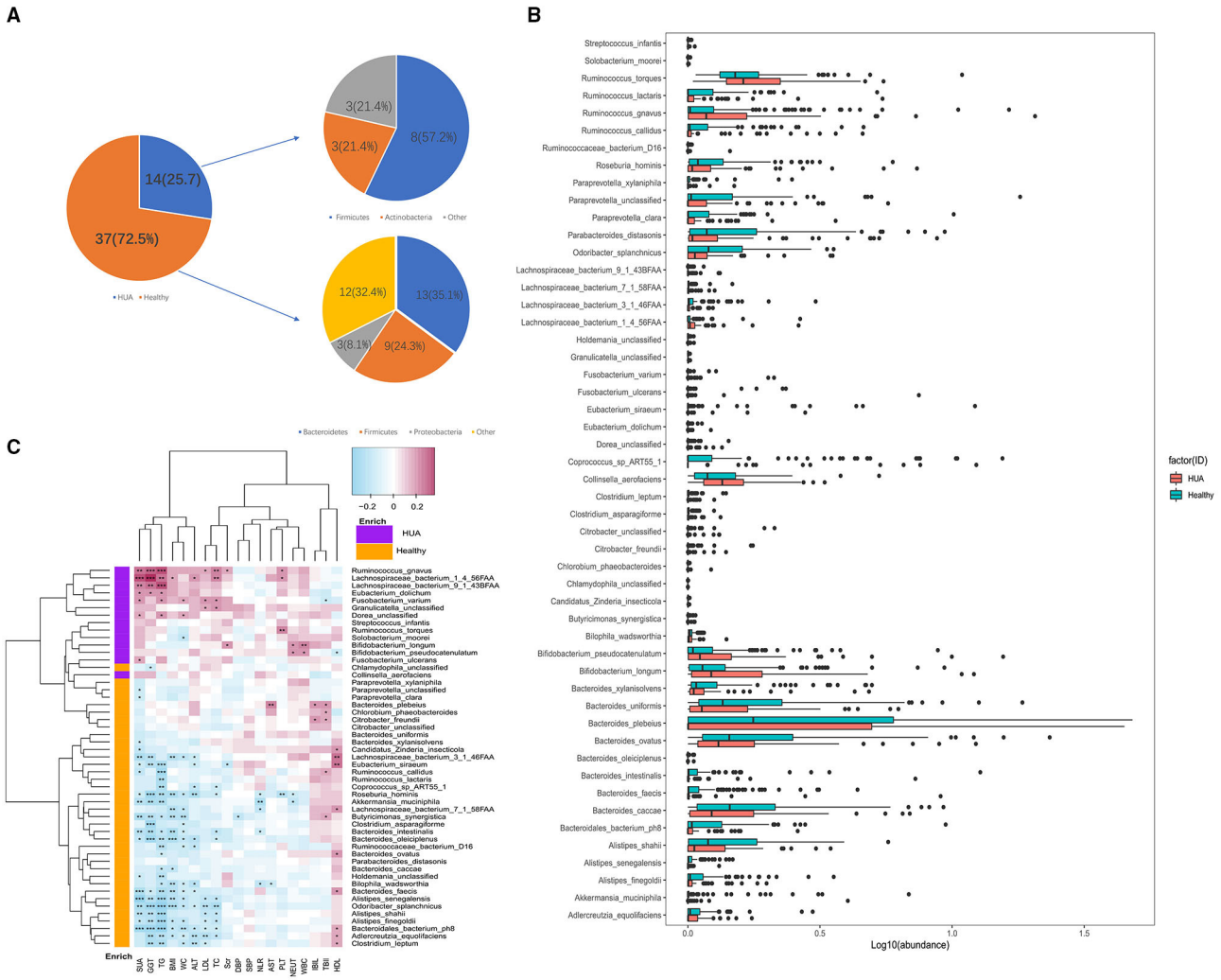

FIGURE 2 | Microbiome differences between HUA group and healthy controls and the correlations with clinical characteristics. **(A,B)** Abundance of bacterial species that were of significant difference between HUA group and healthy controls. **(C)** Correlation matrix for bacterial species and clinical characteristics. Cells in red represented positive correlations while blue indicated negative. Cells with *, **, or *** asterisk represented $P < 0.05$, 0.01, or 0.001 respectively.

and carbohydrate such as colanic acid (COLANSYN-PWY); two were responsible for energy generation (pwy66-398 tricarboxylic acid cycle, TCA) and energy metabolism (PWY-7383); one was related to superpathway of guanosine nucleotides degradation (PWY-6595); one was for the interconversion of arginine, ornithine, and proline (ARGORNPROST-PWY). Within the 16 HUA-enriched pathways, nine were related to the biosynthesis of 5-aminoimidazole ribonucleotide (PWY-6122, PWY-6277, and PWY-6121); pyrimidine deoxyribonucleotides (PWY-6545), aromatic amino acids, and chorismate (COMPLETE-ARO-PWY, ARO-PWY and PWY-6163) and tetrapyrrole (PWY-5188); one was responsible for L-glutamine biosynthesisIII, which is the way of nitrogen remobilization (PWY-6549); one was for purine ribonucleosides degradation (PWY0-1296); one was related to carbohydrate degradation, utilization, and assimilation (PWY-6737); one was involved in L-glutamate degradation (P162-PWY); one was for energy generation (PWY-5913); one was for the biosynthesis superpathway of L-lysine, L-threonine, and L-methionine II (PWY-724); and one was related to C4 photosynthetic carbon assimilation cycle (PWY-7117).

Correlation Between Microbial Pathways and a Panel of Clinical Characteristics

Spearman's correlation analyses were applied for the analysis of MetaCyc pathways with significant difference and a panel of clinical characteristics, and then heatmaps were constructed ($P < 0.05$) (**Figure 4**, **Supplementary Table 5**). In **Figure 4**, we could see that except HDL, most of the pathways enriched in HUA group had highly positive correlations with the clinical characteristics, while those pathways enriched in the healthy controls mostly had significant negative correlations with these clinical indexes. Especially, SUA, TC, TG, LDL, BMI, and GGT had highly negative correlations with the pathway of producing SCFAs, such as PWY-6590 and CENTFERM-PWY (*P*

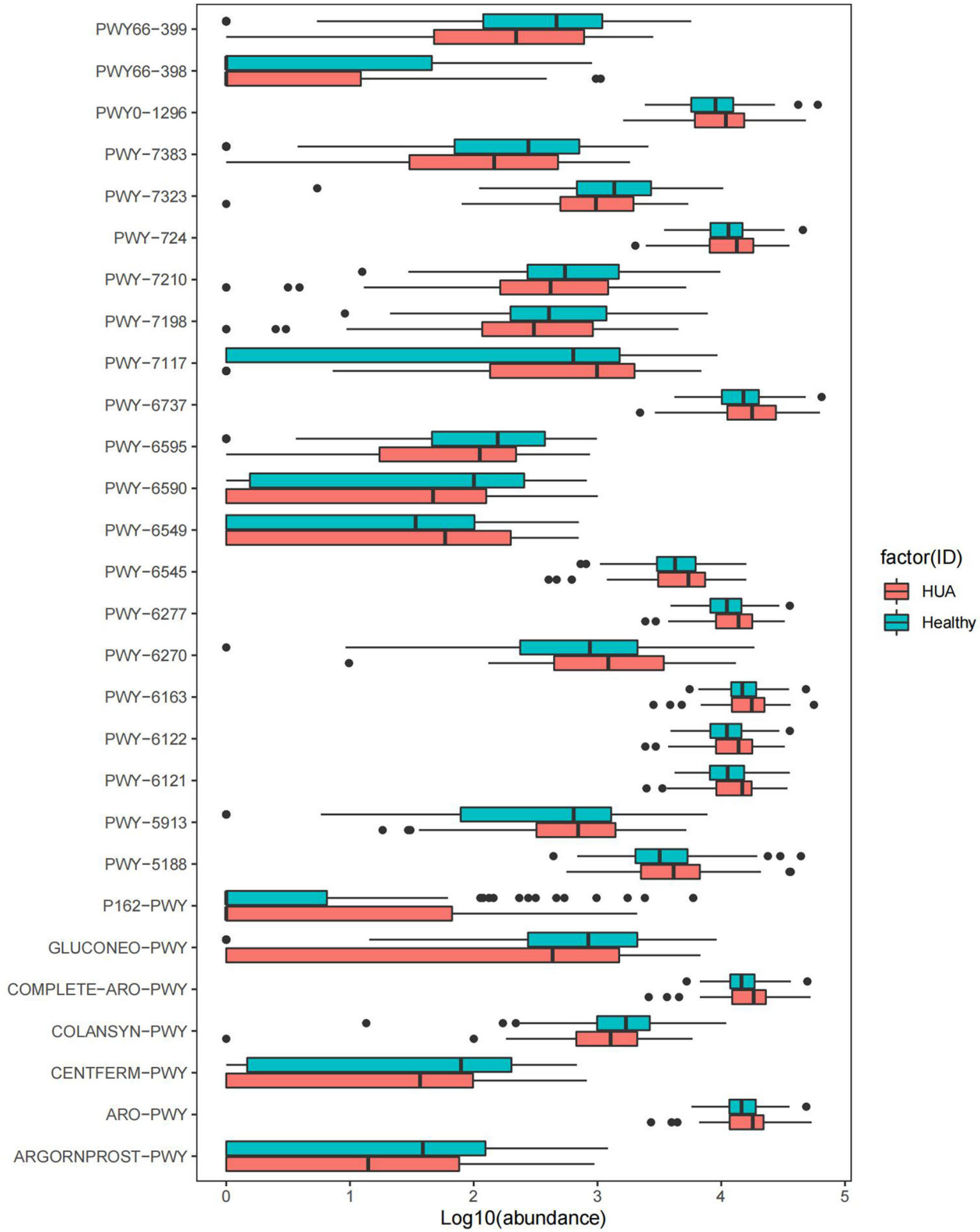

FIGURE 3 | MetaCyc pathways with significant difference abundance between HUA group and healthy controls (Wilcoxon rank sum test, $P < 0.05$).

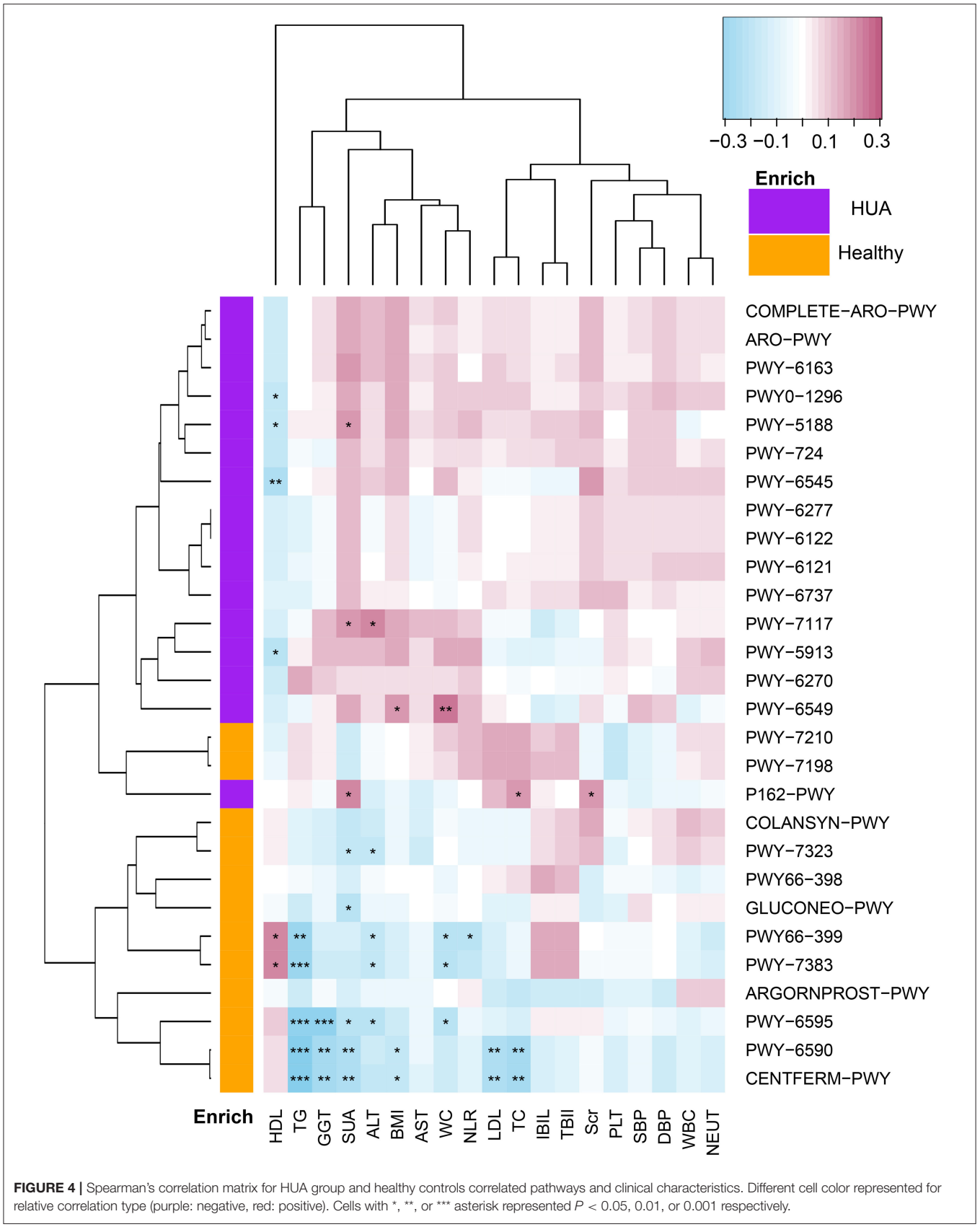

FIGURE 4 | Spearman's correlation matrix for HUA group and healthy controls correlated pathways and clinical characteristics. Different cell color represented for relative correlation type (purple: negative, red: positive). Cells with *, **, or *** asterisk represented $P < 0.05$, 0.01, or 0.001 respectively.

< 0.05). Most of the above clinical index also had significantly negative correlation with PWY-6595, which is responsible for the degradation from purine to allantoin ($P < 0.05$). Besides, TG, ALT, and WC had highly negative correlations with the pathway of PWY66-399 and PWY-7383, while HDL is just the opposite ($P < 0.05$).

Correlation Between Microbial Pathways and a Panel of Bacterial Species

Spearman's correlation analyses were applied to analyze the correlations between bacterial species and microbial pathways ($P < 0.05$) and heatmaps were constructed with Spearman's correlations (**Figure 5**, **Supplementary Table 6**). Apparently, most of the bacterial species enriched in healthy controls had highly positive correlations with the pathways enriched in healthy group, and negative correlations with the pathways enriched in HUA group, while those pathways enriched in the HUA group had the opposite correlation trend. In particular, pwy-6590 and TFERM-pwy pathways had the stronger correlation with most of the bacterial species that can produce butyric acid enriched in healthy controls such as *Odoribacter splanchnicus, Roseburia hominis, Bacteroides uniformis, Lachnospiraceae bacterium 3_1_46FAA, Ruminococcus callidus,* and *Butyricimonas synergistica*. Besides, Lachnospiraceae bacterium 1_4_56FAA, *Ruminococcus gnavuswas and torques, Streptococcus infantis,* and *Solobacterium moorei* had significantly positive correlation with the pathway of producing 5-aminoimidazole ribonucleotide (pwy-6121, pwy-6122 and pwy-6277) and aromatic amino acids (pwy-6316, ARO-PWY and COMPLETE-ARO-PWY), and most of them are conditional pathogens.

DISCUSSIONS

It has been recognized that disturbances of the normal gut microbiome participate in the pathogenesis and progression of diverse chronic diseases, such as obesity (16), diabetes (17), hypertension (18), and liver cirrhosis (19), and the effect of gut microbiota in HUA has been explored gradually in recent years. For example, the current known interactions between the gut microbiota and HUA include that certain microbiota can regulate the synthesis and catabolism of purine and uric acid (such as *Escherichia coli* and *Lactobacillus*), produce some SCFAs or change the number and distribution of uric acid transporter. All of those studies have shown a strong correlation between the gut microbiota and HUA. However, most of them are animal experiments and the microbiota sequencing method is 16S rRNA, specific alterations of the gut microbiota composition and function between HUA (especially in males with HUA and high levels of liver enzymes) in asymptomatic people for physical examination have been rarely reported.

In view of the fact that most patients with HUA are asymptomatic and the intestinal microecology can change correspondingly in other disease states, our study is the first to select asymptomatic people for physical examination as the research subjects to explore the gut microbiome disturbances in males with HUA and high levels of liver enzymes and choose whole-genome shotgun sequencing technology, which are the two highlights and advantages of this research. Our results elucidated that there may do exist some targeted biomarkers of the gut microbiota and have the potential to be used as non-invasive, safe, and easy diagnostic tools for HUA. The new targeted biomarkers could be applied as a supplement to the traditional HUA diagnostic method to some extent.

In this study, we found that the level of SUA accompanied by WC, BMI, AST, ALT, GGT, Scr, and TG in the HUA group were significantly higher than that of the control group, while HDL was lower than the control group. Besides, the levels of FPG, blood pressure, TC, LDL, and inflammation indicators (WBC, PLT, NEUT, and NLR) were slightly, but not significantly, higher in the HUA group than in the healthy controls, TBIL and IBIL were of the opposite trend. Previous studies have confirmed that HUA is closely related to the occurrence of gout, metabolic syndrome, type 2 diabetes, hypertension, cardiovascular disease, chronic kidney disease, and so on, and it is an independent risk factor for the disease (20), which is consistent with our results. Numerous studies have shown that HUA is closely related to metabolic syndrome (obesity, hypertension, hyperlipidemia, insulin resistance), and the two factors are mutually causal and form a vicious circle (21–24). This comorbidity of chronic diseases suggests that there is a certain correlation between chronic diseases. Our results also suggest that HUA is associated with obesity and metabolism-related indicators, such as high levels of liver enzymes (5, 25), worse homeostasis in lipid and bilirubin metabolism, and poorer kidney function. Although the mean hepatic function was within the "current consensus normal reference range," it had been already at a high level. Study has verified that when ALT exceeds 26 U/L, the occurrence of liver fibrosis increased, and it is suitable to adjust the reference range of liver function (26). In short, HUA is a metabolic disease related to habits, customs, age, gender, and inherited factors, and HUA is also correlated with obesity, hypertension, diabetes, renal failure, dyslipidemia, and liver diseases (27–29). Of course, the specific mechanism needs further study.

As for the gut microbiotas, we found that the gut bacterial diversity in HUA group was reduced significantly compared with that of healthy controls and the community of the microbiota was also of significant difference between the two groups. Just as Requena et al. (30) had mentioned the diversity of microbiota was closely associated with our health, the result of our study indicated that the gut microbiota may have altered significantly from a normal healthy status to the development of HUA. In the phyla level, compared with healthy controls, the abundance of Bacteroidetes in HUA group decreased [consistent with the previous research result (31)], while Firmicutes and Actinobacteria increased significantly. At the species level, totally 51 species were of significant difference after removing the low occurrence rate and low abundance species. Among them, 37 species were enriched in the healthy controls and 14 species were enriched in the HUA group. This suggests that maybe it is these significantly different populations in abundance that cause the significant changes in the composition of gut microbiota HUA.

Among the 51 species, compared with the species of healthy controls, the relative abundances of Bacteroidetes (Bacteroides

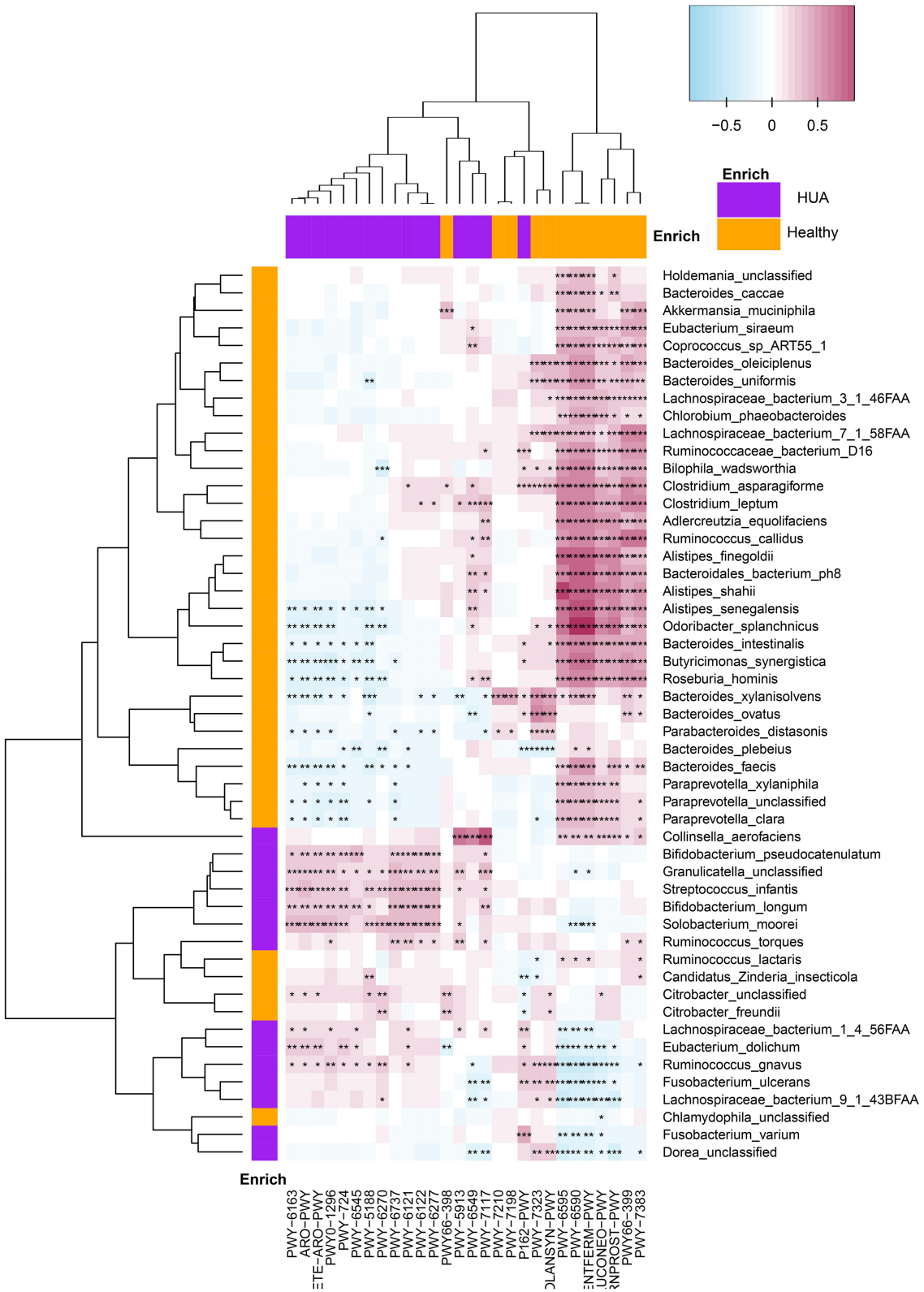

FIGURE 5 | Spearman's correlation matrix for microbial pathways and bacterial species in HUA group and healthy controls. Different cell color represented for relative correlation type (purple: negative, red: positive). Cells with *, **, or *** asterisk represented $P < 0.05$, 0.01, or 0.001 respectively.

uniformis, caccae, ovatus and plebeius, Alistipes finegoldii, senegalensis, and shahii); Firmicutes (Roseburia hominis, Ruminococcus callidus and lactaris, and Lachnospiraceae bacterium 3_1_46FAA); other (Adlercreutzia equolifaciens, Odoribacter splanchnicus, Butyricimonas synergistica, and Akkermansia muciniphila) decreased in the HUA group; while Firmicutes (Ruminococcus gnabus and torques, Lachnospiraceae bacterium 9_1_43FAA); Actinobacteria (Collinsella aerofaciens) increased. Interestingly, most of the gut microbiomes enriched in HUA group not only had significant positive correlations with the clinical characteristics, but also most of them are responsible for the production of SCFAs, while those microbiomes enriched in the healthy controls had highly negatively correlated with these clinical indexes.

In our study, some species that are known as SCFAs (e.g. butanoate) producer [*Roseburia hominis* (32, 33), *Odoribacter splanchnicus* (34), *Ruminococcus callidus* (35), *Lachnospiraceae bacterium 3_1_46FAA* (36), *Bacteroides uniformis* (37) and *Butyricimonas synergistica* (38)], together with the metabolic pathway of producing butanoate (CENTFERM-PWY and PWY-6590) were enriched in the healthy controls. Importantly, both of the two pathways had significantly positive correlations with the above bacterials. Especially, the bacterial of *Odoribacter splanchnicus* had the strongest correlation with them (r = 0.903 and 0.902 relatively) and had frequent correlations with clinical parameters, *Roseburia hominis* was the bacteria that most negatively correlated with clinical parameters and can produce large amounts of SCFAs, which play key protective roles against inflammation (39–41). As is known to all, *Odoribacter splanchnicus* is expressed in human intestine and its complete genome had been sequenced (42), and now it is recognized as a common butyric-acid producing bacterium, which has been reclassified from Bacteroides splanchnicus (34). In a recent study, *Odoribacter splanchnicus* together with *Akkermansia muciniphila*, has been associated with a healthy fasting serum lipid profile (43). The administration of prebiotics and the use of polyphenols have been stated as effective dietary strategies to modulate gut microbiota composition, which could induce the abundance of friendly bacteria, namely *Akkermansia muciniphila*, associated to health benefits in metabolic syndrome (44–46). Our study evidenced that HUA state may reduce or inhibit the growth of those "beneficial" bacterial members.

Besides, another relevant bacterium was *Adlercreutzia equolifaciens*, which was enriched in the healthy controls. *A. equolifaciens* is involved in metabolizing polyphenols and produced bioactive molecules involved in ameliorating metabolic disorders in obesity and diabetes (47). The study had verified that it can convert ingested isoflavones, which is abundant in legumes and soya beans, into equol (48). Equol has a high affinity for the estrogen receptor (49) and may be a selective estrogen receptor modulator. The incidence of HUA is high in men and postmenopausal women, and studies have concluded that it is the estrogen in premenopausal women that reduces its incidence through the follow ways, such as estrogen can directly affect the kidney excretion of uric acid by regulating transporters (49–51); inhibit the xanthine oxidase system (52); reduce the production of uric acid by maintaining the stability of lipid metabolism (53–55). Thus, we can propose *Adlercreutzia equolifaciens* may make contributions to alleviate the damage of HUA to our body in some estrogen-protection way. This finding is of great significance and maybe can provide a direction for the further exploration of new therapeutic targets for HUA based on gut microbiota.

On the contrary, *Lachnospiraceae bacterium 1_4_56FAA, Ruminococcus gnavuswas and torques* were enriched in the HUA group. Not only did they had positive correlations with most clinical indexes, but also the pathways that are significantly enriched in HUA group, such as superpathway biosynthesis of 5-aminoimidazole ribonucleotide (PWY-6122, PWY-6277 and PWY-6121), aromatic amino acids and chorismate synthesis (COMPLETE-ARO-PWY, ARO-PWY and PWY-6163), and L-lysine, L-threonine and L-methionine biosynthesis (PWY-724). Consistent with our results, all of the current studies have shown that the above three bacterials are closely related to immune inflammation in different diseases (56–60), though the inflammation indicators (WBC, PLT, NEUT, and NLR) were slightly, but not significantly, higher in the HUA group than in the healthy controls. Especially, *R. gnavus* can produce specific antigens and stimulates immune cells to produce corresponding antibodies, thus increasing inflammation (61, 62). As to the functional alterations, 5-aminoimidazole ribonucleotide is the key intermediate for purine nucleotide biosynthesis, the increased biosynthesis of 5-aminoimidazole ribonucleotide may increase the production of purines, thereby increase the level of uric acid. Besides, the aromatic amino acids and chorismate can act as substrates in other secondary metabolite pathways, such as indole derivatives and phenolic compounds, which are just the metabolites of our gut microbiotas. So far, no studies have been able to confirm the correlations between these structural and functional alterations of gut microbiota in HUA, but their significant alterations suggest that they may have important physiological significance in the occurrence and development of HUA, which is a research direction worthy for our further study.

There are some limitations in our study. First, this is a cross-sectional study, and it is not able to verify the causality between the discovered species and HUA, hence, further germ-free mice studies are still needed. Second, the study subjects come from the same region, thus, it is better to conduct a multicenter study from different regions owing to the influence of different regions and eating habits on the gut microbiota. Third, the gender in the HUA group is male adult, therefore, it is necessary to be cautious temporarily to apply the results obtained in this article to the female, minors or other population.

In conclusion, our study demonstrated the structural and functional alterations of gut microbiota in males with HUA and high levels of liver enzymes. In our study, the main clues of the correlation mechanisms between HUA and gut microbiome point to the pathways that can produce 5-aminoimidazole ribonucleotide, aromatic amino acids, and chorismate, which were enriched in the HUA group; the pathways that can produce SCFAs and the gut microbiotas that can produce SCFAs and

equol were enriched in healthy controls. Generally, the potential mechanisms underlying the gut microbiome-Equol/SCFAs-uric acid accumulation link remains much for further exploration. The novel correlations between some microbiota species/pathways and uric acid accumulation could provide brand new directions for specific microbiota-targeted therapies.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ebi.ac.uk/ena,PRJEB48022.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee from the First Affiliated Hospital of Zhengzhou University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SD and SS: Conceptualization. JC, YW, and AL: Methodology and formal analysis. QQ, YZ, WL, TL, and XG: Resources. SS: Writing and original draft preparation. SD, LT, and AL: Writing and review and editing. JC and SY: Visualization. All authors have read and agreed to submit the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.779994/full#supplementary-material

Supplementary Table | The detailed experimental results between HUA group and healthy controls.

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REVIEW
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Contribution of the Gut Microbiota to Intestinal Fibrosis in Crohn's Disease

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In Crohn's disease (CD), intestinal fibrosis is a critical determinant of a patient's prognosis. Although inflammation may be a prerequisite for the initiation of intestinal fibrosis, research shows that the progression or continuation of intestinal fibrosis can occur independently of inflammation. Thus, once initiated, intestinal fibrosis may persist even if medical treatment controls inflammation. Clearly, an understanding of the pathophysiological mechanisms of intestinal fibrosis is required to diminish its occurrence. Accumulating evidence suggests that the gut microbiota contributes to the pathogenesis of intestinal fibrosis. For example, the presence of antibodies against gut microbes can predict which CD patients will have intestinal complications. In addition, microbial ligands can activate intestinal fibroblasts, thereby inducing the production of extracellular matrix. Moreover, in various animal models, bacterial infection can lead to the development of intestinal fibrosis. In this review, we summarize the current knowledge of the link between intestinal fibrosis in CD and the gut microbiota. We highlight basic science and clinical evidence that the gut microbiota can be causative for intestinal fibrosis in CD and provide valuable information about the animal models used to investigate intestinal fibrosis.

Keywords: gut microbiota, Crohn's disease, animal model, adherent-invasive *Escherichia coli* (AIEC), intestinal fibrosis

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INTRODUCTION

About a third of patients with Crohn's disease (CD) exhibit a distinct phenotype of intestinal fibrosis and stenosis over a period of 10 years (1). During the clinical course of their disease most of these patients undergo surgery or endoscopic dilation to relieve the symptoms of obstruction. Postoperative recurrence rates are high: 11–32% at 5 years, 20–44% at 10 years, and 46–55% at 20 years (2). This disease course prompted the development of several new biologics, such as vedolizumab, a monoclonal antibody against α4b7 integrin, and ustekinumab, an antibody against interleukin (IL)-12/23, in the field of CD therapy. However, mechanical treatments remain the only practical method to treat obstructive complications (3, 4). Therefore, elucidation of the cellular and molecular mechanisms of intestinal fibrosis in CD is required to improve patients' quality of life.

Why do CD patients develop intestinal fibrosis? It is a generally accepted that inflammatory bowel disease (IBD), consisting of CD and ulcerative colitis, is caused by a loss of tolerance to the gut resident bacteria, which evokes an excessive immune response in a genetically susceptible host (5). Although IBD is a multifactorial disease, human genetic studies support the association of the gut microbiota with the etiology of intestinal fibrosis. CD patients who carry mutations at the nucleotide-binding oligomerization domain 2 (NOD2) locus tend to display the fibrotic phenotype more frequently (6). The dysfunction of bacterial sensing caused by NOD2 mutations implies that

intestinal fibrosis is due to the dysfunction of the recognition of the gut microbiota. In addition, several mouse models have shown that specific bacteria taxa (e.g., *Salmonella* spp.) directly induce a profibrogenic response in the gastrointestinal tract (7).

It has been shown that chronic tissue damage, impaired wound healing, and the expansion of mesenchymal cells are associated with the development of fibrosis. Multiple complex mechanisms involve several cellular components, including mesenchymal cells and immune cells. Physiologically, intestinal fibrosis is the result of an excessive accumulation of the extracellular matrix (ECM). Mesenchymal cells, such as myofibroblasts and stellate cells, serve as the main ECM producers and play a central role in the pathogenesis of fibrosis (8). Studies of cell biology have shown that microbial components affect mesenchymal cell differentiation (9), and that myofibroblasts proliferate at a faster pace in IBD patients compared to healthy individuals (10). These studies highlighted the importance of the gut microbiota in the pathophysiology of intestinal fibrosis.

On the other hand, chronic inflammation is known to be necessary for the initiation of fibrosis, based on the evidence that inflammation promotes mesenchymal differentiation, activation, and proliferation. However, an animal study using mice infected with *Salmonella enterica* serovar Typhimurium showed that eradication of the pathogen using antibiotics in the early phase of the fibrotic process did not prevent intestinal fibrosis formation (11). Despite major therapeutic advances that focus on the suppression of inflammation, the incidence rate of intestinal complications, including stricture and penetration, in CD patients has not markedly changed (12). These observations suggest that the suppression of inflammation does not simply change the clinical consequences of intestinal fibrosis in CD patients. In this context, the gut microbiota can directly activate pro-fibrotic process in myofibroblasts in addition to indirect activation of fibrotic processes through inducing inflammatory responses. However, the notion that inflammation-independent mechanisms may mediate a self-perpetuating intestinal fibrosis has not been elucidated well, leaving a knowledge gap in terms of the precise mechanisms by which the gut microbiota promotes the pathophysiology of intestinal fibrosis.

Herein, we review the insights of clinical and basic science research that link intestinal fibrosis and the gut microbiota. We highlight the cellular and molecular mechanisms by which the gut microbiota induces the formation of intestinal fibrosis.

CLINICAL EVIDENCE

The Gut Microbiota and the Pathogenesis of Crohn's Disease

Clinically, it is known that recurrent CD can be prevented by postoperative diversion of the fecal stream (13–17). Fecal diversion surgery such as ileostomy or colostomy is indicated for patients who have advanced perianal or colorectal CD as it promotes mucosal healing and resolution of perianal disease. Most individuals who undergo fecal diversion surgery exhibit striking clinical improvement within 3–6 months, and a substantial proportion of these patients achieve remission in the long term (13–17). It has also been shown that treatment with antibiotics confers notable benefits on the clinical course of CD (18–25). These studies suggest that the eradication of certain populations of bacteria has a beneficial influence on clinical outcome for patients with CD.

Human genetic studies revealed that individuals who carry variants of the NOD2 gene are more susceptible to CD (26–28). A detailed study including disease subphenotype analysis confirmed that NOD2 has the largest effect on the development of CD and is strongly associated with ileal disease (29). Biologically, NOD2 functions as an intracellular pattern recognition receptor (PRR) for muramyl dipeptide, which is derived from peptidoglycan of both gram-positive and gram-negative bacteria (30). After intracellular stimulation by bacterial products, NOD2 activates the nuclear factor kappa B (NF-κB) pathway and provides a defensive response to protect the host from bacterial infection. A study of mice revealed that variants of the NOD2 gene directly influence intestinal inflammation and bacterial translocation. Maeda and colleagues reported that mice carrying the homozygous NOD21007fs variant have an increased activation of NF-κB after exposure to muramyl dipeptide, which increases susceptibility to bacteria-induced intestinal inflammation, thereby compromising the integrity of the intestinal barrier (31). The study of intestinal biopsies from patients with CD revealed that the presence of NOD2 variants (especially R702W and 1007fs) is associated with increased NF-κB activation and altered epithelial cell–cell contacts, leading to higher intramucosal levels of endotoxin (32). Notably, these studies indicate that a dysfunctional bacterial sensing mechanism in the host can trigger the development of CD. In addition, given that NOD2 recruits ATG16L1 to the plasma membrane, the failure to do so, as occurs in the presence of NOD2 mutants, ultimately impairs autophagosomal encapsulation of invading bacteria in dendritic cells (33, 34). In line with this function, a single nucleotide polymorphism (SNP) in ATG16L1 (re2241880, Thr300Ala) appears to be associated with an increased risk of CD (35, 36).

The introduction of culture-independent techniques to analyze 16S rRNA gene sequences facilitated a more in-depth analysis of the composition of the gut microbiota (37). It was shown that CD is associated with gut dysbiosis, a condition characterized by an imbalance between protective and harmful bacteria (38). A consistent finding of 16S rRNA gene sequencing analysis was the increase in the abundance of members of the phylum Proteobacteria (gram-negative rods, including *Escherichia* spp.) in CD patients compared with non-IBD or healthy controls, and a decrease in members of the phylum Firmicutes (gram-positive bacteria, including *Clostridium* and *Bacillus* spp.) (39). Many microbiome studies recognized the adherent–invasive strains of *Escherichia coli* (AIEC) within the *Enterobacteriaceae* family, which were often found in ileal biopsies of the patients with active CD (40, 41). Some studies suggested that the decreased abundance of the phylum Firmicutes is directly associated with the pathogenesis of CD by modulating immune functions in the intestine. Animal studies revealed that 17 strains within Clostridia clusters IV,

XIVa, and XVIII can induce regulatory T cells (Tregs) in the intestine (42, 43). In addition, *Faecalibacterium prausnitzii*, which belongs to *Clostridium* cluster IV, was identified as a key player in the dysbiosis associated with ileal CD (44), and shown to produce high amounts of butyrate that has beneficial effects on IBD (45). Intriguingly, a low abundance of *F. prausnitzii* is associated with an increased risk of future flares in CD (44). Moreover, *F. prausnitzii* appears to protect the host mucosa from inflammatory injury by favoring the production of antiinflammatory cytokines, such as IL-10 (44). These data support the notion that the composition of intestinal microbiota is one of the critical factors in the pathogenesis of CD.

The Gut Microbiota and Intestinal Fibrosis in Crohn's Disease

It has been shown that CD patients carrying a NOD2 variant, such as Arg702Trp, Gly908Arg, or the frameshift mutation Leu1007insC, are at increased risk for complications and surgery (46, 47). A metaanalysis showed that carriage of at least one NOD2 variant increased the risk of stricture in CD patients (odds ratio 1.94; 95% confidence interval 1.61–2.34) (48). Further, the presence of two NOD2 mutations predicted a 41% increase in the risk of complicated disease (i.e., the stricturing or fistulizing subtype of CD) and a 58% increase in the risk of surgery (49). These data support the idea that dysfunction of bacterial sensing by NOD2 triggers intestinal fibrosis in CD.

The increased production of microbial antibodies in serum also supports the contribution of the gut microbiota toward the pathogenesis of intestinal fibrosis in CD. The serum antibody to flagellin anti-CBir1, which reflects aberrant adaptive immunity to luminal commensal bacteria, is significantly elevated in CD patients (50). Anti-CBir1 has been shown to react with flagellins from *Clostridium* species in the gut (50). It is known that flagellins are important molecules, located on the bacterial surface and involved in both adhesion and motility (51). In addition, flagellin interacts with its toll-like receptor TLR5, leading to the activation of NF-κB and the subsequent transcriptional induction of many proinflammatory cytokines (50, 52). Dubinsky and colleagues showed that children with CD who have anti-CBir1, anti-*E. coli* outer-membrane protein C antibodies (anti-OmpC), anti-*Pseudomonas*-associated sequence I2 antibodies (anti-I2), and anti-*Saccharomyces cerevisiae* antibodies (ASCA) are at an 11-fold higher risk of developing strictures and fistulas compared to those who are seronegative (53). Also, an Irish study reported a significant association between serum anti-CBir1 positivity and a complicated disease behavior as well as ileal location (54). These results suggest that immune responses against gut microbes may contribute to the development of intestinal fibrosis.

In addition, microbiome analysis of CD patients provides information about the relationship between the gut microbiota and intestinal fibrosis. As mentioned, Sokol and colleagues reported the association of a reduced abundance of *F. prausnitzii* with an increased risk of postoperative recurrence of CD (55). Another study showed that the increased risk of CD recurrence after bowel resection was associated with enriched diversity in members of the *Enterobacteriaceae* family, and the maintenance of remission was associated with increased diversity in members of the *Lachnospiraceae* family, which reside within *Clostridium* cluster XIVa (56). Therefore, although clinical evidence of the direct influence of the gut microbiota on intestinal fibrosis has been insufficient, several studies suggest that specific microbiota contribute to the pathogenesis of intestinal fibrosis in CD.

Moreover, it is reported that intestinal myofibroblasts display different functional capacities between normal individuals and patients with IBD, particularly CD (10). Myofibroblasts isolated from CD patients proliferated faster than those derived from normal individuals and UC (10). Also, the expression patterns of TGF-β isoforms differ in CD compared to normal or UC. In CD myofibroblasts, TGF-β_3 is significantly reduced, while TGF-β_2 is enhanced compared to normal or UC (10). These results indicate that the differential functional capacity of myofibroblasts in CD may lead to the development of intestinal fibrosis. However, the involvement of the gut microbiota in the regulation of TGF-β isoforms remains unclear.

EXPERIMENTAL EVIDENCE THAT SUPPORTS THE ROLE OF MICROBIAL STIMULATION IN FIBROSIS

Direct Activation of Fibroblasts Through Microbial Ligands

Excessive ECM synthesis is a fundamental factor in the development of fibrostenosis. In IBD, myofibroblasts originate from numerous sources, including the cells of Cajal and subepithelial myofibroblasts. It is known that bacterial components directly activate intestinal myofibroblasts (**Figure 1**). Activated myofibroblasts are modified fibroblasts with smooth muscle–like features and considered to be responsible for the development of intestinal fibrosis. Once activated, myofibroblasts synthesize large quantities of ECM components; primarily collagen, glycosaminoglycans, tenascin, and fibronectin (57).

It is known that microbe-derived pathogen-associated molecular patterns (PAMPs) are sensed by pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) and Nod-like receptors (NLRs), expressed in intestinal immune and nonimmune cells (58). Likewise, mesenchymal cells in the intestine also express TLR1–9 and NOD1–2 (59). Among several receptors, TLR4 functions as the signaling receptor for lipopolysaccharide (LPS), the major component of the outer membrane of gram-negative bacteria (60), whereas TLR2 is activated by the cell wall components of gram-positive bacteria (61–63). Cultured intestinal myofibroblasts, once activated, secrete cytokine after TLR2 or TLR4 ligand stimulation (59). There is also evidence that intestinal fibroblasts respond to LPS by activating NF-κB signaling, which enhances collagen contraction (64).

As well as TLR2 and 4, TLR5 signaling is associated with the pathogenesis of intestinal fibrosis. Zhao et al. reported that a profibrogenic phenotype of intestinal fibroblasts is triggered exclusively by the TLR5 ligand flagellin (present in all flagellated

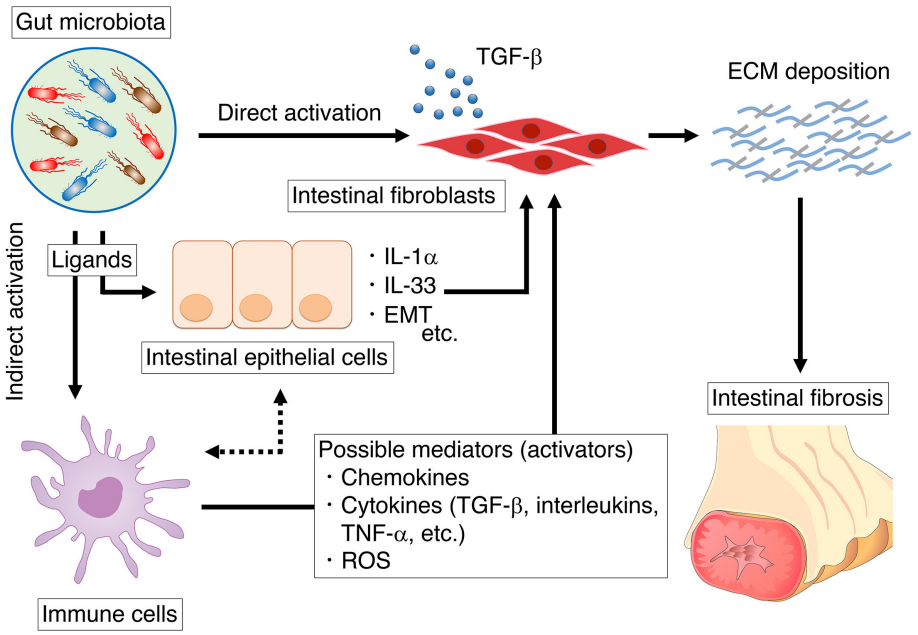

FIGURE 1 | The summary of mechanisms by which the gut microbiota influences intestinal fibroblasts. We summarize the direct influence of microbiota on intestinal fibroblast (section direct activation of fibroblasts through microbial ligands). In addition, we also summarize the indirect influence of microbiota on intestinal fibroblast *via* epithelial cells or immune cells (section indirect activation of fibroblasts through the microbial ligands).

bacteria), and this event is TGF-β1–independent and post-transcriptionally regulated (65) (**Figure 2**). In this study, the role of myofibroblasts to directly sense PAMPs in intestinal fibrosis was confirmed *in vivo*, as the selective deletion of MyD88 (the adaptor molecule for all TLRs except TLR3) in cells expressing α-smooth muscle actin (α-SMA) ameliorated intestinal fibrosis (65). Furthermore, the TLR5 ligand appears to promote cell cycle entry and proliferation of mesenchymal cells *in vitro* (67). Consistent with intestinal fibrosis, there is evidence that the gut microbiota promotes liver fibrosis. Elevated LPS levels have been measured in the systemic and portal circulation of patients with cirrhosis (68, 69). These studies suggest that the microbial ligand of LPS arriving from the portal vein or bacteria translocated to the liver promotes a liver fibrogenic response *via* TLR4 (68, 69). In accordance with clinical study, it has been shown that hepatic stellate cells, are activated by TLR4 ligands and mediate various fibrogenic effects (70, 71). In addition, TLR4 ligands indirectly contribute to liver fibrogenesis, rendering hepatic stellate cells more susceptible to TGF-β1 through downregulation of the TGF-β1 decoy receptor BAMBI (70). In line with the results of these studies, it has been shown that selective decontamination of the intestinal microbiota using an antibiotic agent inhibits experimental liver fibrosis with a decreased level of plasma LPS (70, 72).

Indirect Activation of Fibroblasts Through the Microbial Ligands

In addition to intestinal fibroblasts, the gut microbiota influences immune cells and epithelial cells, which may also serve as possible cell mediators for the development of intestinal fibrosis (**Figure 1**). Microbial stimulation induces chemokines, cytokines, and reactive oxygen species (ROS) production by immune or epithelial cells, which in turn promote the activation of intestinal fibroblasts.

Cytokines are mediators that send a signal from a cell by binding to receptors on themselves or another cell surface. Several types of immune cells in lamina propria exert their function by producing specific cytokines, which can affect the intestinal fibroblasts. It is well known that TGF-β plays a crucial role in the machinery of intestinal mesenchymal cell activation and ECM production. The canonical TGF-β intracellular signal transduction pathway is mediated by Smad proteins as TGF-β receptor activation phosphorylates Smad2 and Smad3 and induces binding with Smad4 (73). The Smad2/3-Smad4 complex translocates into the nucleus, regulating TGF-β target genes. The Smad-dependent pro-fibrotic effects of TGF-β can result in myofibroblast activation and ECM accumulation (collagen production).

Several other cytokines are also involved in the formation of intestinal fibrosis. For instance, IL-1β can be the mediator to connect the microbiota-immune cells-intestinal fibroblasts interactions. In line with the previous studies, our laboratory showed that specific microbes accelerate IL-1β production by mononuclear cells in the lamina propria (74). It is also known that IL-1β is mainly produced by mononuclear phagocytes, acting as a pro-inflammatory effector cell of intestinal inflammation (75). In turn, IL-1β promotes the secretion of collagens I and IV, IL-8, monocyte chemoattractant protein (MCP)-1, and MMP-1 from colonic subepithelial myofibroblasts (76). These findings imply the possibility that the gut microbiota contributes

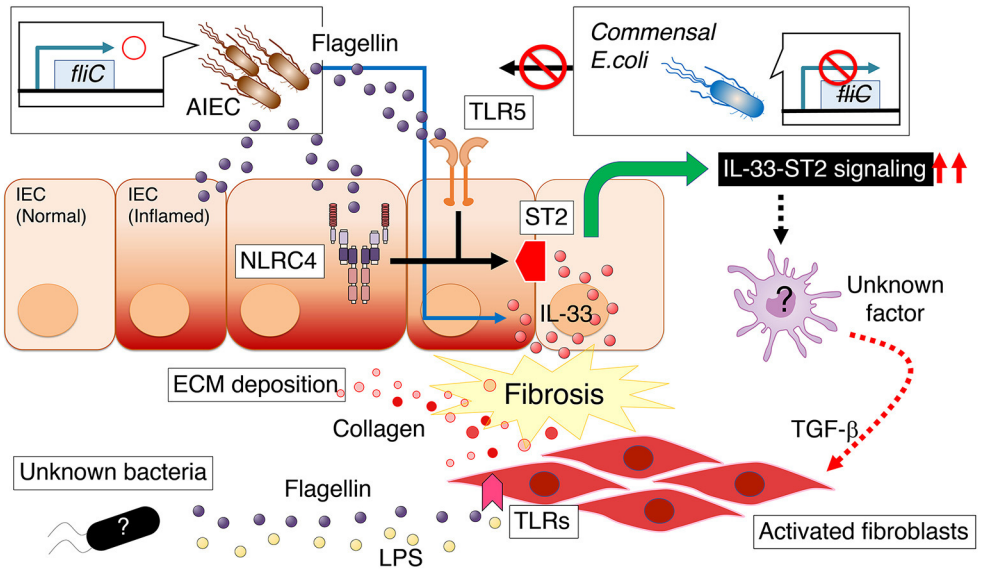

FIGURE 2 | Persistent intestinal colonization of AIEC strain LF82 potentiates the development of intestinal fibrosis, a common and potentially severe complication of intestinal colitis (66). Flagellin produced by AIEC promotes the expression of interleukin 1 receptor–like 1 (IL1RL1, also known as ST2) in intestinal epithelial cells (IECs), which depends on flagellin ligands TLR5 and NLRC4 on IECs. ST2 expression augments IL-33 signaling, thereby promoting intestinal fibrosis. Conversely, there is a mechanism by which intestinal myofibroblasts directly respond to flagellin with enhanced fibronectin or collagen production in a MyD88-dependent manner (65). AIEC, adherent–invasive *Escherichia coli*; ECM, extracellular matrix; LPS, lipopolysaccharide; ST2, interleukin 1 receptor–like 1 (IL1RL1, also known as ST2).

to the development of intestinal fibrosis *via* induction of IL-1β from immune cells, albeit there is another contradictory report showing IL-1β inhibits collagen synthesis and induces collagenase and TIMP-1 production in intestinal smooth muscle cells (77, 78). Furthermore, TL1A, a protein encoded by TNFSF15, binds to death domain receptor 3 (DR3) and is expressed by various cell types, including immune cells. Primary intestinal myofibroblasts express DR3 and respond to TL1A, increasing collagen deposition (79). Consistently, it was reported that constitutive expression of TL1A in either lymphoid or myeloid cells leads to the acceleration of intestinal and colonic fibrosis (80, 81). Importantly, TL1A-mediated intestinal fibrosis and fibroblast activation are dependent on specific microbial populations (9). It is generally believed that Th1 cell-associated cytokines drive inflammation, whereas uncontrolled type 2 and type 17 cell responses might drive tissue fibrosis through the excessive deposition of ECM (75). IL-17 cytokines, primarily produced by Th17 cells, consists of six related proteins: IL-17A (also called IL-17), IL-17B, IL-17C, IL-17D, IL-17E (also called IL-25), and IL-17F, which signal through five receptor subunits IL-17RAIL17RE (82). It has been shown that pathogenic IL-17A-dependent immune responses are induced by microbial stimulation of DCs through NOD2, and therefore the deletion of NOD2 prevents the development of colitis (83). In addition, IL-17A enhances the production of collagen I and heat shock protein 47 (HSP47) in subepithelial myofibroblasts, which is significantly elevated in the intestinal tissues of patients with active CD (84). In line with these findings, the colonization by adherent-invasive *Escherichia coli* (AIEC) induces Th17 responses, heightens proinflammatory cytokines and fibrotic growth factors, with transmural inflammation and fibrosis (85). Previous studies have also shown that another mediator is chemokines that are leukocyte chemo-attractants that cooperate with profibrotic cytokines in fibrogenesis by recruiting myofibroblasts, macrophages, and other critical effector cells to sites of tissue injury (86). Blockade of CC- and CXC chemokine receptors decreases fibrosis in association with decreased IL-4 and IL-13 (86).

In addition to immune cells, intestinal epithelial cells also act upstream of intestinal fibroblasts, thereby contributing to intestinal fibrosis development. Epithelial cells are located at the interface of the inner lumen of the digestive tract and inside the intestinal wall. Like immune cells, epithelial cells also express various receptors for microbial ligands and produce several cytokines. For instance, IL-1α is constitutively expressed in epithelial cells, although it can be expressed by other cell types, such as macrophages, monocytes, and endothelial cells (87). Previously, it was reported that intestinal epithelial cell-derived IL-1α induces cytokine production by human intestinal fibroblasts (HIFs) (88). In addition, it has been known that IL-1α and TNF-α also increase TGF-β1 and TIMP-1 production by colonic epithelial cells (89). Also, IL-1α acts as a profibrotic cytokine in other organs, as IL-1α -deficient mice exhibit reduced collagen deposition in response to bleomycin treatment in lung fibroblasts (90).

In addition to these mechanisms, intestinal epithelial cells are involved in the pathogenesis of intestinal fibrosis *via* the machinery of epithelial-mesenchymal transition (EMT). It is well known that EMT is the primary mechanism in the development and progression of cancer and fibrosis (91). While EMT, epithelial

cells change their morphology to spindle-shape, down-regulating the expression of epithelial molecules, such as E-cadherin, and gain mesenchymal characteristics, including vimentin and alpha-smooth muscle actin (α-SMA). Wang and colleagues have shown the role of the NLRP3 inflammasome in the EMT process. Interestingly, the EMT process is independent of the NLRP3 inflammasome complex formation but requires the presence of the NLRP3 protein (92).

Other mechanisms that cause intestinal fibrosis due to intestinal microbiota can be evoking oxidative stress. It has been known that polymorphonuclear leukocytes (PMNs) migrate to the site of infection or injury, engulf invading pathogens, and secrete ROS (93). ROSs are small molecules, including oxygen radicals (superoxide and hydroxyl) and non-radicals such as hypochlorous acid, singlet oxygen, and hydrogen peroxide. In the intestine, PMNs are the primary sources of ROS and reactive nitrogen species (RNS), as these cells express nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzymes (NOX/dual oxidase), the mitochondrial electron transport chain (mETC), and nitric oxide synthases (NOSs). It has also been shown that certain intestinal epithelial cells rapidly generate reactive oxygen species (ROS) in response to microbial signals (94), and subsequently, generated ROS promotes the production of several profibrogenic factors that stimulate the production or inhibit the degradation of ECM (95).

ANIMAL STUDIES

Microbiota-Dependent Animal Models of Intestinal Fibrosis

Radiation-Induced Intestinal Fibrosis Mouse Model

Clinically, it is known that radiation of the small bowel and colon induces severe intestinal fibrosis. A model of radiation-induced intestinal fibrosis in rats, and to a lesser extent in mice, has been widely used to study the mechanisms of intestinal fibrosis. This model reproduces the events responsible for the intestinal fibrosis in humans observed during radiation therapy. Morphological and pathological findings in this model include radiation-induced thickening of the bowel wall, accompanied by an enlarged submucosa, increased proliferation rates of fibroblasts and smooth muscle cells, as well as enhanced accumulation of collagen and other ECM components (96). Some genetically engineered animal models of IBD do not develop intestinal fibrosis when maintained in germ-free (GF) conditions (97). Mice colonized with only *Bacteroides thetaiotaomicron* and *E. coli* have similar outcomes to the GF mice (97). Consistent with GF mice, Zhao and colleagues showed that antibiotic cocktail pretreatment before radiation effectively reduces the content of LPS and inhibits the TLR4/MyD88/NF-κB signaling pathway in the ileum (98). Antibiotic treatment also significantly improves the survival rate and attenuates intestinal injury of the mice after radiation by reducing inflammation and preventing intestinal fibrosis (98). These results indicate that the intestinal microbiota plays an important role in this model (**Table 1**).

IL-10–Deficient Intestinal Fibrosis Mouse Model

It was reported that interleukin 10 (IL-10)-deficiency aggravates intestinal (106) and renal fibrosis (107). It is well known that the interleukin 10 (IL-10)–deficient mouse shares several characteristics in common with CD (106). Nevertheless, this model has not been extensively adopted to study intestinal fibrosis. However, it has been shown that if IL-10–deficient mice undergo ileocecal resection, they develop postsurgical fibrosis in the small intestine, distant from the site of surgery, with an associated increase in procollagen-α1(I) (COL1A1) mRNA expression (99). In addition, consistent with CD patients, intestinal fibrosis develops on the proximal side of the anastomosis (108). Therefore, this model is clinically relevant and useful to study postoperative recurrence as occurs in CD. As for the machinery of intestinal fibrosis associated with IL-10 deficiency, the prohibition, which serves as a chaperone involved in stabilizing mitochondrial proteins (92), might be associated with the pathogenesis of intestinal fibrosis in IBD (109). It is well known that IL-10–deficient mice show no inflammation when housed in specific pathogen-free (SPF) conditions, but they develop intestinal inflammation on transfer to regular housing conditions (110). Interestingly, IL-10–deficient mice housed in GF conditions fail to develop inflammation or fibrosis even after ileocecal resection, suggesting that this response also depends on the presence of the gut microbiota (99) (**Table 1**).

SAMP1/Yit Intestinal Fibrosis Mouse Model

Spontaneous models of intestinal fibrosis are particularly promising because they do not depend on exogenous stimulations. The senescence accelerated mouse (SAM) P1/Yit strain was originally generated by selective breeding of the SAMP1 line (100). The SAMP1/YitFc substrain (SAMP1/Fc) was developed in Fabio Cominelli's laboratory (111) and shown to share more histomorphological features in common with human CD than the SAMP1/Yit mouse line. The SAMP1/Yit mouse develops spontaneous enteric inflammation in the ileum within 10 weeks after birth and reveals a 100% penetrance of fibrosis by 30 weeks after birth (100). In addition, a fraction of these mice spontaneously develops perianal fistulas and accumulate ECM in the small bowel and colon with thickening of the muscularis mucosa, predominantly in the terminal ileum, a feature closely resembling intestinal stricture in CD patients (111). Like the two intestinal fibrosis models just described, the SAMP1/Yit mice do not exhibit inflammation in GF conditions (100). However, the GF SAMP1/Yit mice reconstituted by transfer of the gut microbiota from SPF SAMP1/Yit mice do develop intestinal disease (100). Evidently unknown host–microbial interactions amplify the severity of intestinal disease in this model (**Table 1**).

Constitutive TL1A Expression–Induced Intestinal Fibrosis Animal Model

TL1A (a protein encoded by the TNFSF15 gene) is a member of the tumor necrosis factor (TNF) superfamily that can bind to death domain receptor 3 (DR3). A TNFSF15 haplotype appears to be associated with higher TL1A production, increased risk of CD, intestinal fibrostenosis, and greater need for surgery (112–114). In accordance with clinical data, constitutive TL1A expression

TABLE 1 | Summary of animal models of intestinal fibrosis associated with the gut microbiota.

Model	Method	Site	Other issues	Ref.
1. Microbiota-dependent animal models of intestinal fibrosis				
Radiation-induced intestinal fibrosis	Mice are exposed to radiation	This model depends on the site of irradiation	· Intestinal fibrosis in this model resembles the appearance in CD · Intestinal fibrosis is radioresistant to total body irradiation with 10–22 Gy · Antibiotic treatment prevents intestinal fibrosis	(97, 98)
IL-10$^{-/-}$ mouse	Spontaneously induced	Colon, primarily Small intestine, less common	· IL-10–deficient mice housed in GF conditions fail to develop inflammation or fibrosis	(99)
SAMP1/Yit mouse	Spontaneously induced	Small bowel, primarily (terminal ileum) in early and late disease	· Intestinal histology resembles CD · SAMP1/Yit mice do not exhibit inflammation under GF conditions	(100)
TL1A overexpression-induced fibrosis	Spontaneously induced	Ileum and colon	· TL1A–Tg mice raised in GF conditions do not display an increased number or proportion of activated fibroblasts in the cecum · The major advantages of the TL1A–Tg fibrosis models are the obvious relevance to human CD	(9)
2. Animal models of intestinal fibrosis induced by microbial components				
PG–PS-induced intestinal fibrosis	Injection of PG–PS into the subserosa of cecal or small bowel wall	Small and large bowel	· Transmural granulomatous enterocolitis and severe transmural fibrosis in ileum and colon may be observed · Time-consuming and technically demanding technique, requiring a surgical laparotomy	(101)
Intestinal microbiota (feces)–induced intestinal fibrosis	Injection of a filtered fecal suspension into the wall of the left colon during laparotomy	Colon	· A focal and aggressive colitis with severe transmural fibrosis, elevated collagen levels, and frequent colonic strictures may be observed · Technical difficulty limits the availability of this model	(102)
3. Animal models of intestinal fibrosis induced by bacterial infection				
Salmonella spp. infection–induced intestinal fibrosis	Mice are given streptomycin orally 24 h before infection with bacteria by oral gavage	Cecum and colon	· C57BL/6 (B6) mice are extremely sensitive to wild-type *Salmonella* infection, resulting in increased mortality within the first week of infection · Use of the attenuated *S. enterica* ser. Typhimurium ΔaroA mutant causes colitis and severe fibrosis without significant mortality	(7)
AIEC (LF82) infection–induced intestinal fibrosis	Mice with DSS-injured colon (or *Salmonella* infection-injured) are orally challenged with bacteria	Colonic mucosa	· Flagellin (ligand for TLR5 or NLRC4) is necessary to exacerbate DSS-induced mouse colitis · LF82 adhesion is mediated by binding of the type 1 pili of AIEC to the host glycoprotein CEACAM6 on IECs · Flagellin produced by AIEC is a key molecule that promotes the expression of IL1RL1 in IECs, which associates with intestinal fibrosis	(66, 103–105)
AIEC (NRG857c) infection–induced intestinal fibrosis	Mice are given streptomycin orally 24 h before infection with AIEC NRG857c	Colon and cecum	· This model shows ileal and colonic inflammation that involves Th1 and Th17 immune responses · Cecal and colonic fibrosis (transmural fibrosis) in multiple mouse strains may be observed · This model shares significant similarities with CD	(85)

AIEC, adherent-invasive Escherichia coli; CD, Crohn's disease; DSS, dextran sulfate sodium; GF, germ-free; IECs, intestinal epithelial cells; PG–PS, peptidoglycan–polysaccharide.

in mice increases collagen deposition in the colon without detectable histologic colitis, whereas the ileum exhibits increased collagen deposition with spontaneous ileitis (80, 81, 115, 116). In addition, colitogenic conditions induced by chronic dextran sulfate sodium (DSS) treatment or adoptive T-cell transfer increase collagen deposition with fibrostenotic lesions that cause intestinal obstruction in this model (117) (**Table 1**).

Jacob and colleagues showed that the profibrotic and inflammatory phenotype resulting from constitutive TL1A expression is abrogated in the absence of the resident microbiota (9). Although an increased proportion of intestinal myofibroblasts can be observed in TL1A–transgenic (Tg) mice raised in conventional SPF conditions (79), TL1A–Tg mice raised in GF conditions do not display an increased number of activated fibroblasts in the cecum (9). Colonic fibroblasts isolated from the TL1A–Tg mice also displayed a significantly higher migratory capacity compared with those isolated from wild-type mice in a scratch cell migration assay; however, the

enhanced rate of fibroblast gap-closure observed in TL1A–Tg mice raised in native conditions was eliminated in GF conditions (9). Furthermore, reconstitution with intestinal microbiota from SPF mice, but not human donor microbiota, resulted in increased intestinal collagen deposition and fibroblast activation in TL1A–Tg mice (9). Thus, these results indicate that TL1A-mediated intestinal fibrosis and fibroblast activation are dependent on specific microbial populations (**Table 1**).

Chemically-Induced Intestinal Fibrosis Animal Model

It has been well known that mice drinking the sugar polymer of DSS for several days develop highly reproducible colitis with bloody diarrhea, ulcerations, and weight loss (118). In the same way, chronic administration of DSS for several cycles results in intestinal fibrosis in certain strains (119). It was reported that DSS administration induces colitis in GF mice to the same extent or even more severely compared with conventionally housed mice (120), indicating that resident gut microbiota is not required for DSS-induced colitis.

However, mice develop more severe intestinal fibrosis when colonized with a pathobiont AIEC (66), suggesting that specific, most likely pathobiont-type microbiota contributes to the development of fibrosis in this model. Consistently, deletion of MyD88 results in the amelioration of intestinal fibrosis in this model (65). In contrast, a probiotic *Lactobacillus acidophilus* strain reduces the severity of DSS-induced intestinal fibrosis (121).

Likewise, the trinitrobenzene sulfonic (TNBS) acid-induced intestinal fibrosis model is one of the most commonly applied chemically-induced intestinal fibrosis models (122). Repetitive rectal TNBS application results in chronic colitis accompanied by intestinal fibrosis with luminal stenosis and bowel dilatation. TNBS administration disrupts the epithelial barrier, thereby leading to the invasion of luminal bacteria into the colonic wall in conventionally-housed animals (123). In contrast, no colitis occurs when TNBS is administered after eradication of the colonic microbiota by antibiotics (124), and some bacteria, including *Lactobacillus casei* (DN 114-001 strain), may even have protective properties in TNBS mouse model (123). Thus, the gut microbiota regulates the pathogenesis of TNBS-induced colitis and intestinal fibrosis.

Animal Models of Intestinal Fibrosis Induced by Microbial Components

Animal Model of Intestinal Fibrosis Induced by Peptidoglycan–Polysaccharide

Peptidoglycan–polysaccharide (PG–PS) is a polymer composed of sugars and amino acids that is found in the bacterial cell wall. Transmural enterocolitis in rats can be observed after injection of purified sterile PG–PS derived from bacteria (e.g., *Streptococcus pyogenes*) into the subserosa of the cecal or small bowel wall during laparotomy (101). In this model, the initial insult is characterized by intense transmural inflammation and avid infiltration of acute inflammatory cells, including polymorphonuclear leukocytes. After several weeks, the acute inflammatory response becomes a patchy, chronic granulomatous inflammation, which has similarities to the chronic inflammation in CD. The affected intestinal wall becomes thickened and intraabdominal adhesions can develop (125), while areas of granulomatous inflammation express increased levels of collagen-α1 (COL1A1), TGF-β1, and IL-6 mRNA (126). Moreover, significant fibrosis and abundant mesenchymal cells surround the granulomas in this model. The mesenchymal cells have morphological and immunostaining patterns consistent with myofibroblasts, which are the key effector cells in intestinal fibrosis (126). The PG–PS model shows that the infiltration of nonviable bacterial components into the intestinal wall is sufficient to trigger inflammation and initiate intestinal fibrosis, and this infiltration can be enacted by bacterial components in the healthy intestinal lumen (**Table 1**).

Animal Model of Intestinal Fibrosis Induced by Feces and Bacterium Injection

This model shares many technical similarities to the PG–PS model. An injection of a filtered fecal suspension into the wall of the left colon of rats during laparotomy causes a focal and aggressive colitis with severe transmural fibrosis, elevated collagen levels, and frequent colonic strictures (102). A subserosal injection of a single organism suspension of intestinal anaerobes, but not aerobes, reproduces similar findings (102). The treated animals show signs of chronic inflammation and fibrosis with stricture development, significantly elevated levels of mucosal and serum TGF-β and increased collagen deposition. In this model, the increased production of TGF-β1 stimulates Smad2/3 phosphorylation and enhanced ALK5, TIMP-1, and COL1A2 gene expression (127). In addition, it has been shown that use of anti-TGF-β antibodies significantly abrogate collagen deposition in this model (102). These observations emphasize the impact of commensal intestinal bacteria on TGF-β1, collagen production, and intestinal fibrogenesis (**Table 1**).

Intestinal Fibrosis Induced by *Salmonella* spp. Infection

Nontyphoidal *Salmonella enterica* spp., such as *Salmonella enterica* serovar Typhimurium, are intestinal pathogens that can infect a wide range of animals, including humans (128, 129). It is known that certain *Salmonella* serovars are host restricted, whereas others have a broad host range. In humans, *S. enterica* ser. Typhimurium and Paratyphi can cause typhoid characterized by systemic infection, fever, and often, gastrointestinal symptoms such as diarrhea. In contrast, *S. enterica* ser. Typhimurium causes enterocolitis in humans and cattle, but systemic infection in mice (**Table 1**).

Serovars of *S. enterica* spp. are widely used in laboratory studies to gain an understanding of the basis of mucosal immune responses and intestinal diseases such as gastroenteritis and typhoid. It is known that oral infection with *S. enterica* ser. Typhimurium leads to spread *via* the gut-associated lymphoid tissue (GALT) to systemic sites in genetically susceptible mice. The bacteremia and lesions in the systemic organs of these mice are akin to typhoidal salmonellosis in humans; hence, this phenotype is known as mouse typhoid (130). As for *Salmonella* colonization in the mouse intestine, it is known that strain-dependent genetic susceptibility affects the host response to

Salmonella infection. It has been shown that *S. enterica* ser. Typhimurium can cause chronic infection of systemic organs in some genetically resistant inbred mouse strains (e.g., 129SvEv, Nramp1$^{+/+}$) (131). Consequently, these mice are useful animal models of persistent *Salmonella* systemic infection (131). On the other hand, several studies have aimed to improve *Salmonella* colonization in the mouse intestine. It is known that the intestinal tract of conventional SPF mice is poorly colonized by *S. enterica* ser. Typhimurium ($\sim 10^4$ CFU/g of contents) (132, 133). However, pretreatment of SPF mice with an antibiotic agent results in abundant colonization of *S. enterica* ser. Typhimurium in the cecum and colon, and susceptibility to colitis (132); a technique that is now widely used in this murine model of *Salmonella* spp. infection (**Table 1**).

In addition to studies of enterocolitis, oral administration of live *S. enterica* ser. Typhimurium has been used to study the pathogenesis of intestinal fibrosis. The severity of disease and intestinal fibrosis has been shown to depend on the genetics of the mouse strain (7). 129sv/J mice pretreated with antibiotics and chronically infected with *S. enterica* ser. Typhimurium strain SL1344 can serve as a robust model of intestinal fibrosis (7). In contrast, the C57BL/6 mouse strain is extremely sensitive to wild-type *Salmonella* infection, resulting in increased mortality within the first week of infection (7). Grassl and colleagues reported that to use C57BL/6 mice for an intestinal fibrosis model using *Salmonella* infection, the attenuated *S. enterica* ser. Typhimurium mutant strain ΔaroA (attenuated by a block in the synthesis of aromatic amino acids) can be used. These C57BL/6 mice have severe fibrosis without significant mortality (7). Thus, infection with the *S. enterica* ser. Typhimurium ΔaroA mutant allows analysis of the mechanisms that contribute to intestinal fibrosis in knockout mouse models maintained on a C57BL/6 background. So, which mouse strain and bacterial strain are the best to study intestinal fibrosis? Johnson and colleagues reported that although the severity of fibrosis in the *Salmonella* infection models varies depending on the host and bacterial strain, CBA/J mice infected with the *S. enterica* ser. Typhimurium SL1344 strain may be the optimal model for intestinal fibrosis (134) (**Table 1**).

This model has provided several insights that attribute the pathogenesis of intestinal fibrosis to the intestinal microbiota. First, the *Salmonella* virulence factors such as *Salmonella* pathogenicity islands (SPI)-1 and−2 are essential for the induction of intestinal fibrosis in this model (7). The resulting extensive transmural inflammation, primarily evident in the cecum but also in the colon, is accompanied by an upregulation of T helper 1 (Th1) cytokines, fibrotic growth factors, and procollagen type I. These profibrotic profiles are consistent with CD, which is associated with strong Th1 immune responses, including elevations in proinflammatory cytokine TNF-α expression. Further, early blockade of inflammation by eradicating the *S. enterica* ser. Typhimurium infection with levofloxacin ameliorates intestinal fibrosis, but does not abolish subsequent fibrosis, suggesting that once initiated, intestinal fibrosis in this model is self-propagating (11). Finally, an animal study using *Rora*$^{sg/sg}$ BMT mice (i.e., group 2 innate lymphoid cell (ILC2)–deficient mice) showed that collagen deposition is associated with IL-17A and RORα-dependent innate lymphoid cells (ILCs) (135), affirming ILC involvement in intestinal fibrosis in this model (**Table 1**).

Intestinal Fibrosis Induced by Adherent–Invasive *E. coli*

Members of the *E. coli* family constitute a normal component of the healthy intestinal microbiota. It is known that *E. coli* strains can acquire virulence factors to adapt to harsh circumstances in the host. Many studies have reported that some *E. coli* strains isolated from the ileal lesions of CD patients exhibit adherent and invasive capabilities in gastrointestinal epithelial cells and macrophages (136–141); hence, termed adherent–invasive *Escherichia coli* (AIEC) (136). Cell biological studies showed that AIEC phagocytosed by macrophages are more resistant to xenophagy and capable of inducing a persistent inflammatory response by releasing large amounts of TNF-α (141, 142). Interestingly, monocytes from CD patients who carry homozygous or heterozygous NOD2 polymorphisms display reduced secretion of IL-1β, IL-6, and IL-10 after AIEC infection *in vitro* compared with monocytes from CD patients without NOD2 polymorphisms (143). In addition, clinical studies showed that AIEC strains are preferentially observed in ileal CD (40, 137, 144).

To date, there are two well-characterized prototypic AIEC strains: LF82 and NRG857c (85, 136, 145). The prototype AIEC strain LF82 colonizes the intestinal mucosa and induces proinflammatory cytokines during acute DSS-induced colitis (103–105). In this model, flagellin (ligand for TLR5 and NLRC4) is necessary for the AIEC strain LF82 to exacerbate DSS-induced mouse colitis, while the nonflagellated LF82 mutant strain behaves like the nonpathogenic *E. coli* strain K12 (103). Mechanistically, it has been shown that its adhesion is mediated by binding of the type 1 pili of AIEC to the host glycoprotein carcinoembryonic antigen–related cell adhesion molecule (CEACAM) 6 on the intestinal epithelial cells (IECs) (104, 146). Barnich and colleagues reported that CD patients with ileal disease have an abnormal ileal expression of CEACAM5 and 6, and that only CEACAM6 acts as a receptor for AIEC (146). Intriguingly, *in vitro* studies demonstrated that CEACAM6 expression is increased in cultured IECs after infection with AIEC, indicating that AIEC promotes its own colonization through induction of CEACAM6 expression in the host (146). In addition to these mechanisms, it has been shown that bacterial adhesion to IECs is mediated *via* chitin-binding domains in bacteria, encoded by bacterial chitinase ChiA, that interact with human chitinase CHI3L1 expressed on IECs in inflammatory conditions (105) (**Table 1**).

As well as LF82, a human CD isolate of AIEC strain NRG857c was used to develop a chronic AIEC infection mouse model to study intestinal inflammation and fibrosis (85). Like the *S. enterica* ser. Typhimurium infection model, mice were pretreated with oral streptomycin prior to infection with NRG857c (85). After NRG857c infection, this model showed that ileal and colonic inflammation involves Th1 and Th17 immune responses (85). The resulting inflammation leads to cecal and

colonic fibrosis in multiple mouse strains, in varying degrees, and progresses to transmural fibrosis (85). This model shares significant similarities with CD (**Table 1**).

Our laboratory has shown that persistent intestinal colonization of AIEC strain LF82 potentiates the development of intestinal fibrosis in conditions of *Salmonella*-induced or DSS-induced colitis (66) (**Figure 2**). In this model, flagellin produced by AIEC, a principal component of bacterial flagella, is a key molecule that promotes the expression of interleukin 1 receptor–like 1 (IL1RL1, also known as ST2) in intestinal epithelial cells (IECs), which depends on flagellin ligands TLR5 and NLRC4 on IECs (66). Further, it has been shown that ST2 expression in IECs augments IL-33 signaling, thereby promoting intestinal fibrosis, as the blockade of IL-33–ST2 signaling by anti-ST2 antibody significantly ameliorates intestinal fibrosis (66). Therefore, therapeutic approaches that target AIEC or its downstream IL-33–ST2 signaling pathway would benefit CD patients with intestinal fibrosis (**Table 1**).

MICROBIOTA-TARGETED THERAPY FOR INTESTINAL FIBROSIS

Accumulating evidence suggests that interventions against the gut microbiota may regulate the prognosis of intestinal fibrosis in CD. However, we have not had any therapeutical options using microbiota-targeted interventions that specifically treat intestinal fibrosis (147). Although we have various strategies that modulate the gut microbiota (e.g., antibiotics, probiotics, fecal microbiota transplantation [FMT]), it is challenging to evaluate the effects of interventions on intestinal fibrosis. This is due to the unavailability of quantification methods for intestinal fibrosis. Albeit several diagnostic tools, such as ultrasound, computer tomography, magnetic resonance, and gastrointestinal endoscopy, are available to estimate the developmental status of intestinal fibrosis in CD, there are no modalities to quantify the degree of intestinal fibrosis without conducting the surgical resection of the affected intestine (147). In addition, because intestinal fibrosis gradually progresses in CD in several decades, a much longer time should be required to certificate its effectiveness of action. Another possible reason is that it is also challenging to target pathobionts selectively without affecting other bacteria in the healthy intestine. Although antibiotics have provided significant advances in therapies for infectious diseases, several bacterial species susceptible to the agents will be affected by the treatment.

On the other, there are some success stories of "microbiota-targeted therapy" in fields other than intestinal fibrosis. For instance, several researchers reported that FMT could be a promising treatment to induce remission in UC with active disease (148, 149). In addition, probiotics may be the candidate for the treatment because it has suppressed UC relapse (150). These data might indicate that microbiota-targeted therapy is promising, and therefore we hope that practical application of microbiota-targeted therapies for intestinal fibrosis come on the stage in the near future.

CONCLUSION

The cellular and molecular mechanisms of intestinal fibrosis are the focus of intense investigation. Clearly, patients who suffer from intestinal fibrosis need novel and more effective treatments that target this common, often severe, complication of CD. Compared to the enormous advances in the development of new therapies to control intestinal inflammation, such as anti-TNFs, anti-integrins, and kinase inhibitors, progress to develop therapeutic modalities that may prevent or reverse intestinal fibrosis in CD is limited. As reviewed, the gut microbiota may have a considerable impact on the pathophysiology of intestinal fibrosis in CD. Of note, several animal models enable the investigation of the precise role of the gut microbiota in the development of intestinal fibrosis. Also, technical advances provide access to the global data associated with the alterations of gene expression and gut microbial composition during the process of intestinal fibrosis. These research tools may identify specific microbes or microbial components and virulence factors that affect intestinal fibrosis development. A rational identification of microbes and microbial factors could lead to effective therapies for preventing and attenuating intestinal fibrosis.

AUTHOR CONTRIBUTIONS

DW and NK wrote the manuscript and approved it for publication.

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Gut Mucosal Microbiome Signatures of Colorectal Cancer Differ According to BMI Status

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Background: Carrying excess body weight is a strong risk factor for colorectal cancer (CRC) development with ∼11% of CRC cases in Europe linked to being overweight. The mechanisms through which excess body weight influences CRC development are not well understood but studies suggest the involvement of the presence of chronic low-grade inflammation and changes in the gut microbiota are involved.

Aim: To compare the mucosal associated microbiota of patients with CRC to understand whether carrying excess body weight was associated with a unique CRC microbial signature.

Methods: Microbiota signatures from colonic mucosal biopsies of CRC lesions and adjacent normal mucosal samples from 20 patients with overt CRC were compared with 11 healthy controls to see if having a BMI of >25 kg/m^2 influenced colonic microbial composition.

Results: Colonic mucosa samples from patients with CRC confirmed previously reported over-abundance of Fusobacteria associated with CRC but also an increase in Fusobacteria and *Prevotella* were associated with a BMI of >25 kg/m^2. Correlation analysis of bacterial taxa indicated co-exclusive relationships were more common in CRC patients with a BMI >25 kg/m^2 with an increase in transphylum relationships also seen in this patient group.

Conclusions: The findings suggest that gut microbiota composition in patients with CRC is influenced by BMI status. Further understanding/defining these differences will provide valuable information in terms of developing novel pre-onset screening and providing post-manifestation therapeutic intervention.

Keywords: colorectal cancer, gut microbiota, high throughput sequencing, increased body mass index, colonic mucosa

INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths globally, with ∼860,000 recorded deaths per year (1). CRC incidence is rising in parallel with the proportion of people carrying excess body weight (2, 3). Whilst several genetic factors have been shown to have an aetiologic role in CRC (4), the majority of sporadic CRC is largely attributable to environmental

factors, such as obesity, smoking, and dietary factors (5–7). Being overweight (BMI > 25 kg/m^2) is a well-known risk factor for cardiovascular disease and metabolic disorders, such as diabetes (8–10). However, a growing number of epidemiological studies show that carrying excess body weight, in the form of body fat, is associated with an increased risk of cancer, such as CRC (3, 11). As the global prevalence of obesity continues to rise, this will potentially lead to a further increase in the global incidence of CRC.

Suggested mechanisms linking excess body weight and CRC risk include the chronic low-grade inflammation which is associated with both conditions (12–15). It is commonly accepted that the gut microbiota strongly influences host health (16) and there is growing evidence to show that the gut microbiota is able to initiate inflammation as well as being linked to excess body weight and CRC development (17–20), with the gut microbiota known to be influenced by many factors, such as diet, environmental exposures, genetics, health status, and lifestyle (16, 17, 21, 22). We set out to assess whether carrying excess body weight was associated with a unique microbial signature in CRC. We performed 16S rRNA gene sequencing on paired colonic mucosal biopsies (adjacent normal mucosa and CRC tissue) from patients undergoing surgical resection for CRC and compared the findings to microbial signatures from colonic mucosal biopsies from healthy individuals. We focussed on identifying distinct taxonomic configurations as well as exploring co-occurrence networks.

METHODS

Subject Recruitment

Study participants were recruited from subjects who had presented for the screening colonoscopy as part of the national colorectal cancer screening or patients undergoing colonic resection for CRC. Samples from colonoscopy patients were collected from 11 patients who had no colonic microscopic or macroscopic pathology (subsequently referred to as healthy subjects). All participants were from the same demographic as the patients with adenoma and CRC, and all had undergone a similar bowel cleansing procedure. No subjects had taken antibiotics in the 6 months prior to sampling. All samples were taken from the sigmoid colon. Participants were stratified based on body mass index (BMI) and classified as Healthy Weight (BMI of 20–25 kg/m^2) or Overweight (BMI > 25 kg/m^2). Sequence data from the healthy subjects were published previously and re-analysed within this study (23). All participants were from the Scottish Colorectal Cancer Screening Program, who had been invited to attend for colonoscopy following a positive faecal occult blood test (24). No colonoscopy participants had received antibiotics for 6 months prior to their endoscopy procedure. Surgical resection samples were collected from twenty-eight patients and none of them had received pre-operative therapy and all had the tumour surgically excised.

Sample Collection

Biopsies were collected during colonoscopy using standard endoscopic forceps (Boston Scientific Nanterre Cedex France). Pinch biopsies were either fixed for histological assessment or placed directly into a 1.5 ml Eppendorf tube and snap-frozen in liquid nitrogen and transferred to a −80°C freezer until further analysis; within 1 month. All surgical resection samples were provided by the Grampian Biorepository who provided snap frozen tissue from both normal and tumour.

DNA Extraction

Genomic DNA was extracted from colonic samples using the QIAamp DNA Mini Kit (Qiagen, Crawley, UK) using minor modifications of the manufacturer's instructions. Biopsy samples were kept frozen until the addition of ATL buffer before allowing biopsies to equilibrate to room temperature, an additional 10 μl of Proteinase K was added for an initial lysis period of 18 h to ensure complete lysis of the biopsy material prior to the DNA extraction (25). A series of blank samples were included which comprised DNA extraction kit reagent blanks as well as sterile water blanks.

PCR Amplification and Sequencing

All participant samples and blanks were subjected to 16S rRNA gene sequencing. The V3-V4 region of the 16S rRNA gene was amplified using BAKT_341F (CCTACGGGNGGCWGCAG) and BAKT_805R (GACTACHVGGGTATCTAATCC) primers. The primers were designed with the Illumina adapter overhang already included. Amplification was performed using the Q5 polymerase kit following the instructions of manufacturer (New England Bio, Ipswich, MA, USA). Post-amplification, samples were purified using AMPure XP (Beckman Coulter, Brea, CA, USA) according to protocols of manufacturer. The samples were then indexed using the Nextera XT Index Kit V2 (Illumina, San Diego, CA, USA) and KAPA HiFi Hotstart ReadyMix (Kapa Biosystems, Cape Town, South Africa) with a short cycle PCR step followed by a clean-up with AMPure XP. The libraries were quantified using Quant-iT^{TM} dsDNA Assay Kit HS (Thermo Fisher Scientific, Waltham, MA, USA) and analysed on a FLUOstar Omega plate reader (BMG LABTECH, Ortenberg, Germany). The library size was determined using the Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA, USA). The libraries were pooled at equimolar concentrations in preparation for sequencing. Sample sequencing was performed using an Illumina MiSeq sequencer (Illumina, San Diego, CA, USA) using Illumina V3 chemistry and paired-end 2 × 300 base pair reads by the Centre for Genome Enabled Biology and Medicine, University of Aberdeen.

Bioinformatics Analysis

QIIME version 1.9.0 (26) was used to merge paired end reads, quality philtre, pick open reference operational taxonomic units (OTUs) against the GreenGenes 13.8 database (27, 28) based on a 97% similarity, align representative sequences, remove singleton OTUs, and assign taxonomy. A series of blank samples were included from DNA extraction through library preparation and sequencing. Blank samples had a total of 278 sequences, equating to 70 ± 8 (mean ± SEM) reads per blank sample. This number was sufficiently low enough for us to determine that the contamination of samples had not occurred during the library preparation and sequencing.

For all sample cohorts, diversity was assessed using QIIME. Alpha and Beta diversity metrics; Observed OTUs, Chao (29), Shannon (30), Simpson (31), Good's Coverage, Bray Curtis (32), and Jaccard (33) were calculated using a subsampling depth of 3,689 sequences per sample. Rarefaction curves demonstrated that this subsampling level was sufficient to capture ample sample diversity (**Supplementary Figure 1**). Community structures were compared using the principal coordinates analysis (PCoA) plots generated using the Bray Curtis distance metrics and visualised using Emperor. Linear discriminant analysis effect size (LEfSe) analysis (34) was carried out for the identification of discriminative biomarkers associated with meta-data categories. Statistical analysis of stratification by metadata category was performed using PERMANOVA *via* the compare_categories script of QIIME using the Adonis function with 999 permutations. Subsequent statistical analysis was done in R 3.2.2 (35). Differential taxonomic abundance testing of the healthy and CRC sample set and figure generation was performed by converting the OTU table to a PhyloSeq object (36) and testing for changes in abundance using DESeq2 (37). Heatmaps were produced using the heatmap.plus package for R. All other figures were created using the ggplot2 package for R. Colours palettes from the R package RColorBrewer were used within plots.

Co-occurrence Analysis

Rarefied taxa abundances at the species level were used to calculate the co-occurrence metrics for the CRC sample set using SparCC (38). Within group taxon-taxon correlation coefficients were calculated as an average of 20 inference iterations and pseudo p were calculated using 1,000 permuted datasets. The values of p were corrected for multiple testing using the Benjamini–Hochberg method. Taxon-taxon correlations >0.6 and < -0.6 with an adjusted $p < 0.05$ were visualised using Cytoscape.

RESULTS

Mucosal Microbial Communities Are Affected by BMI Status as Well as the Adenoma-Carcinoma Sequence

The 16S rRNA gene sequence data were processed using TrimGalore! to remove the primer sequences. Within the CRC cohort, the total number of raw paired read sets was 27,536,992 with a mean number of sets of paired reads per sample of 162,584 (**Supplementary Table 1A**). The healthy sample 16S rRNA gene sequence data had a total of 1,019,169 raw paired read sets, with an average of 92,651.73 paired reads per sample (**Supplementary Table 1B**). After trimming with TrimGalore!, CRC samples had a mean number of paired reads of 60,389 and the healthy samples had a mean number of paired reads of 85,714.55.

To determine associations of colonic microbiome profiles with BMI status, we performed 16S rRNA gene sequencing on subjects with CRC. We compared the microbiome profiles with sequence data from healthy subjects who had attended for CRC screening on the basis of a positive faecal occult blood test but were subsequently confirmed to have no macroscopic or microscopic evidence of colonic disease (**Tables 1A,B**) (23). For subjects with CRC, tissue was available from both the tumour and adjacent normal mucosa. Tissue samples from the 11 healthy subjects were collected from the sigmoid colon as the majority of CRC and adjacent normal mucosa samples were from the distal colon and previous studies have confirmed that there are limited differences in the microbial diversity across the colon. We stratified subjects into 2 groups based on the BMI

TABLE 1A | Study cohort information for patients with colorectal cancer.

CRC patient cohort	All subjects	Sequencing cohort
Number of patients (*N*)	28	20
Gender (% M:F)	39:61	35:65
Average age, in years, at procedure (range)	72 (47, 88)	71.5 (47, 87)
BMI status (kg/m^2)		
20–25	12	10
>25	16	10
Sample location (%)		
Caecum	25	30
Ascending colon	17.9	5
Transverse colon	10.7	25
Splenic flexure	3.6	15
Sigmoid colon	35.6	5
Rectum	7.2	20
Extramural venous invasion (% yes:no)	39:61	35:65
Dukes stage (%)		
A	7	5
B	57	55
C (C1:C2)	36 (32:4)	40 (35:5)
TNM staging (%)		
T2N0	2 (7%)	1 (5%)
T3N0	13 (46%)	8 (40%)
T3N1	6 (21%)	6 (30%)
T4N0	3 (10.5%)	3 (15%)
T4N1	2 (7%)	1 (5%)
T4N2	1 (3.5)	1 (5%)

Participants were stratified based on body mass index (BMI) and classified as Healthy Weight (BMI of 20–25 kg/m^2) or Overweight (BMI > 25 kg/m^2). Eight of the microbial profiles of patients with CRC were discarded due to low sequencing depth in either the CRC or adjacent normal mucosa sample.

TABLE 1B | Study cohort information for healthy patients.

Healthy patient cohort metadata	
Number of patients (*N*)	11
Gender (% M:F)	9:91
Average age, in years, at procedure (range)	58 (52, 67)
BMI status (kg/m^2)	
20–25	5
>25	6
Sample location (%)	
Sigmoid colon	100

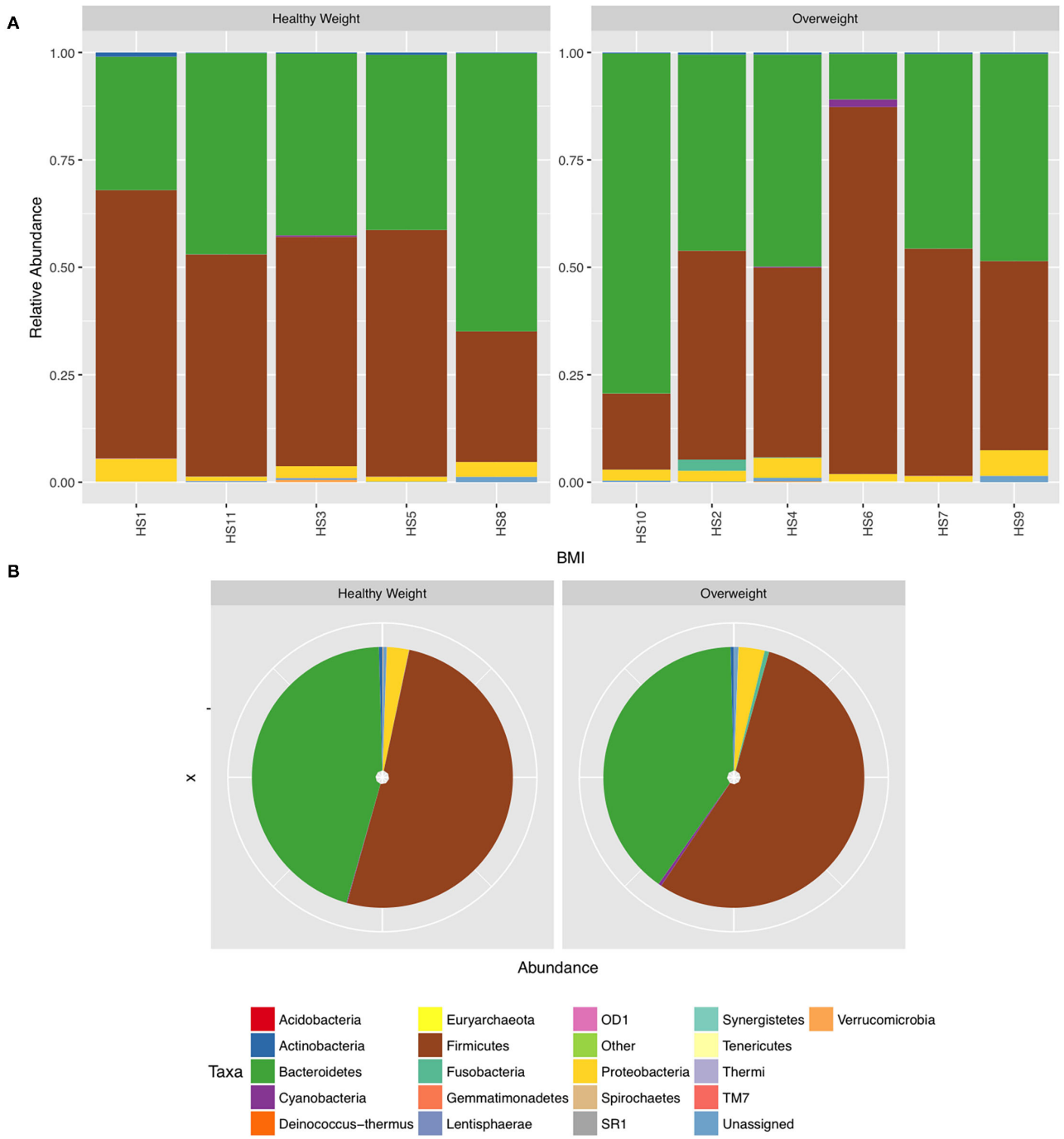

FIGURE 1 | Relative abundance at phylum level for healthy samples stratified by body mass index (BMI). **(A)** Individual subject stacked bar charts. **(B)** Collective pie chart representation.

status: (1) Healthy weight with a BMI of 20–25 kg/m^2, and (2) Overweight with a BMI of >25 kg/m^2. The effect of BMI on alpha diversity was assessed, across all sample types—normal mucosa (healthy subjects), normal mucosa (CRC patients), or CRC tissue based on OTU richness, diversity, and evenness. We studied the healthy subjects comparing alpha diversity based on the BMI status. The CRC patient groups were initially stratified by (a) tissue type (lesion vs. adjacent normal mucosa) or (b) BMI status and then further analysed to encompass both tissue type and BMI status. No differences in the alpha diversity were seen across any comparisons made (**Supplementary Figures 2, 3**; $p > 0.05$, for all analyses, Wilcoxon rank test). In addition,

we assessed alpha diversity between normal mucosa samples from the healthy subjects compared with CRC patients. A marginal but non-significant decrease in the alpha diversity was observed in the normal mucosa samples of CRC patient compared with healthy subjects (**Supplementary Figure 4**, $p > 0.05$, for all analyses, Wilcoxon rank test), demonstrating that bacterial richness in normal mucosa was similar between healthy subjects and patients with CRC. Further stratification by BMI status failed to demonstrate differences suggesting that there was comparable community evenness between the subject groups (**Supplementary Figure 4**, normal weight individuals $p = 0.440$, overweight individuals $p = 0.181$, Wilcoxon rank test of observed species).

We next conducted relative abundance analysis which indicated that the dominant phyla between the 2 subject groups varied. Firmicutes was the dominant phyla in healthy subjects (median 51.66%; interquartile range [IQR] 44.09%, 55.36%); followed by Bacteroidetes (median 45.61%; IQR 41.54%, 48.76%); Proteobacteria (median 2.54%; IQR 1.50%, 4.03%); and Actinobacteria (median 0.34%; IQR 0.23%, 0.51%; **Figure 1**; **Table 2**; **Supplementary Figure 5**). This profile was independent of BMI status. When a similar comparison was undertaken for CRC patient samples, there was a shift in the dominant phyla with Fusobacteria replacing Actinobacteria as the fourth most abundant phylum (**Figure 2**; **Supplementary Figure 6**; **Table 3**). Similar to the healthy subject group, Firmicutes were the most abundant phyla. When stratified according to sample type, there was a notable overabundance of Fusobacteria in CRC samples (8.6% in tumour tissue compared with 0.8% in adjacent normal mucosa $p = 0.009$, Wilcoxon rank-sum test; **Table 3**), an observation which confirms previous findings (39–42). Interestingly, the increased abundance in CRC samples was only present in the samples of overweight patient ($p = 0.029$, Wilcoxon rank sum test) and not in healthy weight individuals ($p > 0.05$, Wilcoxon rank sum test). Fusobacteria presence in the samples of the healthy controls was determined, with a relative abundance of less than the normal mucosa of CRC patient (0.25% compared with 0.8%). When stratified according to BMI, Fusobacteria abundance was higher in the overweight healthy control group (0.46%) compared with the normal weight group (0.004%), although this was not statistically significant.

Further interrogation of the CRC patient cohort, based on BMI status, showed that overweight individuals had higher levels of Bacteroidetes than their lean counterparts (31.17% in the samples of normal weight patient, 40.35% in the samples of overweight patient; $p = 0.049$; **Table 3**). In particular, an increase in *Prevotella* was observed in patients within the higher BMI group (**Supplementary Figure 7**). To further interrogate the influence of BMI and sample type in the samples of CRC patients, differential abundance analysis was conducted using DESeq2. This methodology has been shown to reduce false positive rates in discovery of significant abundance differences when compared with typical rarefaction methods (43). DESeq2 analyses further supported these taxonomic differences with *Fusobacterium* identified as differing between adjacent normal mucosa and CRC samples (adj $p < 0.005$, **Figure 3A**; **Supplementary Table 2**), and *Prevotella copri* seen to have significant differences in abundance between healthy weight and overweight patient samples (adj $p < 0.05$, **Figure 3B**; **Supplementary Table 2**).

TABLE 2 | Mean difference in the relative abundance of the phyla Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria in healthy subjects stratified according to BMI status. Healthy weight (BMI of 20–25 kg/m^2), overweight (BMI of > 25 kg/m^2).

Phylum	Mean relative abundance ± SEM		Difference in mean relative abundance ± SEM	*p*-value
	Healthy weight	Overweight		
Firmicutes	51.04 ± 5.49	48.79 ± 8.89	2.24 ± 3.40	0.429
Bacteroidetes	45.14 ± 5.53	46.37 ± 8.88	1.23 ± 3.35	0.537
Proteobacteria	2.69 ± 0.79	3.10 ± 0.74	0.41 ± 0.05	0.792
Actinobacteria	0.42 ± 0.16	0.49 ± 0.23	0.07 ± 0.01	0.792

The value of p based on Wilcoxon rank-sum test (to 3 d.p.). Healthy weight (BMI of 20–25 kg/m^2), overweight (BMI of > 25 kg/m^2).

We used LEfSe to identify OTUs that were driving the differences between BMI stratified samples in the two subject groups. There were no significant differences between healthy weight and overweight subject samples in the healthy subject cohort. Discriminant feature analysis of the CRC cohort showed *Bacteroides* and Tissierellaceae were over-represented in adjacent normal mucosa in overweight patients, compared with adjacent normal mucosa in healthy weight patients, and *Lactobacillus zeae*, was over-represented in adjacent normal mucosa in the healthy weight patients with CRC (**Figure 4A**; **Supplementary Table 3**). *Acinetobacter* was increased in CRC samples from the overweight group. Similar to the adjacent normal mucosa samples, *Lactobacillaceae zeae* was seen in higher abundance in healthy weight CRC patient samples (**Figure 4B**; **Supplementary Table 3**).

We further interrogated the dataset to allow us to identify which specific cohort parameters were influencing the observed microbial diversity changes. We used Bray Curtis and Jaccard distance measures, which revealed that samples did not cluster strongly by BMI status in the healthy subject cohort ($p > 0.05$; PERMANOVA; **Table 4**; **Supplementary Figure 8**). However, interrogation of the CRC cohort suggested that BMI status significantly contributed to the distance between samples ($p < 0.05$; PERMANOVA; **Table 4**; **Supplementary Figure 9**).

Microbiome Interaction Networks Are Influenced by BMI Status as Well as Disease State

We next inferred all pairwise taxonomic correlations between adjacent normal mucosa and CRC samples, with BMI status as a classifier, using the SparCC algorithm. After correcting for spurious correlation coefficients and controlling for false discovery rates, we were able to see that BMI status impacted on the number of observed taxonomic correlations (**Figure 5**;

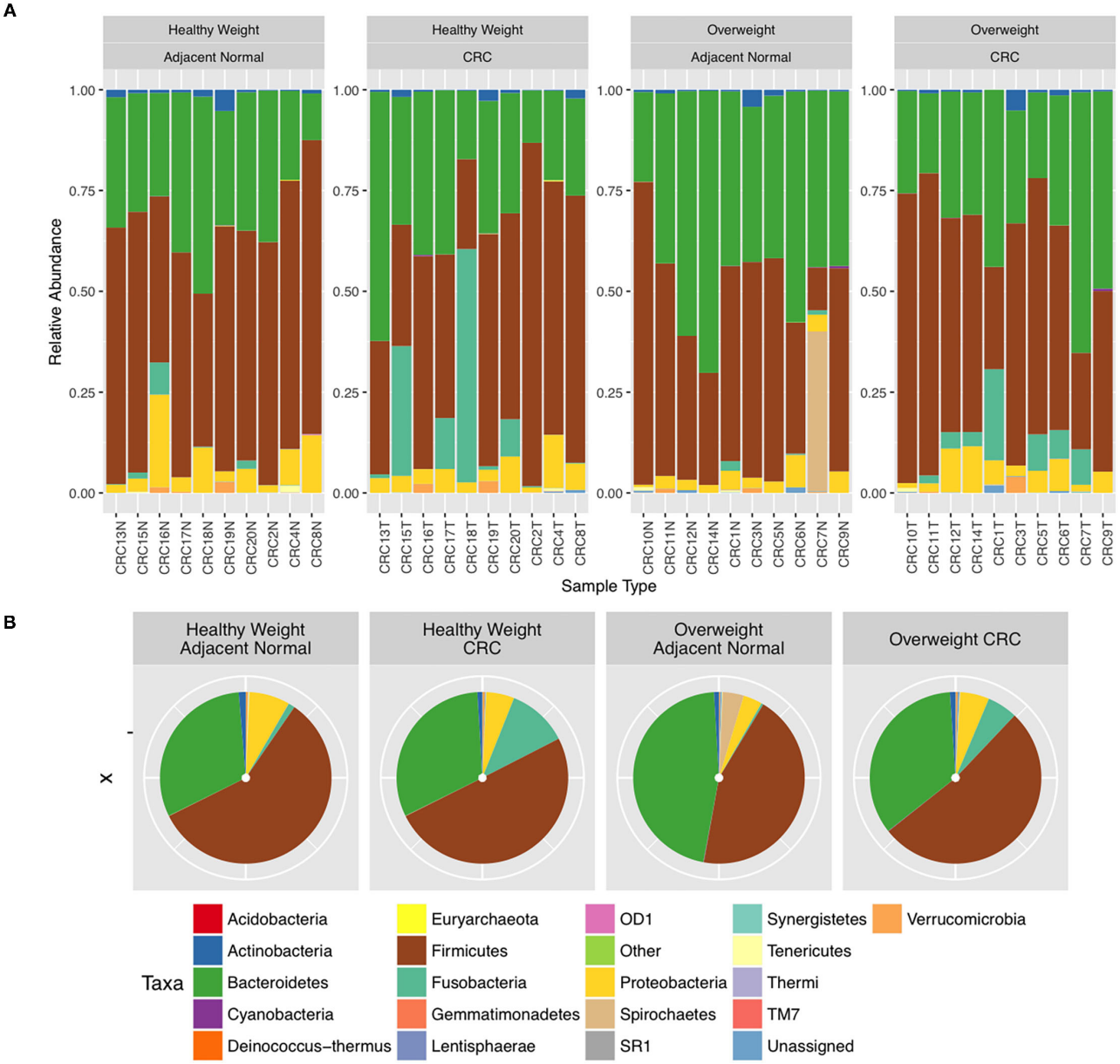

FIGURE 2 | Relative abundance at phylum level for colorectal cancer (CRC) tissue and paired adjacent normal mucosa samples. **(A)** Individual subject stacked bar charts. **(B)** Collective pie chart representation.

Additional File 1). We found the highest number of significant positive correlations in the samples of overweight patient with CRC (BH adjusted $p < 0.05$; **Figure 5**). Samples from healthy weight patients with CRC had a total of 88 significant correlations (81 co-occurrence and 7 co-exclusion) across 70 taxa. When additionally stratified by sample type (adjacent normal mucosa or CRC), adjacent normal mucosa samples had 41 significant correlations compared with only 15 in the CRC samples. When a similar assessment was undertaken on the samples from the high BMI CRC patient group, the number of significant correlations increased dramatically to 184 (108 co-occurrence and 76 co-exclusion), although the number of taxa did not increase indicating the increase observed reflected an increase in networking within a similarly rich community. Additional stratification based on the sample type showed that adjacent normal mucosa samples had 25 correlations compared with 29 in CRC samples. Trans-phylum relationships, with strong correlation, (0.6 or above) were much more common in the higher BMI group compared with the samples of normal weight patient with CRC, however, no difference was seen based on the sample type indicating that the co-occurrence networks were driven by BMI status rather than the presence of CRC. Similar to findings from Nakatsu et al. (19), Firmicutes members were more likely to form strong co-occurring relationships indicating

TABLE 3 | Mean difference in the relative abundance of the 4 major phyla Firmicutes, Bacteroidetes, Proteobacteria, and Fusobacteria from the CRC sample cohort between polyp and paired normal samples, and between normal weight and overweight patient samples.

Phylum	Mean relative abundance between CRC and Adjacent normal mucosa ± SEM		Difference in mean relative abundance ± SEM	*p*-value	Mean relative abundance between Healthy and High BMI groups ± SEM		Difference in mean relative abundance ± SEM	*p*-value
	Tumour	Adjacent normal mucosa			Healthy weight	Overweight		
Firmicutes	51.19 ± 3.96	51.12 ± 3.60	0.07 ± 0.36	0.862	54.11 ± 3.50	48.20 ± 3.94	5.91 ± 0.44	0.201
Bacteroidetes	32.96 ± 3.07	38.56 ± 3.12	5.59 ± 0.05	0.165	31.17 ± 2.68	40.35 ± 3.26	9.18 ± 0.58	**0.049**
Proteobacteria	5.36 ± 0.80	5.61 ± 1.20	0.25 ± 0.40	0.659	6.46 ± 1.22	4.51 ± 0.69	1.95 ± 0.53	0.265
Fusobacteria	8.57 ± 3.21	0.79 ± 0.41	7.78 ± 2.80	**0.009**	6.31 ± 3.21	3.05 ± 1.23	3.26 ± 1.98	0.675

The value of p based on Wilcoxon rank-sum test (to 3 d.p.).

that specific gut microbiota members can form niche-specific relationships, which in our study appear to be a response to the increased BMI status. Network analysis identified very little overlap in the co-occurrence networks between healthy weight and overweight patient sample sets indicating that the increases seen reflected a progressive alteration from healthy weight to overweight patient samples. The strongest interactions were among various Firmicutes belonging to *Bulledia, Dorea* and *Ruminococcus* co-occurring with Bacteroidetes members, such as *Prevotella* and *Rikenella,* although there was evidence of a co-exclusion relationship between *Ruminococcus* and *Prevotella* (**Additional File 1**). Other associations included uncultured Oscillospira forming strong co-occurrence relationships with *Coriobacteria* as well as *Barnsiella.* Interestingly, a number of Firmicutes, such as *Faecalibacterium, Ruminococcus,* and *Blautia* were shown to co-occur with *Bifidobacterium*. The strongest co-exclusion networks were seen between *Selenomonas* and other Firmicutes, such as *Lachnospiraceae* and *Ruminococcaceae* but also Bacteroidetes and Actinobacteria. These were only seen in overweight patients.

DISCUSSION

It is expected that CRC burden will substantially increase in the next two decades as a consequence of adoption of a western lifestyle (1). However, to date there is limited information related to whether BMI status influences the microbial composition in the context of CRC. In this study, we investigated how a BMI of >25 kg/m^2 influenced mucosal associated microbial communities in patients with CRC. We compared the findings to control subjects of equivalent BMI status, from the same demographic area but with no colonic pathology. The findings demonstrate that BMI status influences the microbial community structure in patients with CRC. In particular, we show that in patients with CRC, an increased BMI was associated with more dynamic microbial networks evidenced by the increased numbers of co-occurring and co-exclusion relationships between microbes which may highlight a BMI-directed colonic tumour environment. Previous studies have demonstrated that differences in gut microbial communities are present through the various stages of the adenoma-carcinoma sequence (19, 44, 45). These changes have been proposed to happen, at least in part, in response to the changing colonic environment as carcinogenesis progresses, such as increased inflammatory activity, altered host energy metabolism, and increased cell turnover (46–48). In addition, our findings show that BMI status contributes to the mucosa-associated microbial community shifts, in particular having a BMI of >25 kg/m^2 was associated with an overabundance of *Prevotella* in patients with CRC. *Prevotella* sp. have been repeatedly demonstrated to be associated with obesity induced disease (49–51) as well as being under-represented in non-obese subjects (52), and being detected in CRC microbial signatures (53). Recently *Prevotella copri* was shown to be associated with fat accumulation in pigs (54). Whilst the majority of information linking *Prevotella* abundance with weight gain has been in the context of obesity, our study has extended the findings to a CRC cohort with BMI of >25 kg/m^2. This means that increased *Prevotella* abundance is present prior to obesity. *Prevotella* are known to play a role in carbohydrate fermentation, producing exogenous short-chain fatty acids, such as succinate, as well as producing sialidases which degrade mucin affecting the mucosal barrier integrity (54–57). It has been shown that hydrogen-producing *Prevotella* can coexist with hydrogen-oxidising methanogenic Archaea in the gastrointestinal tracts of individuals with a high BMI (55). This syntrophic relationship may increase the host energy extraction from indigestible carbohydrates, as an increase in hydrogen-oxidising methanogenesis facilitates fermentation. Therefore, Prevotellaceae populations may be an important factor in the association among increased BMI status, CRC, and the gut microbiota. It remains to be determined how such changes in the gut microbiota, and the accompanying impact on microbial function affect the host during CRC development. Future studies focussing on defining the tumour-promoting potential of *Prevotella* are warranted to assess how individual species interact and contribute to the tumourigenic process. This is particularly pertinent as there have been conflicting reports of the beneficial as well as deleterious effects of *Prevotella* species, depending on the nature of the environment (58, 59).

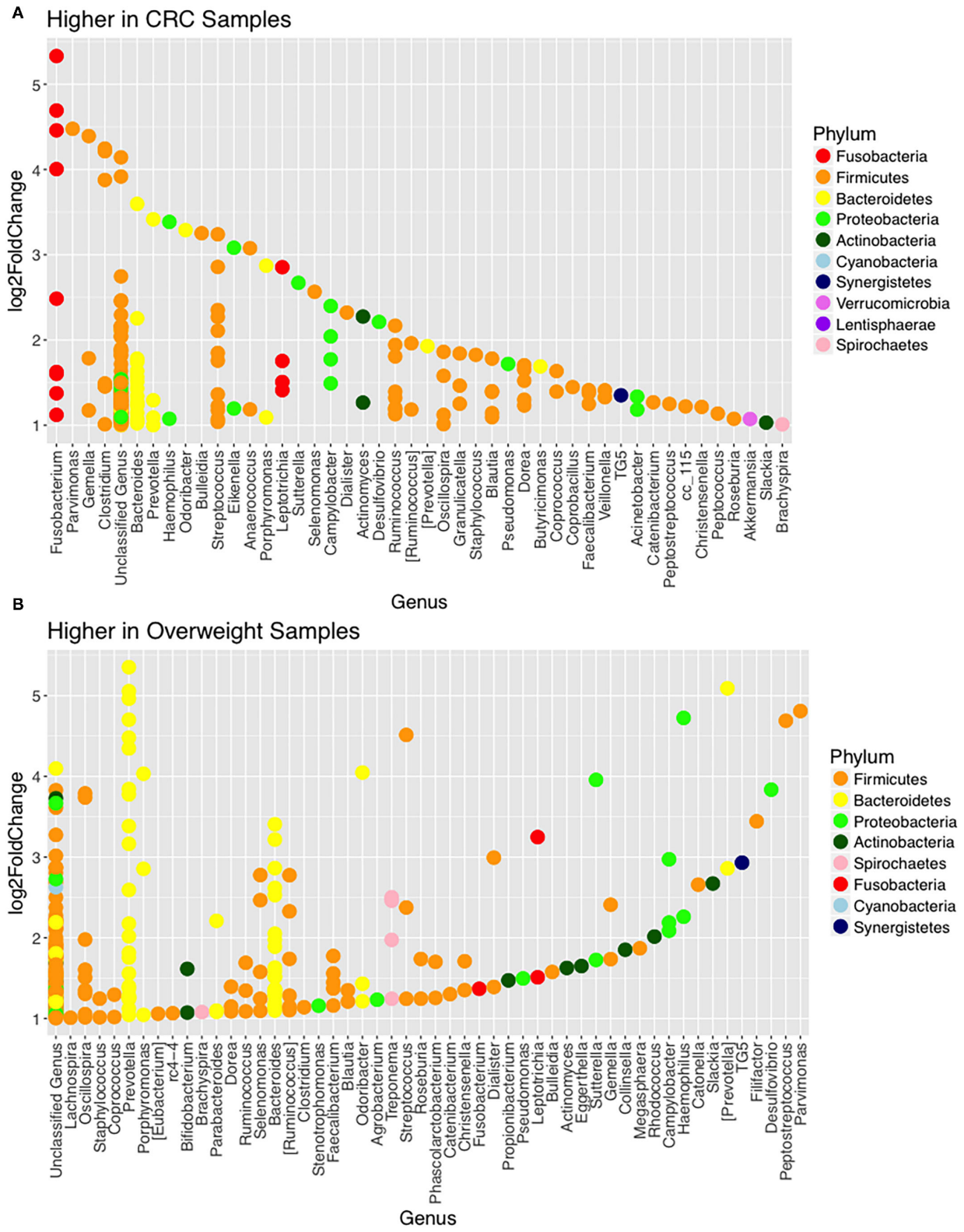

FIGURE 3 | Changes in relative abundance of operational taxonomic units (OTUs). **(A)** OTUs with LogFC > 1 with higher abundance in CRC samples (when compared with adjacent normal samples) including *Fusobacterium* (adj $p = 0.014$; DESeq2) and **(B)** OTUs with LogFC > 1 with higher abundance in overweight samples (when compared with healthy weight), in the CRC patient cohort, including *Prevotella copri* (adj $p = 0.042$; DESeq2).

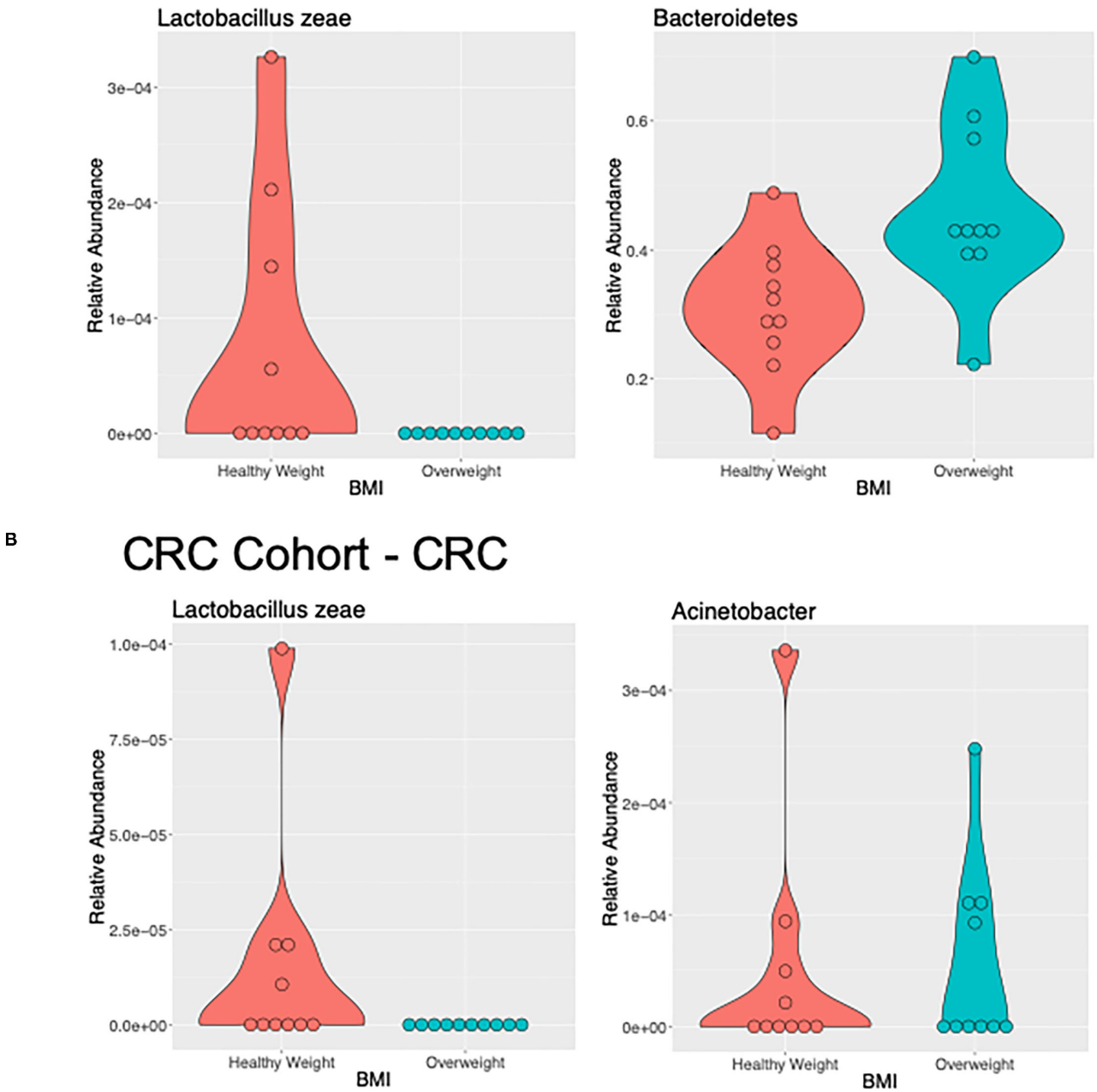

FIGURE 4 | Differentially abundant genera of interest between the samples of healthy weight and overweight patient from **(A)** adjacent normal mucosa samples from patients with CRC, and **(B)** CRC samples by LefSe (LDA > 2).

Additionally, our findings demonstrate an overabundance of Fusobacteria in CRC samples, with CRC tissue harbouring a higher Fusobacteria load compared with adjacent normal mucosa which confirms previous findings (39, 60, 61) with a lower Fusobacteria abundance seen in the mucosa of healthy subject. Comparison of Fusobacteria abundance in normal mucosa between patients with CRC and healthy subjects in the same geographical cohort is scarce. Our findings agree with a previous quantitative PCR study which compared Fusobacteria abundance in the mucosal samples of patients with CRC and healthy subjects (62). Our findings of increased BMI, independent of disease status, also correlating with increased Fusobacteria, is novel and worthy of further exploration. Although Fusobacteria abundance in healthy subject levels was lower than the levels

of patients with CRC, the same trend of increased Fusobacteria abundance correlating with increased BMI was seen between both groups. To our knowledge, this is the first report of an association specifically with overweight patients with CRC. Fusobacteria, particularly *Fusobacterium nucleatum*, along with enterotoxigenic *Bacteroides fragilis* and *Escherichia coli* have been described as putative bacterial oncogenic drivers of CRC and their role in colorectal tumorigenesis has been repeatedly demonstrated, with the presence of *F. nucleatum* especially reported to contribute to disease progression, chemoresistance, and metastatic disease (53, 63, 64). Recent attention has focussed on the oncogenic potential of collective gut microbial communities, such as the role of bacterial biofilms, rather than individual contributors, with oncogenic driver organisms known to be the key constituents of these polymicrobial biofilms (65–67). Within biofilms, microorganisms become resistant not only to host defence mechanisms, but also to anti-microbial strategies with invasive polymicrobial bacterial biofilms being a known driver of tissue inflammation. It has been previously

TABLE 4 | PERMANOVA results produced by Adonis of the R package Vegan showing the contribution of each metadata category to the sample distances.

Metadata category	p-value for Bray Curtis distance matrix	*p*-value for Binary Jaccard distance matrix
Healthy sample cohort		
BMI	0.258	0.147
CRC cohort		
Sample type	0.437	0.996
BMI	0.025	0.009
Patient	0.069	0.015

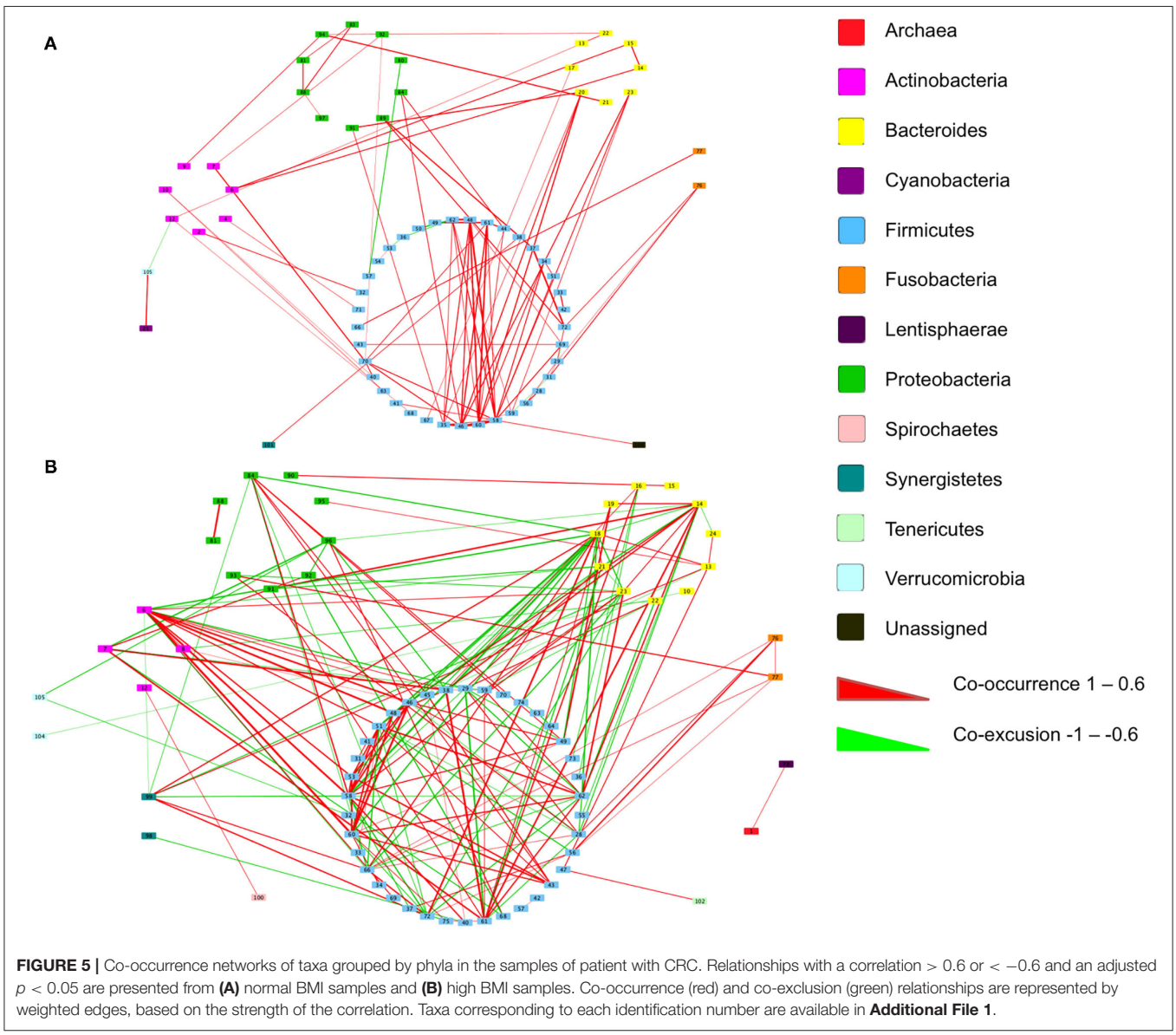

FIGURE 5 | Co-occurrence networks of taxa grouped by phyla in the samples of patient with CRC. Relationships with a correlation > 0.6 or < −0.6 and an adjusted $p < 0.05$ are presented from **(A)** normal BMI samples and **(B)** high BMI samples. Co-occurrence (red) and co-exclusion (green) relationships are represented by weighted edges, based on the strength of the correlation. Taxa corresponding to each identification number are available in **Additional File 1**.

demonstrated that *F. nucleatum* plays a central role in oral biofilm formation, mediating coaggregation between strains including various *Prevotella* species (68). Whether *F. nucleatum* plays such a pivotal role in gut biofilms remains to be determined, however, a recent study has shown that *Fusobacterium* and its associated microbiome—such as *Bacteroides, Selenomonas*, and *Prevotella* species, present in CRC primary lesions are also present in distal metastases suggesting that *Fusobacterium* has some ability to direct its environment (53).

A strength of our experimental design was the inclusion of paired samples, with histologically normal mucosa, from near the CRC site of the lesion. By using paired samples, each individual acted as their own control, providing a higher level of comparability. Selected previous studies, have also opted to use this approach (19, 39, 61), although most other studies either use healthy individuals as controls or more often relying on the faecal sample comparison between individuals. The paired patient sample approach provides the best benchmark of microbial diversity for each individual as it is widely appreciated that there is no "gold standard" definition of the microbial composition of the healthy or normal gut microbiota. Limitations of our study include small sample size and also the inevitable effect of bowel cleansing preparation on the mucosa-associated microbiota. However, this is a caveat of all studies which look to obtain colonic samples and the assumption would be that all subjects were affected to a similar extent as they have undergone almost identical procedures. A further limitation of the study was the fact that no anthropomorphic assessment of patients was undertaken to define whether the increased BMI status was due to increased body fat or muscle mass. Previous studies assessing gut microbial communities in athletes with an increased BMI compared with individuals with an increased BMI due to carrying excess body fat, have shown that gut microbial communities differ dramatically depending on the body composition (69, 70). Based on the age range of our cohort, which was adults with an average age of >60 years old, we anticipate that the increased BMI cohort was reflective of the population demographic from which they were recruited.

CONCLUSION

In summary, our study has shown that carrying excess body weight influences mucosal microbial community structure in patients with CRC. We anticipate that evaluating the mucosal microbial community composition and progression alongside host responses will provide a clearer picture of how carrying excess bodyweight influences the CRC development. Although further confirmation of our findings is needed, studies are warranted to define the mechanistic link between *Prevotella* overabundance and increased BMI status in the context of CRC. This information may enable earlier screening to predict patients at risk of developing CRC and allow prevention strategies to be implemented.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/ena, PRJEB15003, and PRJEB22039.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the North of Scotland Research Ethics Service (Study Codes 09/S0802/106, 12/NS/0061). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GH conceived the study. SS and SB performed all the analyses. JT collected clinical samples. EE-O collected clinical samples and provided critical evaluation of the manuscript. GM assessed colonic pathology of biopsy and surgical resection samples. GH and GM secured funding for the study through a grant awarded by Friends of Anchor. SS and GH interpreted the data and wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.800566/full#supplementary-material

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Refining a Protocol for Faecal Microbiota Engraftment in Animal Models After Successful Antibiotic-Induced Gut Decontamination

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Background: There is mounting evidence for the therapeutic use of faecal microbiota transplant (FMT) in numerous chronic inflammatory diseases. Germ free mice are not always accessible for FMT research and hence alternative approaches using antibiotic depletion prior to FMT in animal studies are often used. Hence, there is a need for standardising gut microbiota depletion and FMT methodologies in animal studies. The aim of this study was to refine gut decontamination protocols prior to FMT engraftment and determine efficiency and stability of FMT engraftment over time.

Methods: Male *C57BL/6J* mice received an antibiotic cocktail consisting of ampicillin, vancomycin, neomycin, and metronidazole in drinking water for 21 days *ad libitum*. After antibiotic treatment, animals received either FMT or saline by weekly oral gavage for 3 weeks (FMT group or Sham group, respectively), and followed up for a further 5 weeks. At multiple timepoints throughout the model, stool samples were collected and subjected to bacterial culture, qPCR of bacterial DNA, and fluorescent *in-situ* hybridisation (FISH) to determine bacterial presence and load. Additionally, 16S rRNA sequencing of stool was used to confirm gut decontamination and subsequent FMT engraftment.

Results: Antibiotic treatment for 7 days was most effective in gut decontamination, as evidenced by absence of bacteria observed in culture, and reduced bacterial concentration, as determined by FISH as well as qPCR. Continued antibiotic administration had no further efficacy on gut decontamination from days 7 to 21. Following gut decontamination, 3 weekly doses of FMT was sufficient for the successful engraftment of donor microbiota in animals. The recolonised animal gut microbiota was similar in composition to the donor sample, and significantly different from the Sham controls as assessed by 16S rRNA sequencing. Importantly, this similarity in composition to the donor sample persisted for 5 weeks following the final FMT dose.

Conclusions: Our results showed that 7 days of broad-spectrum antibiotics in drinking water followed by 3 weekly doses of FMT provides a simple, reliable, and cost-effective methodology for FMT in animal research.

Keywords: antibiotics, faecal microbiota transplant, microbiome, gut decontamination, gut engraftment

INTRODUCTION

The human gut harbours trillions of microbes which include bacteria, archaea, viruses, and eukaryotes. Bacteria and archaea make up most of the gut microbiome, and studies have demonstrated a role for the gut microbiota in influencing disease processes in many organs (1, 2). Unsurprisingly, alterations in the richness, diversity, composition, and function of the gut microbiota have been associated with risks of a broad range of diseases, ranging from gastrointestinal inflammatory and metabolic conditions to neurological, cardiovascular, and respiratory illnesses (2–6). Hence, targeted manipulation of the gut microbiota for personalised nutrition and precision medicine has emerged as an attractive and potential therapeutic strategy (6–9). Indeed, increasing evidence suggests gut manipulation through faecal microbiota transplantation (FMT) has the potential to be beneficial in many diseases (10–12).

Animal models are increasingly used to study the effect of gut microbiome manipulation by FMT on disease pathogenesis, and gut based therapeutic interventions. Although microbial similarity between mice and humans at the phylum level is remarkable, at the genus level 85% of murine sequences represent species that have not been detected in humans (13). "Humanization" of the mouse microbiota is frequently used to overcome this limitation, and it has been shown that mice humanised with different human donors generate a similar microbiome composition and metabolomic profile to that of the donor, with preservation of individual-specific features (14). Thus, for effective translation of therapeutics targeting the gut microbiota, and to explore the mechanisms underlying host-microbe interactions in animals, reconstitution of animals with human donor samples through FMT is key to translational research in this area.

For animal-based FMT studies, recipient animals are required to be either germ free (GF) or depleted of gut bacteria using antibiotics. Germ free mice are considered the gold standard as they are completely devoid of microbes and are therefore perfect candidates for receiving FMT. However, GF animals display an immature and underdeveloped lymphoid system compared with animals living in a conventional microbiological environment (15). In addition, maintaining GF animals requires specialised facilities and may not be feasible in many institutions. Furthermore, GF mice administered FMT also harbour chronic alterations that result in reduced intestinal absorptive function (16). As such, GF animals may not be suitable for all studies, especially when assessing the impact of FMT on host physiology and immunology.

The antibiotic-based approach is usually preferred, as it avoids several issues seen in GF models, allowing mice to undergo normal colonisation and full postnatal development of their immune system (17, 18). However, a consistent protocol is needed for the composition, dose, frequency, length, and mode of antibiotic administration, as highlighted by Kennedy, King (17). Most studies based on antibiotic-induced microbiota depletion of gut use a cocktail comprising of vancomycin, neomycin, ampicillin, and metronidazole. Nonetheless, the duration of antibiotic administration in drinking water varies substantially from 3 days up to 10 weeks (8, 17, 19–21). Prolonged antibiotic use can have an adverse impact on animal health and potentially alter host physiology (19). Hence, it is important to determine the minimum duration of antibiotic treatment required to achieve gut clearance with minimal side-effects.

Similarly, in animal studies, the frequency and duration of FMT administration have varied from a single administration (22), to once daily for 3 days (16), to three doses a week for 3 weeks (23), or once or twice weekly for 4 weeks (22) and up to twice daily for 12 weeks (20). Furthermore, not all studies have analysed microbial composition after completion of FMT administrations to ensure that engraftment of the human microbiota was stable. Consequently, a simple, quick, cost-effective, reproducible, and translational model for reconstituting the animal gut with human gut microbiota is required.

In this study we aimed to address these gaps in the literature, by developing a gut decontamination protocol and determining the minimum duration of antibiotic treatment required to efficiently deplete mice of their commensal gut microbiota. This then enables us to determine the effectiveness of FMT and engraftment stability of human donor microbiota until the end of our follow up period.

MATERIALS AND METHODS

Animals

Four-week-old male *C57BL/6J* mice were obtained from Australian Biological Resources (NSW, Australia) and allowed to acclimatise for 7 days. Mice were co-housed in a temperature-controlled facility (22–24°C), 12:12 hrs light/dark cycle, with standard irradiated chow (Mouse Maintenance Diet; Speciality Feeds, WA, Australia) and water available *ad libitum*. All mice ($n = 20$) received antibiotic treatment for 21 days. Day 0 corresponded to pre-antibiotics phase. After antibiotic treatment mice were randomised to faecal microbiota transplant (FMT) or Sham groups ($n = 10$ mice per group) (**Figure 1A**). Animals received either FMT or saline by weekly oral gavage for 3 weeks (FMT group or Sham group, respectively). Mice were monitored and samples collected for a further 5 weeks after oral gavage.

Animal experiments were approved by the Animal Care and Ethics Committee (UNSW Sydney, Ethics ID: 18/79B) and conducted in compliance with the NSW Animal Research Act 1985 and the National Health and Medical Research Council (24).

Antibiotic Treatment Protocol

Mice received an antibiotic cocktail consisting of Ampicillin 1 g/L, Vancomycin 0.5 g/L, Neomycin 1 g/L, and Metronidazole 1 g/L, in drinking water supplemented with 10% sucrose for 21 days *ad libitum*. This combination of antibiotics is the most commonly used cocktail in literature (17) (reviewed by) and is effective in depleting the microbiome with minimal effects on the morbidity and mortality of the animal (21, 25). The antibiotic solution was changed three times a week. Water intake was monitored throughout the 21 days of antibiotic administration. Following completion of the antibiotic protocol,

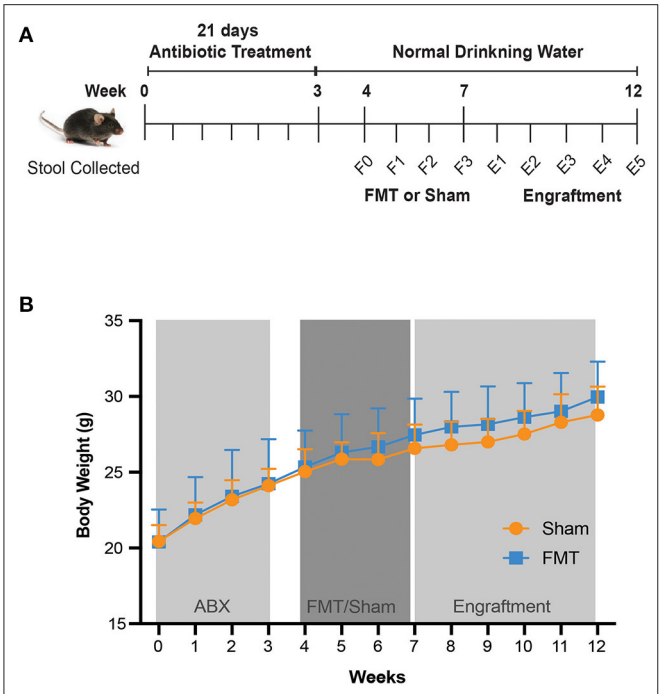

FIGURE 1 | Study design and mouse body weight. **(A)** Study design. From five weeks of age, mice were given antibiotics in drinking water for 21 days. FMT of human donor stool sample (FMT group) or Saline (Sham group) was then orally administered weekly for 3 weeks, followed by a 5 week follow up period. Stool was collected every 3–4 days from day 0 until day 21, and then collected weekly until the completion of the experiment. F0–F3, FMT (Faecal Microbiota Transplant) weeks 0–3; E1–E5, Engraftment weeks 1–5. **(B)** Mouse body weight over the course of 12 weeks, with different treatments. Values shown are average ± SD (n =10 mice/group).

regular drinking water was provided *ad libitum* for the remainder of the experiment.

Faecal Microbiota Transplant (FMT) Protocol

Faecal microbiota solution was prepared from fresh human stool sample. Stool was collected and homogenised in phosphate buffered saline (PBS) (0.1 g/mL) within an hour of collection. The mixture was passed through a 100 μm cell strainer, followed by a 40 μm cell strainer, and was stored in 15% glycerol/PBS at −80°C. After 21 days of antibiotics, ∼0.2 mL (a maximum volume of 10 mL/Kg) of faecal solution (FMT group) or an equivalent volume of Saline (Sham group) was administered to mice via oral gavage once a week for 3 weeks.

Murine Stool Collection

Stool samples from individual mice (at least three pellets per mouse) were collected aseptically for bacterial culture, fluorescence *in-situ* hybridisation (FISH), qPCR and 16S rRNA sequencing. Stool samples were collected before starting antibiotics on day 0 and every 3–4 days during antibiotic treatment (days 3, 7, 10, 14, 17 and 21). Stool samples were also collected before inoculation of faecal microbiota (F0) and after each of the three doses of FMT which were administrated once a week for 3 weeks (F1, F2, F3, respectively). Post FMT completion, to determine efficiency and stability of gut engraftment over time, stool samples continued to be analysed weekly, for a further 5 weeks, corresponding to the engraftment time points (E)1-E5 (**Figure 1A**). Stool pellets were weighed individually, and frozen at −80°C until used.

Stool Culture

To assess bacterial levels in stool pellets, freshly collected pellets were homogenised with 20x volume of PBS, using Qiagen Tissuelyzer II (30 Hz for 1 min). The homogenate was then centrifuged at 300 x *g* for 2 min and the supernatant resuspended in 15% glycerol. A 1:1000 dilution of the stool homogenate was prepared and streaked onto blood agar plates [Columbia Agar Base (Sigma Aldrich Australia) + horse blood (10% vol/vol)] and incubated at 37°C under aerobic, anaerobic and microaerophilic conditions for 48 h before being observed for growth.

Fluorescent *in-situ* Hybridisation—Fish

A universal 16S rRNA probe for bacteria was used for hybridisation (EUB338). Stool pellets were initially homogenised with 20x volume of PBS. FISH was performed as described previously (26, 27). In brief, samples were fixed in 4% PFA (final dilution of 1:100). For slide preparation, 10 μL of sample was added to a gelatine coated slide and left to air dry at room temperature. Slides were then fixed in 95% ethanol for 10 min. Another 10 μL of fixed sample was added to the slide and dried in a hybridisation oven at 50°C. Bacteria was fixed by soaking the slide for 3 min each in 60, 80 and 95% ethanol. Finally, hybridisation master mix was prepared with 1 μL of probe and 9 μL of hybridisation buffer (0.9 M NaCl; 20 mM Tris pH 8.0; 0.1% SDS). Ten microliters of probe/hybridisation mix were added on top of the cells and hybridised for 90 min at 50°C. Slides were washed in dH_2O, air dried, and mounted with soft mounting media.

DNA Extraction

DNA from stool samples was extracted using the PSP Spin Stool DNA plus kit (Stratec Molecular, Berlin, Germany) according to the manufacturer's instructions. Total genomic DNA concentration was determined fluorometrically using Qubit dsDNA broad range assay kit (Qubit 3.0 Fluorometer; Thermo Fisher Scientific, United States).

Quantitative PCR

Assessment of bacterial load remaining following antibiotic treatment was performed by qPCR. This was performed using PowerUp SYBR Green Master Mix (Life Technologies, TX, USA) with universal 16S primers 926F and 1062R (5'-AAACTCAAAKGAATTGACGG-3' and 5'-CTCACRRCACGAGCTGAC-3', respectively) on the QuantStudio 7 Flex (Life Technologies, CA, USA) with the following cycling conditions: initial denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, with a ramp rate of 1°C/s. This was followed by a melt curve analysis from 65 to 95°C at a rate of 0.5°C/s. Each sample was

analysed in duplicate. A positive and negative control were run in each plate. Based on the amplification curve, Ct values for each sample were calculated by the instrument when Ct values were below 40.

16S rRNA Sequencing

Library preparation and sequencing was performed using 341F and 805R primers (5'-CCTACGGGNGGCWGCAG-3' and 5'-GACTACHVGGGTATCTAATCC-3', respectively) for the 16S V3–V4 rRNA region on the Illumina MiSeq platform with paired 300 bp reads at Ramaciotti Centre for Genomics (UNSW Sydney, Australia).

Microbiome Analysis

16S rRNA gene forward and reverse reads were imported into Qiime2 (28). The DADA2 pipeline (29) was used for detecting and correcting Illumina amplicon sequences, removal of primers and chimeric reads, and assembly into sequence variants (SV)/operational taxonomic units (OTUs) (30). Taxonomy was assigned using a naïve Bayes classifier trained on the RefSeq database (31). Alpha diversity metrics investigated included Faith's Phylogenetic Diversity (PD), Evenness, Operational Taxonomy Units (OTUs) and Shannon's diversity index which were calculated using qiime2-q2-diversity. Beta-diversity metrics were calculated using Qiime2. Distance metrics calculated included weighted and unweighted UniFrac50 and Bray–Curtis dissimilarity index. Data was visualised using principal coordinate analysis (PCoA) plots and alpha diversity plots generated within R version 4.0.2 using ggplot2 (32) and phyloseq (33). Other packages used included dplyr (34) and qiime2R (35).

Statistical Analysis

Results are presented as mean ± SD. Statistical analyses for DNA quantification and qPCR were assessed by one-way ANOVA and *post-hoc* Tukey's multiple comparisons test. P values of <0.05 were considered statistically significant. Statistical analyses were performed with Prism v8.4 (GraphPad Software, Inc.).

A Kruskal–Wallis test with Dunn's *post-hoc* test was used to identity individual variations in alpha diversity between treatment groups. Distance based permutation multivariate analysis of variance (PERMANOVA) (36) was performed to test the null hypothesis that there were no differences in the microbial community structure across treatment at a significance level of $P = 0.05$ based on 999 permutations. Resulting P values were false discovery rate (FDR) corrected with 0.05 as the cut off. Corrected P values are presented as q-values.

RESULTS

Broad-Spectrum Antibiotic Regimen Did Not Impact General Health of Animals

Throughout the 3 weeks of antibiotic treatment, there were no adverse effects nor animal deaths. Animal well-being was also confirmed by measuring body weight and water intake. All mice gained weight (**Figure 1B**) and had a stable water intake (**Figure S1A**), indicating that general health of animals was maintained. Stool weight was unchanged for the first 7 days of antibiotic treatment, with subsequent increase in stool weight observed from day 7 to day 17 compared to day 0 (baseline) ($p < 0.001$) (**Figure S1B**). At day 21, stool weight had returned to baseline (**Figure S1A**). No observable change in stool consistency was recorded throughout this period. Importantly, there were no differences in body weight (**Figure 1B**), water intake (**Figure S1A**) or stool weight (**Figure S1B**) when comparing animals that would subsequently receive FMT or Sham, thus providing a stable baseline for subsequent treatment comparisons.

Broad-Spectrum Antibiotic Regimen Efficiently Decontaminated the Gut After Seven Days

Next, we determined the optimal antibiotic treatment period for gut decontamination. To evaluate the efficacy of broad-spectrum antibiotic on bacterial depletion, bacterial culture, qPCR and FISH of stool aliquots were undertaken to measure bacterial load. Prior to antibiotic treatment, at day 0 (baseline), measures of high bacterial presence were observed across all assays as expected. Numerous colony forming units (CFUs) were observed on blood agar plates (**Figure 2A**), DNA concentration was 2.6 ± 0.8 ng/mg stool (**Figure 2B**), an average qPCR threshold cycle (Ct) of 16.8 was obtained (**Figure 2C**), and bacteria of various morphologies were confirmed by FISH (**Figure 2D**).

At day 3 of antibiotic treatment, no CFUs were visible on aerobic and microaerophilic plates, while a dramatic reduction in CFUs was observed on anaerobic plates (**Figure 2A**). Total stool DNA decreased significantly (96% reduction) when compared to baseline ($P < 0.00001$; **Figure 2B**), and average Ct values of 34.2 indicates a 100,000-fold reduction in bacterial DNA ($p < 0.0001$; **Figure 2C**). In addition, limited numbers of fluorescent bacterial cells were observed using FISH assay (**Figure 2D**), indicating a significant reduction in bacterial presence at day 3 of antibiotic treatment.

From day 7 of antibiotic treatment, there were further reductions in bacterial presence, which remained constant until day 21. At all 5 timepoints between day 7 and day 21 (inclusive), no bacterial growth was observed (**Figure 2A**), total DNA concentration was reduced by 98% compared to baseline (**Figure 2B**), average Ct values of 39.3 were equivalent to negative controls (**Figure 2C**), and no bacterial cells were visible in FISH assay (**Figure 2D**). Our results showed that broad-spectrum antibiotic cocktail was effective at gut decontamination following 7 days of antibiotic treatment and was maintained thereafter.

Recolonisation of the Gut After Three Doses of Human Donor FMT Remained Stable for Five Weeks

Following antibiotic gut decontamination, we next investigated the degree of recolonisation by oral administration of either donor faeces (FMT) or saline (Sham) for 3 weeks. There was no impact of FMT or Sham on body weight (**Figure 1B**).

In animals that received FMT, the microbial composition shifted away from the initial community structure that was present prior to oral gavage (F1–3 vs F0; $q < 0.05$; **Figure 3A**)

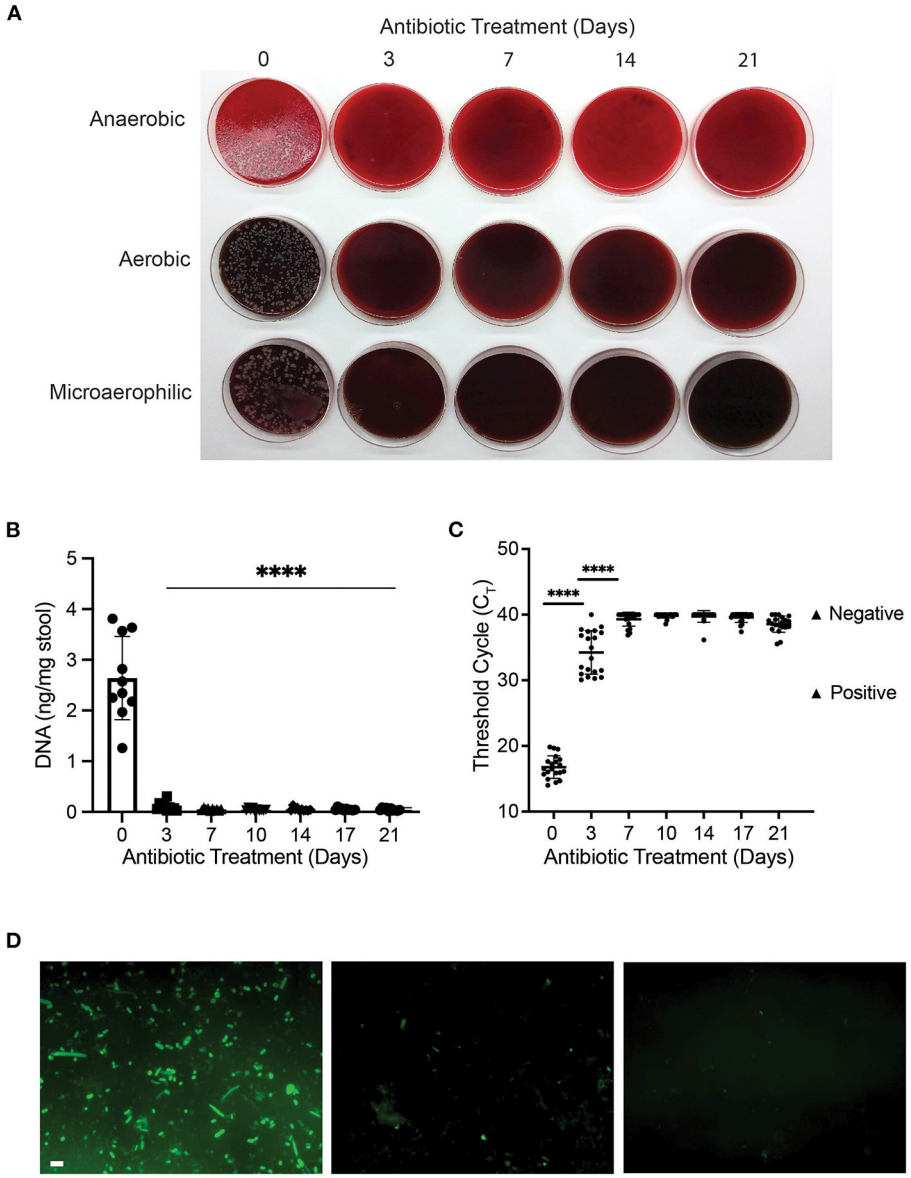

FIGURE 2 | Antibiotic treatment reduced bacterial content of mouse gut after three days. **(A)** Bacterial culture of stool samples on 10% horse blood agar plates; **(B)** DNA concentration (ng/mg of stool) of stool samples during the 21-day course of antibiotic treatment with ampicillin, vancomycin, neomycin and metronidazole (****$P < 0.0001$, One-way ANOVA); **(C)** Variation in Ct value of 16S rRNA gene amplification during antibiotic treatment (****$P < 0.0001$, One-way ANOVA). Positive and negative controls for 16S are represented in the graph; **(D)** Representative pictures of fluorescent *in-situ* hybridisation at day 0 (D0; left panel), day 3 (D3; middle panel) and day 7 (D7; right panel) of antibiotic treatment. Scale bar 2 μm.

and most resembled the donor stool (Bray Curtis dissimilarity index) after the first FMT dose, at F1 (**Figures 3A,B**). After subsequent FMT doses (F2 and F3), the gut microbiota composition became more dissimilar to the donor stool sample, with subsequent stabilisation during the engraftment period (E1–5) (**Figure 3B**). Following FMT administration weekly for 3 weeks, the community diversity was increased compared to F0 ($q < 0.05$) and was sustained for a further 5 weeks to the end of the study (**Figures 3C,D**). Taxonomic profiling of the FMT group at 5 weeks engraftment (E5) confirmed the presence of dominant taxa originating from the donor, including Firmicutes, Bacteroides, Verrucomicrobia and Proteobacteria (**Figure 3E**), indicating successful FMT engraftment that was stable for 5 weeks.

The microbial community of the Sham group also differed from the initial community structure that was present prior to oral gavage ($q < 0.05$) (**Figure 3A**), indicating recolonization of the gut irrespective of FMT treatment. Since all animals received antibiotic gut decontamination, this is likely due recovery of endogenous gut microbiota following antibiotic

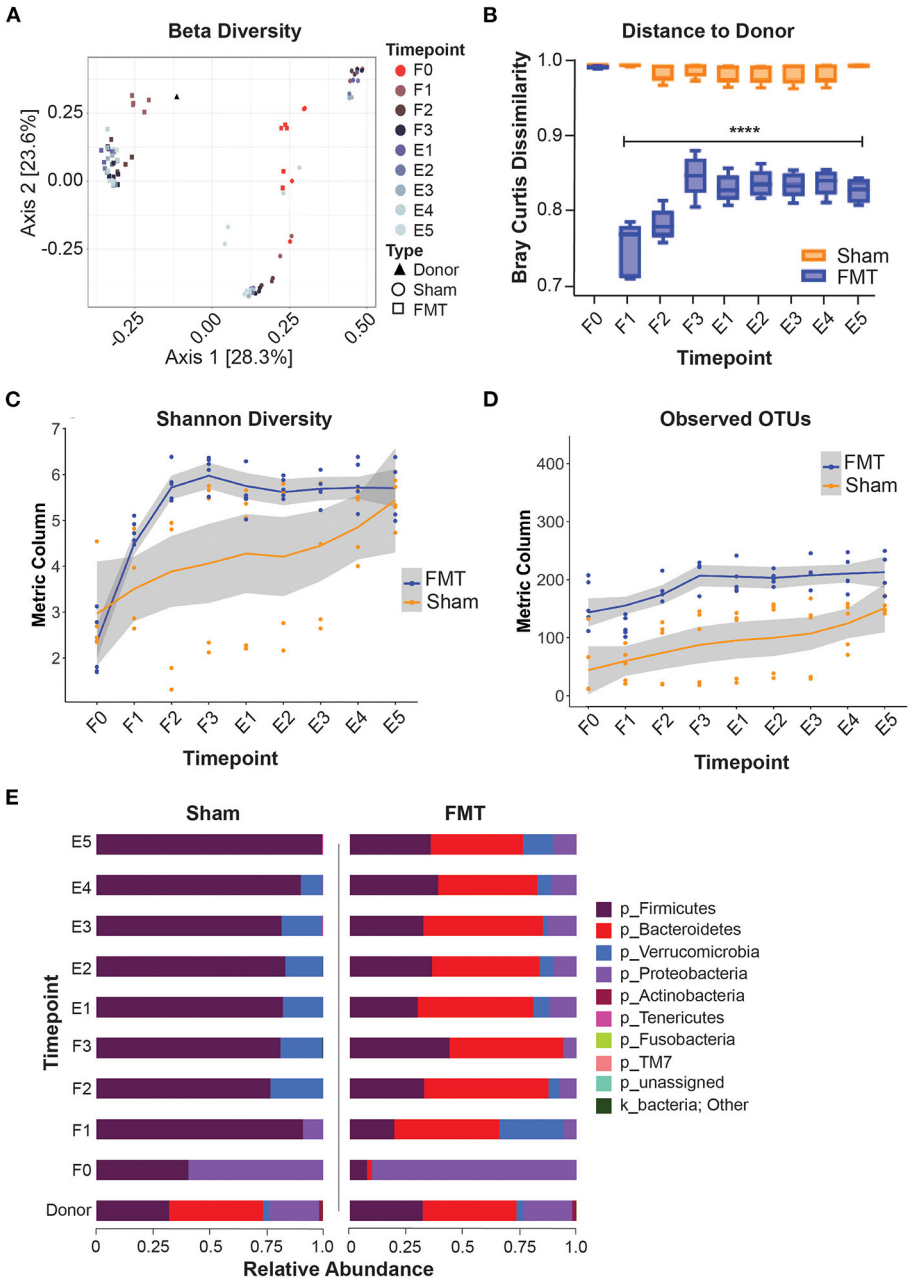

FIGURE 3 | Microbiome composition shift and engraftment after FMT. **(A)** PCoA plot showing beta-diversity of different treatment groups. **(B)** Bray Curtis dissimilarity index comparing distances to donor microbiota. **(C,D)** Alpha-diversity indices during and after FMT. **(E)** taxonomy data (Phylum level) showing successful engraftment of donor microbiota. F0–F3: FMT weeks 0–3; E1–E5, Engraftment weeks 1–5; OTUs, operational taxonomic units.

decontamination. The microbial community composition of the Sham group differed from that of the FMT group at all comparative timepoints ($q < 0.05$), indicating no cross-contamination of the control mice from environmental input (**Figure 3B**). Taxonomic composition of the Sham group showed near total domination by Firmicutes in the following five weeks post antibiotics treatment (**Figure 3E**), again suggesting recovery of the gut microbiota following antibiotics.

DISCUSSION

GF mice are ideal models for gut microbiota studies. However, GF models harbour immunological defects, and the feasibility of additional housing requirements render it challenging for many studies. Thus, broad-spectrum antibiotic depletion of the gut microbiota in animals is used as an alternative. However, there are inconsistencies in the method, dose, and duration of

treatment regimes. In this study, we determined the optimal duration required for effective gut decontamination as well as frequency of FMT required to achieve effective gut engraftment by donor FMT. We tested for the presence of faecal bacteria at multiple timepoints by multiple modalities using bacterial culture, qPCR, and FISH analysis. Our results showed that remnants of bacteria were present after three days, and that the optimal duration of antibiotic treatment for gut decontamination was seven days. We also showed that three weekly doses of FMT were sufficient for successful engraftment following antibiotic gut decontamination, and that following FMT, bacteria from the donor persisted in animals for the entire five weeks of follow up.

Indeed, antibiotic depletion of microbiota is necessary for the successful engraftment of FMT (37) as microbiota depleted mice have been shown to exhibit equivalent or better engraftment than GF mice (16, 37). However, long periods of antibiotics may lead to unwanted side effects. For instance, 12 days of antibiotic treatment can lead to a significant overgrowth of fungi in mouse faeces (8). Hence, a shorter course of antibiotics is potentially a better approach. Our results supported the previous report indicating that a 7-day course of ampicillin, vancomycin, neomycin, and metronidazole was a viable method to deplete the gut of its native microbiota prior to FMT (8). This was achieved without any observed side-effects, such as dehydration, diarrhoea, or loss of body weight, that may be associated with extended antibiotic treatment.

We observed a reduction in bacteria (both bacterial growth and reduction in bacterial DNA load) within three days with qPCR assessment confirming optimum bacterial DNA depletion was achieved following seven days of antibiotic therapy. This was in concordance with other studies that administered antibiotics via oral gavage or drinking water and observed depletion of the gut microbiota within four days. In this setting, extending the antibiotic regimen beyond seven days did not improve the efficiency of bacteria depletion (8). Notably, the mode of delivery was also important, with similar efficacy when antibiotics were administered via drinking water or twice daily doses via oral gavage. However, a single daily dose via oral gavage did not sufficiently deplete the gut microbiota (8). We opted to deliver the antibiotic cocktail via drinking water, which minimised discomfort and stress to the animals while also being simple and consistent to perform. Taken together, our antibiotic treatment regime combined frequently used antibiotics with a simple delivery method that, when administered for seven days, was highly effective at depleting the gut of bacteria.

We noted that bacteria that remained following antibiotic treatment were not those that were initially abundant in untreated mice, but rather represented residual taxa that were resistant to antibiotic treatment. This supported previous observations that antibiotic treatment exerted a selective pressure on bacterial communities (38), leading to enrichment of communities that were initially scarce. These residual indigenous species had been shown to be able to outcompete a considerable proportion of the human donor microbiota within the first week following FMT (37).

Addressing the question of the frequency and intervals of FMT to achieve successful engraftment is also important. A singular FMT inoculation was shown to elicit no clear improvements in microbial diversity when compared to saline alone (20, 39). Therefore, multiple FMT doses were necessary to maintain a stable engrafted microbiome. Previous concerns were raised that FMT dosing at short intervals, such as twice a week for several weeks, might disturb the newly established ecosystem and impair its equilibrium (22). Our results indicated that three weekly doses of FMT was able to effectively engraft the decontaminated gut with abundances of Firmicutes, Bacteroides, Verrucomicrobia and Proteobacteria similar to that of the donor. Importantly, this similarity in microbiome composition to the human donor sample remained for the entire five weeks of follow up, suggesting that the frequency and interval tested, were successful in achieving stable engraftment.

Despite our results showing good engraftment of donor microbiota, the study does have limitations. A longer follow up period could have determined the length of persistence of successful engraftment in mice. Although there is compelling evidence for the use of antibiotics in successful FMT engraftment, we did not compare our results to mice without antibiotic decontamination, nor to germ free mice due to feasibility.

Although our gut depletion protocol is likely applicable across different groups of mice, other factors need to be taken into consideration when interpreting results utilising gut depletion and subsequent FMT. We have only used young, male C57Bl/6J mice, which are the most widely used inbred, general-purpose strain of mouse as they are a stable strain and easy to breed (40, 41). Nevertheless, the gut microbiota is likely to be different in different strains and in female mice, which may affect the development of disease (42). A number of studies have also demonstrated that the gut microbial structure and diversity changes with age (43–45). However, few studies utilise other mouse strains in gut depletion studies and even fewer have validated the efficacy of gut depletion following antibiotic treatment. Effectiveness of the protocol can easily be confirmed in different strains, ages, and sexes using the same methods in this study. Assessment of the colony-forming units (CFUs) from faecal samples plated in aerobic and/or anaerobic conditions on non-selective media, and qPCR of the 16S rRNA gene allows for culture-independent assessment of gastrointestinal bacterial load. These techniques are easily accessible and reproducible to quickly assess the efficacy of gut microbiota depletion.

The aim of this protocol was to deplete gut bacteria, which is arguably independent of the mouse strain, age and sex. One caveat would be the residual gut microbiota post-antibiotics may be different between mice of different strains, ages and sexes which is an inherent limitation of antibiotic-mediated gut depletion protocols. This would arise from differences in the initial colonisation of the gut microbiota prior to antibiotics treatment. These differences may result in different immune and inflammatory responses (46) and lingering effects of initial host-microbiota interactions cannot be discounted. Depletion of the gut microbiota with broad spectrum antibiotics also changes host immunology and has been associated with altered immune cell

populations. For example, secretory IgA and various immune cell types were depleted in the intestine, while in the spleen, dendritic cells and neutrophils were depleted, but basophils were enriched (17) (reviewed by). Thus, care should be taken when planning studies to ensure the gut microbiota is profiled at different timepoints to ensure these factors are taken into consideration. Nevertheless, this protocol is likely applicable to multiple strains of mice of differing ages and sexes, but factors inherent to antibiotic-mediated gut depletion need to be considered.

CONCLUSION

We describe a viable and efficient protocol for gut decontamination and microbiota manipulation studies, consisting of seven days of antibiotic administration in drinking water, followed by three weekly doses of faecal transplant into recipient mice. This approach to decontaminate the gut prior to FMT was cost-effective and less invasive, providing an alternative to using GF mice. Standardising methodologies for FMT in animal research are required for the effective translation of pre-clinical findings.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA765053.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Ethics Committee, UNSW Sydney.

AUTHOR CONTRIBUTIONS

This study was performed in collaboration among all authors. NA designed the study, drafted the manuscript, and performed the statistical analyses. NA and AR collected the animal data. NA, AR, and SKh performed the experimental work. NA and EM performed the microbiome analyses. NA, GH, and AZ analysed the results and supervised the study. SS and SKo revised the manuscript and made significant changes during the review process. All authors contributed to revision of the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.770017/full#supplementary-material

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Alcohol-Related Elevation of Liver Transaminase Is Associated With Gut Microbiota in Male

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Alcoholic liver damage has become a widespread health problem as alcohol consumption increases and is usually identified by elevated liver transaminase. We conducted this study to investigate the role of the gut microbiome in the individual susceptibility to alcoholic liver injury. We divided the participants into four groups based on alcohol consumption and liver transaminase elevation, which were drinking case group, drinking control group, non-drinking case group, and non-drinking control group. The drinking case group meant participants who were alcohol consumers with elevated liver transaminase. We found that alpha and beta diversities of the drinking case group differed from the other three groups. Species *Faecalibacterium prausnitzii* and *Roseburia hominis* were significantly in lower abundance in the drinking case group and were proved the protective effect against inflammatory liver damage in the former study. *Ruminococcus gnavus* exhibited the most positive association to alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and contributed to liver inflammation.

Keywords: gut microbiome, alcohol, liver transaminase, whole-genome sequencing, individual susceptibility, inflammation

INTRODUCTION

Alcohol abuse is an important public risk factor for a variety of health problems and is related to more than 200 diseases, among which the liver is a major target organ (1–3). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are the most commonly used and the most easily available liver injury serum markers (4). When hepatocyte necrosis occurs, ALT and AST can be released into the blood circulation, resulting in increased levels of serum ALT and AST (5).

The earlier investigations uncovered several mechanisms of alcohol-induced liver injury. Ethanol-induced oxidative stress (6) and the toxic product of ethanol metabolism acetaldehyde were found to contribute to liver damage (7). Notably, the emerging evidence confirmed the importance of gut microbial dysbiosis in the progress of alcoholic liver damage. The altered colonic microbiome was observed in alcoholism: the gut microbiome dysbiosis in alcoholic liver disease is long-lasting and serum endotoxin levels were higher than the healthy individuals (8). Alcohol can

improve intestinal permeability through transepithelial permeability and paracellular permeability (9). The elevated intestinal permeability, namely a gut leaky, is associated with liver injury, which leads to the translocation of bacterial toxins into the bloodstream (10, 11). Endotoxin can enter the liver through the blood circulation, activate Kupffer cells, and induce liver damage (12).

Individual susceptibility plays an important role in hepatocyte necrosis (13). Only about 35% of those who have alcohol use disorders develop early liver disease. Many risk factors contribute to liver damage susceptibility. Felix Stickel et al. found that the PNPLA3 rs738409 variant was associated with alcoholic liver cirrhosis and elevated aminotransferase levels in Caucasians (14). Mice that received the intestinal microbiota from severe alcoholic hepatitis patients developed more serious liver inflammation, compared to mice harboring intestinal microbiota from no alcoholic hepatitis, which indicated that the gut microbiome contributed to the individual susceptibility of alcoholic liver disease (15). Therefore, we aimed to discover the association between the gut microbiome and individual susceptibility in alcoholic liver damage.

Considering that normal ranges of ALT for men and women were different, and the number of women who drink alcohol is relatively low (16), we only included men in this study. In our study, we found that with similar drinking behaviors (which meant there was no statistical difference in drinking behaviors between the two groups), 23 participants developed elevated aminotransferase, and 55 participants had normal aminotransferase. We hypothesized that the individual susceptibility of alcoholic liver damage is associated with the gut microbiome and firstly used whole-genome sequencing (WGS) to explore the relationship.

METHODS

Study Population

In this cross-sectional study, participants were recruited at the First Affiliated Hospital of Zhengzhou University, Eastern District of the Hospital between January 2018 and April 2019. Totally 531 individuals who had undergone routine physical examinations and had stool specimens collected in the Physician Health Center were included in our study. These were the exclusion criteria: (1) females, (2) participants under 18 years old, (3) cancer or systemic diseases patients, (4) hepatic cyst patients, (5) participants with histories of diarrhea in the previous 3 months, (6) participants who took any antibiotics 3 months prior to the collection of stools, (7) participants who have HBsAg positive illness, and (8) participants who drank alcohol only once a month in the drinking group (**Figure 1A**).

To clarify how alcohol regulates gut microbiota to cause liver damage, we designed four groups based on two variables: alcohol consumption and liver aminotransferase, namely ALT and AST. The four groups are as follows: (1) non-drinking control group: non-drinkers with normal ALT and AST; (2) non-drinking case group: non-drinkers with elevated ALT or AST; (3) drinking control group: alcohol drinkers with normal ALT and AST; and (4) drinking case group: alcohol drinkers with elevated ALT or AST. The four groups were matched with age and body mass index (BMI).

Drinking Behaviors Assessment

We collected participants' lifestyle information and personal history including drinking behaviors and smoking status *via* questionnaires. Drinking behaviors were evaluated according to drinking or not, drinking years, drinking frequency, drinking types, and alcohol consumption. If drinking frequency in the questionnaire showed once or twice a month, we calculated it by 1.5 a month. The past week was used as the time period for drinking types and alcohol consumption because individuals could recall their alcohol usage more precisely. Drinking types included liquor, wine, beer, and wine and liquor. Alcohol consumption was assessed based on the alcohol content of various drinking types and consumption of alcoholic beverages. To assess ethanol consumption, we selected the most frequent alcohol by volume for beer, liquor, and wine to calculate ethanol consumption, which are 5, 45, and 12%, respectively. For example, the calculation for beer is: ethanol consumption = beer consumption (ml) $\times$ 45% $\times$ 0.8 (g/ml).

Clinical Characteristics Assessment and Stool Sample Collection

Written informed consent was obtained from the participants. Participants were required to fast the night before the physical examination to draw blood tests and collect stool samples. Questionnaires, blood tests, and stool samples were collected on the same day. Blood samples were detected by Roche Cobas 8000 automatic biochemical analyzer (Mannheim, Germany). Both ALT and AST had normal ranges of 0–40 U/L. Stool samples were collected on the day of the physical examination. Indexes controlled attenuation parameter (CAP) and liver stiffness measure (LSM) were assessed by FibroTouch of HISKY Medical Technologies (Wuxi, China). The normal ranges for CAP and LSM are $<$240 dB/m and $<$7.3 kPa, respectively. A total of 20 participants performed the FibroTouch examination. The numbers of participants who underwent the examination in non-drinking control, non-drinking case, drinking control, and drinking case were 2, 1, 10, and 7, respectively. We obtained the liver function and FibroTouch information from the management system of the hospital. Stool samples from participants were all freshly collected at the hospital and stored at -80°C within 30 min after subpackaging.

Genomics DNA Extraction

Following the manufacturer's instructions, DNA from the microbial population was extracted using the MagPure Stool DNA KF Kit B (Magen, Guangzhou, China). DNA was quantified with a Qubit Fluorometer by using the Qubit dsDNA BR Assay Kit (Invitrogen, USA), and the quality was checked by running an aliquot on 1% agarose gel.

Library Construction

About 1 μg genomic DNA was randomly fragmented by Covaris (Woburn, Massachusetts, USA). The fragmented DNA was selected by magnetic beads to an average size of 200–400 bp.

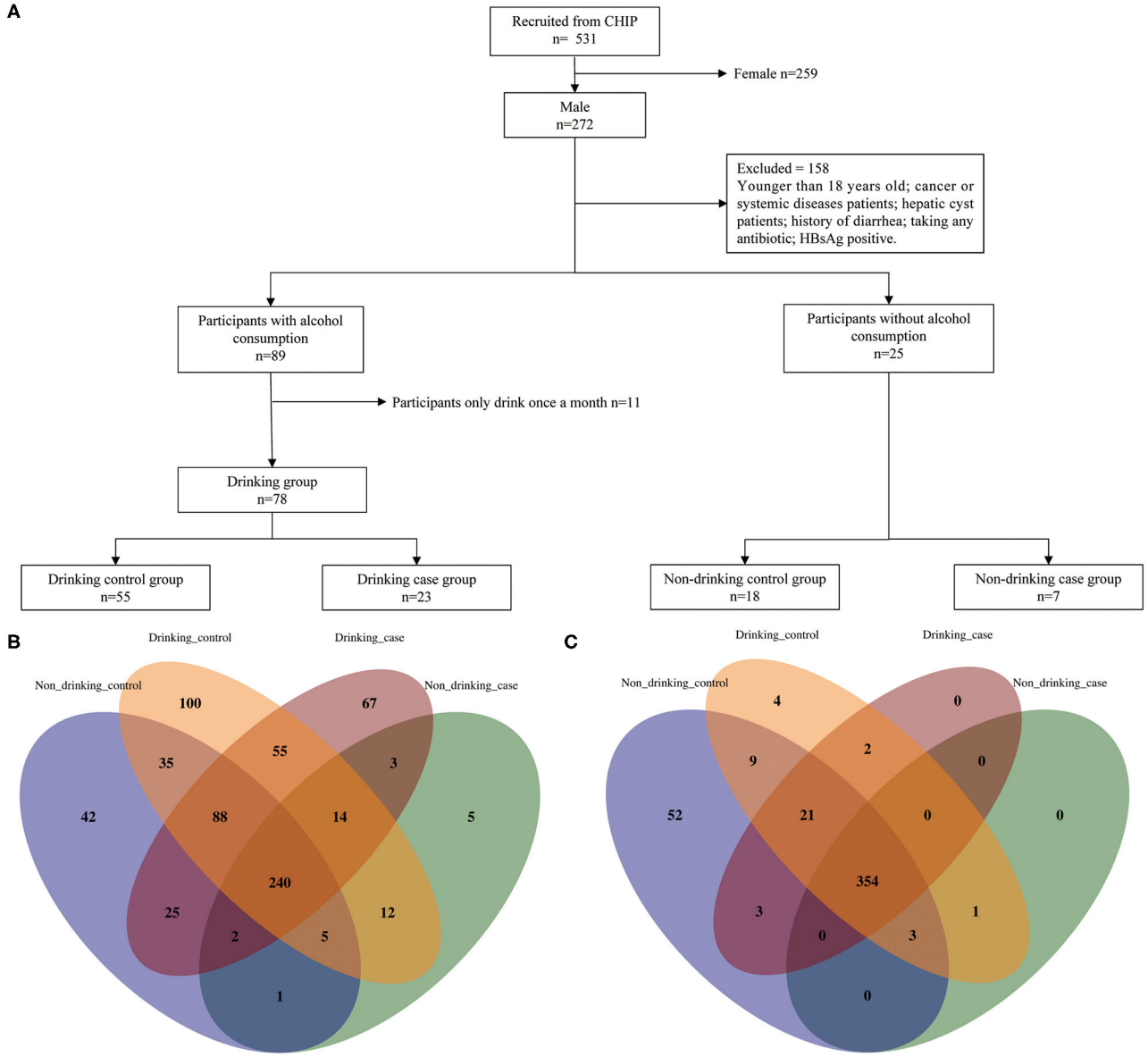

FIGURE 1 | Enrollment flow and distribution of species and metabolic pathways. **(A)** Enrollment flow; **(B)** species distribution; and **(C)** metabolic distribution.

The selected fragments were through end repair, 3′ adenylated, adapters ligation, PCR amplifying, and the products were purified by the magnetic beads. The double-stranded PCR products were heat-denatured and circularized by the splint oligo sequence. The single-strand circle DNA (ssCir DNA) was formatted as the final library and qualified by QC. The qualified libraries were sequenced on BGISEQ 500 platform (BGI, Shenzhen, China).

Quality Control

Questionnaires and fecal samples were all collected on the same day. Women were excluded from our study due to gender variations in ALT and AST, and gut microbiota. The participants who had undergone physical examinations at the Physician Health Center of the First Affiliated Hospital of Zhengzhou University fasted the night before the examination. We only included drinkers who drank more than once a month to draw a clearer differentiation between the drinking groups and non-drinking groups.

Statistical Analysis

Statistical analyses were all performed on R version 3.6.1. Continuous variables in demographic characteristics shown as mean (SD) were compared using the Wilcoxon rank-sum test between two groups. Continuous variables in drinking behaviors shown as median (interquartile range, IQR) were compared

using the Wilcoxon rank-sum test between two groups. A two-tailed p-value < 0.05 was considered statistically significant. The difference of species and pathways of the two groups were calculated by the one-tailed Wilcoxon rank-sum test. Categorical variables, shown as counts and percentages [n (%)], were compared using Fisher's exact test, and a two-tailed p-value < 0.05 was considered statistically significant. Spearman's rank test was used for the correlation analysis.

Microbiota Diversity Analysis

The Shannon index, the Simpson index, and the Gini index were used to estimate alpha diversity using the "vegan" package and "ineq" package of R. Beta diversity was assessed by the Pearson distance and Bray–Curtis distance, which were calculated by "vegan" package. We performed principal coordinate analysis (PCoA) to represent the statistically and visually microbial community profile differences using "ade4" package. Alpha diversity indexed and beta diversity distances were performed using the one-tailed Wilcoxon rank-sum test, with the statistically significant p-value <0.05.

Venn diagrams conducted by "VennDiagram" packages were plotted to reveal the common and unique species or pathways in multiple samples and find differential species or pathways, displaying the similarity and overlap among the four groups.

RESULTS

Demographic Characteristics of the Cohorts

After a strict inclusion and exclusion process, a totally of 103 participants were enrolled in our study, which included 4 groups:

TABLE 1 | Demographic characteristics of the study participants.

Feature	Non-drinking control ($n = 18$)	Non-drinking case ($n = 7$)	P_1 value	Drinking control ($n = 55$)	Drinking case ($n = 23$)	P_2 value	P_3 value
Demographic mean (SD)							
Age (year)	41.778 (8.257)	41.714 (4.572)	0.855	41.527 (9.309)	38.609 (7.953)	0.273	0.268
BMI (kg/m^2)	26.772 (3.532)	28.041 (2.713)	0.397	26.856 (3.112)	27.075 (2.773)	0.493	0.327
DP (mmHg)	81.333 (12.551)	81.714 (8.056)	0.738	82.455 (10.136)	80.304 (9.716)	0.316	0.623
SP (mmHg)	128.111 (14.373)	128.429 (6.051)	0.952	131.709 (12.704)	131.043 (15.032)	0.755	0.864
PP (mmHg)	46.778 (8.822)	46.714 (4.309)	0.879	49.255 (8.859)	50.739 (10.037)	0.709	0.476
Liver function mean (SD)							
ALT (U/L)	23.222 (7.659)	61.571 (18.347)	<0.001	24.145 (7.077)	59.478 (32.517)	<0.001	0.447
AST (U/L)	20.667 (4.79)	35.857 (14.871)	0.006	21.127 (3.977)	36.174 (19.853)	<0.001	0.768
ALP (U/L)	70.056 (16.232)	65.857 (5.757)	0.363	70.582 (16.156)	72.217 (21.405)	0.852	0.540
GGT (U/L)	25.833 (12.552)	49 (30.111)	0.042	41.345 (33.264)	72.913 (61.854)	0.001	0.404
ALB (g/L)	48.578 (2.537)	50.571 (1.996)	0.034	48.802 (2.287)	49.883 (2.677)	0.143	0.508
GLO (g/L)	26.006 (3.614)	29.714 (3.986)	0.032	26.682 (3.929)	25.87 (4.124)	0.393	0.062
TBIL (μmol/L)	13.829 (5.048)	9.904 (3.185)	0.064	11.792 (3.986)	15.013 (5.897)	0.015	0.029
DBIL (μmol/L)	5.342 (2.135)	4.136 (1.31)	0.244	4.819 (1.277)	5.69 (2.104)	0.070	0.050
IBIL (μmol/L)	8.489 (3.362)	5.757 (2.148)	0.069	6.973 (3.06)	9.326 (4.203)	0.016	0.031
GLU (mmol/L)	5.399 (0.454)	5.659 (1.228)	0.751	5.427 (0.86)	5.364 (0.879)	0.361	0.462
FibroTouch [*n* (%)]							
CAP							
Normal	0/2 (0.0)	0/1 (0.0)	1.000	2/10 (20.0)	2/2 (28.6)	1.000	1.000
High	2/2 (100.0)	1/1 (100.0)		8/10 (80.0)	5/5 (71.4)		
LSM							
Normal	2/2 (100.0)	1/1 (100.0)	1.000	10/10 (100.0)	6/7 (85.7)	0.412	1.000
High	0/2 (0.0)	0/1 (0.0)		0/0 (0.0)	1/7 (14.3)		
Smoking or not							
Yes	6/18 (33.3)	1/7 (14.3)	0.626	14/54 (25.9)	7/22 (31.8)	0.587	0.635
No	12/18 (66.7)	6/7 (85.7)		40/54 (74.1)	15/22 (68.2)		

P_1, Comparisons between non-drinking control group and non-drinking case group.

P_2, Comparisons between drinking control group and drinking case group.

P_3, Comparisons between non-drinking case group and drinking case group.

Continuous variables were compared using the Wilcoxon rank-sum test between two groups. Categorical variables were compared using Fisher's exact test. Statistical analyses were performed using R (Version 3.6.1).

BMI, body mass index; DP, diastolic pressure; SP, systolic pressure; PP, pulse pressure; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyltransferase; ALB, albumin; GLO, globulin; TBIL, total bilirubin; DBIL, direct bilirubin; IBIL, indirect bilirubin; CAP, controlled attenuation parameter; LSM, liver stiffness measure.

non-drinking control group, non-drinking case group, drinking control group, and drinking case group, containing 18, 7, 55, and 23 participants, respectively (**Figure 1A**). Participants' age and BMI were all matched among four groups. The clinical characteristics of the four groups are shown in **Table 1**. The average age of the drinking case group was 38.609 years old. Apparently, ALT and AST were at high levels in the two case groups. Total bilirubin (TBIL), direct bilirubin (DBIL), and indirect bilirubin (IBIL) were all higher in the drinking case group than the drinking control group and non-drinking case group. Globulin tended to be lower in the drinking case group compared to the non-drinking case group. No difference was found in smoking status among the four groups.

Table 2 depicts the drinking behaviors of the two drinking groups. We employed some indexes including the drinking years, drinking frequency, drinking type, and ethanol consumption. There was no significant difference in drinking behaviors between the drinking control group and the drinking case group.

Gut Microbiota Distribution and Diversity of Four Groups

Totally 449 pathways and 694 species (including 215 viruses) were detected in the whole participants. Venn diagrams display the overlaps among the four groups (**Figures 1B,C**). The four groups shared 240 species and 354 pathways, with 397 species and 377 pathways shared by the drinking control group and the drinking case group. Notably, compared to the other 3 groups, there were 67 species unique to the drinking case group.

Species alpha diversity indicated by Shannon index and Simpson index was decreased in drinking case group compared with non-drinking control group. Although there was no statistically significant difference, species Shannon index and Simpson index in the drinking case group tended to be a downward trend in microbiome diversity compared with the non-drinking case group and drinking control group (**Figures 2A,B**). The Gini index was higher in the drinking case group than that of the other 3 groups, indicating the less equal microbiota distribution for the drinking case group, although only the difference between the drinking case group and non-drinking control group attained statistical significance (**Figure 2C**). Differences among the non-drinking case group, drinking control group, and drinking case group in alpha diversity were not observed. The alpha diversity of metabolic pathways yielded similar results (**Figures 2D–F**).

TABLE 2 | Drinking behaviors.

	Drinking control ($n = 55$)	Drinking case ($n = 23$)	*p*-value
Drinking year	15 (7.25–27.5)	15 (10–15)	0.374
Drinking frequency	6 (1.5–6)	1.5 (1.5–6.0)	0.818
Drinking type			
Beer	4/33 (12.12)	0/13 (0.00)	0.186
Liquor	27/33 (81.81)	10/13 (76.92)	
Wine	1/33 (3.03)	2/13 (15.38)	
Wine and liquor	1/33 (3.03)	1/13 (7.69)	
Ethanol consumption (g)	54 (36–99)	72 (54–90)	0.438

Data are median (interquartile range, IQR) or n/N (%). Continuous variables were compared using the Wilcoxon rank-sum test between two groups. Categorical variables were compared using Fisher's exact test.

Altered Overall Gut Microbiota in Drinking Case Group

We performed beta diversity calculated by PCOA to display the overall diversity in microbiome composition among four groups. For species diversity, the Pearson distance and Bray–Curtis distance revealed substantial variations in the microbial community between the drinking case group and the other three groups (**Figure 3**). Samples from the drinking case group (red dots) separated from other groups along the direction of the second axis for the Pearson distance and Bray–Curtis distance, explaining 19.4 and 14.6% of the total variations, respectively.

Metabolic pathway beta diversity was also assessed using the same analysis (**Supplementary Figure 1**). The drinking case group and the other 3 groups demonstrated significant differences in PCoA based on the Bray–Curtis distance. Drinking case participants could separate from the other three groups along the first axis for the Pearson distance and the second axis for the Bray–Curtis distance. Species and pathway beta diversity confirmed that gut microbial communities were different among the drinking case group and the other three groups.

Differential Gut Microbiota in the Drinking Case Group

To compare the gut microbial communities at phylum and species level among the drinking case group and other three groups, microbial significant differences were analyzed by the one-tailed Wilcoxon rank-sum test. The average compositions and relative abundance at the phylum level were displayed (**Figure 4A**). Apparently, phylum *Bacteroidetes* was reduced in the drinking case group vs. the drinking control group, whereas phylum *Firmicutes* was increased (**Figures 4B,C**).

To demonstrate the difference at the species level, we further used Venn diagrams to show the statistically differential overlaps in the distribution of gut microbial species and metabolic pathways (**Figures 4D,E**). Overall, there were 2 differential species and 12 pathways related to the drinking case group. Furthermore, there was no statistical difference among the non-drinking control group, non-drinking case group, and drinking control group in the 2 species and 12 pathways (**Supplementary Table 1**).

The distribution of the statistically differential species and metabolic pathways among the four groups was demonstrated in the Boxplot diagrams (**Figure 5**). Compared to the other three groups, the drinking case group had a lower abundance of differential species, *Faecalibacterium prausnitzii*, and *Roseburia hominis*. Eight of the 12 differential metabolic pathways were enriched in the drinking case group, and the other 4 pathways were decreased in the drinking case group. For the 8 pathways enriched in the drinking case group, three

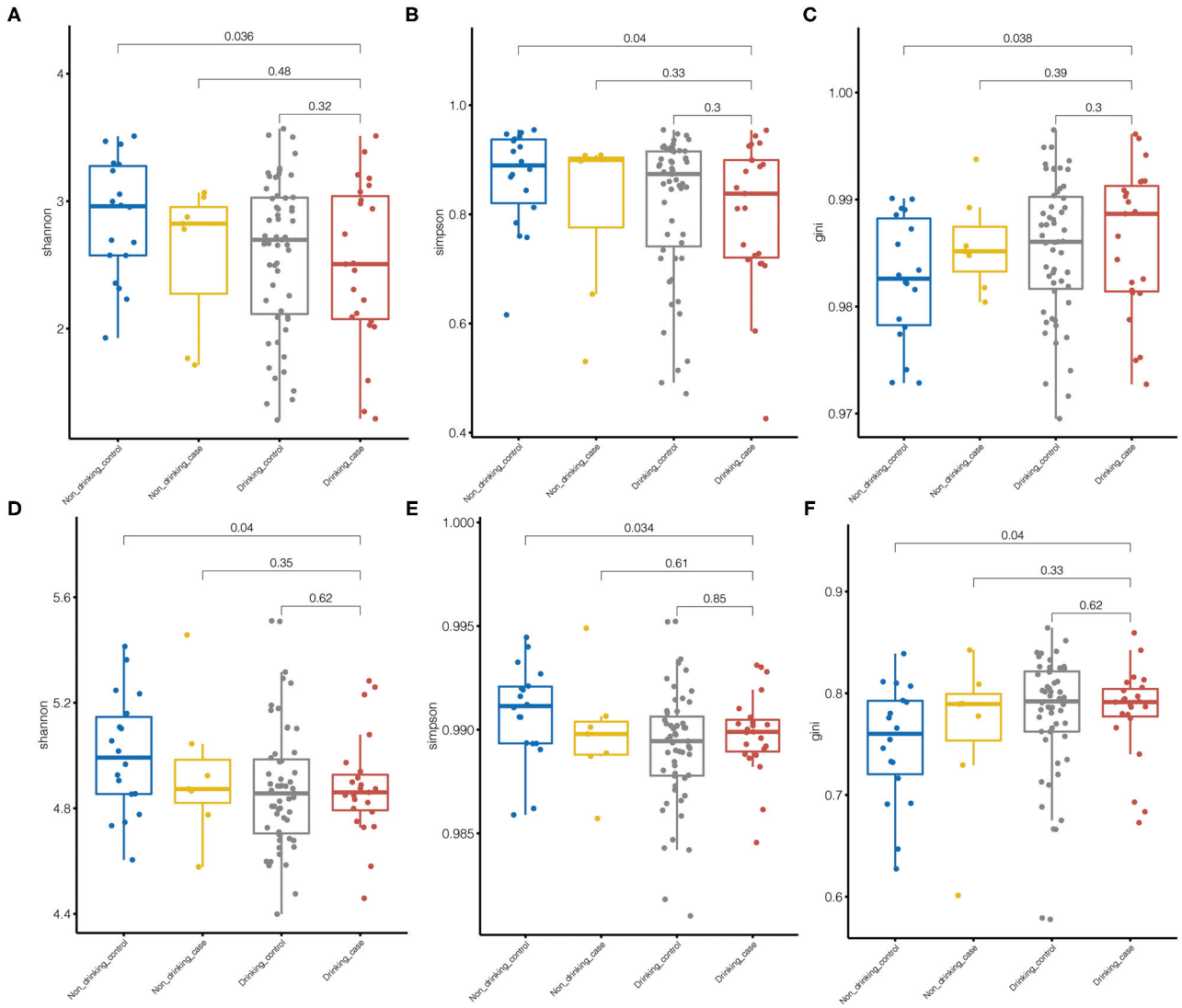

FIGURE 2 | Microbiome diversity. **(A–C)** Species alpha diversity was estimated by the Shannon index, Simpson index, and Gini index, respectively. **(D–F)** Pathway alpha diversity was estimated by the Shannon index, the Simpson index, and the Gini index, respectively.

are associated with nucleoside and nucleotide biosynthesis (PWY-7197, PWY-7208, and PWY_7228), one is associated with amine and polyamine degradation (GLCMANNANAUT-PWY), one is associated with pentose phosphate pathways (NONOXIPENT-PWY), one is associated with carrier biosynthesis (PWY_7371), one is associated with tetrapyrrole biosynthesis (PWY_5188), and one is associated with nucleoside and nucleotide degradation (PWY0-1296). For the four pathways decreased in the drinking case group, one is associated with galacturonate and glucuronate catabolism (GALACT-GLUCUROCAT-PWY), one is associated with β-D-glucuronide degradation (GLUCUROCAT-PWY), one is associated with D-galacturonate degradation (GALACTUROCAT-PWY), and one is associated with mixed acid fermentation (FERMENTATION-PWY).

Correlations Between the Gut Microbiome and Liver Damage

To identify the correlations between clinical variables and microbiota, associations between the gut microbiome and continuous clinical variables were calculated by the Spearman correlation coefficient in all participants. Species heatmap revealed the correlation between species and clinical variables (**Figure 6A**). *R. hominis* showed a negative correlation with ALT and significantly decreased in the drinking case group. Among all the species in the heatmap, *Ruminococcus gnavus* showed the most positive correlation with ALT and AST.

For pathways, we noticed that NONOXIPENT-PWY, GLCMANNANAUT-PWY, PWY-5188, and PWY-7197 were all positively correlated with ALT or AST, and they were increased in the drinking case group (**Figure 6B**).

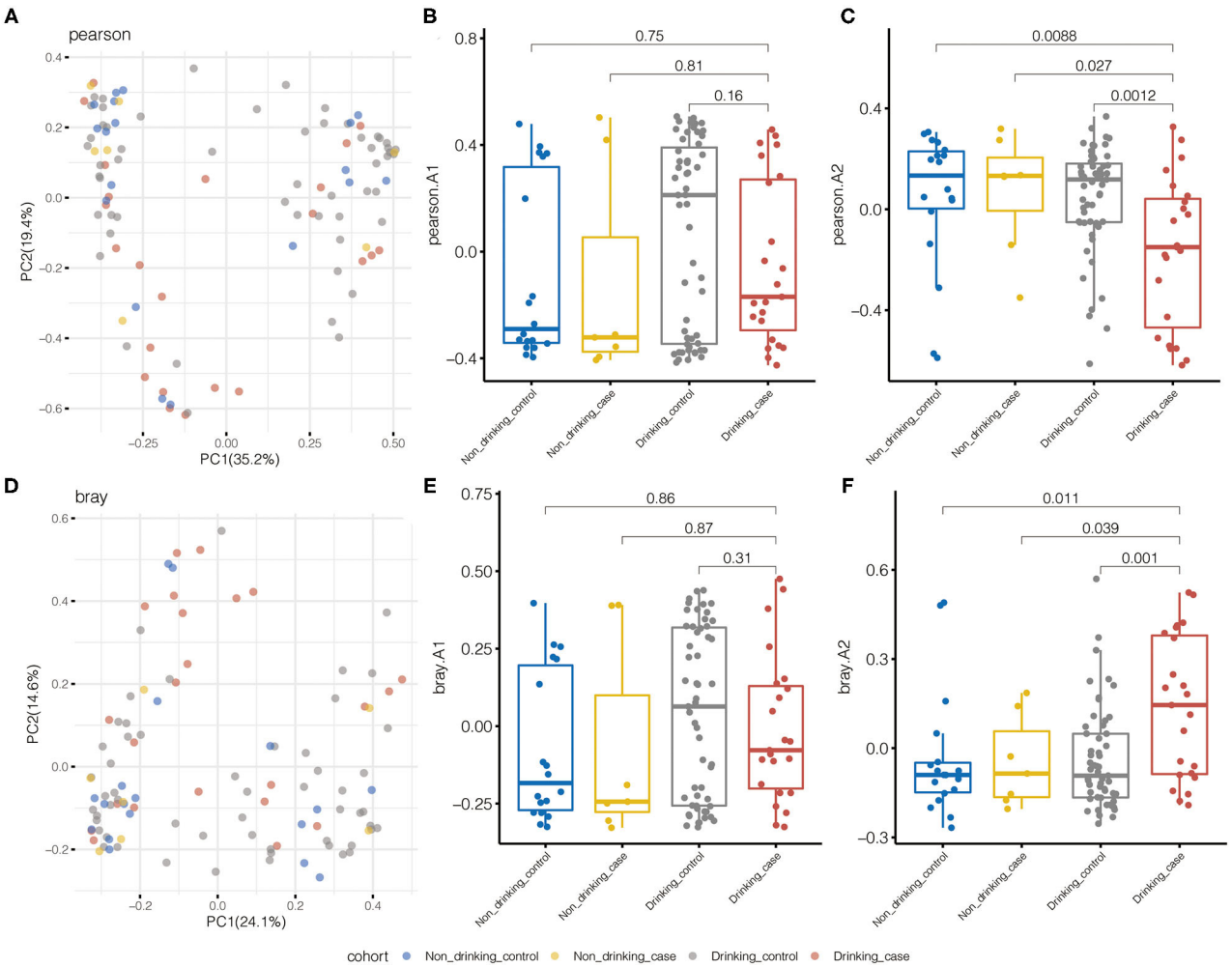

FIGURE 3 | Pearson distance and Bray–Curtis distance illustrated significant differences in the microbial community for species beta diversity. **(A)** Principal coordinate analysis (PCOA) diagram by the Pearson distance. **(B,C)** The first and second principal components based on the Pearson distance. **(D)** PCOA diagram by Bray–Curtis distance. **(E,F)** The first and second principal components are based on the Bray–Curtis distance.

DISCUSSION

We firstly performed WGS in the comprehensive groups to explore the association between the gut microbiome and individual susceptibility in alcohol-related liver transaminase elevation. In our study, although the two drinking groups showed no differences in drinking behaviors, one group had raised transaminases and the other group had normal transaminases—indicating individual variability reflecting the liver injury caused by alcohol consumption. In our study, we observed fecal microbiome community in the drinking case group differed from the other groups and described the significantly different species and metabolic pathways.

Liver function indexes ALT and AST are the easiest markers to assess hepatocellular damage and usually are the screen tests for liver disease (5). Compared to ALT, AST is more widely distributed in many tissues—including liver, skeletal muscle, heart, etc. (17). Considering the weak specificity of AST distribution, we ruled out participants with cancers, any systemic diseases, and any patients with liver disease. ALT and AST also can be influenced by genetic contribution (18), gender differences (19), exercise (20), late sleep, and other environmental factors. Men have higher levels of ALT and AST than women, according to the large cohort studies (16, 21). Considering the possible analytical errors, we only included male participants in this study and also established the non-drinking case group (participants who had no alcohol consumption with elevated ALT and AST).

Microbiome alpha diversity of the drinking case group based on the Shannon index and the Simpson index was lower compared to HC participants. Alcohol use disorder patients were found to have a decreased alpha microbiome diversity in the former study (22). The reduction microbial diversity was one of the major types of gut disease-associated dysbiosis (23) and was documented in many diseases, such as IBD (24), autoimmune

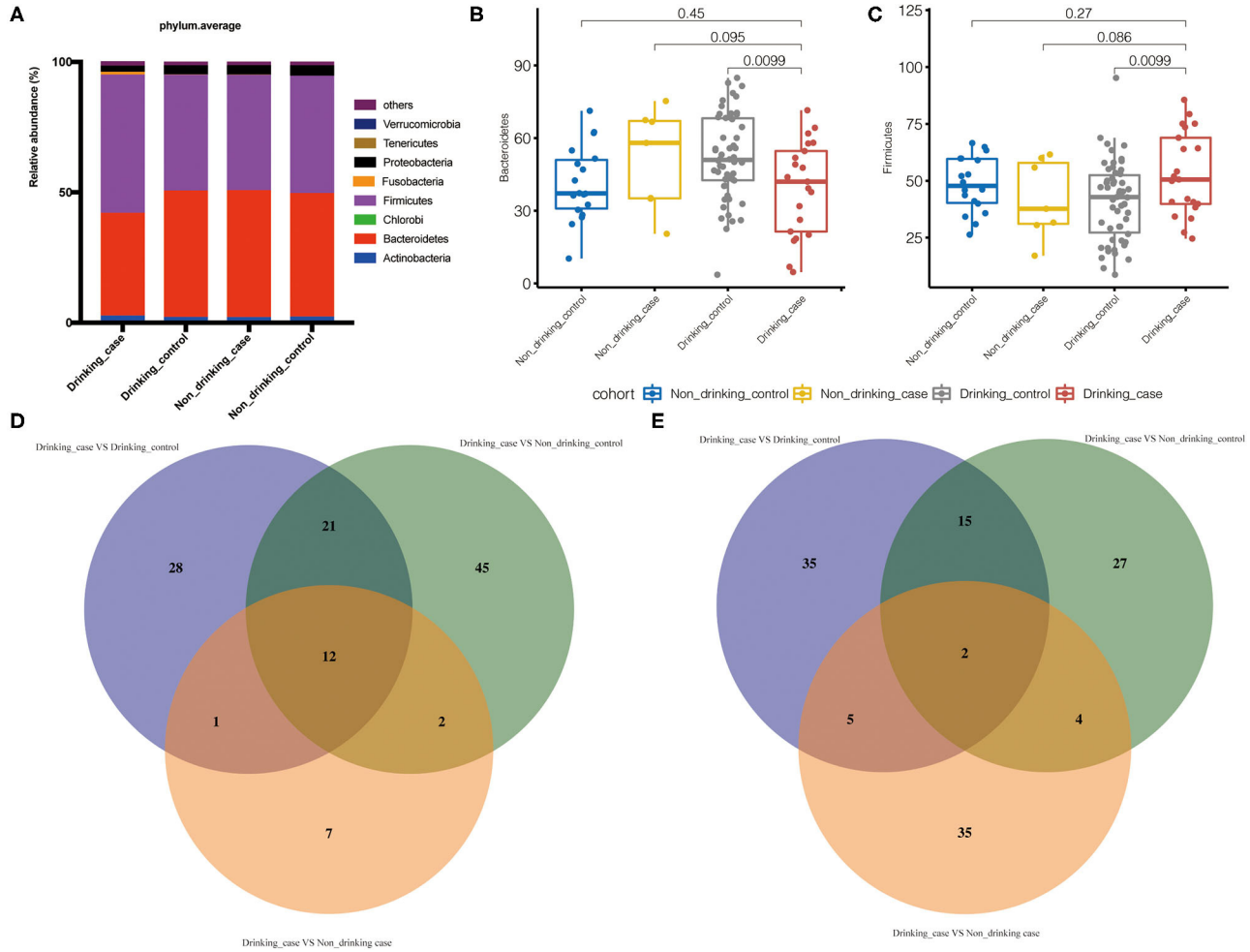

FIGURE 4 | Differential gut microbiota in the drinking case group. **(A)** The average compositions and relative abundance at phylum level of the four groups; **(B,C)** the abundance of phyla *Bacteroidetes* and *Firmicutes* in the four groups; and **(D,E)** the statistically differential overlaps in the distribution of gut microbiome metabolic pathways and species.

hepatitis (25), and type 1 diabetes (26). The prior research proved that individuals with decreased microbial richness have more tendency to develop low-grade inflammation (27, 28). The alcohol consumers who have elevated ALT and AST may develop dysbiosis in gut microbiome composition and more possibility to develop inflammation.

In our study, we found elevated phylum *Firmicutes* and decreased phylum *Bacteroidetes* in the drinking case group compared to the drinking control group. Phyla *Firmicutes* and *Bacteroidetes* are dominant and account for the majority of human gut microbiota (29). Former studies reported lower *Bacteroidetes* in individuals with alcoholism and cirrhosis, which was consistent with our findings (8, 30). Ethanol treatment in mice induced the decrease of *Firmicutes* and *Bacteroidetes,* especially *Bacteroidetes* (31). *Bacteroidetes* was found to be significantly reduced following the ethanol feeding to mice in this 8-week investigation. The different findings of phylum *Firmicutes* may be the result of the difference of observed objects and events.

The intersections of bacteria with statistical differences were obtained using the Venn diagram, and finally, two species were obtained, which were *F. prausnitzii* and *R. hominis*, and both were significantly reduced in the drinking case group. Increased gut permeability permits the translocation of macromolecules including endotoxins, contributing to the alcoholic liver damage (11). *F. prausnitzii* and *R. hominis* are both important butyrate producers and contribute to gut integrity (32). *F. prausnitzii* is one of the most common bacteria in human gut flora (33). Microbial anti-inflammatory molecule (MAM) was discovered to be the production of *F. prausnitzii* and could inhibit the NF-κB pathway in the intestinal epithelial cells, therefore, preventing mice from colitis (34, 35). Activation of NF-κB pathway participates in the breakdown of the intestinal barrier caused by alcohol (36). *R. hominis* significantly decreased in the drinking case group and also showed a negative correlation with ALT. *R. hominis* treatment could relieve colitis and reduce inflammatory markers including interleukin (IL)1-β, IL6, and

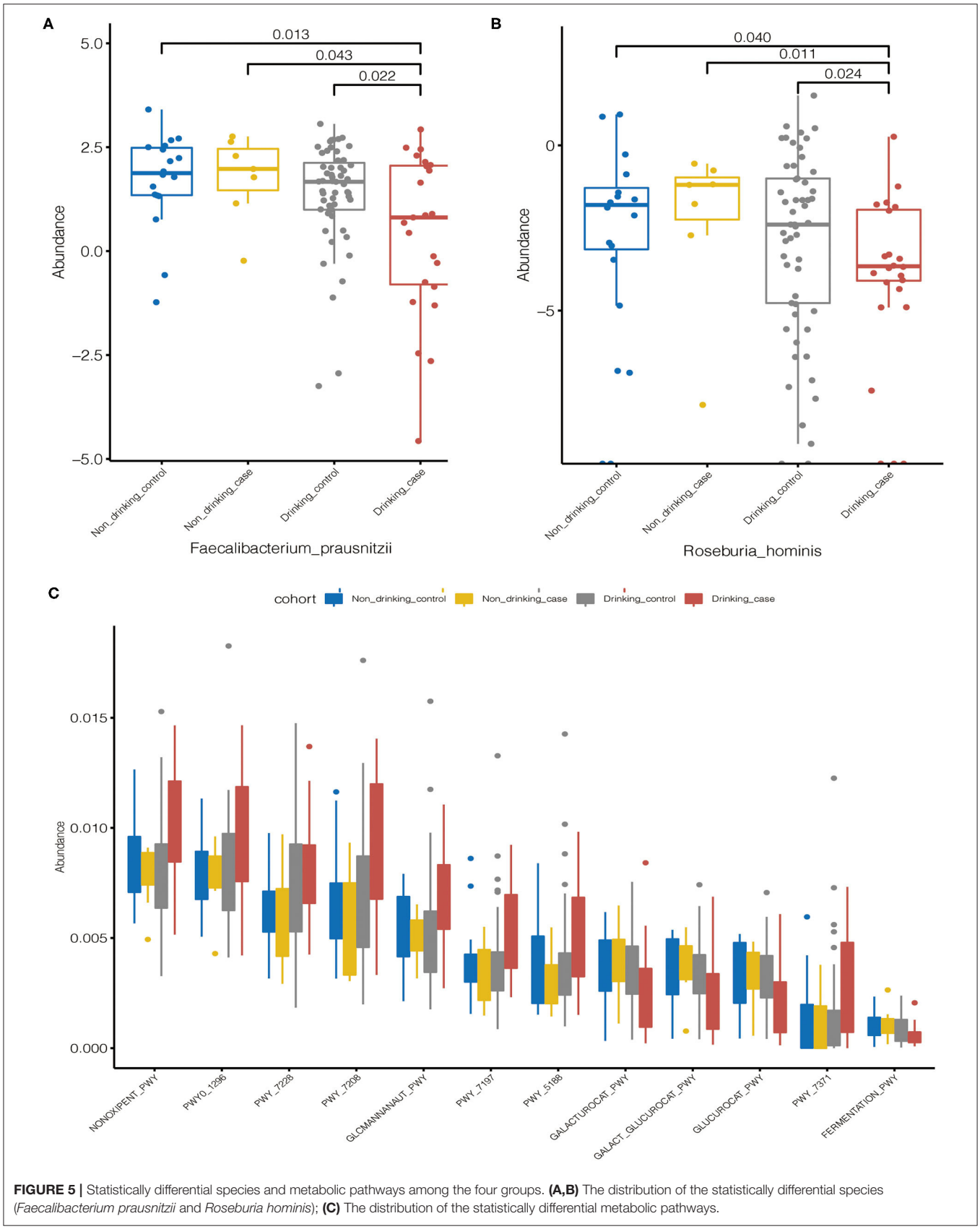

FIGURE 5 | Statistically differential species and metabolic pathways among the four groups. **(A,B)** The distribution of the statistically differential species (*Faecalibacterium prausnitzii* and *Roseburia hominis*); **(C)** The distribution of the statistically differential metabolic pathways.

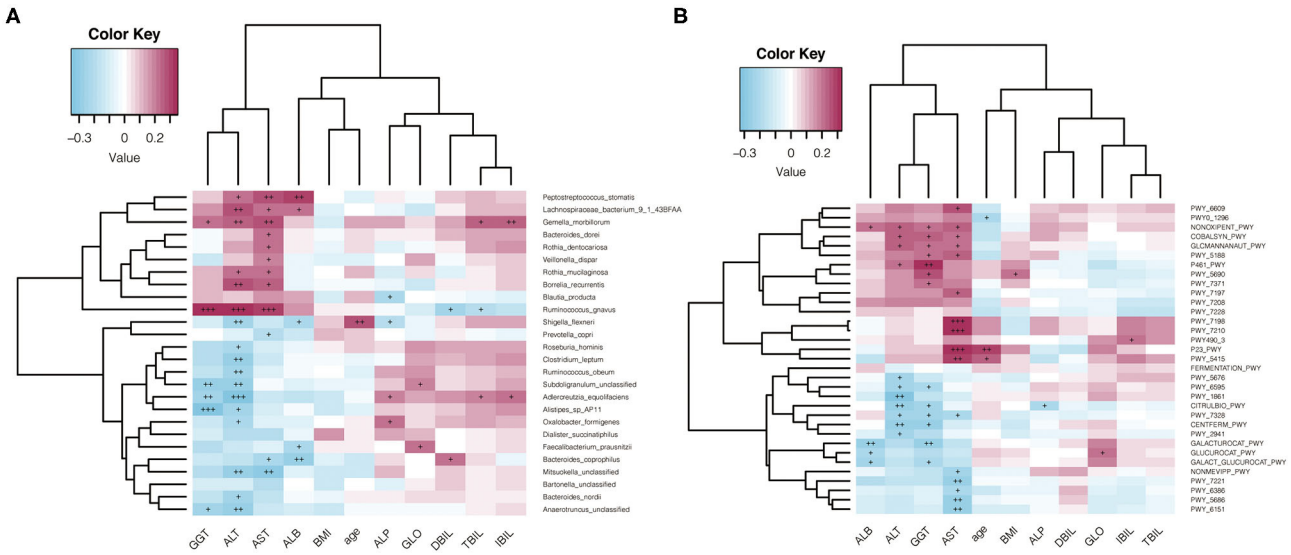

FIGURE 6 | Spearman correlation analysis. **(A)** Spearman analysis of species abundance and clinical indexes; **(B)** Spearman analysis of metabolic pathways and clinical indexes.

tumor necrosis factor (TNF)-α (37). TNF-α is an important inflammatory cytokine and can promote alcoholic liver damage, mostly produced by liver Kupffer cells (38, 39). *Ruminococcus gnavus* showed the most positive correlation, both with ALT and AST. *Ruminococcus gnavus* was reported as a potential pathogen in infectious disease and exhibited an increased abundance in inflammatory bowel disease (40, 41). In 2019, a study reported that *Ruminococcus gnavus* was the producer of inflammatory polysaccharides, a promotor in TNF-α secreted by dendritic cells (42). Therefore, *R. hominis* and *Ruminococcus gnavus* showed opposite effects on the TNF-α production. Alcohol enhances the sensitivity of Kupffer cells to product TNF-α (37, 43), which can be decreased by *R. hominis* and increased by *Ruminococcus gnavus*.

We discovered 12 metabolic pathways that were statistically different among the drinking case group and the other three groups. Among the eight pathways, which increased in the drinking case group, 3 pathways were associated with nucleoside and nucleotide biosynthesis and 1 pathway was associated with nucleoside and nucleotide degradation. The activation of the processes might associate with the dysbiosis of gut microbiome and the possible bacterial overgrowth in patients with alcoholic liver injury (44, 45). The other alterations of metabolic pathways may be generated by the dysbiosis of the gut microbiome.

These are the advantages that only the participants without underlying diseases were included, and we designed four groups based on the alcohol consumption and liver aminotransferase. However, due to the strict exclusion criteria, the number of subjects was limited, and we only included male participants. Some participants forgot their drinking behavior resulting in missed information. In our study, the most important was that we discovered three altered species, which were related to alcoholic liver injury. The finding should be further verified through large-scale studies and animal experiments.

CONCLUSION

Alcoholic liver damage is usually noted early in the clinic with elevated aminotransferase levels. Our findings suggested that the gut microbiome contributes to the susceptibility of individuals to develop liver injury after alcohol consumption. Species *F. prausnitzii* and *R. hominis* exhibited a protective effect on the liver, and *Ruminococcus gnavus* showed a liver-damage effect. Further verification is needed in future studies. Since the three meaningful species were discovered in this study, we believe that after further verification, the probiotic administration or dietary patterns can be used to regulate intestinal flora, and thus protect the liver of alcohol drinkers.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number (s) can be found below: https://db.cngb.org/search/project/CNP0002336/.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Review Committee of Scientific Research Projects in The First Affiliated Hospital of Zhengzhou University. The ethics approval number is 2018-KY-56. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AL, SD, and JL contributed to the conception and design of the study. MJ performed the analysis and wrote the manuscript. MJ, SY, and AL performed the data analysis. MJ and JL analyzed the participants' data. QS, YLiu, and YLi helped collect the data and edit the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.823898/full#supplementary-material

Supplementary Figure 1 | Pearson distance and Bray–Curtis distance illustrated significant differences in the microbial community for pathway beta diversity. **(A)** Principal coordinate analysis (PCOA) diagram by the Pearson distance. **(B,C)** The first and second principal components are based on the Pearson distance. **(D)** PCOA diagram by the Bray–Curtis distance. **(E,F)** The first and second principal components are based on the Bray–Curtis distance.

Supplementary Table 1 | Statistically differential species and metabolic pathways related to the drinking case group.

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Endotoxin Translocation and Gut Barrier Dysfunction Are Related to Variceal Bleeding in Patients With Liver Cirrhosis

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Background: Bacterial infections are associated with the risk of variceal bleeding through complex pathophysiologic pathways.

Objectives: The primary objective of the present case-control study was to investigate the role of bacterial translocation and intestinal barrier dysfunction in the pathogenesis of variceal bleeding. A secondary objective was to determine independent predictors of key outcomes in variceal bleeding, including bleeding-related mortality.

Methods: Eighty-four ($n = 84$) consecutive patients participated in the study, 41 patients with acute variceal bleeding and 43 patients with stable cirrhosis, and were followed up for 6 weeks. Peripheral blood samples were collected at patient admission and before any therapeutic intervention.

Results: Child-Pugh (CP) score (OR: 1.868; $p = 0.044$), IgM anti-endotoxin antibody levels (OR: 0.954; $p = 0.016$) and TGF-β levels (OR: 0.377; $p = 0.026$) were found to be significant predictors of variceal bleeding. Regression analysis revealed that albumin (OR: 0.0311; $p = 0.023$), CRP (OR: 3.234; $p = 0.034$) and FABP2 levels (OR:1.000, $p = 0.040$), CP score (OR: 2.504; $p = 0.016$), CP creatinine score (OR: 2.366; $p = 0.008$), end-stage liver disease model (MELD), Na (OR: 1.283; $p = 0.033$), portal vein thrombosis (OR: 0.075; $p = 0.008$), hepatocellular carcinoma (OR: 0.060; $p = 0.003$) and encephalopathy (OR: 0.179; $p = 0.045$) were significantly associated with 6-week mortality.

Conclusions: Bacterial translocation and gut barrier impairment are directly related to the risk of variceal bleeding. Microbiota-modulating interventions and anti-endotoxin agents may be promising strategies to prevent variceal bleeding.

Keywords: cirrhosis, variceal bleeding, bacterial translocation, intestinal barrier, liver-gut axis

INTRODUCTION

Acute variceal bleeding is a serious complication of liver cirrhosis associated with significant morbidity and mortality (1–3). Current treatment guidelines (4–6), which include early administration of vasoactive drugs, antibiotic prophylaxis, and band ligation, have resulted in increased survival (2).

The inclusion of prophylactic antibiotic treatment in current treatment guidelines is based on the notion that infection is a prognostic mortality factor (7, 8) and can also trigger variceal bleeding via a complex cascade of pathophysiologic events, including endotoxin release, endothelin activation, and portal hypertension (7, 9, 10). There is strong evidence that antibiotic use significantly reduces bacteremia and the incidence of spontaneous bacterial peritonitis, and that prophylactic antibiotic therapy prevents rebleeding and improves mortality (11–13). Moreover, bacterial infection has been shown to be strongly associated with variceal bleeding and failure to control bleeding (7). Nevertheless, the role of bacterial translocation in the pathogenesis of variceal bleeding has not been fully elucidated (14).

The aim of the present study was to determine whether bacterial translocation is more common in patients with acute variceal bleeding compared with stable cirrhotic patients. To this end, we examined indices of bacterial translocation and gut barrier integrity along with markers of systemic inflammatory response, including cytokine levels, in patients on admission to the hospital before any therapeutic manipulation that might increase the risk of infection and thus confound our results. Previous studies have shown indirect evidence of a contribution of bacterial translocation to variceal bleeding through activation of endogenous heparin-like activity (15, 16). However, there are no data directly linking bacterial translocation and endotoxin release to the risk of variceal bleeding. A secondary objective of the present study was to identify independent predictors of bleeding control failure, risk of rebleeding, and bleeding-related mortality.

MATERIALS AND METHODS

Patients

The present case-control study was conducted in the Division of Gastroenterology, Department of Internal Medicine, Patras University General Hospital (PUH), Patras, Greece. All study participants or their legal guardians provided written informed consent before participating in the study. The study protocol was approved by the Scientific Review Board and the Ethics Committee of PUH as part of a general application to collect biological samples from patients attending the hepatology clinics to study factors involved in the pathogenesis of liver cirrhosis. PUH adheres to the 1975 Declaration of Helsinki on Ethical Principles for Medical Research Involving Human Subjects.

We studied 41 consecutive patients with cirrhosis and variceal bleeding and 43 stable consecutive patients with cirrhosis who visited the hepatology outpatient clinics from February 2016 to February 2018. Patients were followed for 6 weeks. Patients with variceal bleeding were treated according to the guidelines at that time (5, 6). The presence of infection was investigated with blood and urine cultures, paracentesis and culture of ascites, and chest radiography. The presence of active infection at any site was an exclusion criterion because we wanted to focus on the role of bacterial translocation in variceal bleeding.

Diagnosis

Liver cirrhosis was diagnosed on the basis of histologic findings, clinical evaluation, laboratory data, or imaging findings. Variceal bleeding was diagnosed based on hematemesis or melena with either bleeding varix (active bleeding or clot adherent to the varix or variceal ulceration) or, if there was no other source of bleeding, during upper gastrointestinal endoscopy. Severe bleeding was defined as arterial hypotension (systolic blood pressure $<$ 100 mm Hg) or/and hemoglobin $<$ 8 g/dl on admission. Failure of bleeding control was defined as recurrence of bleeding during the 5 days of somatostatin infusion. Rebleeding was defined as the recurrence of bleeding between days 5 and 42 after the initial bleeding. Bleeding-related mortality was defined as death within 6 weeks of the bleeding episode (17).

Markers of Systemic Inflammation, Bacterial Translocation, and Gut Barrier Integrity

Peripheral blood samples (3-4 ml) were collected at patient admission before therapeutic intervention. Blood samples were centrifuged, and sera were collected and stored aliquoted at -80°C until further use.

Systemic inflammation was assessed by measuring C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), nitric oxide (NO), soluble CD14, and pro- and anti-inflammatory cytokine levels (IL-1β, IL-6, IL-8, IL-12, TNF-α, IL-10, TGF-β). The presence of bacterial translocation was estimated by measuring serum lipopolysaccharide (LPS), anti-endotoxin immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies, lipopolysaccharide-binding protein (LBP), and bacterial DNA. Gut barrier integrity was assessed by measuring fatty acid binding protein 2 (FABP2) in serum.

Measurement of Serum Levels of Cytokines, LBP, Endotoxin, and NO

Serum levels of cytokines IL-1β, IL-6, IL-8, IL-10, IL-12p70, and TNF-α were determined by a cytometric bead array (CBA) method using the Human Inflammatory Cytokine CBA kit (BD Bioscience, San Diego, USA) as described (18). Measurements were performed using a BD FACS Array Bioanalyzer as described (18). Raw data were analyzed using Flow Jo V7.5 software (Tree Star Inc., Ashland, OR, USA). Serum TGF-β and LBP concentrations were determined using a multispecies TGF-β ELISA kit (Invitrogen Corporation, CA, USA) and a human LBP ELISA kit (SunRed Biological Technology, Shanghai) respectively, as described in (18). NO levels were measured using a nitric oxide quantification kit (Active Motif, Belgium), as described in (18). Endotoxin levels were measured using the Limulus amebocyte lysate chromogenic endpoint assay (Hycult

Biotech, The Netherlands), as described in (18). Data analysis was performed using Curve Expert 1.4 software. The detection limits of the methods used were: IL-1β = 7.2 pg/ml, IL-8 = 3.6 pg/ml, IL-10 = 3.3 pg/ml, IL-6 = 2.5 pg/ml, IL12p70 = 1.9 pg/ml, TNF-α = 3.7 pg/ml, TGF-β = 15.6 pg/ml, LBP = 0.135 μg/ml, NO < 1 μM, endotoxin = 0.04 EU/ml.

Detection of Bacterial DNA in Serum

Bacterial DNA was detected by PCR using a pair of universal primers (F: 5′-AGAGTTTGATCATGGCTCAG-3′, R: 5′-ACCGCGACTGCTGCTGGCAC-3′) as described in (18, 19).

Statistical Analysis

Numerical data were expressed as medians and interquartile ranges (IQR) and categorical data as counts and percentages. All variables were tested for normal distribution using the Kolmogorov-Smirnov test. According to this analysis, the values of variables did not follow a normal distribution. For this reason, comparisons between groups were made using the Mann-Whitney test, which is a non-parametric test. Differences between groups in demographic, clinical, and laboratory parameters were assessed with the χ^2 test for categorical variables. A number of clinically relevant variables that differed significantly between bleeders and stable cirrhotics entered multivariate stepwise logistic regression analysis to determine which parameters better predicted bleeding risk. Collinearity among the independent variables was tested using variance inflation factors and tolerances for each variable. Given the presence of statistical collinearity, individual variables required for calculation of the end-stage liver disease model (MELD) or Child-Pugh (CP) score were excluded from multivariate analysis. $P < 0.05$ was considered to indicate a statistically significant difference. The SPSS statistical package (version 19.0 for Windows; SPSS Inc., Chicago, Illinois, USA) was used. Graphical representation was performed using GraphPad Prism v. 8.3.1 software.

RESULTS

Eighty-four consecutive patients participated in the study, 41 patients with acute variceal bleeding and 43 patients with stable cirrhosis. Three patients with variceal bleeding had evidence of active infection, 2 had urinary tract infection, and 1 had a positive blood culture; these patients were excluded from further analysis. Six patients (15.8%) with variceal bleeding had active bleeding at endoscopy. As expected, patients with variceal bleeding were more likely to have been treated with beta-blockers compared to the control group ($p = 0.021$). Similarly, patients with variceal bleeding were more likely to have higher Child-Pugh ($p = 0.042$) and MELD scores ($p < 0.001$).

Table 1 shows the demographic and clinical parameters of stable cirrhotic patients compared to patients with variceal bleeding. Patients with variceal bleeding had significantly lower hemoglobin and albumin levels and significantly higher white blood cell counts compared to controls. In addition, variceal bleeders had significantly lower IgM anti-endotoxin antibody,

TABLE 1 | Demographic, clinical, and laboratory data of study subjects.

	All study subjects	Variceal bleeders	Stable cirrhotics	*P*-value
N	84	38	43	
Gender, male (%)	62 (76.5)	32 (84.2)	30 (69.8)	0.126
Smoking, yes (%)	29 (43.9)	17 (58.6)	12 (32.4)	0.052
Past variceal bleeding, yes (%)	28 (43.1)	18 (50.0)	10 (34.5)	0.209
Use of beta-blockers, yes (%)	38 (46.9)	23 (60.5)	15 (34.9)	**0.021**
HCC, yes (%)	12 (14.8)	8 (21.1)	4 (9.3)	0.137
Portal vein thrombosis, yes (%)	9 (11.3)	6 (16.2)	3 (7.0)	0.192
Ascites, yes (%)	34 (42.0)	20 (52.6)	14 (32.6)	0.068
Encephalopathy, yes (%)	12 (14.8)	9 (23.7)	3 (7.0)	**0.035**
		Median (IQR)		
Age	60 (52, 65.5)	57.5 (47.5, 66.5)	61 (54, 65)	0.656
Child-Pugh score	7.0 (6, 9)	8 (7, 9)	6 (5, 8.8)	**0.042**
MELD score	12 (10, 16)	14.5 (11, 16.25)	10 (8, 13)	**<0.001**
MELDNa score	15 (12, 18)	16 (14, 19)	13 (9, 15.5)	**0.001**
Hemoglobin (g/dl)	10.8 (9.2, 13.5)	9.2 (7.9, 10.0)	12.8 (10.9, 14.7)	**<0.001**
WBC (cells/μl)	6355 (4602.3, 9522.5)	9160 (6730, 13350)	4830 (4200, 6252.5)	**<0.001**
Albumin (g/dl)	3.4 (2.8, 4.0)	3 (2.6, 3.4)	3.7 (3.2, 4.2)	**<0.001**
ESR (mm/hour)	40.0 (35-60)	40.0 (30.5-62.5)	45 (40, 50)	0.798
CRP (mg/L)	0.8 (0.4-2.0)	1.3 (0.4-2.3)	0.4 (0.1, 0.8)	0.231
Bacterial DNA	ND	ND	ND	ND

HCC, hepatocellular carcinoma; IQR, interquartile range; MELD, model of end-stage liver disease; WBC, white blood cells; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; ND, not detected. The bold values correspond to statistical significant differences between the examined variables.

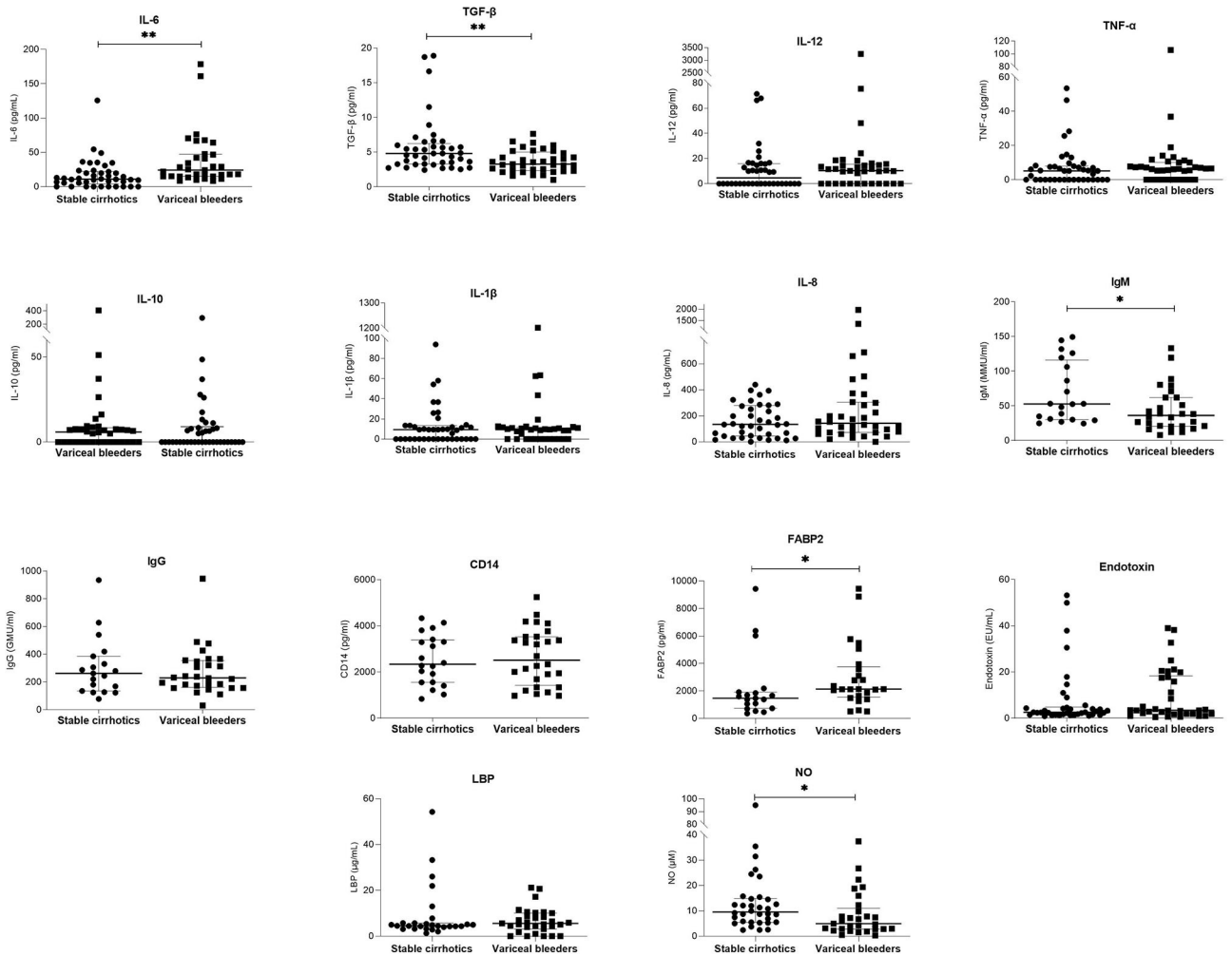

FIGURE 1 | Comparison of serum laboratory parameters between patients with stable liver cirrhosis and patients presenting with variceal bleeding. Bars and plots represent the median and interquartile range, respectively. Asterisks indicate statistical significance (*$p < 0.05$; **$p < 0.01$).

NO, and TGF-β levels and higher FABP2 and IL-6 levels (**Figure 1** and **Supplementary Table 1**). Bacterial DNA was not detected in any of the serum samples.

Multivariate stepwise regression was then calculated to predict variceal bleeding risk based on Child-Pugh score, beta-blocker treatment, IgM anti-endotoxin antibodies, serum levels of TGF-β and IL-6. The results (**Table 2**) show that Child-Pugh score, IgM anti-endotoxin antibodies, and TGF-β levels remained as significant predictors of variceal bleeding and explained 48.9% of the variance in variceal bleeding risk.

Failure to Control Bleeding and Rebleeding

Bleeding control was unsuccessful in 4 (10.5%) patients. After initial bleeding control, rebleeding occurred in 3 (7.9%) patients between day 5 and day 42. **Table 3**; **Figure 2** and **Supplementary Table 2** show the demographic, clinical, and laboratory parameters of patients whose bleeding could not be initially controlled compared with those whose bleeding was successfully controlled. **Table 4** shows the demographic, clinical, and laboratory parameters of the patients in whom rebleeding occurred compared with the patients in whom rebleeding did not occur. Multivariate regression analysis to determine independent predictors of bleeding control failure and risk of rebleeding was not performed because of the small sample size.

6-Week Mortality

Eight (21.1%) of variceal bleeders died within 6 weeks of the bleeding episode. **Table 5**; **Figure 3** and **Supplementary Table 3** show a comparison of demographic, clinical, and laboratory data between the 6-week survivors and non-survivors in the variceal bleeding group. Binary logistic regression analysis was performed to determine the variables that significantly predicted death within 6 weeks in the variceal bleeders group. After univariate analysis, the levels of albumin, CRP, and FABP2, Child-Pugh score, Child-Pugh creatinine score, MELDNa, presence of portal vein thrombosis, and hepatocellular carcinoma were significantly associated with 6-week mortality. **Table 6** shows the coefficients

TABLE 2 | Independent predictors of variceal bleeding.

	Univariate analyses	Multivariate stepwise regression analysis		
	***P*-value**	**OR**	**95% CI for exp (B)**	***P*-value**
IgM (GMU/ml)	0.049	0.954	0.917-0.991	**0.016**
TGF-β (pg/ml)	0.009	0.377	0.159-0.891	**0.026**
Child-Pugh score	0.126	1.868	1.016-3.433	**0.044**
IL-6 (pg/ml)	0.024	-	-	0.204
Beta-blockers treatment	0.023	-	-	0.320
FABP2 (pg/ml)	0.250	-	-	0.511
Regression statistics		$X^2 = 22.895$, df = 4, $p < 0.001$ Nagelkerke $R^2 = 0.583$		

OR, odds ratio; CI, confidence interval; FABP2, fatty acid-binding protein 2. The bold values correspond to statistical significant differences between the examined variables.

TABLE 3 | Demographic, clinical and laboratory parameters of patients with uncontrolled bleeding vs. patients with controlled bleeding.

Variceal bleeders	**Failure to control bleeding**	**Controlled bleeding**	***P*-value**
N	4	34	
Gender, male (%)	4 (100)	28 (82.4)	0.360
Smoking, yes (%)	2 (50)	15 (60.0)	0.706
Past variceal bleeding, yes (%)	0 (0)	18 (54.5)	0.070
Use of beta-blockers, yes (%)	3 (75)	20 (58.8)	0.482
HCC, yes (%)	2 (50)	6 (17.6)	0.133
Portal vein thrombosis, yes (%)	1 (33.3)	5 (14.7)	0.401
Ascites, yes (%)	3 (75)	17 (50.0)	0.344
Encephalopathy, yes (%)	2 (50)	7 (20.6)	0.191
		Median (IQR)	
Age	61.5 (53.3, 70.5)	57.5 (44.5, 66.5)	0.536
Child-Pugh score	9 (8.3, 9.8)	8 (6.8, 9)	0.100
Child-Pugh creatinine score	9 (8.3, 9.8)	8 (6.5, 9)	0.124
MELD	16 (12, 17)	14 (11, 16)	0.444
MELDNa	19 (17, 21.8)	16 (14, 18.3)	0.069
Hemoglobulin (g/dl)	10.0 (8.9, 11.9)	9.1 (7.8, 10.0)	0.208
WBC (cells/μl)	14800 (9995, 18450)	8630 (6470, 13000)	0.058
Albumin (g/dl)	2.6 (2.3, 2.9)	3.1 (2.7, 3.5)	**0.048**

HCC, hepatocellular carcinoma; IQR, interquartile range; MELD, model of end-stage liver disease; WBC, white blood cells. The bold values correspond to statistical significant differences between the examined variables.

and odds ratios for each variable included in the univariate regression analysis for 6-week mortality.

DISCUSSION

In the present study, the vast majority of variceal bleeders had no evidence of overt infection on hospital admission before diagnostic or therapeutic intervention. However, we observed significantly lower levels of IgM anti-endotoxin antibodies and TGF-β in bleeders compared to non-bleeders. The absence of a difference in anti-endotoxin IgG antibodies between variceal bleeders and non-bleeders suggests a chronic exposure of both cirrhotic groups to low-grade endotoxemia, whereas the significantly lower anti-endotoxin IgM levels in variceal bleeders are consistent with a pulse of recent endotoxin release leading to antibody consumption (20, 21). This is the first study to provide direct evidence that bacterial translocation is associated with variceal bleeding, possibly suggesting that the latter is a critical precursor to increased bleeding risk (22).

Bacterial translocation is commonly found in cirrhotics (23) and has been associated with hemodynamic changes and portal hypertension (9, 10, 24). The passage of microbes and their products from the intestinal lumen into the mesenteric lymph nodes and systemic circulation stimulates a cascade of intrahepatic immune signals that promote fibrosis and changes in intrahepatic vascular tone, leading to an exacerbation

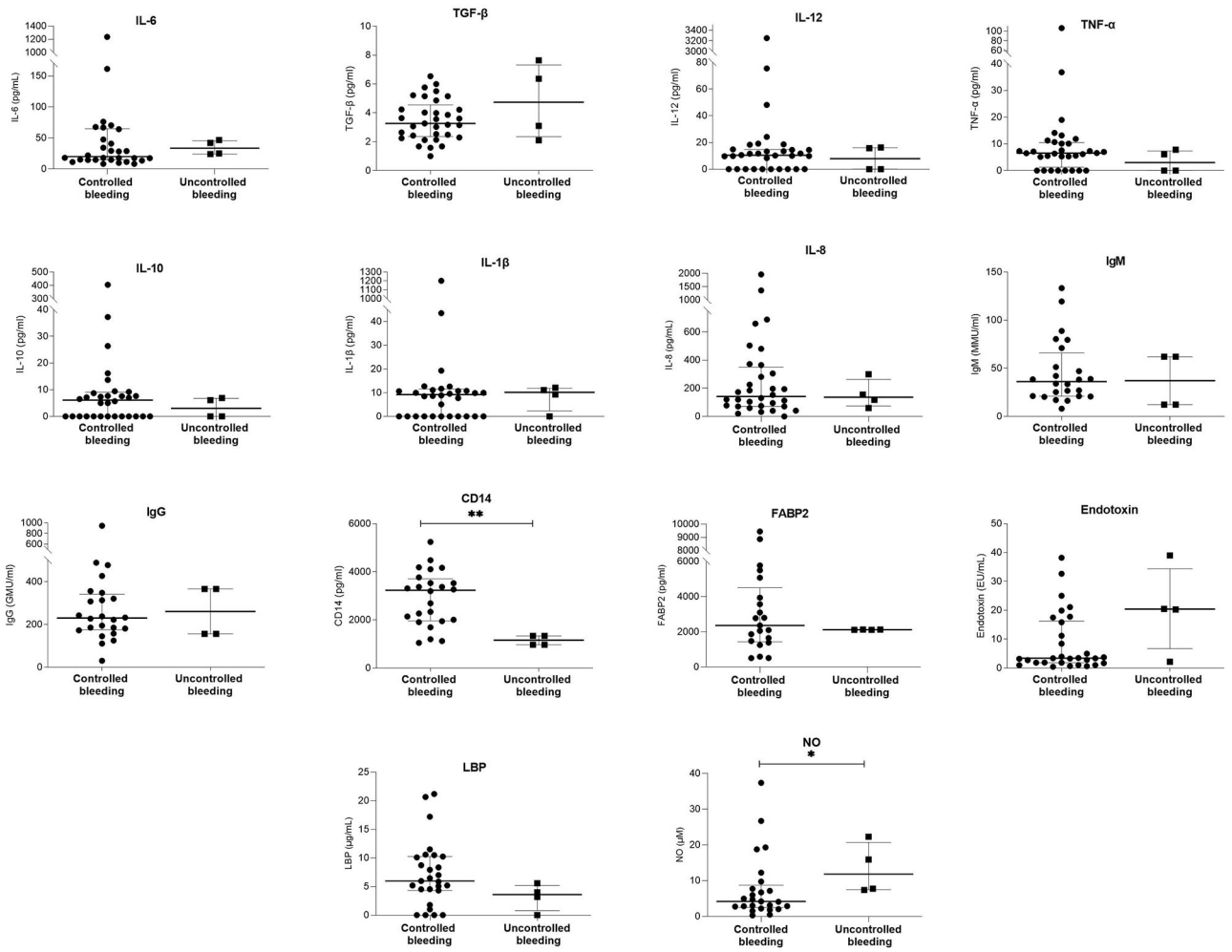

FIGURE 2 | Comparison of serum laboratory parameters between patients with controlled bleeding and patients with uncontrolled bleeding. Bars and plots represent median and interquartile range, respectively. Asterisks indicate statistical significance (*$p < 0.05$; **$p < 0.01$).

of portal hypertension (24). In addition, the presence of bacterial infection in cirrhotic patients has been associated with increased endogenous heparin-like activity, which inhibits platelet aggregation and leads to a higher risk of bleeding (16).

Goulis et al. (25) formulated the hypothesis that bacterial infection induced by bacterial translocation could be the event that triggers variceal bleeding through a combination of physiological phenomena, including impaired coagulation, increased portal pressure, and failure of liver function. This hypothesis is primarily based on a previous study by the same group, which showed that the presence of bacterial infection is an independent predictor of failure to control bleeding in patients with variceal bleeding (7). Previous studies have also shown that bacterial infections affect 20-60% of variceal bleeders (7, 26, 27) and that the presence of bacterial infection independently predicts the risk of rebleeding and mortality (7, 26, 28, 29). However, the methodological design of these studies did not allow for differentiation between infections that were already present at the time of bleeding and infections that occurred as a consequence of bleeding or due to the invasive nature of endoscopic procedures (14). In addition, several studies have reported that the prophylactic use of antibiotics reduces infection rates, the risk of rebleeding, and all-cause mortality (29–31). A recent meta-analysis found that the relevant studies had methodological weaknesses and a high risk of bias, suggesting that further research on this topic is needed (12). In addition, Goulis' hypothesis focused exclusively on the role of infection and did not consider that the phenomena of gut barrier dysfunction and bacterial translocation do not always lead to infection but may still be related to the pathophysiology of variceal bleeding.

In the present study, emphasis was placed on measuring indices of bacterial translocation as close as possible to the bleeding episode and before any intervention, and patients with active infection at any site or bacteremia were excluded to ensure that the endotoxin detected originated from the intestinal lumen. Our results strongly suggest a close pathophysiological relationship between the translocation of bacteria and endotoxins

TABLE 4 | Demographic, clinical and laboratory parameters of rebleeders vs. non-rebleeders.

	Rebleeders	Non-rebleeders	*P*-value
N	3	35	
Gender, male (%)	3 (100)	29 (82.9)	0.435
Smoking, yes (%)	2 (66.7)	15 (57.7)	0.765
Past variceal bleeding, yes (%)	2 (66.7)	16 (48.5)	0.546
Use of beta-blockers, yes (%)	2 (66.7)	21 (60.0)	0.821
HCC, yes (%)	1 (33.3)	7 (20.0)	0.587
Portal vein thrombosis, yes (%)	1 (33.3)	5 (14.7)	0.401
Ascites, yes (%)	1 (33.3)	19 (54.3)	0.485
Encephalopathy, yes (%)	0 (0)	9 (25.7)	0.315
		Median	
Age	36.35	58	0.588
Child-Pugh score	9	8	0.563
Child-Pugh creatinine score	9	8	0.337
MELD	13	15	0.935
MELDNa	17	16.0	0.913
Hemoglobulin (g/dl)	8	9.3	0.616
WBC (cells/μl)	9260	8930	0.953
Albumin (g/dl)	2.4	3.1	0.076
ESR (mm/h)	41.5	40.0	0.921
CRP (mg/L)	4.74	0.97	0.146
IgM (MMU/ml)	36.35	36.0	0.923
IgG (GMU/ml)	193.75	229.8	0.501
FABP2 (pg/ml)	5463.55	2247.2	0.465
CD14 (pg/ml)	3429.95	2768.2	0.386
TGF-β (pg/ml)	5.85	3.2	0.065
IL-1β (pg/ml)	600.82	9.4	0.699
IL-6 (pg/ml)	624.23	23.1	0.730
IL-8 (pg/ml)	420.97	125.9	0.269
IL-12 (pg/ml)	13.49	10.4	0.622
IL-10 (pg/ml)	202.68	5.9	0.616
TNFα (pg/ml)	8.76	6.4	0.530
Endotoxin (EU/ml)	5.15	3.4	0.763
LBP (μg/ml)	8.55	5.4	0.359
NO (μM)	5.96	5.0	0.796

HCC, hepatocellular carcinoma; MELD, model of end-stage liver disease; WBC, white blood cells; ECR, erythrocyte sedimentation rate; CRP, C-reactive protein; FABP2, fatty acid-binding protein 2; LBP, lipoprotein binding protein; NO, nitrogen oxide.

from the intestine and variceal bleeding. Although our results do not provide evidence of causality, the finding that variceal bleeders had decreased levels of IgM anti-endotoxin antibodies in the absence of bacterial DNA at the onset of bleeding suggests that endotoxin translocation may be the initiating event in the pathophysiology of variceal bleeding. Previous studies have shown that increased gut permeability precedes the detection of bacterial DNA in the sera of cirrhotic patients (22, 32). In addition, detection of endotoxin may be a more reliable index of bacterial translocation than bacterial DNA; furthermore, the presence of bacterial byproducts in patients' sera likely represents a later step in the timeline of variceal bleeding pathophysiology (19). Bacterial translocation is a transient phenomenon that does not always lead to variceal bleeding. Our analysis showed that bleeders and the cirrhotic patients had similar levels of IgG anti-endotoxin antibodies suggestive of previous episodes of bacterial translocation. This confirms that cirrhotic patients often experience repeated episodes of bacterial translocation or chronic low-grade endotoxemia. The fact that variceal bleeders exhibited stronger evidence of recent bacterial translocation strengthens the possibility that this is the trigger of the bleeding.

The central role of bacterial and endotoxin translocation (due to a disrupted intestinal barrier) in variceal bleeding is further supported by our finding of increased FABP2 levels in variceal bleeders. Moreover, according to our analysis, FABP levels proved

TABLE 5 | Demographic, clinical and laboratory parameters in 6-week survivors vs. non-survivors.

Variceal bleeders		6-week survivors	Non-survivors	*P*-value
N	30	8		
Cause of death, yes (%)	Bleeding	NA	4 (50)	NA
	Sepsis		1 (12.5)	
	HCC		3 (37.5)	
	Renal failure		4 (50)	
	Liver failure		4 (50)	
Gender, male (%)		24 (80.0)	8 (100)	0.168
Smoking, yes (%)		14 (63.9)	3 (42.9)	0.331
Past variceal bleeding, yes (%)		17 (58.6)	1 (14.3)	**0.035**
Use of beta-blockers, yes (%)		16 (53.3)	7 (87.5)	0.079
HCC, yes (%)		3 (10.0)	5 (62.5)	**0.001**
Portal vein thrombosis, yes (%)		2 (6.7)	4 (57.1)	**0.001**
Ascites, yes (%)		16 (53.3)	4 (50)	0.867
Encephalopathy, yes (%)		5 (16.7)	4 (50)	**0.049**
		Median (IQR)		
Age		54.5 (39.8, 65.3)	66 (59, 74.3)	**0.023**
Child-Pugh score		7.5 (6.0, 8.3)	9 (9, 10)	**0.002**
Child-Pugh creatinine score		7 (6.0, 8)	9.5 (9, 11.75)	**0.001**
MELD		14 (11, 16)	17 (13.5, 18.5)	**0.039**
MELDNa		16 (13.8, 17)	20 (17.25, 21.75)	**0.002**
Hemoglobulin (g/dl)		8.9 (7.8, 9.9)	10 (8.8, 12.4)	0.079
WBC (cells/μl)		8500 (6300, 11410)	13175 (9185, 16525)	**0.036**
Albumin (g/dl)		3.2 (2.8, 3.5)	2.4 (2.2, 2.8)	**0.005**
ESR (mm/h)		45 (36.5, 71.3)	28 (21.5, 39.8)	0.176
CRP (mg/L)		0.6 (0.4, 1.5)	3.62 (1.61, 5.66)	**0.021**

HCC, hepatocellular carcinoma; NA, not applicable; IQR, interquartile range; MELD, model of end-stage liver disease; WBC, white blood cells; ECR, erythrocyte sedimentation rate; CRP, C-reactive protein. The bold values correspond to statistical significant differences between the examined variables.

to be a significant predictor of 6-week mortality in variceal bleeders, further emphasizing the key role of the gut barrier in variceal bleeding and its sequelae. FABP2, an endogenous cytosolic enterocyte protein, is a marker of enterocyte integrity. Because FABP2 is located in the mature epithelium of the villi, it can more easily leak into the bloodstream when enterocytes are damaged. Animal and clinical studies have shown that cirrhosis is associated with gut barrier disruption (33), and increased intestinal permeability appears to be involved in the pathogenesis of cirrhosis-related complications. We have previously reported (34) that the expression of claudin 1 and occludin is decreased in the intestinal epithelial cells of cirrhotic patients, especially in decompensated cirrhosis, indicating gut barrier impairment. Similarly, another study found increased duodenal permeability in patients with decompensated cirrhosis (35). In the same context, in the present study, we found that decreased TGF-β levels were associated with an increased risk of bleeding. TGF-β is an anti-inflammatory cytokine that protects the intestinal mucosal barrier (36); therefore, decreased TGF-β levels could potentially contribute to a disrupted gut barrier. **Figure 4** shows our proposed mechanism for the contribution of bacterial translocation and increased gut permeability to variceal bleeding risk.

It is still unclear whether antibiotics significantly reduce bacterial translocation. Recently, a randomized controlled trial showed that rifaximin had no significant effect on indices of gut permeability and bacterial translocation in patients with decompensated cirrhosis (37, 38). In contrast, a previous study had reported that norfloxacin decreased endotoxin levels and partially reversed the hyperdynamic circulatory state in liver cirrhosis (39). Similarly, lactulose, which accelerates intestinal transit and improves gut permeability, reduced bacterial translocation and intestinal bacterial overgrowth in animal models of liver cirrhosis (40). In addition to antibiotics and lactulose, other anti-endotoxin agents such as bactericidal/permeability-increasing protein (BPI), high-density lipoprotein (HDL), anti-endotoxin antibodies, and LPS antagonists should be investigated as potential treatment strategies to inhibit bacterial translocation and thus reduce the risk of variceal bleeding and bleeding-related mortality in cirrhotic patients. In addition, interventions to modulate the gut microbiota, including prebiotics, probiotics, and transplantation

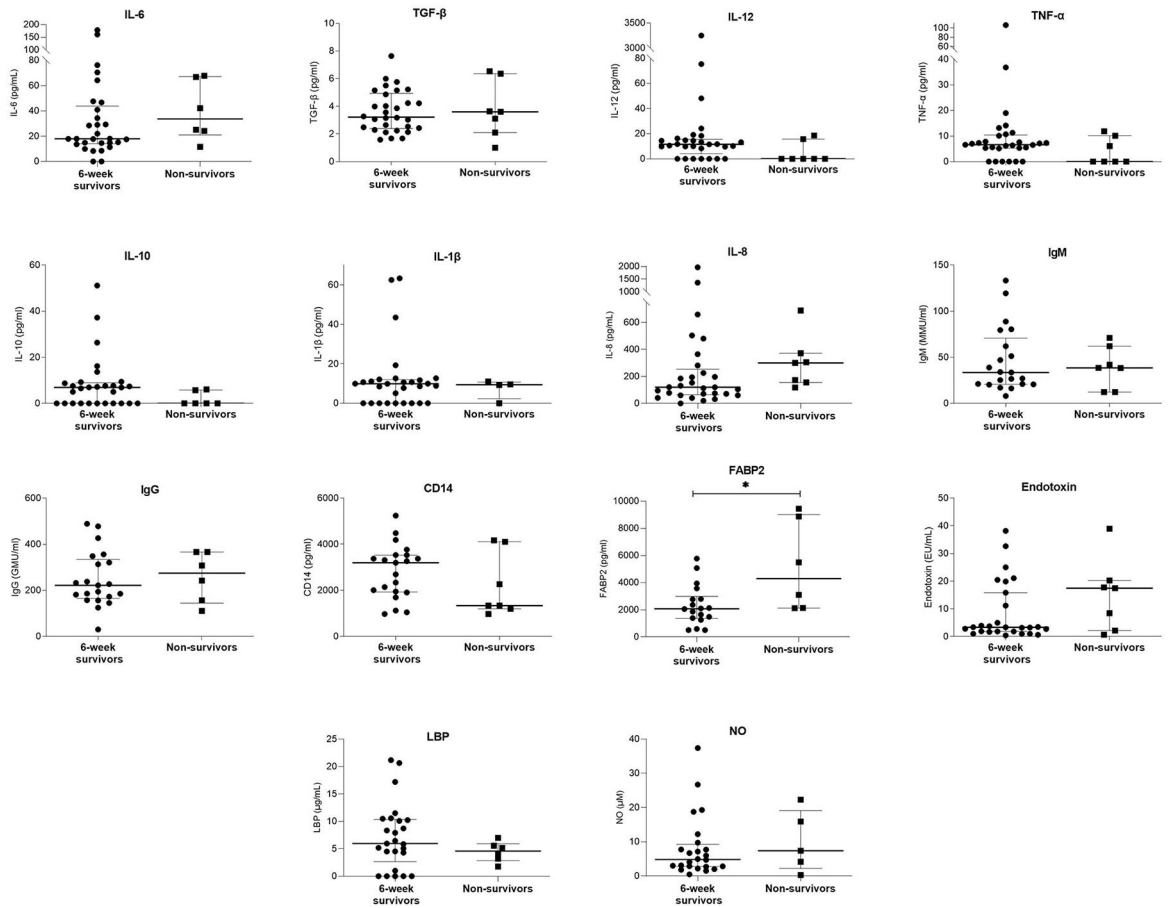

FIGURE 3 | Comparison of serum laboratory parameters between 6-week survivors and non-survivors. Bars and plots represent median and interquartile range, respectively. Asterisks indicate statistical significance (*$p < 0.05$).

TABLE 6 | Independent predictors of 6-week mortality risk.

		Univariate logistic regression analysis		
	Coefficient	**OR**	**95% CI for exp (B)**	***P*-value**
Age (years)	0.078	1.081	1.004-1.165	**0.038**
WBC (cells/μl)	0.000	1.000	1.000-1.000	0.065
Albumin (g/dl)	−3.562	0.028	0.002-0.527	**0.017**
Child-Pugh score	1.204	3.334	1.304-8.522	**0.012**
Child-Pugh creatinine score	1.012	2.751	1.301-5.817	**0.008**
MELD score	0.232	1.262	0.973-1.635	0.079
MELDNa score	0.416	1.515	1.106-2.077	**0.010**
FABP2 (pg/ml)	0.001	1.000	1.000-1.001	**0.040**
CRP (mg/L)	1.128	3.090	1.061-8.999	**0.039**
Past variceal bleeding	−2.140	0.118	0.012-1.108	0.061
Beta-blockers treatment	1.812	6.125	0.669-56.095	0.109
HCC	2.708	15.000	2.328-96.666	**0.004**
Portal vein thrombosis	2.927	18.667	2.348-148.426	**0.006**
Encephalopathy	1.609	5.0	0.926-26.990	0.061

OR, odds ratio; CI, confidence interval; WBC, white blood cells; MELD, model of end-stage liver disease; FABP2, fatty acid-binding protein 2; CRP, C-reactive protein; HCC, hepatocellular carcinoma. The bold values correspond to statistical significant differences between the examined variables.

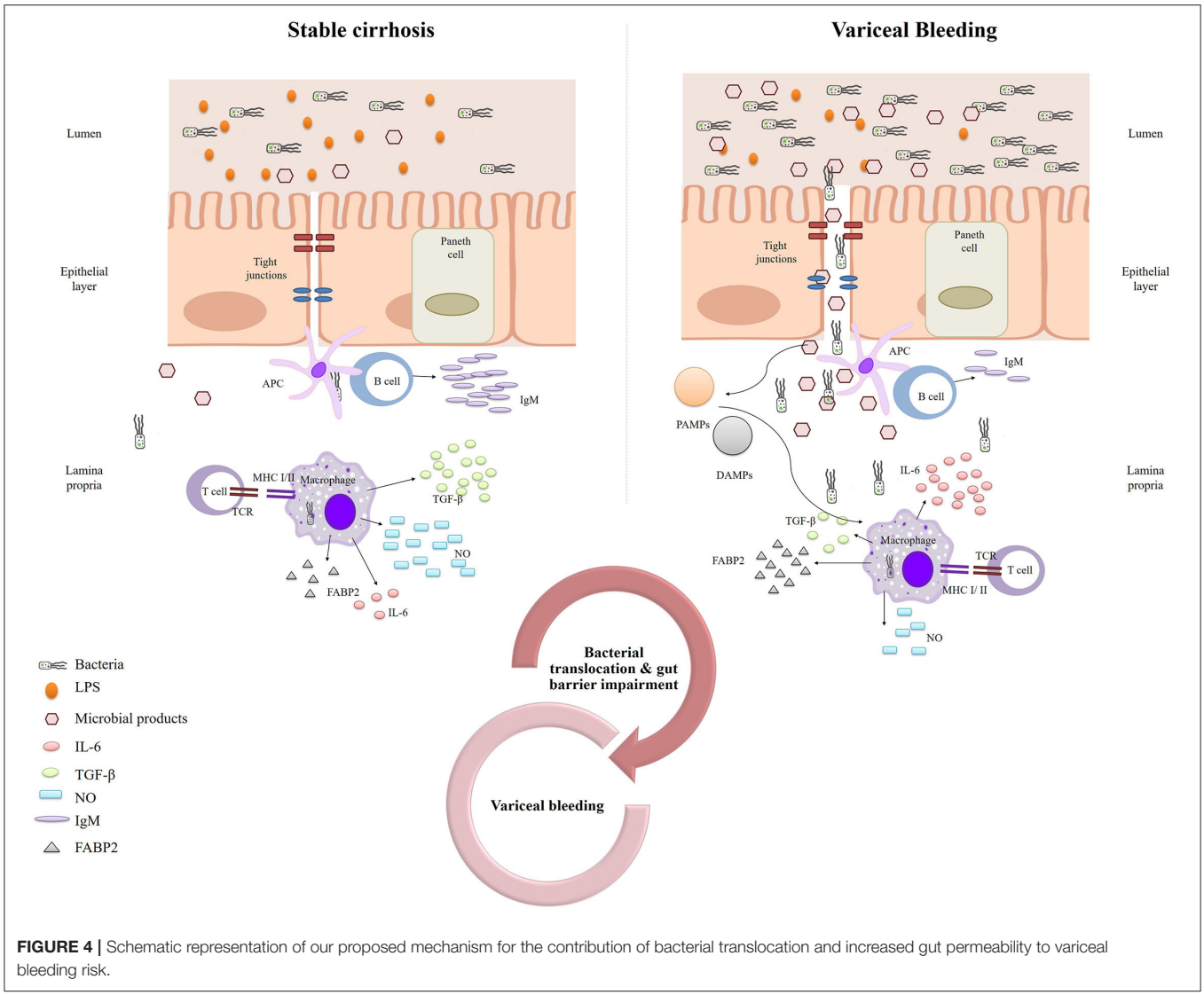

FIGURE 4 | Schematic representation of our proposed mechanism for the contribution of bacterial translocation and increased gut permeability to variceal bleeding risk.

of fecal microbiota, may play a role in preventing bacterial translocation in chronic liver disease (41). Probiotics and prebiotics have been shown to be effective in reducing bacterial translocation in patients with diabetes mellitus (42), HIV (43), and in cancer patients after surgery (44). Horvath et al. (45) studied the effect of probiotic treatment in patients with liver cirrhosis and found minimal benefits in terms of gut barrier function and prevention of bacterial translocation. The critical role of bacterial translocation and gut barrier permeability in the development of variceal bleeding underscores the need for further randomized controlled trials investigating the role of microbiota-modulating treatments in the prevention of variceal bleeding and cirrhosis-related prognosis.

Regarding 6-week mortality, our analysis showed that it correlated with FABP2, albumin, CRP levels, severity of liver disease, and the presence of severe cirrhosis-related complications, including portal vein thrombosis and hepatocellular carcinoma. The presence of hepatocellular carcinoma has been shown to be an independent predictor of early rebleeding or mortality in patients with variceal bleeding (46–48). In a recent study, the presence and stage of hepatocellular carcinoma were identified as strong predictors of 6-week mortality in patients with acute variceal bleeding (49). In parallel, a new model for predicting prognosis in patients with cirrhosis and acute gastrointestinal bleeding, called the CAGIB score, has recently been developed (50). Among other clinical and laboratory variables, hepatocellular carcinoma is a component of this model (50). Considering the prognostic importance of hepatocellular carcinoma for the outcomes of patients with cirrhosis and variceal bleeding, we included patients with cirrhosis and hepatocellular carcinoma in our study. In addition, there was no difference in the presence of cirrhotics with hepatocellular carcinoma between the studied groups (p-value 0.137). Despite the widespread use of Child-Pugh and MELD scores as predictive models of cirrhosis progression, several investigators advocate the

use of alternative laboratory indices, including CRP levels (3, 51, 52). In a recent study, Lee et al. (3) showed that CRP levels strongly predicted 6-week mortality after acute variceal bleeding. The authors suggested that the prognostic value of CRP should be explained by the presence of infection or altered inflammatory response, both conditions associated with elevated CRP levels.

A limitation of the present study was the relatively short follow-up period of survivors of variceal bleeding. A longer follow-up period and additional measurements of indices of gut permeability and bacterial translocation would likely have allowed us to draw more robust conclusions about the pathophysiology of variceal bleeding. Another limitation was the small sample size, which did not allow further analysis to determine significant predictors.

In conclusion, the present study provides direct evidence of the contribution of bacterial translocation and increased gut permeability to variceal bleeding risk. Our findings pave the way for future research to elucidate the role of microbiota in variceal bleeding and to investigate the therapeutic effects of microbiota-modulating interventions and anti-endotoxin agents on bleeding risk and survival of cirrhotic patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Patras University Hospital Scientific Review Board and Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CT had the idea, drafted the article, wrote the article, critically revised the article for important intellectual content, and approved publication of the article. MK, SA, and KK collected the data, analyzed and interpreted the data, wrote the article, and approved publication of the article. AMa, IA, PS, GT, MR, HK, and DG collected the data, analyzed and interpreted the data, and approved publication of the article. SM, CG, DS, GD, AMo, and KT critically revised the article for important intellectual content and approved publication of the article. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.836306/full#supplementary-material

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REVIEW
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The Role of Gut Bacteria and Fungi in Alcohol-Associated Liver Disease

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Cirrhosis and liver cancer caused by alcohol-associated liver disease (ALD) are serious threats to people's health. In addition to hepatic cell apoptosis and liver inflammation caused by oxidative stress during alcohol metabolism, intestinal microbiota disorders are also involved in the onset and development of ALD. Ethanol and its' oxidative and non-oxidative metabolites, together with dysbiosis-caused-inflammation, destroys the intestinal barrier. Changes of several microbial metabolites, such as bile acids, short-chain fatty acids, and amino acid, are closely associated with gut dysbiosis in ALD. The alcohol-caused dysbiosis can further influence intestinal barrier-related proteins, such as mucin2, bile acid-related receptors, and aryl hydrocarbon receptor (AhR), and these abnormal changes also participate in the injury of the intestinal barrier and hepatic steatosis. Gut-derived bacteria, fungi, and their toxins, such as lipopolysaccharide (LPS) and β-glucan translocate into the liver through the damaged intestinal barrier and promote the progression of inflammation and fibrosis of ALD. Thus, the prevention of alcohol-induced disruption of intestinal permeability has a beneficial effect on ALD. Currently, multiple therapeutic treatments have been applied to restore the gut microbiota of patients with ALD. Fecal microbial transplantation, probiotics, antibiotics, and many other elements has already shown their ability of restoring the gut microbiota. Targeted approaches, such as using bacteriophages to remove cytolytic *Enterococcus faecalis*, and supplement with *Lactobacillus*, *Bifidobacterium*, or *boulardii* are also powerful therapeutic options for ALD.

Keywords: gut dysbiosis, fungi, alcohol-associated liver disease, gut-liver axis, intestinal barrier

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INTRODUCTION

According to the most recent WHO data, the burden of alcohol-associated liver disease (ALD) is growing (1). The natural disease course of ALD ranges from asymptomatic liver steatosis, alcoholic hepatitis to the development of cirrhosis, and liver cancer. Alcohol-mediated reactive oxygen species (ROS) formation and hepatic inflammation are the main pathophysiologies of ALD (2). Recently, the role of the gut–liver axis in ALD development and progression has attracted the attention of researchers. Germ-free mice receiving microbiota from patients with alcohol hepatitis gained more serious liver inflammation and disruption of the intestinal integrity, while the mice receiving microbiota from patients without alcoholic hepatitis could reverse the alcohol-caused liver injuries (3).

Cytotoxic effects of ROS during ethanol metabolism are the pathological basis of ALD, and the injuries are exacerbated by hypoxia, inflammation, and bacterial translocation (4). Studies about

the role of intestinal flora in ALD have made breakthroughs in the past few years. On one hand, dysbiosis induces intestinal barrier injury, promotes lipopolysaccharide (LPS) and other pathogen-associated molecular patterns (PAMPs) translocation and aggravates inflammatory damage in the liver (5). On the other hand, dysbiosis related exotoxins, such as cytolysin from *Enterococcus faecalis* (6) and *Candidalysin* from *Candida albicans* (7) directly cause hepatocyte death and liver injury. Among the intestinal microorganism, bacteria and fungi are the most studied in ALD. Therefore, we review the interaction between alcohol associated liver disease and the gut microbiome and/or mycobiome to evaluate the contribution of intestinal dysbiosis to ALD. In addition, therapeutic options to restore the intestinal micro-ecosystem will be discussed.

GUT-LIVER CROSS TALK

About 70% of liver's blood supply come from the gut through the portal vein. In addition to bringing nutrients through the portal vein, there is also a chance for intestinal microbiota and their products to enter the liver, especially with increased intestinal permeability. Liver inflammation caused by PAMPs translocated from intestine is the key progression factor of ALD (8) (**Figure 1**).

Intestinal Barrier Function

The integrity of the gut barrier plays a key role in prohibiting harmful intestinal materials being translocated into the bloodstream (**Figure 1**). All patients with various degrees of ALD have disruption of the gut barrier (9, 10). The oxidative and nonoxidative metabolites of ethanol produced by gut bacteria and intestinal epithelial cells disrupt the gut barrier by degrading the tight junction proteins or destroying the interaction of claudin-1 and ZO-1 (11–13). In chronic alcohol feeding mice, enteric dysbiosis-related intestinal inflammatory response causes intestinal barrier injury, and the restoration of eubiosis using non-absorbable antibiotics inhibits intestinal inflammation and barrier dysfunction (14). However, researchers find that mouse colonized with harmful *C. albicans* and cytolytic *E. faecalis* has no effect on intestinal permeability when exposed to alcohol (6, 7). To sum up, the relationship between intestinal dysbiosis and barrier function needs further studies.

The gut vascular barrier (GVB) is another gatekeeper that prevent bacteria translocation across the gut to reach the portal vein. *Salmonella typhimurium* broke the GVB in a manner that is dependent on the declined Wnt/β-catenin signaling in gut vascular endothelial cells (15). In experimental cirrhosis and non-alcoholic fatty liver disease (NAFLD), farnesoid X receptor (FXR) agonists modulate the GVB to reduce bacterial translocation through driving β-catenin activation in endothelial cells (16, 17). Leakage of intestinal vascular endothelia has been observed in experimental ALD mice and patients with ALD (18, 19).

Gut Microbiota

The intestinal bacteria are significantly altered in patients with ALD and experimental animals. At the phylum level, mice fed with alcohol diet have relatively higher abundances of *Bacteroidetes* and *Verrucomicrobia* when compared to mice fed with a control diet, whereas the mice fed with a control diet have a relative predominance of *Firmicutes* (20). However, alcoholics, with decreased communication of the microbial network in the colon (21) (**Table 1**), have the lower median abundances of *Bacteroidetes* and the higher median abundances of *Proteobacteria* than the healthy subjects (22). The commensal microbiota is exhausted in ALD patients with and without cirrhosis (23). At the species level, when compared with the healthy group, the numbers of *Bifidobacteria*, *Lactobacilli*, and *Enterococci* are significantly reduced in the alcoholics (24). Patient with alcoholic cirrhosis have 27 times more *Enterobactericaea* in their feces than healthy volunteers, and *Enterobactericaea* is the most common liver translocated bacterium in patients with cirrhosis (25).

The severity of ALD closely relates to the degree of intestinal flora alternations (26). Alcohol dependence is negatively correlated with levels of butyric-producing clostridium species (23). Severe patients with alcoholic hepatitis have increased *Bacilli*, *Lactobacillales*, *Veillonella*, and decreased *Eubacterium*_g23, *Oscillibacter* and *Clostridiales* in the fecal compared with the healthy controls (27). The reduced *Akkermansia* and increased *Bacteroides* are used to identify alcohol use disorder patients with an accuracy of 93.4% (28). Cytolysin-positive *E. faecalis* is correlated with the severity of liver disease and mortality in patients with alcoholic hepatitis (34). However, a research from Arun J Sanyal el at. points out that when compared with heavy drinkers, alcoholic hepatitis have more obvious microbiome characteristics, while the bacterial signature between moderate and severe alcoholic hepatitis is not differential (35).

Gut Fungi

Except for bacteria, gut microorganisms consist of fungi, archaea, and viruses. The role of intestinal fungi in ALD has caught researchers' attention recently. Compared with the control subjects, alcoholic patients have lower fungal species richness and diversity (31). An increased systemic immune response to fungi and their products is associated with increased mortality in patients with alcoholic hepatitis (32). The overgrowth of *Candida*, especially *C. albicans*, is observed in patients with ALD compared with non-alcoholic controls, where *Penicillium* is dominant in the gut of non-alcoholic controls (7, 31, 32). Moreover, the species of *C. albicans* are significantly decreased in alcohol abusers after 2 weeks of abstinence (33). In chronic ethanol diet feeding mice, the commensal fungus *Meyerozyma guilliermondii* is significantly increased compared with mice fed with control diet (36).

MECHANISMS OF INTESTINAL DYSBIOSIS IN THE DEVELOPMENT OF ALD

Pathogenic microorganism-related signals and metabolites produced by bacteria or fungi, such as short-chain fatty acids, bile acids, and β-glucan, are involved in the pathology of ALD (37). Restoring intestinal dysbiosis has been found to improve alcoholic liver injury and inflammatory response in patients and experimental mice. We will discuss the mechanism of

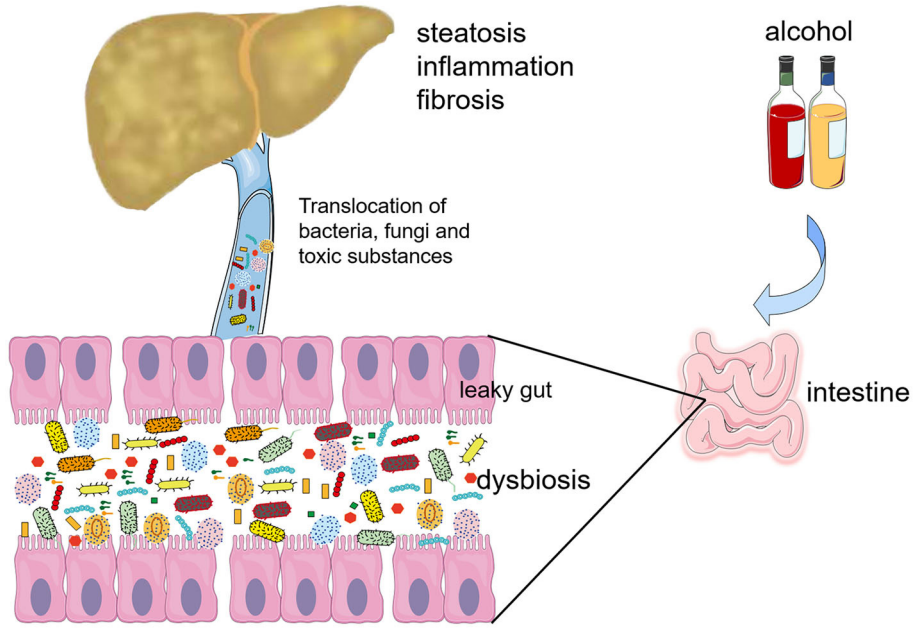

FIGURE 1 | Gut-liver cross talk in alcohol-associated liver disease (ALD). Alcohol induces the gut dysbiosis, mainly manifested as changes in the quantitative and qualitative of intestinal microbiota and fungi. The oxidative and non-oxidative metabolites of ethanol and gut dysbiosis all destroy the intestinal barrier. Gut-derived bacteria, fungi and their toxins, such as lipopolysaccharide (LPS) and β-glucan translocate into the liver though the damaged intestinal barrier and promote the progression of inflammation and fibrosis of ALD.

intestinal dysbiosis in promoting ALD development in the aspects of dysfunction of the intestinal barrier, translocated harmful materials, fatty acid metabolism immunity, bile acid homeostasis, FXR signaling, and AhR signaling (**Figure 2**).

Dysfunction of Intestinal Barrier

In addition to the direct effects of alcohol, several molecules have been identified as important factors that contribute greatly to alcohol-related intestinal barrier dysfunction. *Hif1a* knockout in mice intestinal epithelial cells results in a significantly decrease of the *Firmicutes*/*Bacteroidetes* ratio and the *Lactobacillus* level, exacerbating gut leakiness (38). Mucin 2, secreted by goblet cells, protects against pathogens penetrating the inner mucus layer. However, *Muc2* knockout protects mice from alcohol-induced liver injury, prevents the intestinal bacterial overgrowth, and enhances the expression and activity of Reg3b and Reg3g (39). *Mmp7*$^{-/-}$ mouse with a deficiency in active α-defensins of intestinal paneth cells promotes PAMP translocation and worsens the liver damage of alcohol-exposed mice (40).

Immune dysfunction is another important cause of alcohol-related intestinal barrier dysfunction. Intestinal flora is essential for maintaining the immune homeostasis of the gut-liver axis (41). On one hand, the innate and adaptive immune systems influence the component and diversity of intestinal microorganisms (42). On the other hand, gut microbiota has the potential to model intestinal immune responses in healthy and disease states (43). Alcohol feeding significantly upregulates the expression of proinflammatory cytokines interleukin-1 (IL-1) beta, tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), high mobility group protein box-1 (HMGB-1), interleukin-17 (IL-17), interleukin-23 (IL-23), and inducible nitric oxide synthase (iNOS) in the intestine (44, 45). High TNF-α ruptures the tight junctions by phosphorylating the myosin light chain kinase (MLCK) of intestinal epithelial cells through TNF-receptor I (14). Mice fed with a chronic ethanol diet have decreased the level of fecal immunoglobulin A (IgA) but increased the systemic level of IgA increases compared with the control mice (46). Binge-on-chronic alcohol reduces the number and maturation of mucosa-associated invariant T cells in mice intestines (47). Gut high permeability and dysbiosis result in the highly compromised antibacterial defense of mucosa-associated invariant T cells in patients with ALD (48). Ethanol-induced dysbiosis lower type 3 innate lymphoid cells' production of interleukin-22 (IL-22), and restore IL-22 produced by engineered bacteria in the intestine protecting mice from ethanol-induced steatohepatitis (49). In addition, IL-22 activates the hepatic signal transducer and activator of transcription 3 (STAT3), decreases the hepatic expression of fatty acid transport protein, and ameliorates alcohol related liver inflammatory injury and hepatic oxidative stress (50).

Translocated Intestinal Bacteria and Their Products

The translocation of intestinal bacteria and their constituent parts, such as peptidoglycan, LPS, flagellin, and CpG DNA, play an important role in ALD progression. Most studies have reported a change in LPS. LPS translocation through

TABLE 1 | Changes in intestinal bacteria or fungi and associated metabolites in patients with alcoholic liver disease.

Groups	Different bacteria or fungi			Metabolites	Reference
	Phylum	Family	Genus/Species		
Alcoholics with liver disease ($n = 19$) vs. healthy controls ($n = 18$)	*Bacteroidetes*	*Bacteroidaceae*↓			(21)
Alcoholics without liver disease ($n = 28$) vs. healthy controls ($n = 18$)	*Bacteroidetes*	*Bacteroidaceae*↓			
patients with chronic alcohol ($n = 24$) vs. control ($n = 18$)	*Proteobacteria*↑	*Enterobactericaea and Desulfovibrionaceae*↑	*Faecalibacterium* (genus) ↓ *Sutterella*, *Clostridium, and Holdemania* (genus) ↑	Butyric acid(%) of total SCFA concentration↓	(22)
Alcoholism without advanced liver disease ($n = 72$) vs. control ($n = 60$)			*Klebsiella, Lactococcus* (genus) ↑ *K. pneumoniae, Lactobacillus salivarius, Citrobacter koseri, Lactococcus lactis subsp. Cremoris* (species) ↑ *Akkermansia, Coprococcus, unclassified Clostridiales* (genus) ↓		(23)
Alcoholism with advanced liver disease ($n = 27$) vs. control ($n = 60$)			*Bifidobacterium, Streptococcus Lactobacillus* (genus) ↑ *Prevotella, Paraprevotella, Alistipes* (genus) ↓		
Alcoholic patients ($n = 66$) vs. healthy controls ($n = 24$)			*Bifidobacteria, Lactobacilli, Enterococci* (species) ↓		(24)
Alcoholic cirrhotics ($n = 13$) vs. Healthy control ($n = 7$)			*Enterobactericaea, Enterobacter, Bacteroides* (species) ↑		(25)
Alcoholic hepatitis with bilirubin higher than 14.1 mg/dl ($n = 36$) vs. alcoholic hepatitis with bilirubin less or equal 14.1 mg/dl ($n = 37$)			*Veillonella, Enterococcus* (species)↑ *Akkermansia* (species) ↓		(26)
Alcoholic hepatitis with MELD higher than 21 ($n = 54$) vs. alcoholic hepatitis with MELD score lower or equal than 21 (n=18)			*unclassified Clostridales, unclassified Prevotellaceae, Anaerostipes* (species) ↓		
Severe alcoholic hepatitis ($n = 24$) vs. healthy controls ($n = 24$)	*Bacteroidetes, Verrucomicrobia* ↓ *Fusobacteria*↑ *Firmicutes/Bacteroidetes ratio*↑	*Bacilli*↑	*Veillonella* (genus) ↑ *Eubacterium*_g23 (genus) ↓		(27)
Bacteria-derived extracellular vesicles (EVs) of severe alcoholic hepatitis ($n = 24$) vs. bacteria-derived EVs of healthy controls ($n = 24$)	*Bacteroidetes, Verrucomicrobia* ↓ *Fusobacteria*↑ *Firmicutes/Bacteroidetes ratio*↑		*Veillonella* (genus) ↑ *Eubacterium*_g23 (genus) ↓		
Alcohol use disorder ($n = 36$) vs. controls ($n = 36$)			*Sutterella, Haemophilus, Staphylococcus, Paraprevotella, Eubacterium, Streptococcus, Odoribacter, Veillonella, Enterococcus, Lactobacillus el at* (genus) ↑ *Akkermansia, Blautia, Bifidobacterium, Coprococcus, Dorea, Anaerostipes, Adlercreutzia, Ruminococcus* (genus) ↓		(28)
Alcoholic hepatitis ($n = 13$) vs. control subjects ($n = 17$)				Indole-3-acetic acid, Indole-3-lactic↓	(29)
Active alcohol abuser ($n = 15$) vs. non-alcoholic individuals ($n = 6$)			*Lactobacillus* (species)*	Long-chain fatty acids, C15:0 and C17:0*	(30)

(Continued)

TABLE 1 | Continued

Groups	Different bacteria or fungi			Metabolites	Reference
	Phylum	Family	Genus/Species		
Alcoholic hepatitis ($n = 82$) vs. controls ($n = 25$)			*Enterococcus faecalis* ↑	Cytolysin ↑	(6)
Alcoholic hepatitis ($n = 82$) vs. alcohol use disorder ($n = 38$)			*Enterococcus faecalis* ↑	Cytolysin ↑	
Alcoholics($n = 20$) vs. controls ($n = 8$)			*Candida* (genus) ↑ *Epicoccum, unclassified fungi, Galactomyces, Debaryomyces* (genus) ↓		(31)
Alcoholic hepatitis ($n = 91$) vs. controls ($n = 11$)			*Candida albicans* ↑	Candidalysin↑	(7)
Alcoholic hepatitis ($n = 91$) vs. alcohol use disorders ($n = 42$)			*Candida albicans* ↑	Candidalysin↑	
Alcohol use disorder ($n = 15$) vs. non-alcoholic controls ($n = 11$)			*Candida* (genus) ↑ *Penicillium, Saccharomyces, Debaromyces* (genus)↓		(32)
Alcoholic hepatitis patients ($n = 59$) vs. non-alcoholic controls ($n = 11$)			*Candida* (genus) ↑ *Penicillium, Saccharomyces, Debaromyces* (genus) ↓		
Alcohol use disorder ($n = 66$) vs. control subjects ($n = 18$)			*Candida, Debaryomyces, Pichia, Kluyveromyces, Issatchenkia, Scopulariopsis* (genus) ↑ *C. albicans, Candida zeylanoides, Issatchenkia orientalis, and Scopulariopsis cordiae* (species) ↑ *Aspergillus* (genus) ↓ *Kazachstania humilis* (species) ↓		(33)

**There is significantly correlation between Lactobacilli species and levels of long-chain fatty acids, and their metabolites C15:0 and C17:0 in the fecal samples of active alcohol abusers but not in controls. ↑ means increased, ↓ means decreased.*

the leaky intestine results in circulating endotoxemia and aggravates alcohol-induced liver inflammation through TLR4 signaling in liver (51). Activation of the LPS-TLR4 signaling pathway promotes the release of pro-inflammatory factors, such as TNF-α and IL-6. Hepatocyte TLR4 deficiency decreases lipogenic genes expression, enhances fatty acid oxidation, and reduces inflammatory genes expression of white adipose tissue, then prevents mice from alcohol-induced liver injury (29). Chronic ethanol exposure sensitizes hepatic macrophages to LPS and enhances liver inflammatory damage through TLR4 signaling (52). The absence of MyD88, one of TLR4 signaling adaptor, prevents the progression of hepatic steatosis and inflammation in chronic ethanol-exposed mice (53). However, TLR7-mediated signaling suppresses hepatic injury, steatosis, and inflammation of chronic binge ethanol fed mice (54). In addition, TLR9 signaling protects chronic alcohol exposure mice from hepatic oxidative stress but worsens hepatic inflammation.

Increased exposure to bacterial exotoxins and reduced toxin clearance in the liver also aggravate alcohol-related liver injury and inflammation. Cytolysin from *E. faecalis*, which caused hepatocyte death, are correlated with the severity of liver disease and mortality in patients with alcoholic hepatitis (34). The lack of α1-2-fucosylation in the intestine might contribute to increase the cytolytic *E. faecalis* in chronic ethanol exposed mice (55). Chronic ethanol feeding impairs the hepatic clearance of translocated pathobionts due to the reduced complement receptor of immunoglobulin (CRIg) expression in Kupffer cell (56).

Fatty Acid Metabolism

Fatty acid metabolism is significantly altered in ALD. Short-chain fatty acids (SCFA), the fermented product of dietary fiber, are the energy source for intestinal epithelial cells and for maintaining barrier integrity (57). The 16S rRNA gene and whole genome shotgun metagenomic analysis have showed the damage of acetyl-coenzyme A (CoA) butyrate synthesizing pathway in butyrate-producing bacterial genera which caused a decreased intestinal level of butyrate by chronic ethanol feeding (58). Tributyrin can inhibit ethanol-induced intestinal barrier and liver injury (59, 60). Saturated long-chain fatty acids are decreased in ethanol intragastric mice, and maintaining the levels of saturated fatty acids in the intestine promote commensal *Lactobacillus* growth and stabilize gut barrier (30). The n3-polyunsaturated fatty acids (PUFAs) can attenuate experimental ALD through decreasing neutrophil chemoattract (61).

Bile Acid Homeostasis

Bile acid homeostasis is disturbed in ALD. Gut bacteria modulate bile acid metabolism as bile acid diversity is

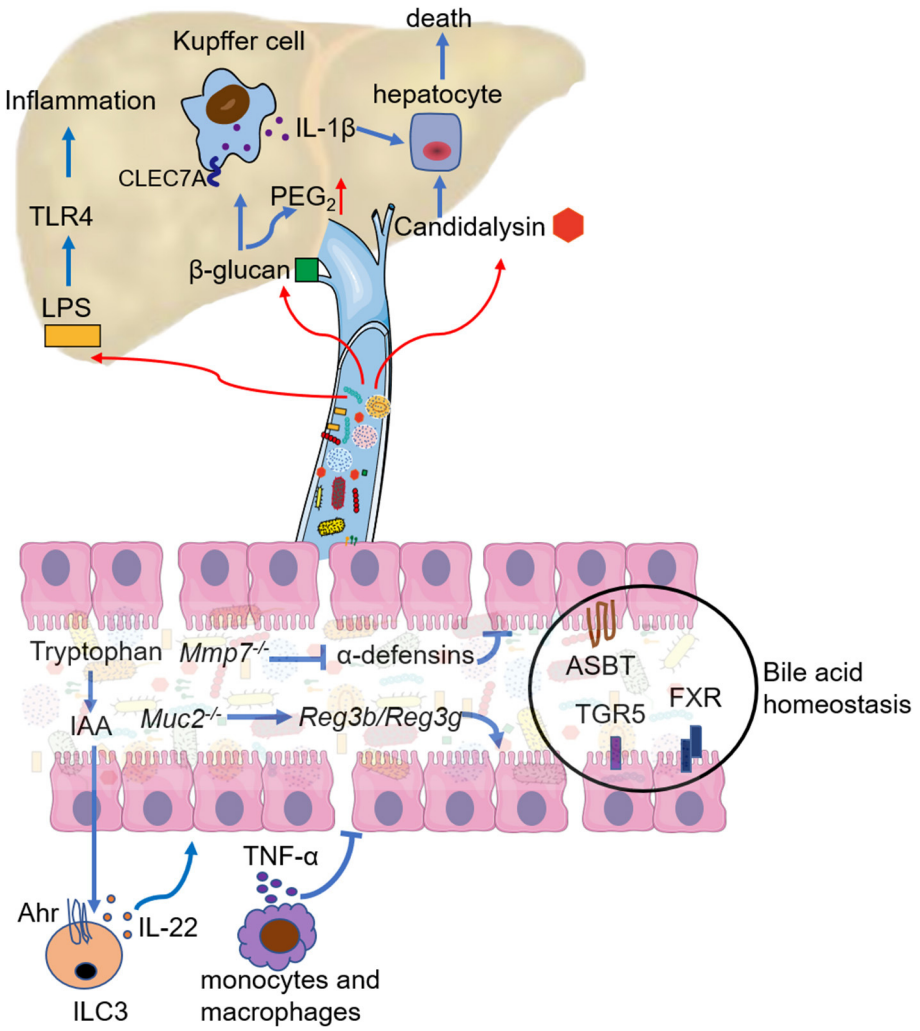

FIGURE 2 | Mechanistic contribution of the gut dysbiosis to ALD. Gut dysbiosis modulates the response of intestinal immune cells, mainly manifest as decreased IL-22 secretion by innate lymphoid cell 3 (ILC3) and increased TNF-α secretion by intestinal monocytes and macrophages. Both lead to the breakdown of the intestinal barrier function. Deficiency in active α-defensins of intestinal paneth cells (Mmp7 knockout) promotes pathogen associated molecular pattern (PAMP) translocation, but mucin2 deficiency enhances the expression and activity of Reg3b and Reg3g. Bile acid homeostasis is disturbed in ALD, and the regulation of apical sodium-dependent bile salt transporter (ASBT), bile acid receptor (TGR5), and farnesoid X receptor (FXR) could restore bile acid homeostasis and ethanol-associated dysbiosis. LPS from the intestine aggravates liver inflammation mainly through TLR4 signaling. 1,3-β-glucan from the overgrowth of fungi on the one hand binds to the C-type lectin domain family 7 member A (CLEC7A) of Kupffer cells and promotes liver inflammation, on the another hand increases PGE_2 production in the liver. *Candidalysin* from *Candida albicans* has directly cytotoxic to hepatocytes.

lower and the proportion of taurine-conjugated bile acids is increased in germ-free rats compared with conventional subjects (62). Chronic alcohol administration induces the high expression of choloylglycine hydrolase that is responsible for the deconjugation of bile acid in mice bacteria and the increased levels of unconjugated bile acids, such as cholic acid and muricholic acid in the ileum and plasma, compared with control mice (63). Persons with chronic alcohol consumption have increased bile acid synthesis and bile acid pool in the liver (64). The bile-acid receptor TGR5 maintains biliary homeostasis, as TGR5 deficiency mice have intestinal microbiota dysbiosis, higher plasma and liver levels of secondary bile acids, and greater liver steatosis and inflammation when fed with ethanol diet than WT mice (65). The apical sodium-dependent bile salt transporter (ASBT) inhibitor GSK2330672 attenuates the liver injury of chronic plus binge alcohol mouse by decreasing intestinal bile acid accumulation, and increasing hepatic CYP7A1 expression (66). Restoring bile acid homeostasis by a proliferator-activated receptor-delta agonist seladelpar (MBX-8025) could reduce chronic ethanol-induced liver damage and improve ethanol-associated dysbiosis (67).

FXR Signaling

The FXR plays an important role in energy metabolism and bile acid synthesis, and thus are extensively studied in ALD.

The importance of FXR is different in the gut and the liver in ALD. Intestine-specific FXR knockout mice are more susceptible to ethanol-induced liver steatosis and inflammation compared with WT mice (68, 69). The intestine-restricted FXR agonist fexaramine mitigates hepatic injury and inflammation of chronic ethanol-fed mouse (63). However, FXR deletion in hepatocytes has no effect on the severity of steatosis, inflammation, or liver fibrosis in chronic plus binge alcohol feeding mice, although slight liver lipid deposition and collagen accumulation are increased (70). Nevertheless, an administration of ursodeoxycholic acid (UDCA) attenuates NF-kB activation and inflammatory infiltrations in the liver of FXR knockout mice exposed to ethanol (71). These demonstrate the significance of FXR interaction with gut microbiota in the pathophysiology of ALD.

Tryptophan and AhR

Aromatic amino acids are important factors to maintain the homeostasis of intestinal flora, among which tryptophan is fully studied in ALD. Tryptophan and tryptophan-derived metabolites are reduced in the fecal and serum of alcoholic hepatitis patients with cirrhosis when compared with nonalcoholic controls, despite that the ability of microorganisms to synthesize tryptophan has improved (72). In ALD animal experiment, *Lactobacillus rhamnosus* GG derived exosome enriched with bacterial metabolites of tryptophan can improve intestinal barrier function through AhR signaling that promotes IL22 production by intestinal immune cells (73). Alcohol-induced intestinal dysbiosis reduces intestinal IL-22 production and indoce-3-acetic acid (IAA) levels. IAA supplementation, as a microbial-derived ligand of AhR, protects mice from ethanol-induced liver steatohepatitis by preventing bacterial translocation to liver (49). Intestinal epithelial cell-specific *Ahr* knockout exacerbates ethanol-induced liver injury through promoting the translocation of *Helicobacter hepaticus* and *Helicobacter ganmani* to liver (74). Ficz (6-formyllindolo (3, 2-B) carbazole), an AhR agonist, reduces ALD liver injury with similar effects to prebiotics. Moreover, alcohol-fed *Ahr* knockout mice eliminates the beneficial effects of prebiotics (75).

Gut Fungi Relevant Mechanism

The 1,3-β-glucan from the overgrowth of fungi in chronic alcohol-fed mice binds to the C-type lectin domain family 7 member A (CLEC7A) of Kupffer cells and promotes liver inflammation (31). Patients with alcoholic hepatitis have an intense immune response to fungus, as serum anti–*Saccharomyces cerevisiae* antibodies are significantly high compared to patients with alcohol use disorder and nonalcoholic controls (32). *Candidalysin* positive *C. albicans* exacerbate ethanol-induced liver injury not dependent on the further impairment of intestinal barrier function through *Candidalysin*, but dependent on directly cytotoxic to hepatocytes (7). The commensal fungus *M. guilliermondii* induced alcoholic hepatic steatosis is probably due to translocated β-glucan increasing PGE_2 production in the liver (36).

TREATMENTS

Fecal Microbiota Transplantation

Comparing with patients treated with the standard of care, fecal microbiota transplantation from family members attenuates the disease severity and improves the survival rate of severe patients with alcoholic hepatitis (76). In mice models, fecal microbiota transplantation from alcohol-tolerant donor mice to alcohol-sensitive recipient mice can correct alcohol-induced dysbiosis and prevent alcohol-induced liver injury (77).

Probiotic Bacteria

Many probiotic therapies have been carried out in human patients with ALD and experimental ALD mice where they have received inspiring results. Patients with alcoholic hepatitis who receive 7 days of *Lactobacillus subtilis*/*Streptococcus faecium* have reduced gut-derived microbial LPS and TNF-α level (78). Different species of *Lactobacillus*, such as Lactobacillus plantarum (79), *Lactobacillus acidophilus* (80), *Lactobacillus fermentum* (81), and *L. rhamnosus* GG (82) are all reported to protect against alcohol-induced liver injury through improving intestinal barrier function, modulating gut bacteria, and balancing T_{reg} and T_H17 cells in peripheral blood of mice. *Akkermansia muciniphila* supplementation decreases ethanol-induced gut leakiness and hepatic injury (83). *L. plantarum* LC27 and *Bifidobacterium longum* LC67 inhibit the activation of NF-κB mediated by LPS, restore the disturbed intestinal flora, and ultimately reduce alcoholic steatosis in mice (84). *Bacillus subtilis* relives alcohol-induced liver damage by reducing bacterial endotoxin translocation and liver inflammation (34). A new strain of *Pediococcus pentosaceus* alleviates ethanol-induced liver injury by increasing the abundance of bacteria that produce SCFAs and strengthening tight junctions of intestinal epithelial cells (85). *Faecalibacterium prausnitzii* and potato starch supplementation attenuate chronic-binge ethanol-induced liver injury by increasing propionate abundance in mice cecum and mitigating the losses of SCFA transporter in the proximal colon (86). VSL#3 treatment prevents intestinal bacteria and their products from spreading to portal circulation and downregulates liver inflammation mediated by TNF-α (87).

Probiotic Fungi

Saccharomyces cerevisiae var. *boulardii* which is anti-carcinogenic, antibacterial antiviral, antioxidant, and able to reduce serum cholesterol level, has been used for treating various gut-related diseases (88). *Saccharomyces boulardii* administration attenuates acute liver injury (89). Besides, *S. boulardii* administration attenuates hepatic steatosis, low-grade inflammation, and changes the gut microbiome (90). *Hanseniaspora osmophila*, *Lachancea thermotolerans*, and *S. cerevisiae* strains are proved to have the most potential as health-promoting probiotics (91). However, it has also been reported that *Clostridium difficile* colitis and neutropenic patients have S. *cerevisiae* fungemia after treatment with *S. boulardii* as probiotic (92, 93). For future treatments, doctors should concern about the potential risk when prescribing fungal probiotics, especially to immunocompromised patients.

Prebiotics

Some amino acids, fatty acids, and probiotic fermentation are found to alleviate alcoholic liver disease. Ethanol feeding exhausts protein thiols, raises oxidized protein thiols in mice gut, while glutamine complement can attenuate the protein thiol oxidation of distal colonic mucosa (94). What's more, glutamine prevents the ethanol-induced disruption of the tight junction by EGFR-dependent mechanism (95, 96). L-cysteine attenuates acetaldehyde-induced transepithelial electrical resistance (TEER), and inhibits the ROS injury of Caco-2 cells (11). Tributyrin supplementation has been found to attenuate both acute and chronic-binge ethanol induced intestinal leakage and liver damage (60, 97, 98). For chronic alcohol intragastric mice, supplementation with saturated fatty acids can enhance the intestinal barrier and reduce alcohol-induced liver injury (30). The fermentation broth of the mixture of *Pueraria lobata*, *Lonicera japonica*, and *Crataegus pinnatifida* by *L. rhamnosus* 217-1 is reported to alleviate alcohol-induced intestinal microbiome disorders, and reduce oxidative stress and inflammatory signals in the liver (99).

Lifestyle and Medical Intervention

Diet Regulation

Dietary inulin and flaxseed oil treatment both attenuate the hepatitis of chronic alcohol exposed mice *via* modulating liver inflammatory response and restoring of the gut microbiota dysbiosis (100, 101). Dietary okra seed oil consumption attenuates lipid metabolic disorder and gut dysbiosis of ALD mice (46).

Traditional Medicine

Water-insoluble polysaccharide from *Wolfiporia cocos* reduce liver steatosis caused by chronic ethanol feeding, and suppress the overgrowth of intestinal fungi and *Proteosbacteria* (36). Pomegranate prevents intestinal leakage and liver inflammatory damage caused by alcohol abuse through inhibiting the gut oxidative and nitrative stress (102). Kaempferol alleviates acute alcoholic liver injury in mice by regulating intestinal tight junction protein, butyric acid receptor, and butyric acid transporter expressions (103). Ginkgo biloba compound and puerarin ameliorate experimental alcoholic liver injury by downregulating the expressions of TNF-α, lipopolysaccharide binding protein (LBP), CD14, and TLR4 in liver, and upregulating the expression of tight junction proteins in the intestine (104, 105). The rice bran phenolic extract relieves alcohol caused intestinal microbiota dysbiosis, barrier dysfunction, and liver inflammation (106).

CONCLUSIONS

Gut dysbiosis promotes the development of ALD. The role of fungi in ALD is also important, which deserves further study. As an important barrier for pathogenic microorganisms from the intestine to the portal vein, the mechanism of intestinal blood barrier injury in ALD needs to be clarified. A research recently pointed out chronic alcohol exposure will result in insufficient anti-bacterial immunity of the body, as the number of MAIT cells in peripheral blood of patients with alcohol-related cirrhosis was significantly reduced and their function was impaired (48). Attention should be paid to the high risk of bacteria infection in ALD patients. There is a clear causal link between intestinal translocation PAMPs and liver inflammation (40). Therefore, it is of great significance to further elucidate the mechanism of intestinal barrier injury in ALD. Microecological disorder is an important cause of intestinal barrier impairment, and microbiota-based treatments are the powerful therapeutic options for ALD (107).

AUTHOR CONTRIBUTIONS

HC, LY, and XH designed the review and revised the manuscript. LC collected the data and drafted the manuscript. YZ revised the manuscript. All authors have approved the final version.

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Characterization of Gut Microbiota and Exploration of Potential Predictive Model for Hepatocellular Carcinoma Microvascular Invasion

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Background: The association between gut microbiota and microvascular invasion (MVI) in patients with hepatocellular carcinoma (HCC) remains unclarified. Hence, the microbiome analysis of patients with HCC might predict MVI development as an accurate, non-invasive, and convenient assessment. The aim of this study was to investigate the characteristics of gut microbiota in patients with HCC-MVI and establish a microbial prediction model of HCC-MVI based on a microbiome study.

Methods: Fecal samples were collected from 59 patients with HCC (24 of the total with MVI disease and 16 healthy controls) and were further analyzed by 16S rRNA amplicon sequencing followed by a comprehensive bioinformatic analysis. The diagnostic performance of microbiome characteristics in predicting MVI was assessed by receiver operating characteristic (ROC) curves. The correlation between gut microbiota and tumor microenvironment (TME) in the HCC-MVI group was further analyzed by using immunohistochemistry and immunofluorescence assay.

Results: A significant differentiation trend of microbiota composition and structure was observed between the HCC-MVI group and those without vascular invasion (HCC-NVI). Compared with HCC-NVI group and healthy controls, gut bacteria *Klebsiella*, *Proteobacteria*, *Prevotellaceae,* and *Enterobacteriaceae* were significantly enriched, whereas *Firmicutes, Ruminococcus,* and *Monoglobaceae* were significantly decreased in patients with HCC-MVI. *Klebsiella* was considered to be the key microbiome signature for patients with HCC-MVI. The area under the curve (AUC) of the established HCC-MVI microbial prediction model was 94.81% (95% CI: 87.63–100%). The percentage of M2-type tumor-associated macrophages (TAMs) was increased in the HCC-MVI group compared with the HCC-NVI group ($p < 0.001$). M2-type TAMs in TME were negatively correlated with Shannon and Simpson index of HCC-MVI gut microbiota (all $p < 0.01$).

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In addition, predicted KEGG pathways showed that the functional differences in the metabolic pathways of microbiota varied among the groups.

Conclusion: The results indicated that differences existed in the fecal microbiome of patients with HCC-MVI and healthy controls. The prediction model of HCC-MVI established with certain gut bacterial signatures may have the potential to predict HCC-MVI outcome, and the characteristics of the fecal microbiome in patients with HCC may be associated with TME, though future larger-cohort studies are required to validate this supposition.

Keywords: hepatocellular carcinoma, microvascular invasion, gut microbiota, predictive model, tumor microenvironment, M2-type TAM, mTOR signaling pathway

INTRODUCTION

Liver cancer is considered to be the fifth leading cause of cancer-related death (1, 2). The risk of recurrence after resection is up to 70% after 5 years, and the 5-year survival rate is as low as 18% (3). Hepatocellular carcinoma (HCC) is the most prevalent form of liver cancer (4), and most patients with HCC have metastatic disease or vascular invasion at their initial diagnosis (5–7). HCC tumors are heterogeneous, and patients at the same disease stage may have very different treatment outcomes (1).

Vascular invasion, including microvascular invasion (MVI), is one of the most critical factors in determining long-term survival and tumor recurrence for patients with HCC (8, 9). MVI is defined as a histological feature that is based on postoperative pathology (10–14). Once MVI develops, the tumor invades the blood vessels, and the current postoperative diagnosis of MVI leaves not much time window for therapeutic intervention. Accurate preoperative tests to predict MVI are thus urgently needed to reduce recurrence and improve the survival of patients with HCC. However, no such related methods, especially in a non-invasive manner, established to date have adequate sensitivity, specificity, or reproducibility for this purpose (15).

More than 100 trillion gut microorganisms form a complex microbial community, namely microbiota, that has a major impact on human health (16). Accumulating evidence has suggested a link between the microbiome, the metagenome of microbiota, and liver diseases. It is estimated that gut microbiota could be associated with 15–20% of patients with HCC (17). The gut–liver axis refers to the bidirectional relationship between the gut with its microbiota and the liver, which enables the transport of gut-derived products including microbial metabolites directly to the liver, and the liver feedback route of bile and antibody secretion to the intestine (18, 19). Previously, interactions between the intestinal microbiome and liver diseases were also reported in several studies, which showed the predictive value of gut microbiome in tumor prognosis, the effect on chemotherapy, and immunotherapy responses (20–22). Gut microbiota can regulate corresponding miRNAs through impacting circRNA expression and play a role in microbiota-mediated cancer metastasis (23). Li et al. identified CTSK as a mediator between the dysbiosis of gut microbiota and CRC metastasis (24). Gut or mammary duct colonization with enterotoxigenic bacteria *Bacteroides fragilis* triggers epithelial hyperplasia and augments breast cancer growth and metastasis (25).

The underlying mechanism of MVI in patients with HCC which induces metastasis and recurrence is very complicated and remains largely unclear (26). To the best of our knowledge, the correlation between gut microbiota and HCC-MVI remains uninvestigated, despite numerous reports linking gut dysbiosis with HCC pathogenesis. We hereby hypothesized that gut microbiota has a potential influence on HCC-MVI development, which may give credit to its role in cancer metastasis. Therefore, in this clinical study, we wanted to further investigate the relationship between the gut microbiota and HCC-MVI to gain a better understanding of the underlying interaction. To validate the previous research and provide deeper insights into the mechanism of fecal microbiome affecting HCC patients' MVI development, the current study was intended to further apply 16S rRNA amplicon sequencing and microbiome analysis to investigate the differences in taxonomic and derived functional profiles in the fecal microbiota from patients with HCC and healthy controls, clarify the predictive role of a key microbial signature in HCC-MVI, and further demonstrate its relevance to host immunity.

METHODS

Subject Recruitment and Sample Collection

In this study, we identified 123 patients between January 2020 and December 2020 who had an initial presentation of hepatitis B virus (HBV)-related HCC at the Department of Liver Cancer Center, Tianjin Medical University Cancer Institute and Hospital. HBV-related HCC was defined here as seropositive for hepatitis B surface antigen (HBsAg), non-hepatitis C, and non-alcohol-related liver disease. The initial diagnosis of primary HCC is determined by pathology. Macrovascular invasion (MaVI) diagnosis is based on imaging (CT/MRI). The diagnosis of MVI is based on the postoperative pathological diagnosis. Patients with HCC without vascular invasion are grouped as HCC-NVI. Gender- and age-matched healthy volunteers with no history of diseases were enroll (1) concomitant with other

malignant tumors; (2) nonnative patients who have received antitumor therapy; (3) combined with chronic diseases such as hypertension, diabetes, and gastrointestinal diseases; (4) use or take antibiotics, proton pump inhibitors, probiotic products 1 month before sampling; (5) missing clinical parameters. This study was approved by the Medical Ethics Committee at the Tianjin Cancer Hospital and written informed consent was obtained from all participants.

DNA Isolation From Fecal Samples and 16S rRNA Amplicon Sequencing

Fecal specimens (10 ml) were thawed and centrifuged at 7,500 *g*, 4°C for 10 min. Total genomic DNA was extracted by DNA Extraction Kit (Tiangen, China) from fecal samples. The 16S rRNA genes of distinct regions (16S V3-V4) were amplified using specific primers with the barcode. The PCR reactions were performed by 0.2 μM of forward and reverse primers, 15 μl of Phusion® High-Fidelity PCR Master Mix (New England Biolabs, USA), and 10 ng of template DNA. PCR cycling was carried out by the following program: 98°C 1 min, 25 cycles of 98°C 15 s, 55°C 30 s, 72°C 30 s, and 72°C 5 min. After the PCR process, the products were detected by operating electrophoresis on a 2% agarose gel. Then, Qiagen Gel Extraction Kit (Qiagen, Germany) was used to purify the mixture PCR products. Sequencing libraries were produced by TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following the manufacturer's recommendations, and the index codes were added. At last, the library was sequenced on an Illumina NovaSeq platform and 250 bp paired-end reads were generated.

Immunohistochemical Staining

The HCC tissues were serially sectioned at a thickness of 3–5 mm. Then, the tissues were dewaxed, deparaffinized in xylene, and rehydrated. The samples were then boiled for 15 min in a microwave oven for the purpose of increasing antigen retrieval. Then, 3% hydrogen peroxidase was used to block the endogenous peroxidases for 30 min. The slides were incubated with primary antibody $CD8^+$ (PeproTech, 67786-1, 1:8000) overnight. Biotin-free horseradish peroxidase-labeled polymer of the Envision Plus detection system (Dako, Denmark) was used in the process of detection. Double immunofluorescence staining was performed as previously described. Paraffin-embedded sections were incubated with the following primary antibodies: F4/80 (Santa Cruz Biotechnology, SC-365340, 1:500), CD206 (PeproTech, 18704-1-AP, 1:800), and CD86 (CST, 91882S, 1:100) at 4°C overnight. Then, the sections were incubated at 37°C with the matched fluorescently labeled secondary antibodies (1:500; Invitrogen; CA, USA) for 30 min.

Bioinformatics and Statistical Analyses

According to the QIIME (V1.9.1) quality control process, quality filtering of raw tags was conducted under specific filtering conditions for the purpose of obtaining the high-quality clean tag. Uparse software (Uparse v7.0.1001) was used for the analysis of the sequences. Similar sequences higher than 97% were defined by the same operational taxonomic units (OTUs). Silva Database (http://www.arb-silva.de/) based on the Mothur algorithm was applied to annotate taxonomic information of each representative sequence. All the indices were calculated with QIIME and displayed with R Software (Version 2.15.3). Cluster analysis was preceded by principal component analysis (PCA), which was used to reduce the dimension of the original variables by FactoMineR package and ggplot2 package. Principal coordinate analysis (PCoA) was displayed to get principal coordinates and visualized with the WGCNA package, stat packages, and ggplot2 package. To determine the enrichment in the assigned taxonomic and functional profiles, LEfSe analysis was performed. Taxonomic levels with LEfSe values higher than 4 at a $p < 0.05$ were statistically significant. Operating characteristic curves (receiving operational curve, ROC) were constructed, and then the area under the curve (AUC) was calculated to evaluate the discriminatory ability of the random forest model. General statistical analysis was performed in Prism v8.2.1 (GraphPad Software, Inc.), and one-way ANOVA with Tukey's multiple

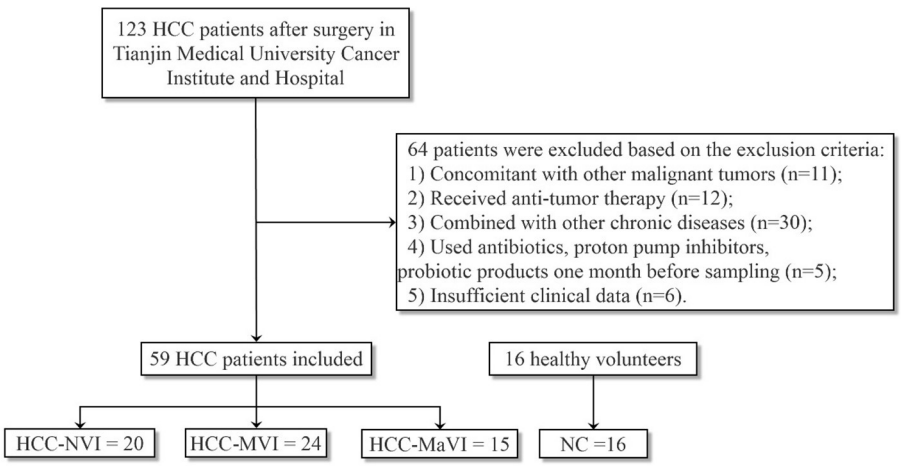

FIGURE 1 | Flow diagram describing the selection of the study population.

TABLE 1 | Clinical characteristics of all participants.

	NC	HCC-NVI	HCC-MVI	HCC-MaVI	*P-value*
	n = 16	*n* = 20	*n* = 24	*n* = 15	
Gender (Male)	10 (62.5%)	12 (60.0%)	20 (83.3%)	12 (80.0%)	0.252
Age, years	53.0 (8.0)	57.4 (8.6)	59.8 (12.1)	53.3 (11.4)	0.131
BMI, kg/cm^2	24.4 (2.6)	24.6 (3.1)	24.4 (3.7)	24.8 (2.8)	0.980
Cirrhosis (yes)	-	13 (65.0%)	16 (66.7%)	12 (80.0%)	0.588
Etiology (HBV)	-	20 (100.0%)	24 (100.0%)	15 (100.0%)	NA
ALB, g/L	43.8 (2.0)	43.3 (3.8)	42.3 (4.6)	41.8 (6.6)	0.559
TBIL, mmol/L	14.4 (4.5)	15.0 (5.8)	15.7 (5.0)	20.5 (6.7)	0.011
AFP, ng/ml	9.23 (4.1)	4,957 (14,905.0)	6,807 (19,171.0)	40,324 (89,308.0)	0.035
Child-Pugh class (A/B)	-	20 (100.0%)/0 (0.0%)	24 (100.0%)/0 (0.0%)	13 (86.7%)/2 (13.3%)	0.061
AST (U/L)	32.0 (20.3)	32.5 (19.5)	35.6 (28.8)	41.0 (21.7)	0.685
PLT (10^9/L)	197.56 (45.14)	185.00 (86.70)	173.83 (69.86)	195.33 (76.53)	0.717
Tumor largest size (cm)	NA	5.58 (3.23)	6.24 (4.06)	7.51 (2.87)	0.279

AFP, alpha-fetoprotein; ALB, albumin; AST, aspartate aminotransferase; BMI, body mass index; HBV, hepatitis B virus; TBIL total bilirubin. Quantitative value was presented as Mean (SD).

comparisons test was used to determine differences between the groups. Spearman's rank correlation was performed in R (v3.6.1) to analyze the relationships between the microbiome and disease status.

All *p*-values were corrected using Benjamini–Hochberg multiple test correction. *p*-values of <0.05 were considered statistically significant.

RESULTS

Baseline Clinical Characteristics of the Participants

Fecal samples were collected from a total of 75 subjects including 16 healthy controls (NC), 20 patients with HCC-NVI, 24 patients with HCC-MVI, and 15 patients with HCC-MaVI (**Figure 1**). The demographic and clinical data of the four groups of participants including 59 patients with HCC and 16 healthy controls were analyzed and displayed in **Table 1**. Based on the published clinical studies employing microbiome analyses of four or more groups, we performed sample size calculation using G*power software and estimated that at least 15 participants in each group would provide an estimated effect size of 0.405, which is adequate to detect clinically meaningful differences between the subgroups with a power of at least 80% with a two-tailed *p*-value of <0.05. Comparisons of all four groups and three HCC groups demonstrated significant differences among the levels of total bilirubin (TBIL), which is regarded as an indicator for liver dysfunction (**Supplementary Figure 1A**) (1). Although there was no significant difference in alpha-fetoprotein (AFP) level among the three HCC groups, a statistically significant difference in AFP level among all the four groups was observed when healthy controls were taken into consideration (**Supplementary Figure 1B**). No significant difference in age, gender, BMI, and other available clinical parameters was elicited among these four groups.

Alpha and Beta Diversities of Fecal Microbiome

Richness and evenness analysis was performed in the fecal microbiome of the patients with HCC and healthy controls (NC) using the Shannon and Simpson index to display the alpha diversity (**Figures 2A,B**). No significant difference was detected. When compared with the NC or HCC-NVI group, the microbial alpha diversity within the HCC-VI (HCC-MVI or HCC-MaVI) group showed significant statistical differences (**Figures 2C,D**). However, the beta diversity in the patients with HCC and NC group exhibited significant differences under the multiple response permutation procedure (MRPP; $A = 0.0291$, $P = 0.001$). The result of MRPP in NC, HCC-MVI, and HCC-MaVI groups all showed significant differences (all $A > 0$, $P < 0.001$).

The PCA and unweighted Unifrac principal coordinate analysis (PCoA) analysis indicated that the NC and HCC groups had no clustering with members of their own group (**Figures 3A,B**). Subgroup analysis demonstrated that no identified microbiota clusters were observed in NC, HCC-NVI, HCC-MVI, and HCC-MaVI groups with PCA and unweighted Unifrac PCoA analysis (**Figures 3C,D**).

Taxonomic Profiles of the Gut Microbiome

According to the relative abundance of the microbiota in the NC group, HCC samples, and their subgroups, classification and analysis were carried out at the levels of phylum, class, order, family, and genus. At the phylum level, the relative abundance of Firmicutes and Actinomycetes in the NC group was significantly greater than that of the HCC group ($P = 0.004$; 0.002), while the Bacteroidetes was significantly enriched in patients with HCC ($P < 0.001$; **Figure 4A** and **Supplementary Figure 2**). Firmicutes showed a significant decrease in the HCC-VI group when compared with the HCC-NVI group and the NC group. The relative abundance of Proteobacteria in the HCC-MVI group was greater than that of the HCC-NVI group and the NC group; the

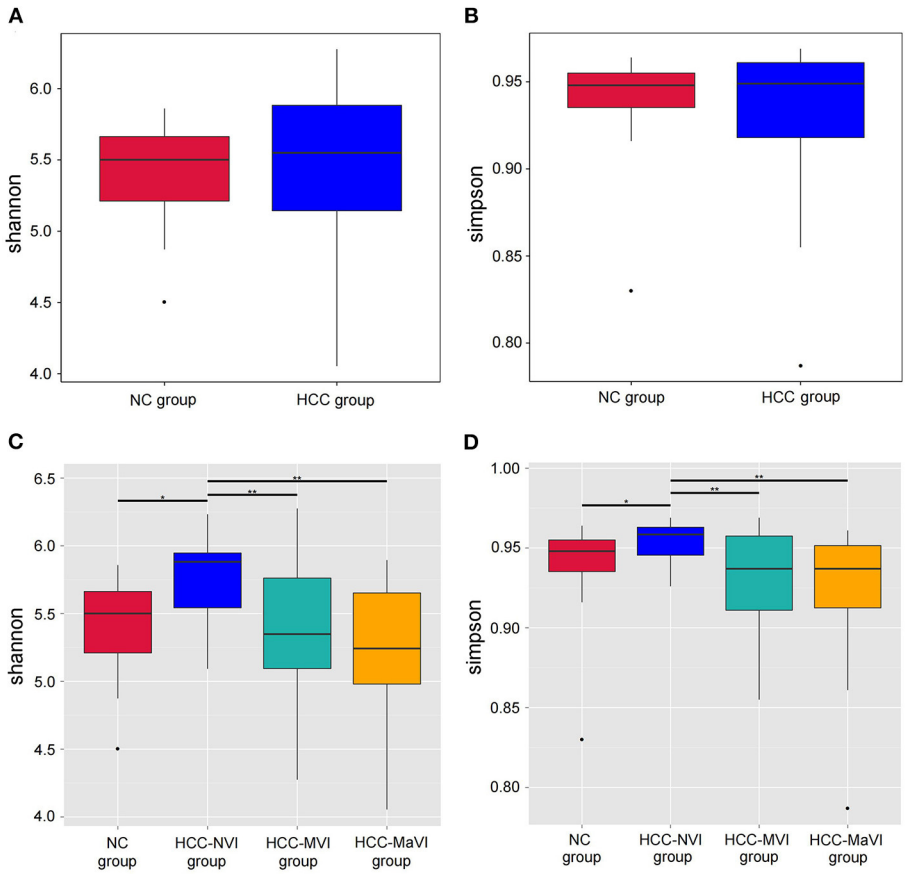

FIGURE 2 | Comparison of alpha-diversity of the gut microbiota between patients with HCC and the NC group. Shannon **(A)** and Simpson **(B)** indexes were used to evaluate the evenness and richness in the HCC and NC groups; Shannon **(C)** and Simpson **(D)** indexes were used for comparing the evenness and richness in the NC, HCC-NVI, HCC-MVI, and HCC-MaVI groups. Difference in microbiota composition between the groups based on Bray-Curtis metrics was displayed by box-plot according to the Wilcoxon rank-sum test, $^{*}P < 0.05$, $^{**}P < 0.01$.

relative abundance of Bacteroides in the HCC-MaVI group was higher than that of the HCC-NVI group and the NC group, while the relative abundance of *Actinobacteria* was lower (**Figure 4B** and **Supplementary Figure 3**).

Under the phylum level, the abundance of *Bifidobacterium* was decreased in patients with HCC (P = 0.002), while *Prevotella* and *Klebsiella* were increased compared with the NC group. Compared with the HCC-NVI group and the NC group, the abundance of *Prevotellaceae* and *Enterobacteriaceae* was significantly increased in the HCC-MVI group while that of *Ruminococcaceae* was decreased. The abundance of *Ruminococcaceae*, *Bifidobacteriaceae,* and *Veillonellaceae* was significantly decreased in the HCC-MaVI group while *Bacteroidaceae* showed a substantial increase (**Figure 4C**). Compared with the HCC-NVI group and the NC group, the abundance of *Prevotella* and *Klebsiella* was significantly increased in the HCC-MVI group. The abundance of *Bifidobacterium* significantly decreased and *Prevotella* significantly increased in the HCC-MaVI group (**Figure 4D**). Differential abundance of microbes at the class, order, family, and genus levels are displayed in **Supplementary Figures 4–10**. Briefly, *Clostridia* and *Bacterodia* exhibited higher abundance in HCC groups at the class level, *Bacteroidales* was significantly increased in HCC groups at the order level, the abundance of *Prevotellaceae* in HCC groups was significantly increased while *Bifidobacteriaceae* was significantly decreased at the family level, and *Faecalibacterium* showed the highest abundance in either NC group or HCC groups at the genus level.

Predicted Functional Profiles of the Gut Microbiome

In addition to diversity, the LEfSe analysis was applied to identify microbiome signatures that were differentially enriched among the NC, HCC-MVI, and HCC-MaVI groups. The result showed that genus_*Agathobacter*, genus *Citrobacter*, genus_*Klebsiella,* and species_*Klebsiella_pneumoniae* were significantly enriched in HCC-MVI samples. Accordingly, several microbes, such as *Prevotellaceae*, *Bacteroidia*, *Prevotella*, *Bacteroidales,* and *Bacteroidota* exhibit enrichment in patients with HCC-MaVI ($p < 0.05$, LDA score > 4; **Figures 5A,B**).

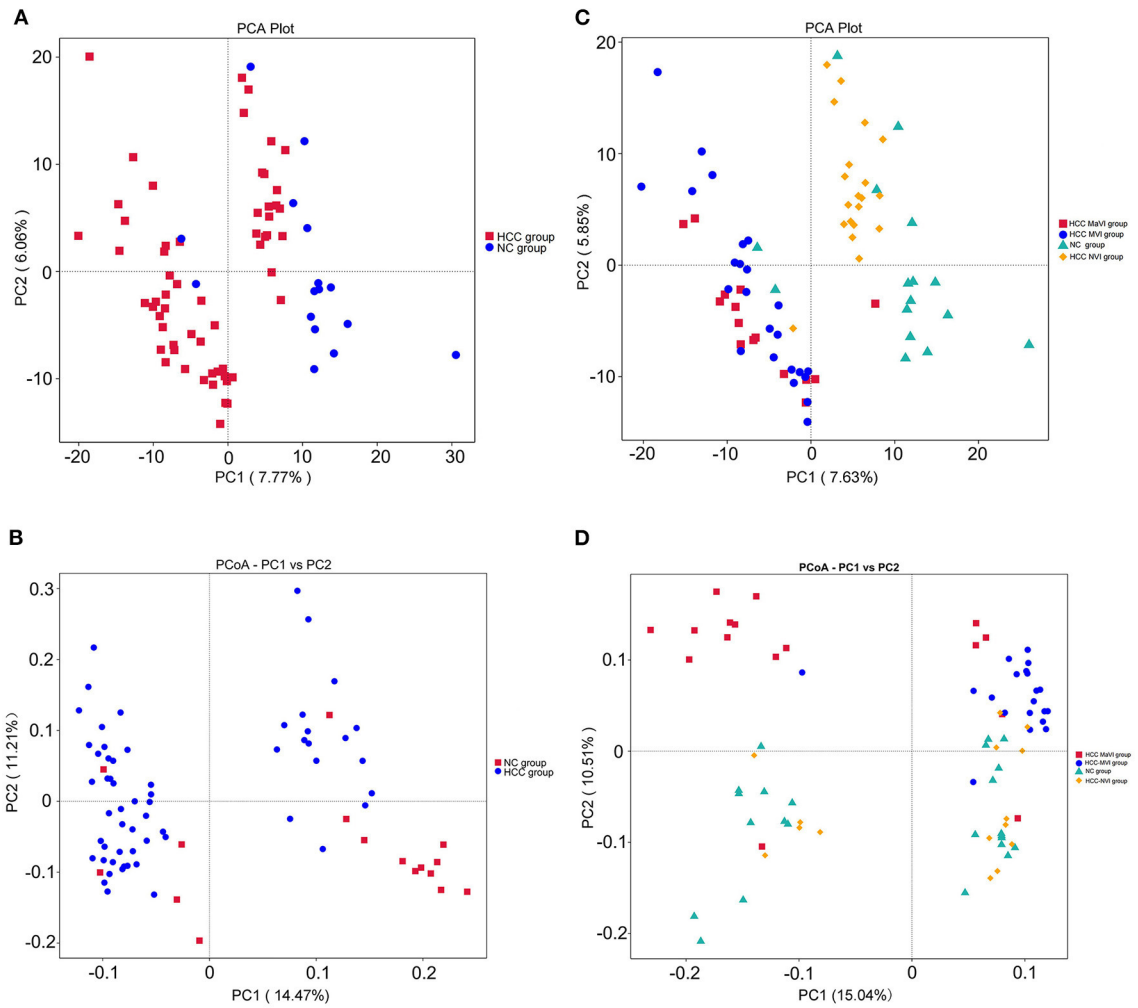

FIGURE 3 | Comparison of beta diversity by principal component analysis (PCA) and principal coordinate analysis (PCoA) plots. PCA **(A)** and PCoA analysis of variation based on unweighted UniFrac distances **(B)** of NC and HCC groups. Red and blue dots represent HCC and normal controls, respectively. Each point represents an individual sample. PCA **(C)** and PCoA analysis of variation based on unweighted UniFrac distances **(D)** of NC, HCC-NVI, HCC-MVI, and HCC-MaVI groups. Red dots represent HCC-MaVI, blue dots represent HCC-MVI, yellow dots represent HCC-NVI, and green dots represent the NC group, respectively. Each point represents an individual sample.

The Potential Microbial Model Established for HCC-MVI Prediction

A random forest model was applied to predict HCC-MVI using 20 bacterial families (including *Clostridiaceae, UCG_010, Tannerellaceae, Ruminococcaceae, Eubacterium coprostanoligenes group, Synergistaceae, Monoglobaceae, Rikenellaceae, unidentified Gastranaerophilales, Rhodobacteraceae, Dysgonomonadaceae, Bifidobacteriaceae, Burkholderiaceae, Enterobacteriaceae, Morganellaceae, Atopobiaceae, Marinifilaceae, Erysipelatoclostridiaceae, Bacteroidaceae, and Desulfovibrionaceae*) with a significant difference among groups, which was filtered out based on mean decrease accuracy and mean decrease gin analysis. The receiver operating characteristic (ROC) analysis was performed to validate the prediction accuracy of this potential model for HCC-MVI with the calculated area under the curve (AUC) being 94.81% (95% CI: 87.63–100%), illustrating a good predictive performance of this model (**Figure 6** and **Supplementary Figure 11**).

Gut Microbiota Associated With TME Changes in HCC-MVI Patients

Intratumoral interactions between the cellular and structural components of the tumor microenvironment (TME) can regulate cancer cell survival, local invasion, and metastatic dissemination (27). Immunohistochemical staining was thus applied to analyze the positive rate of $CD8^+$ T cells in the TME group of liver cancer with or without MVI. Compared with the HCC-NVI group, the positive rate of $CD8^+$ T cells in the HCC-MVI TME was significantly decreased (41.3 vs. 20.8% $P = 0.001$; **Figures 7A,B**).

An immunofluorescence study was applied to further analyze M1 and M2 tumor-associated macrophage (TAM) cells in the TME of patients with HCC. The results indicate that compared

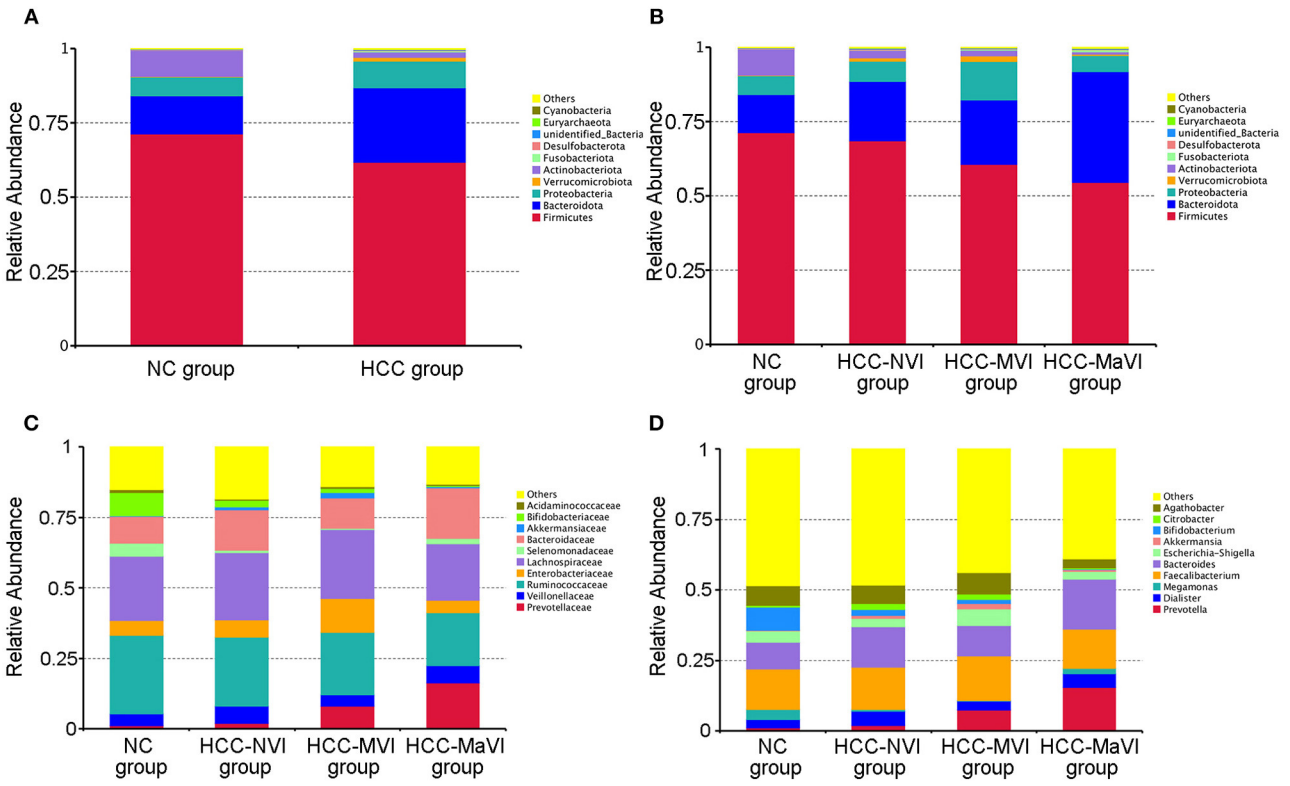

FIGURE 4 | Relative abundance of microbiome across different groups. **(A)** The heatmaps of the NC and HCC bacteria groups at the phylum level. Heatmaps of the NC, HCC-NVI, HCC-MVI, and HCC-MaVI bacteria groups at the phylum level **(B)**, at the family level **(C)**, at the genus level **(D)**.

with the HCC-NVI group, the positive rate of M1 TAM in the HCC-MVI group was significantly lower (32.5 vs. 54.0%, $P < 0.001$), whereas the positive rate of M2-type TAM cells in the HCC-MVI group was significantly increased (51.1 vs. 31.0%, $P < 0.001$; **Figure 8**).

Correlation Analysis Between the Intestinal Microbiome and TME Indicators

Cumulative contribution analysis (CCA) is used to explore the correlation among the positive rate of $CD8^+$ T cells, M1- and M2-type TAM cells in TME, and the intestinal microbiome of different vascular invasions. As shown in **Figure 9A**, the cumulative contribution of TME indicators is 37.29% for CCA1 and 22.17% for CCA2, and the results indicate that there is a significant correlation between M2-type TAM and HCC-MVI group intestinal microbiome.

Based on Spearman correlation analysis, correlation among $CD8^+$ T cells, M1/M2-type TAM cells, and HCC-MVI group intestinal microbiome was explored in TME. The positive rate of M2-type TAM cells is negatively correlated with the alpha diversity of the HCC-MVI intestinal microbiome ($P < 0.01$, **Figure 9B**). The positive rate of M2-type TAM in its TME increases once the diversity of the HCC-MVI intestinal microbiome decreases or the microbiome becomes disordered (dysbiosis).

Functional Analysis of HCC–MVI Microbiome and Its Correlation With M2-type TAM

Based on Tax4Fun, we further explored the functional alterations of the microbiome in the HCC-MVI and HCC-NVI groups (**Supplementary Figure 12**). The function of the HCC-MVI intestinal microbiome was significantly concentrated in pathways, including replication and repair, translation, energy metabolism, nucleotide metabolism, glycan biosynthesis, and metabolism, while the intestinal microbiome function of HCC-NVI patients was significantly enriched in pathways such as membrane transport, signal transduction, and cell motility (all $p < 0.05$). Based on the KEGG database, we further explored the correlation between the functional pathway of HCC-MVI microbiome and the positive rate of M2-type TAM in TME, and the mTOR signaling pathway was found to be positively correlated with a positive rate of M2-type TAM in TME (correlation coefficient 0.37, $P = 0.01$; **Table 2**). Taken together, these results suggest that the increase of TAM M2 positive rate in HCC-MVI TME may be involved in the mTOR signaling pathway.

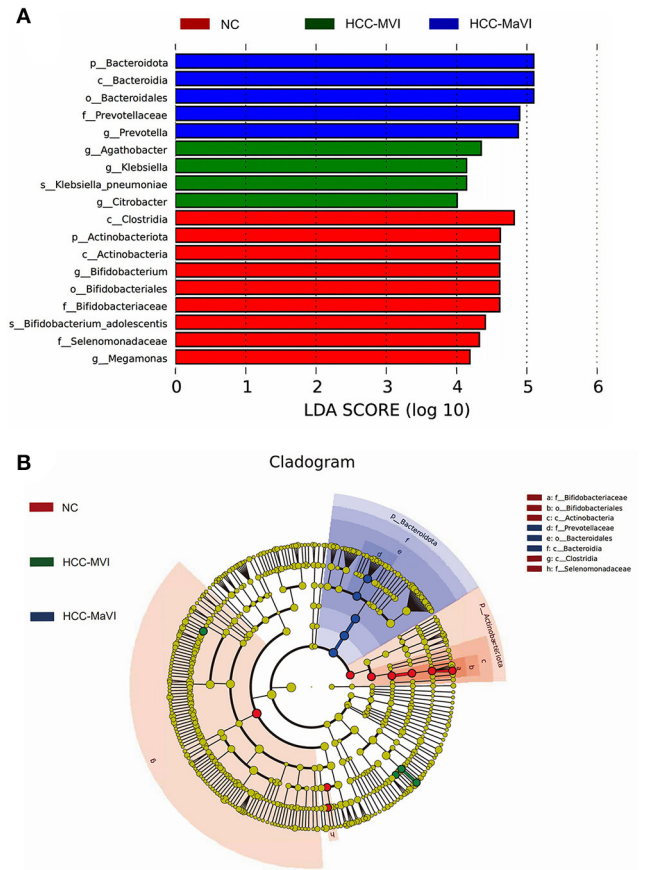

FIGURE 5 | LEfSe analysis of gut microbiota. **(A)** LefSe analysis showed significant bacterial differences among NC, HCC-MVI, and HCC-MaVI groups; **(B)** A cladogram of different taxonomic compositions among NC, HCC-MVI, and HCC-MaVI groups.

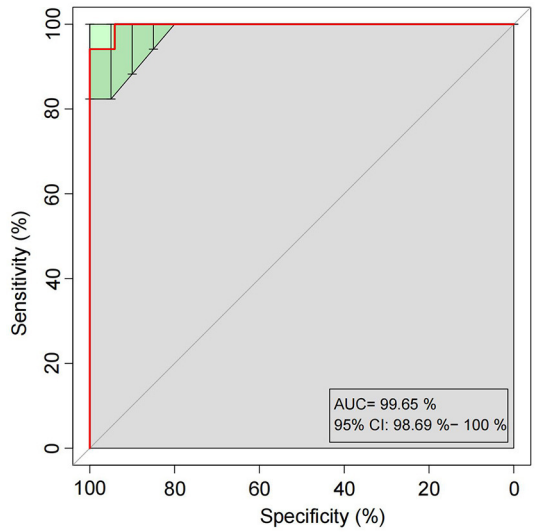

FIGURE 6 | Receiver operating characteristic (ROC) curves built with twenty bacterial families for HCC-MVI prediction.

DISCUSSION

In this study, we comprehensively explored the effects of the fecal microbiome on vascular invasion in patients with HCC, correlating them with clinical data and assessing whether a microbiome study can gain insight into a potential HCC-MVI prediction model. We showed that HCC-MVI significantly affects the relative abundance of several gut bacteria and that a microbial prediction model (based on 20 significant signature microbes) has the potential for predicting and discriminating high-risk patients with HCC-MVI. We also demonstrated that the characteristics of the fecal microbiome in patients with HCC may be associated with TME.

Several studies have shown that gut microbiota plays a crucial role in tumor prognosis and response to immunotherapy/chemotherapy (28). However, the effects of gut microbiota on MVI are largely unknown. As an important prognosis factor of HCC, current MVI assessment mainly depends on pathology following surgery. Therefore, preoperative prediction of MVI, especially in a non-invasive manner, is of great significance. Several studies have evaluated the performance of MVI predictive models constructed with clinical and molecular indicators, and the AUC of these models ranged from 0.73 to 0.81, suggesting that higher specificity and sensitivity are crucial for future studies (29–31). Intestinal microbiome analysis becomes an important direction for predictive model construction considering its convenience and non-invasive sample collecting requirements. Previously, Zheng et al. (32) screened out six important microbial markers at the genus level among hepatitis B virus (HBV)-related patients with HCC and healthy controls. They constructed predict models to explore the predictive value of intestinal microbiome on posthepatectomy recurrence and survival. The AUC of 2-year RFS survival predicted value was 68%, and the 5-year OS predicted AUC was 81%. Currently, there is no research that can predict HCC-MVI based on intestinal microbiome analysis.

In the present study, we performed comprehensive fecal microbiome analysis among the healthy controls (NC) and patients with HCC with different vascular invasion statuses (the HCC-NVI, HCC-MVI, and HCC-MaVI groups). The major microbiome component of healthy controls is found to be *Firmicutes* (with the percentage of 50–80%), followed by *Bacteroides, Proteobacteria, Actinomycetes, and Verrucomicrobia.* Our research indicated that the abundance of *Bacteroides* significantly enriched in patients with HCC while *Firmicutes* were reduced, which is consistent with previous studies (32–34). Compared with the HCC-NVI group and NC group, the abundances of *Prevotella* and *Klebsiella* were significantly increased in the HCC-MVI group. *Prevotella* was enriched in hepatitis C virus-related HCC and regarded as an independent prognostic biomarker in ESCC patients (30, 35). *Prevotella's* lipopolysaccharides (LPS) have a proinflammatory effect and its abundance *is* negatively correlated with interleukin (IL)-17A

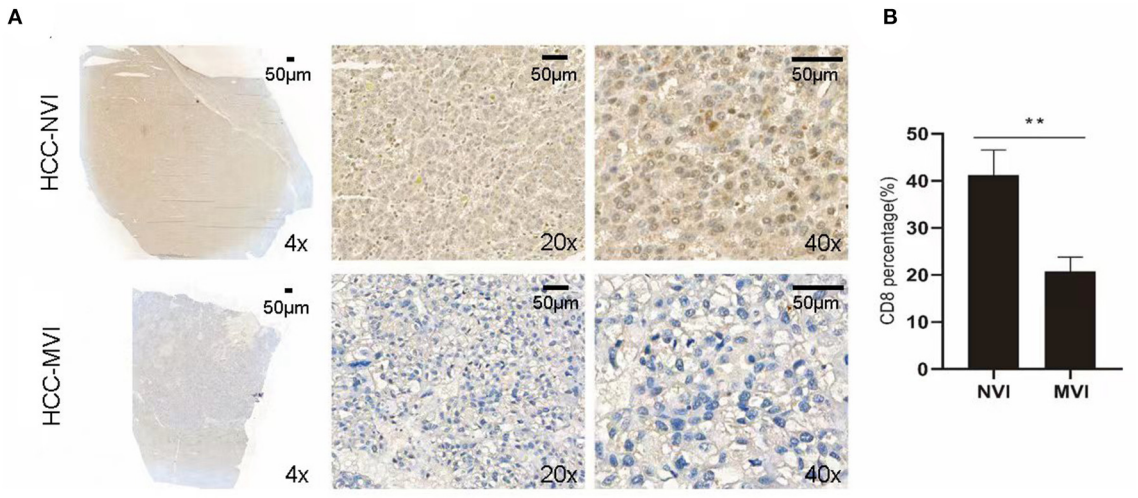

FIGURE 7 | Immunohistochemical profiles of HCC. **(A)** Representative immunohistochemistry images reveal increased expression of $CD8^+$ T cell in the HCC-MVI and HCC-NVI group (4×, 20× and 40× magnification, respectively). Scale bar: 50 μm. **(B)** Percentage of $CD8^+$ T cell in HCC-MVI and HCC-NVI groups, $^{**}P < 0.01$ by t-test.

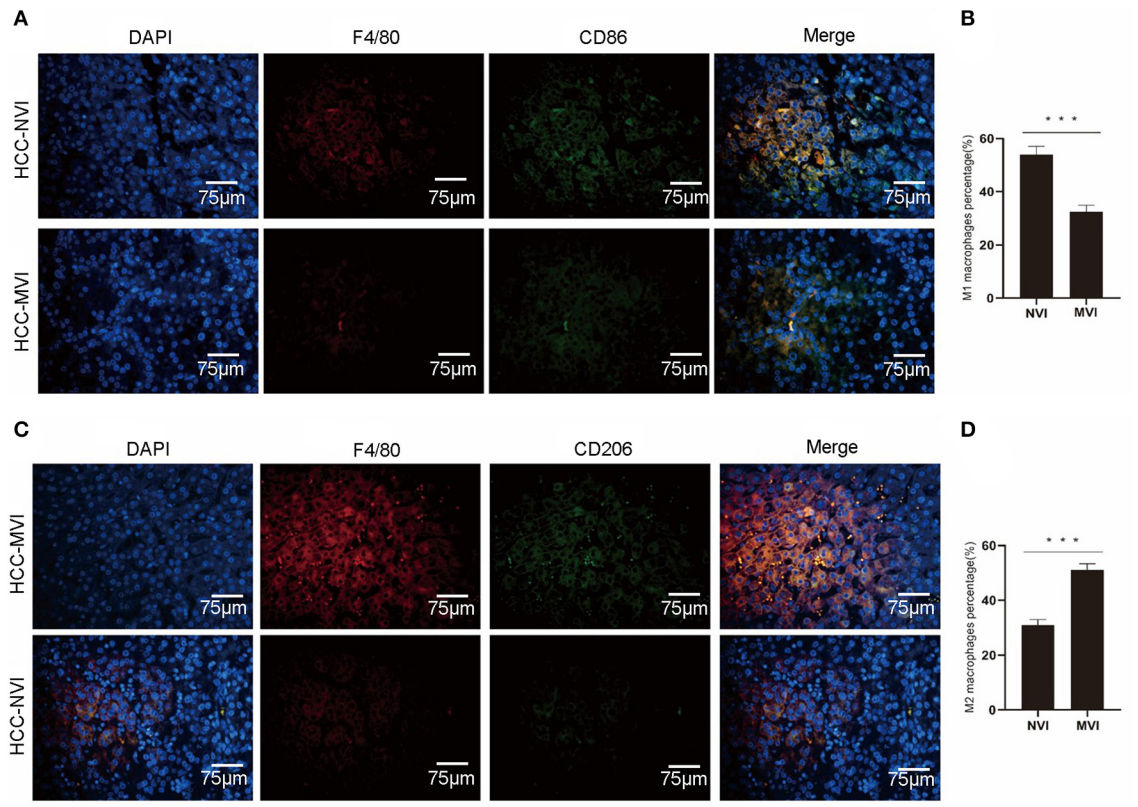

FIGURE 8 | Immunofluorescence profiles of HCC. Representative image **(A)** and calculation **(B)** of M1 TAM positive rate in the HCC-MVI and HCC-NVI groups (40× magnification). Representative image **(C)** and calculation **(D)** of the M2-type TAM positive rate in the HCC-MVI and HCC-NVI groups (40× magnification). Scale bar: 75 μm, $^{***}P < 0.001$ by t-test.

in colorectal cancer samples while the correlation with IL-9 is positive (36). *Klebsiella* has been shown to have increased abundance in patients with a variety of cancers including pancreatic cancer, colon cancer, and esophageal cancer (37–39). It was also related to the lymphatic metastasis and prognosis of pancreatic cancer (38). *Klebsiella pneumoniae* may

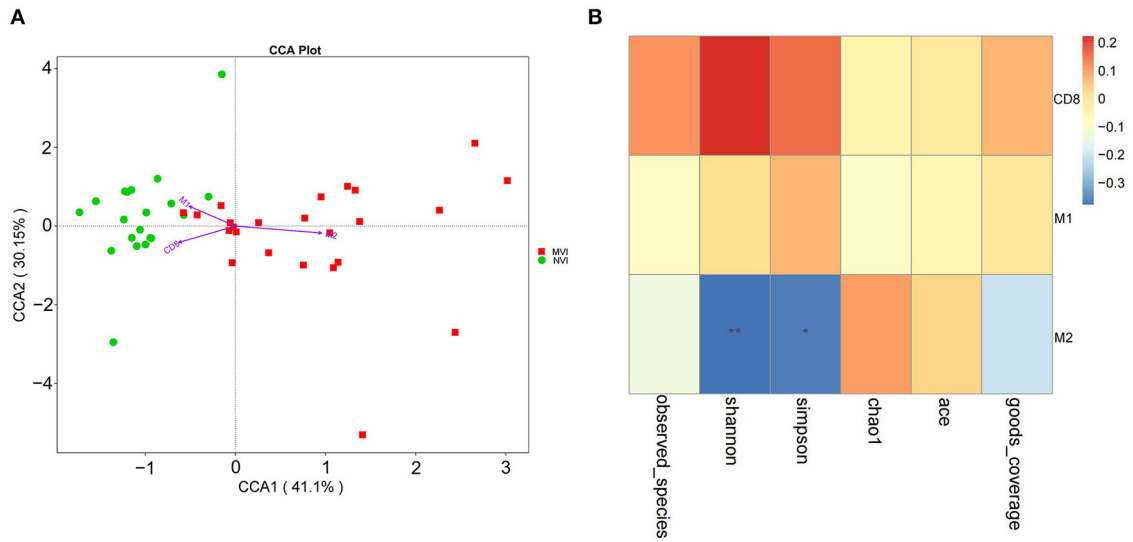

FIGURE 9 | Correlation analysis of TME indicators and intestinal flora. **(A)** CCA analysis of intestinal flora and CD8$^+$ T cells, M1 and M2-type TAM cells in HCC-MVI and HCC-NVI groups; **(B)** Spearman correlation analysis of CD8$^+$ T cells, M1 and M2 type TAM cells in HCC-MVI group.

promote chemoresistance to adjuvant gemcitabine treatment while following intervention with antibiotic quinolone can improve survival (40). Interestingly, our results indicated that *Monoglobales*, a member of microbiota which has not been reported in liver disease or cancer study, shows gradually decreased abundancy in the four subgroups according to differential vascular invasion status (highest in NC, then in HCC-NVI, HCC-MVI, and lowest in HCC-MaVI). Therefore, it may serve as a potential biomarker for negatively predicting HCC vascular invasion. However, with a limited study reported and finite evidence, the role of gut bacteria *Monoglobales* in HCC-MVI needs to be further explored.

As an emerging investigative field, microbiome study has some advantages over clinical or molecular indicators, especially when patients prefer non-invasive and convenient sample collection procedures. Wang et al. conducted a nomogram that identified four risk factors (tumor size, number of tumors, neutrophils, and serum α-fetoprotein) for MVI prediction (29). Circulating tumor DNA (ctDNA) was also identified in operable patients with HCC, suggesting that ctDNA may function as an independent risk factor for MVI and facilitate precise treatment strategies (41). Compared with aforesaid MVI prediction methods, the microbial model we constructed for HCC-MVI prediction obtained better performance with higher AUC (94.81%). The advent of high throughput next-generation sequencing (NGS) technologies has revolutionized microbiome study, especially when combined with advances in bioinformatics analysis and machine learning. Thus, the gut microbiome should be considered as a viable diagnostic and therapeutic target, which can be applied for assessing the clinical impact of these findings in future studies.

Currently, the mechanisms underlying the effects of microbiota on tumor prognosis remain unclear. Several clinical and subcutaneous preclinical studies revealed that gut microbiota was associated with systemic inflammation, including inflammatory responses in tumors (42–46). Our study revealed that the M2-type TAM positive rate was significantly higher in the HCC-MVI group than that of the HCC-NVI group. Moreover, once the diversity of the HCC-MVI microbiome decreases and the intestinal microbiome becomes disordered, the M2-type TAM positive rate in TME shows a further increase. These results are in accordance with previous studies which have shown that high expression of M2-type TAM in tumor tissue can promote tumor invasion and metastasis, resulting in poor prognosis (24, 47). The correlation between functional pathways and TME M2-type TAM was further explored, and the results suggest that the increase of M2-type TAM positive rate may be involved in the mammalian target of rapamycin (mTOR) pathway. The mTOR is frequently deregulated in tumorigenesis, activating somatic mutations of mTOR, which were recently identified in several types of cancer, and hence mTOR is therapeutically targeted (48).

There are certain limitations in the present study. First, some of the results obtained were achieved based on limited sample size and/or lack of further validation, therefore future validation in larger cohorts may reduce the false positive value and unreliability during HCC-MVI screening. Although the M2-type TAM and mTOR metabolic pathways are initially predicted to be involved based on the microbiome data, the related mechanism of their interaction needs to be further explored. However, the fact that these correlations were evident even in this limited sample size testifies to the potentialities of the proposed model. Interpreting changes was not the aim of our current study, thus we did not assess other clinical parameters such as intestinal permeability, inflammatory status, dietary habits, comorbidities, comedications, and portal hypertension,

TABLE 2 | The correlation between the functional pathway of microbiome and the positive rate of M2 type TAM in HCC-MVI group based on the KEGG database.

Signaling pathway	r	*P-value*
Adipocytokine signaling pathway	0.17	0.27
AMPK signaling pathway	0.02	0.89
Calcium signaling pathway	0.065	0.66
cAMP signaling pathway	0.0061	0.97
HIF-1 signaling pathway	−0.15	0.32
IL-17 signaling pathway	0.088	0.55
mTOR signaling pathway	**0.37**	**0.01**
NF-kB signaling pathway	−0.071	0.64
NOD-like receptor signaling pathway	0.025	0.87
p53 signaling pathway	0.079	0.6
Phosphatidylinositol signaling system	−0.0093	0.95
PI3K/Akt signaling pathway	0.055	0.71
PPAR signaling pathway	0.11	0.47
Ras signaling pathway	0.073	0.63
Sphingolipid signaling pathway	0.075	0.62
TNF signaling pathway	−0.071	0.64
VEGF signaling pathway	−0.071	0.64

resulting in insufficient functional understanding of observed microbial changes. Moreover, the present study focused on the differences of gut microbiota resulting from MVI among HBV-related patients with HCC. Involving benign controls with HBV infection in subsequent larger cohort studies may help us refine the prediction model with less variables and higher AUC. Finally, the concomitant fecal metabolomic analysis would be a valuable addition to microbiome data for a more comprehensive understanding, especially on how the observed microbiota changes during MVI development.

However, strengths also are worthy of mention. As far as we know based on a literature search, this is the first clinical study exploring the influence of the gut microbiome on HCC-MVI development, and, as such, its results should be regarded with attention. In particular, we generated the first prediction model for HCC-MVI based on microbiome correlative analysis, which obtains high prediction performance. The microbial model already showed discriminative properties under the current experimental condition. It is likely that expanding the research among a larger cohort as well as other clinical parameters as mentioned above will further disclose the diagnostic potential of this method. Based on the microbiome data, the metabolic pathways that may be involved are initially predicted; however, the mechanism of their interaction needs to be further explored.

In conclusion, the present study highlights that gut microbiome alterations may have an influence on HCC-MVI prediction and relate to M2-type TAM of TME. Moreover, it shows that the microbial prediction model is able to efficiently predict HCC–MVI development. These preliminary results need to be confirmed and expanded in larger HCC cohorts and other cancer populations. The animal study is also needed to understand whether the differential microbiome affects the development of HCC-MVI by exploring possible mechanisms or the other way around. If confirmed, they might open the way to a quick and low-cost non-invasive diagnosis with many potential perspectives not only in hepatic vascular invasion diseases. Therefore, this clinical study provides a strong potential of translational implications from bench to bedside helping the clinicians in terms of disease prediction and diagnosis.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the China National Microbiology Data Center, accession number NMDCX0000120 (https://nmdc.cn/resource/attachment/detail/NMDCX0000120).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Tianjin Medical University Cancer Institute and Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

WLu, EE-O, XZ, and NZ conceived the study. NZ, ZW, JL, and SZ recruited participants. ZW, JL, SW, and YL conducted the analysis. TL and WLi analyzed fecal samples. ZW, LG, and YL wrote the article. All authors revised and approved the written manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.836369/full#supplementary-material

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REVIEW
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Manipulation of Gut Microbiota as a Key Target for Crohn's Disease

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Crohn's disease (CD) is an inflammatory bowel disease (IBD) sub-type characterized by transmural chronic inflammation of the gastrointestinal tract. Research indicates a complex CD etiology involving genetic predisposition and immune dysregulation in response to environmental triggers. The chronic mucosal inflammation has been associated with a dysregulated state, or dysbiosis, of the gut microbiome (bacteria), mycobiome (fungi), virome (bacteriophages and viruses), and archeaome (archaea) further affecting the interkingdom syntrophic relationships and host metabolism. Microbiota dysbiosis in CD is largely described by an increase in facultative anaerobic pathobionts at the expense of strict anaerobic Firmicutes, such as *Faecalibacterium prausnitzii*. In the mycobiome, reduced fungal diversity and fungal-bacteria interactions, along with a significantly increased abundance of *Candida* spp. and a decrease in *Saccharomyces cerevisiae* are well documented. Virome analysis also indicates a significant decrease in phage diversity, but an overall increase in phages infecting bacterial groups associated with intestinal inflammation. Finally, an increase in methanogenic archaea such as *Methanosphaera stadtmanae* exhibits high immunogenic potential and is associated with CD etiology. Common anti-inflammatory medications used in CD management (amino-salicylates, immunomodulators, and biologics) could also directly or indirectly affect the gut microbiome in CD. Other medications often used concomitantly in IBD, such as antibiotics, antidepressants, oral contraceptives, opioids, and proton pump inhibitors, have shown to alter the gut microbiota and account for increased susceptibility to disease onset or worsening of disease progression. In contrast, some environmental modifications through alternative therapies including fecal microbiota transplant (FMT), diet and dietary supplements with prebiotics, probiotics, and synbiotics have shown potential protective effects by reversing microbiota dysbiosis or by directly promoting beneficial microbes, together with minimal long-term adverse effects. In this review, we discuss the different approaches to modulating the global consortium of bacteria, fungi, viruses, and archaea in patients with CD through therapies that include antibiotics, probiotics, prebiotics, synbiotics, personalized diets, and FMT. We hope to provide evidence to encourage clinicians and researchers to incorporate these therapies into CD treatment options, along with making them aware of the limitations of these therapies, and indicate where more research is needed.

Keywords: Crohn's disease, IBD, intestinal microbiota, probiotics, prebiotics, diet, gut health

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INTRODUCTION

Crohn's disease (CD) is a sub-type of an inflammatory bowel disease (IBD) characterized by transmural chronic inflammation of the gastrointestinal tract (1). Research indicates a complex CD etiology involving genetic predisposition and immune dysregulation in response to environmental triggers (1). This disease can affect any part of the intestine, but it is most commonly found in the terminal ileum and colon (2). CD disrupts the body's normal ability to digest and absorb food, including eliminating waste (3). The nature of the inflammation is usually segmental, asymmetrical, and transmural. Most patients present with an inflammatory phenotype at diagnosis, but develop complications over time including strictures, fistulas, or abscesses, which often lead to surgery. Surgery rates for CD have declined due to novel advances in medical therapy, but the 5-year risk of first major abdominal surgery in CD remains at 18% (4). Although biological therapy has significantly improved patient outcomes, this progressively destructive disease can lead to bowel damage and disability, including intestinal failure and short-bowel syndrome (5). Diarrhea, abdominal pain, weight loss, fever, nausea, and vomiting are only some of the symptoms that occur in a relapsing and remitting fashion for patients (1). Up to 47% of patients also experience extra-intestinal manifestations (EIMs) related to joints, skin, liver, biliary tract, and eyes (6, 7). Some of these EIMs are associated with disease course and severity, ultimately contributing to morbidity and mortality (8–10). The financial burden on health systems is related to the direct health care costs of IBD hospitalization, surgery, and medication, especially the rising use of biologics (5).

THE GUT MICROBIOTA

Trillions of microbes comprising bacteria, fungi, viruses, eukaryotes, and archaea colonize the human gut microbiota. The microbiome serves many functions including playing a role in metabolism, immune and nervous system regulation, and colonization resistance (11, 12). Markedly, the gut microbiota has been implicated in the initiation and perpetuation of IBD. Infusion of luminal content into both mice models (13). and into the excluded ileum after a surgical diversion in patients with CD (14). triggers a rapid response in the mucosal immune system. This data suggests that commensal bacteria and dietary components can trigger the inflammatory response seen in CD (13, 14). The chronic intestinal inflammation and subsequent damage of the intestinal mucosa in IBD is associated with dysbiosis in the structure of the microbiota. Studies have confirmed that reduced diversity and dysbiosis of the gut microbiota are more pronounced in CD compared to ulcerative colitis (UC), a different sub-type of IBD (11, 15).

Intestinal Microbiome in CD

The gut microbiome comprising the intestinal bacteria is the most widely studied community. The healthy human commensal intestinal microbiota is composed of bacteria from three major phyla, namely Firmicutes, Bacteroidetes, and Actinobacteria (12). The bacterial signature of CD is reported to have a lower β-diversity (bacterial richness) with a decrease in obligate anaerobes, and an increase in facultative anaerobes (16) (**Figure 1**). This dysbiotic change facilitates the expansion of pathobionts that thrive in the presence of oxygen (16). A decrease in the relative abundance of Bacteroides and Firmicutes, especially Clostridiales butyrate-producing bacteria such as *Faecalibacterium prausnitzii* (17, 18). and *Roseburia* spp is seen in CD (19). The short-chain fatty acid (SCFA) butyrate, produced by these bacteria, acts as an energy substrate for colonocytes which further improves gut permeability by accelerating tight junction formation (12, 17). Butyrate also has anti-inflammatory effects such as inhibition of interleukin (IL)-6 release, lipopolysaccharide (LPS)-induced tumor necrosis factor-α (TNF-α) release, and suppression of the NF-κB inflammatory pathway via TNF-α activation (20). *F. prausnitzii* is a commensal bacterium with anti-inflammatory properties which reduces pro-inflammatory and increases anti-inflammatory cytokines (17). The relative lack of *F. prausnitzii* is associated with postoperative disease recurrence after ileocecal resection and re-anastomosis (18). CD is also characterized by an increased abundance of *Ruminococcus gnavus* and Gammaproteobacteria, such as *Escherichia coli,* which are mucosa-associated adherent-invasive bacteria. These pathogenic strains cross the mucosal barrier, adhere to and invade epithelial gut cells, and reproduce within macrophages to increase secretion of the pro-inflammatory cytokine TFN-α (11, 17, 21).

Intestinal Mycobiome in CD

Although the mycobiome, or the fungal community, is composed of a small fraction of the microbiota ($\sim 10^5$ fungal cells per gram of fecal matters vs. 10^{11} bacterial cells per gram) (22), fungi play a myriad of roles related to host metabolism and host immunity in a broad range of ecosystems. This community is far more variable and dynamic than the bacterial community, responsive to environmental changes, and co-exists with the other microbial communities in the human body. Data suggests that there are three main fungal phyla in the gut, *Ascomycota, Basidiomycota,* and *Zygomycota,* and 10 "core" genera made up of *Candida* (especially *Candida albicans*), *Saccharomyces* (especially *Saccharomyces cerevisiae*), *Penicillium, Aspergillus, Cryptococcus, Malassezia* (especially *Malassezia restricta*), *Cladosporium, Galactomyces, Debaryomyces,* and *Trichosporon.* Importantly, as with bacteria, fungal communities have a spatial organization in the GI tract with luminal subset vs. mucosa-associated subset, the latter being more conservative and well-defined (23). Recently, it was shown that human mucosa-associated fungi, consisting mainly of the "immunoreactive" fungal genera *Candida* spp. and *Saccharomyces* spp. exert immunoprotective effects *via* upregulation of barrier function and transcription of epithelial genes involved in JAK/STAT signaling and DNA repair (23). Namely, in a healthy state the mucosa-associated mycobiota promoted barrier function through induction of $CD4^+$ T helper cell-derived IL-22 and IL-17, resulting in protection against intestinal injury during antibiotic treatment and bacterial infection (23). Meanwhile, studies in patients with CD show an increased fungal burden in conjunction with an increase in abundance of

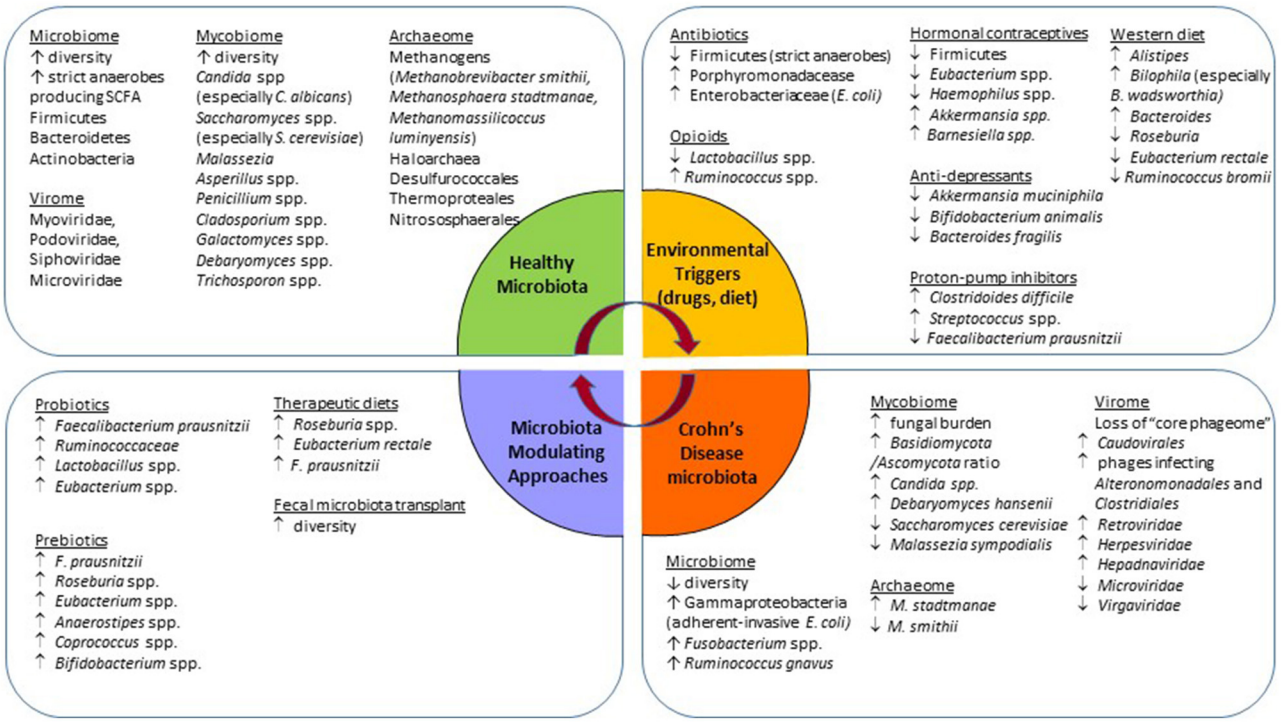

FIGURE 1 | Schematic summary of observed gut microbiota changes associated with Crohn's disease, the usage of common medications, and approaches for microbiota modulation.

the *Basidiomycota/Ascomycota* ratio, *C. albicans, C. tropicalis, Candida glabrata, Gibbrella moniliformis, Aspergillus clavatus, Alternaria brassicola, Cystofilobasidiaceace* family (24), and *Debaryomyces hansenii* (25) (**Figure 1**). *C. albicans*, a prevalent fungal species in the gut of patients with CD, is known as an inducer of T helper 17 (Th17) cells (26). Th17 cells are involved in immunity at the intestinal mucosal barrier. Under pathological conditions, such as IBD, Th17 secretes pro-inflammatory cytokines that aggravate inflammation (27), suggesting a possible pathobiont role of *C. albicans* in the case of IBD. Another study also implied the potential role of *Debaryomyces hansenii* in the perpetuation of chronic inflammation and tissue injury in CD (25). This fungus was found in abundance in patients with CD within the areas of surgical resections and inflamed regions of the intestines. Human isolates of *D. hansenii* effectively impaired colonic crypt repair *in vivo* illustrated by increased mucosal ulcerations and crypt loss (25).

Furthermore, a decrease in *S. cerevisiae* and *Malassezia sympodialis* is observed in CD (24). Sokol et al. demonstrated that *S. cerevisiae* significantly enhances the production of the anti-inflammatory cytokine interleukin (IL)-10 (24). While less is known about the role of *M. sympodialis* in intestinal inflammation, the genus *Malassezia* is often associated with skin disorders such as atopic eczema (28). Recent data suggest that *M. sympodialis* can stimulate mast cells to upregulate the release of cysteinyl leukotrienes and enhance IgE response, which results in a pro-inflammatory effect (29). More research is required to understand the exact role of *M. sympodialis* in CD.

Fungal-bacterial interactions were first recognized by Seelig in 1966 after antibiotic treatment in humans resulted in an overgrowth of *Candida* spp (30, 31). More recently, Sovran et al. demonstrated that antibiotic treatment significantly altered the fungal composition, confirming that bacterial dysbiosis could contribute to fungal dysbiosis (32). This study also showed that administration of *C. albicans* worsened disease severity, while administration of *Saccharomyces boulardii* reduced disease symptoms; however, both effects were lost after antibiotic treatment (32). It is hypothesized that the co-existence of fungi with specific intestinal bacteria is essential for the development/amelioration of colitis. Sovran et al. exemplified this concept in a female C57BL/6J mice model where both *C. albicans* and *S. boulardii* required *Enterobacteriaceace* (modeled with colistin-resistant *E. coli* strains) to trigger their respective detrimental and beneficial effects on DSS-induced colitis (32). To support the role of interdependence between bacteria and fungi, gut fungal dysbiosis has been associated with reduced treatment response to fecal microbiota transplants (FMT) in recurrent *Clostridioides difficile* infections (CDi) (33). The microbiome of patients with CD is characterized by a decrease in fungal-bacterial interactions, as compared to healthy controls. A decreased abundance in *S. cerevisiae* is associated with a reduction in beneficial bacterial genera such as *Bifidobacterium, Blautia, Roseburia,* and *Ruminococcus*, whereas an elevation in *C. tropicalis* is positively associated with opportunistic bacteria such as *Serratia marcescens* and *E. coli* in CD (24).

Intestinal Virome in CD

There is even less information about the role of virome in IBD as compared to the mycobiome, since little information is accessible through the public database, known as "viral dark matter" (34, 35). Gut virome is composed of eukaryotic and bacterial viruses (bacteriophages), including single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), and RNA viruses. The intestinal virome is the most populated niche in the body, consisting of $\sim 10^{15}$ bacteriophages, outnumbering commensal bacteria by a factor of 10 (36, 37). Although large and diverse, the intestinal virome is highly personalized and stable (38). The core "phageome" is mostly composed of dsDNA viruses most commonly from the *Caudovirales* order (*Myoviridae*, *Podoviridae*, and *Siphoviridae* families), and ssDNA viruses from the *Microviridae* family. More recently, a novel crAssphage and crAss-like phages have been identified as a common community in the virome and are associated with the bacterial phylum of *Bacteroidetes* (36, 38, 39).

Specific changes in the virome in patients with CD consist of a loss of the "core phageome" (**Figure 1**). Norman et al. describe a significant expansion of disease-specific *Caudovirales* bacteriophages and disease-and-cohort-specific changes in the virome of patients with CD and UC (40). CD-associated phageome dynamic changes are characterized by an increase in phages infecting *Alteronomonadales* and *Clostridiales* bacterial orders (41), and an inverse shift in *Caudovirales* vs. *Microviridae* bacteriophages (35). As for the eukaryotic virome, an increase in the abundance of *Retroviridae*, *Herpesviridae*, *Hepadnaviridae*, *Hepeviridae* families, and a decrease in the *Virgaviridae* family were observed (36, 37).

Although the exact role of the virome in IBD is not entirely clear, some evidence suggests that it may contribute to intestinal inflammation. Bacteriophages are important to bacteria as they drive bacterial diversity and fitness in the gut (42). They are involved in the horizontal transfer of genetic information between bacteria, including material related to pathogenesis and antibiotic resistance (42–44). In CD, there is an increase in the abundance of temperate phages which shift from lysogenic to lytic replication (43). The normal process of viral reproduction occurs *via* the lysogenic cycle, which involves the fusion of the nucleic acid of a bacteriophage and the host cell leading to proliferation (45). In contrast, the lytic cycle involves the penetration of a cell membrane, nucleic acid synthesis, and lysis of the host cell (37, 43). Lysis of gut bacterial hosts is theorized to release proteins, lipids, pathogen-associated molecular patterns, and antigens that trigger inflammatory pathways leading to pro-inflammatory cytokine induction and tissue damage (40). Additionally, *in vitro* studies have demonstrated that bacteriophages can stimulate macrophages to induce MyD88-dependent pro-inflammatory cytokine production, suggesting its role in innate immunity (46). Bacteriophages may also play a therapeutic role in CD treatment. A cocktail of three bacteriophages was demonstrated to reduce symptoms and significantly reduce fecal adhesive-invasive *E. coli* (AIEC) in DSS-induced colitis in mice. AEIC colonization of ileal mucosa in CD was correlated with disease activity and location, as well as postoperative recurrence (47). This bacteriophage cocktail may thus have therapeutic promise for patients with CD.

Intestinal Archeaome in CD

Archaea are a domain of prokaryotic, single-cell organisms, collectively known as the archeaome (48). The knowledge of the human gut archeaome is limited and is mostly based on methodological concepts biased toward commensal bacteria. The majority of detected archaea in the gut are methane-producing organisms known as methanogens. Methanogens respire H_2 and produce methane gas under anaerobic conditions. They exist in a syntrophic relationship with bacteria; by removing H_2, methanogens improve bacterial fermentation efficiency in the gut and allow complete anaerobic degradation of organic material. Methanogens make up ∼10% of gut anaerobes, as *Methanobrevibacter smithii* is the most common (49–51). Other common species detected in the gut are *Methanosphaera stadtmanae*, *Methanomassiliicoccus luminyensis* (49, 52), as well occasionally several non-methanogenic strains, such as D*esulfurococcales, Sulfolobales, Thermoproteales, Nitrososphaerales,* and *Halobacteriales* (53–55). *M. smithii* has low immunogenic potential, suggesting its commensal role as a gut microbe. *M. luminyensis* plays a beneficial role through the degradation of trimethylamine (TMA) and trimethylamine-N-oxide (TMAO), both byproducts of choline microbial metabolism strongly associated with endothelial dysfunction and increased risk of cardiovascular disease (56). Lastly, *M. stadtmanae* has shown high immunogenic potential (57) and is suggested to be present in high abundance in pathological conditions (58, 59).

Changes in the archeaome related to CD show at least a 3-fold increase in *M. stadtmanae* and a reduction in *M. smithii,* as compared to healthy controls (**Figure 1**). Interestingly, these numbers normalize with IBD remission (49). The "syntrophic imbalance hypothesis" suggests that butyric acid, an SCFA in the gut, is an essential component for the regulation of archaea/bacteria biofilms in the gut. This hypothesis states that dysbiosis is a product of archaeal overgrowth and increased SCFA removal from intestinal biofilms, which in turn triggers bacteria to become endoparasitic and enter intestinal epithelial tissues, initiating and perpetuating chronic inflammation in the gut (60). More research is needed to support the proposed hypothesis, but recall that SCFAs, such as butyric acid, are reduced in patients with IBD, therefore promoting that butyrate-producing bacterial growth to improve intestinal barrier integrity should be a promising target of therapy (61, 62).

CONVENTIONAL THERAPIES IN CD AND THEIR MODULATING EFFECTS ON GUT MICROBIOTA

The goal of CD therapy is to achieve deep remission, that is, to induce and maintain symptomatic and endoscopic remission and mucosal healing. Common anti-inflammatory medications used in CD management include amino-salicylates, immunomodulators, corticosteroids, and biologics (1, 63, 64).

Amino-salicylates are less used in CD due to their lack of efficacy in inducing/maintaining remission and preventing postoperative recurrence. Corticosteroids are used mainly as induction of remission medication but not for maintaining remission, also due to serious side-effects such as osteoporosis, diabetes, hypertension, and increased risk for infections. Immunomodulators are commonly used but also have side effects such as an increased risk for malignancies (such as lymphoma, non-melanoma skin cancers, myeloid disorders, and urinary tract cancers). Anti-tumor necrosis factor (TNF) biologics such as infliximab, adalimumab, and certolizumab, are generally well-tolerated, however; they are expensive and may increase the risk of infections as well as melanoma skin cancer. Vedolizumab (anti-integrin α4β7) and ustekinumab (anti-IL 12/23) are newer biological drugs with improved safety and efficacy profiles but lack long-term data (1, 2).

The efficacy of conventional therapy can be affected by certain factors related to the gut microbiota. For example, patients with IBD who achieve 'early' clinical remission at 14 weeks with anti-cytokine therapy (anti-TNF, anti-IL 12/23) have significantly higher microbial species richness at baseline compared to non-responders (65). The same study also found that nine microbial species at baseline were associated with early clinical remission in patients treated with anti-TNF and that three microbial species were related to response to anti-integrin therapy. Of those species, *Phascolarctobacterium faecium, Agathobaculum butyriciproducens,* and *Clostridium citroneae* were associated with increases in fecal SCFA production to produce anti-inflammatory effects. The decrease in inflammation by anti-TNF therapy is associated with modulation of the gut microbiome toward eubiosis. The microbiome of patients successfully treated with anti-TNF therapy slowly resembles that of healthy individuals. Studies demonstrated a decrease in Enterobacteriaceae (*E. coli* in particular) and *Ruminococcus*, with an increase in abundance of Bacteroidetes and Firmicutes (66).

CONCOMITANT MEDICATIONS USED IN CD AND THEIR MODULATORY EFFECTS ON GUT MICROBIOTA

Recent studies have revealed that many commonly used drugs other than antibiotics also exhibit profound effects on the gut microbiota composition and function (**Figure 1**). An extensive review of the microbiota-modulation effect of non-antibiotic medications and the potential clinical consequences can be found elsewhere (67). Here, we summarize data with respect to drugs commonly used by patients with CD and their potential contribution to the observed intestinal dysbiosis, as well as disease onset and progression.

Antibiotics

Antibiotic exposure has been identified as an environmental stressor contributing to the pathogenesis of IBD. In healthy humans, antibiotic use has demonstrated perturbations and a decrease in colonization resistance of the gut microbiota (68). Broad-spectrum antibiotics can affect the composition of at least 30% of gut microbes to cause a drastic shift in richness, diversity, and evenness (69, 70). Repeated exposure to antibiotics leads to a reduction in diversity, resulting in pathogenic overgrowth and dysbiosis (69, 70), increasing the risk of infection (71). Another mechanism in which antibiotics increase the risk of intestinal infections is related to their role in the thinning of the mucosal layer leading to barrier dysfunction (72). Various studies have found a significant association between prior antibiotic use and the development of CD (73, 74). Both exposures in the first 5 years of life (75) and 2–5 years prior to diagnosis (76). contribute to an increased risk of developing IBD. The association between antibiotic use and CD has been supported through metagenomic analyses, showing a decrease in the abundance of Firmicutes (such as *C. leptum*) and an expansion of gram-negative bacteria such as Porphyromonadaceae, and Enterobacteriaceae (*E. coli*) (77–79).

Hormonal Contraception

The use of oral contraceptive pills (OCP) is associated with a 30% increased risk for the development of IBD in a genetically susceptible host. In particular, there is a 24% higher risk of CD development in those who are exposed to OCP vs. those who are not (80). As OCPs function by using estrogen receptors, van Langen et al. observed that reduced estrogen receptor-β's (ER-β) mRNA expression, the most abundant estrogen receptor, and increased gut permeability preceded the onset of colitis in two animal models. Furthermore, the study found reduced ER-β mRNA levels in colonic biopsies from patients with IBD in relapse. Finally, *in vitro* experiments demonstrated an association between ER-β signaling and epithelial barrier function. They concluded that ER-β signaling has a role in maintaining epithelial barrier function, which in turn is related to IBD risk (81).

Mihajlovic et al. demonstrated that the use of combined hormonal contraceptives (CHC), consisting of natural or synthetic 17-β-estradiol and progesterone, was associated with significantly lower gut microbial diversity and richness due to CHC-induced decrease in endogenous estradiol and progesterone. These authors also identified bacterial groups such as unclassified Firmicutes, *Eubacterium* spp, and *Haemophilus* spp. that were less abundant in the CHC group, while *Akkermansia* and *Barnesiella* were enriched in the CHC group in comparison to healthy controls not using CHC. These data suggest that CHC-induced hormonal changes may affect gut microbiota diversity (82). Lastly, not only are OCPs associated with increased risk for CD but long-term use of OCPs in patients with established CD is also associated with an increased likelihood of surgery and risk of relapse (83). These recent data raise the need for a re-evaluation of the benefits vs. risk of OCPs not only in individuals with established CD but also in those at increased risk for CD, such as their first-degree relatives.

Opioids

Opioids are the most common analgesics prescribed for pain management in IBD. In a study assessing the effect of hydromorphone in both DSS-induced colitis and spontaneous colitis (IL-10 knockout) mouse models of IBD, hydromorphone independently induced barrier dysfunction, bacterial translocation, disruption of tight junction organization, and increased intestinal and systemic inflammation. This effect

was exacerbated with significant microbial dysbiosis in mice receiving hydromorphone in combination with DSS. These data warn against the use of opioids and that clinicians should opt for other methods of pain management in IBD as opioids can accelerate disease progression by dysregulation of the gut microbiota, leading to expansion of pathogenic bacteria, followed by their translocation, leading to worsening immune dysregulation and sustained chronic intestinal inflammation (84). Despite these adverse effects, a recent systematic review and meta-analysis found that 21% of outpatients with IBD and 62% of hospitalized patients with IBD use opioids for pain management. Opioid use was associated with female sex, depression, substance abuse, prior gastrointestinal surgery, biologic use, steroid use, along with a more severe disease course in IBD and increased healthcare use (85). Male C57Bl/6J mice subjected to intermittent morphine treatment display a significant decrease in the relative abundance of *Lactobacillus* spp. and an increase in *Ruminococcus* spp (86). In contrast, mice exposed to sustained morphine treatment show a significant increase in abundance in *Clostridium* spp. and *Rikenellaceace* family (86). The depletion of the gut microbiota in these mice with antibiotic treatment reduced analgesic potency following intermittent morphine treatment (86). These data suggest that an alteration in the gut microbiome following antibiotic therapy or intermittent opioid treatment is detrimental to inflammation (86). Human data on microbiota modulation by opioids is currently not available, hence it is not known if observations made in colitis models can be fully translational in patients with CD.

Anti-depressants

It is well documented that up to 30% of patients with IBD are more likely to experience depression and that depression worsens IBD prognosis. A study assessing six types of antidepressants (phenelzine, venlafaxine, desipramine, bupropion, aripiprazole, and (S)-citalopram) for their microbial activity against 12 commensal bacterial strains revealed that certain antidepressive medications inhibit the growth of beneficial bacteria such as *Akkermansia muciniphila*, *Bifidobacterium animalis,* and *Bacteroides fragilis*. There was a significant reduction in bacterial viability, at a 5 logs cycle reduction (87). These findings demonstrated that certain antidepressants exhibit a strong antimicrobial effect against specific commensal gut bacteria (87), which is often overlooked. Although depression is associated with the onset of CD and UC, another study found that using certain treatments for depression, such as selective serotonin reuptake inhibitor (SSRI) and tricyclic antidepressant (TCA), was associated with disease reduction in CD (88). Therefore, while depression increases the risk of CD development, this risk may be altered by use of specific antidepressants (88). A bi-directional brain-gut axis interaction in patients with IBD refers to psychological disorders such as anxiety and depression and IBD activity. Patients with normal anxiety scores at baseline with active disease were almost 6 times more likely to develop abnormal anxiety scores during follow-up. In contrast, those who had the inactive disease at baseline, but had abnormal anxiety scores, had 2-fold higher rates of the flare of disease activity or need for glucocorticoids and escalation of therapy (89).

Proton Pump Inhibitors

Proton pump inhibitors (PPIs) reduce gastric acid secretion and are widely used in upper GI disorders management, such as peptic ulcer disease, gastroesophageal reflux disease, and non-ulcer dyspepsia (90). Although PPIs exhibit a sound safety profile and have demonstrated significant benefits in acid-related gastrointestinal disorders, there are controversial data regarding their safety in IBD. In an observational analysis of three cohorts made up of 6,40,000 subjects, long-term PPI use was associated with an increased risk of developing IBD (91). This association was also found in multiple other studies correlating PPI use to increased risk of enteric infections such as *Clostridoides difficile* and IBD. A recent metagenomic analysis of three population cohorts (general, IBD, and IBS) also identified that PPI accounted for the largest number of drug-associated microbiota shifts, with a total of 40 altered taxa and 166 altered microbial pathways (92). The mechanisms behind this association are unclear but may be attributed to the reduction in gastric pH, thus introducing oral bacteria and promoting the growth of potentially pathogenic bacteria (as *Streptococcus*) (92, 93) and direct inhibition of certain commensal gut bacteria such as *Faecalibacterium* (92, 93). This leads to a weakening of barrier function and reduction of microbial diversity ultimately leading to dysbiosis (93). This collective data suggest that although PPIs have had profound effects on certain gastrointestinal diseases, they should not be prescribed beyond the minimal dose to induce benefit in patients.

METHODS OF MODULATING THE GUT MICROBIOTA IN CROHN'S DISEASE

Modulation of the gut microbiota is often used as an adjuvant therapy to conventional medication in IBD. Projects such as the Human Microbiome Project (94) and the European Metagenomics of the Human Intestinal Tract (MetaHIT) (95) have had profound contributions to the identification and characterization of the microbes that distinguish health and disease states. Animal models have also been instrumental in the study of the microbiome and IBD. Hernández-Chirlaque et al. (96), confirm that the absence of a microbiome in germ-free (GF) and conventional mice treated with antibiotics cocktail ["pseudo-GF"] experience reduced DSS colonic inflammation but also have impaired barrier function. This data suggests that enteric bacteria are essential for the development of DSS-induced colitis and epithelial barrier function (96). Germ-free mice studies also link the commensal microbiota to the promotion of intestinal immunity through mechanisms such as toll-like receptor (TLR) expression (97). antigen-presenting cells, lymphoid follicles, $CD4^+$ T cells (98, 99), and antibody expression (100). Twin studies demonstrate that despite the heritability of the gut microbiome, environmental factors related to diet, drugs, and lifestyle can be larger determinants of microbiota composition and disease development (101, 102). This data suggests that enteric bacteria are essential for the development of chronic

intestinal inflammation. A logical development then is to attempt to modify the intestinal microbiome (not only its composition but also the microbial metabolic fitness) in order to sustain or even reverse pathophysiological changes observed in CD.

Probiotics in CD

The word probiotic originates from the Latin "pro" and Greek "bios" which translates to "for life" (103). The currently accepted definition is "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (104). This definition encompasses all microbes, although the bacteria of gut microbiota is the most widely studied. The most common probiotic cocktail with proven efficacy in UC and chronic pouchitis is Visbiome® (formerly VSL#3) (105, 106). Visbiome contains eight different bacterial strains from well-known probiotic species *Lactobacillus plantarum* DSM24730, *Streptococcus thermophilus* DSM24731, *Bifidobacterium breve* DSM24732, *L. paracasei* DSM24733, *L. delbrueckii subsp. bulgaricus* DSM24734, *L. acidophilus* DSM24735, *B. longum* DSM24736, and *B. infantis* DSM24737 (107). In particular, bacteria such as *Lactobacilli* and *Bifidobacteria* have been extensively tested for anti-inflammatory effects in colitis and their beneficial effects on gut motility, particularly for the treatment of constipation (108). VSL#3 is not yet shown to be effective in patients with CD, as compared to those with UC (109, 110).

However, Visbiome started within 30 days after ileocecal resection followed by re-anastomosis prevented disease recurrence in a study with 120 patients with CD, suggesting that the timing of this probiotic cocktail after resection is important. However, more studies are needed within this scope (111). *In vitro* supplementation with commensal strains such as *F. prausnitzii* seems to show favorable results (18, 112). Some successes have been demonstrated with the administration of *S. boulardii* in combination with mesalazine in patients with CD, where a significant reduction in the incidence of relapse occurred as opposed to the mesalazine only-treated group, however, this was a small study (113). Although there are few studies assessing the effect of *S. boulardii* in CD, the existing evidence only slightly favors the use of the yeasts as a probiotic in CD in certain populations (such as non-smokers) (114). **Table 1** summarizes various probiotics and their clinical efficacy in CD. Apart from the use of *Saccharomyces boulardii* as a probiotic, there is no evidence that sufficiently supports the use of the current probiotics to induce clinical remission in CD. More large, high-quality well-powered studies are needed to determine the specific factors needed for the efficacy of probiotics in CD.

Prebiotics in CD

Prebiotics are defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit (129)". These health benefits are not necessarily limited to the colon, but also occur in the oral cavity, urogenital tract, lungs and skin (130). Typically, prebiotics were thought to be limited to non-digestible carbohydrate sources, such as fructooligosaccharides, galacto-oligosaccharides, resistant starches, pectin, arabinoxylan, and whole-grains, but an updated definition now includes non-carbohydrate sources, such as polyphenols and certain lipids (130). The most extensively tested type of prebiotic is inulin-type β-fructans, which are naturally derived from food sources such as chicory root, and onions (111, 129).

Prebiotics confer benefit to the host through their fermentation by some commensal microbes in the gut resulting in compositional and metabolic modulations/alterations (111, 131–133). Prebiotics are a non-selective growth substrate, allowing the simultaneous growth of multiple beneficial strains, such as *F. prausnitzii*, *Roseburia* spp., *Eubacterium* spp., *Anaerostipes* spp., *Coprococcus* spp., *Bifidobacterium* spp (134). Furthermore, prebiotics is synergistically co-metabolized by several distinct microbial groups such as butyrate-producing *F. prausnitzii* and acetate-producing *B. adolescents* leading to more efficient co-fermentation (135). However, it is important to note that not all non-digestible carbohydrates can be considered prebiotics. For example, feeding IL-10 knockout mice with dextrin fibers derived from corn resulted in microbiota shifts such as an increase of some Bacteroidetes families (*Porphyromonadaceae* and *Prevotellacea*) vs. reduction of strict anaerobic Firmicutes (*Incertae Sedis XIV, Lachnospiraceae*, *Ruminococcaceae,* and *Lactobacillaceae*). These changes were also seen in conjunction with reduced pro-inflammatory pathways such as IL12p70, IL-6, and chemokine ligand 1 (CXCL) (136). Despite these differences, these mice did not experience any improvement in colonic inflammation—or in other words, no health benefits to the host (136).

Arguably, a major function of prebiotics is their fermentation by commensal microbes into SCFAs. Propionate, acetate, and butyrate are the main SCFAs (20, 137, 138). SCFAs enhance mucus secretion, increase anti-microbial peptides, lower the pH of the colon to decrease oxygen levels, and inhibit the growth of pathogenic anaerobes. SCFAs also upregulate the expression of tight junction proteins to maintain a healthy functional immune system and intestinal barrier (137, 139) and reduce the production of putrefactive substances, such as ammonia, indole, branch-chain fatty acids, and phenol (137). Butyrate acts as the main energy source for colonocytes. This SCFA is of particular interest in IBD, as it is significantly reduced in colonic cells leading to autophagy and energy deprivation (140). Butyrate inhibits NF-κB activation *via* an increase in cytoplasmic inhibit (IKB) thus inhibiting pro-inflammatory cytokines and chemokines, such as interferon-γ (INF-γ), pro-inflammatory chemokine CXCL-8 (IL-8) in Caco-2 cells, and TNF-α (20, 141–143).

In a well-powered placebo-controlled study, Benjamin et al. found that supplementing 120 patients with active CD with 15g/day of inulin-type β-fructans had no clinical benefit (144). However, De Preter et al. and Joossens et al. using 10 g twice daily oligofructose-enriched inulin (OF-IN) in 67 inactive or mild to moderately active CD reported microbiota shifts toward an increase in *B. longum* that positively correlated with an improvement in CD disease activity and that OF-IN intake increased fecal butyrate and acetaldehyde (145, 146). As summarized in **Table 2**, few CD studies with prebiotics have demonstrated inconsistent results, likely due to a dose-dependent effect of prebiotics, disease stage/severity, and adverse effects, such as bloating, which may mask symptom improvement;

TABLE 1 | Use of probiotics in patients with Crohn's disease.

Type of study	Participants (*n*)	Duration (months)	Intervention	Control	Reference	Outcome
Probiotics to induce clinical remission						
Open-label	17	0.5	*Saccharomyces boulardii*	None	(115)	Modest symptomatic improvement
RCT	11	6	*Lactobacillus rhamnosus* GG + Corticosteroids	Placebo + Cortico-steroids	(116)	No benefit
Open-label	10	13	*Bifidobacterium longum*, *B. breve*, *Lactobacillus casei* + Plantago ovata	None	(117)	Symptomatic improvement
RCT	35	6	*B. longum* + FOS/inulin	Placebo	(118)	Symptomatic improvement
Probiotics to maintain clinical remission						
RCT	17	3	*S. boulardii*	Placebo	(115)	Improvement
RCT	28	12	*E. coli Nissle 1917*	Placebo	(119)	No benefit
RCT	35	6	*S. boulardii*	Pentasa	(113)	Prevented relapse
RCT	11	6	LGG	Placebo	(116)	No benefit
RCT	75	42	LGG	Inulin	(120)	Deterioration (NS)
RCT	30	12	VSL #3	Placebo	(109)	Deterioration (NS)
	30	12	*S. boulardii*	Placebo	(121)	No benefit overall; favorable in non-smokers?
RCT	62	1	Symprove (*L. rhamnosus, L. plantarum, L. acidophilus, Enterococcus faecium*)	Placebo	(122)	No Benefit
Observational	200	-	Various	-	(123)	Reduced adverse events
Probiotics to prevent post-operative recurrence						
RCT	40	12	Rifaximin – VSL #3	Mesalamine	(124)	Lower incidence of endoscopic recurrence
RCT	45	12	LGG	Placebo	(125)	Deterioration (NS)
RCT	98	6	*Lactobacillus johnsonii*	Placebo	(126)	No benefit
RCT	30	24	"Synbiotic 2000"	Placebo	(127)	No benefit
RCT	70	3	*L. johnsonii*	Placebo	(128)	No benefit
RCT	120	12	VSL #3	Placebo	(109)	No statistically significant benefit

S. boulardii, Saccharomyces boulardii; LGG, Lactobacillus rhamnosus GG; FOS, fructo-oligosaccharide; NS; not significant; RCT, randomized controlled trial.

therefore, more RCT studies with adequate power assessing objective disease parameters in association with protective mechanisms are needed to discern their effect in various CD phenotypes.

Synbiotics in CD

Synbiotics are a combination of carefully curated prebiotics and probiotics that work together to exert a synergistic effect. While prebiotics encourages the proliferation of beneficial intestinal microbes, probiotics inhibit the growth of pathogenic bacteria to synergistically improve the integrity of the gut barrier (132). In a small placebo-controlled randomized control trial of only 35 patients with active CD, those who received a combination of prebiotic fructo-oligosaccharides/inulin and *Bifidobacterium longum* experienced significant improvements in histological samples after 3 and 6 months of the study. There was also a significant decrease in TNF-α expression at 3 months that was maintained through the rest of the study (118). Similar to studies related to probiotics and prebiotics, more large, high-quality trials are needed to determine the use and efficacy of synbiotics in CD.

Therapeutic Dietary Modifications in CD

Diet is a major lifestyle factor that is significantly linked to the composition/function of the gut microbiota. In an analysis of genotype and microbiome data of healthy individuals, at least

TABLE 2 | Use of prebiotics to induce or maintain clinical remission in patients with Crohn's disease.

Type of study	Participants (*n*)	Duration (months)	Intervention	Control	Outcome	Reference
RCT	41	1	FOS, 15g/d	Maltodextrin, 15g/d	No benefit	(144)
Open label	22	4	Two 8-ounce cans/day of IBDNF	None	Significant decrease in plasma phospholipid levels of arachidonic acid with an increase in eicosapentaenoic acid and docosahexaenoic acid.	(147)
RCT	67	1	OF-IN, 20g	Placebo	Improvement in disease activity associated with increase in *Bifidobacterium longum,* and butyrate	(145, 146)
Observational case-control	303	-	None	None	Patients with active CD presented lower fructan and lower oligofructose intakes than inactive CD or control groups. Negative correlation between HBI wellbeing score and fructan and oligofructose intakes.	(148)

RCT, randomized control trial; FOS, fructo-oligosaccharide; IBDNF, Inflammatory Bowel Disease nutrition formula; OF-IN, oligofructose-enriched inulin; CD, Crohn's disease, HBI, Harvey-Bradshaw Index.

20% of β-diversity is related to environmental factors, such as diet, drugs, and anthropometric measurements (102). In a five-day *ad libitum* dietary intervention, significant alteration in the gut microbiome composition and activity was evident in just 24 h of diet initiation (149). Those consuming an animal-based diet including meat, cheese, and eggs showed an increase in bile-tolerant microbes, such as *Alistipes, Bilophila,* and *Bacteroides*. In particular, the increase in *Bilophila wadsworthia* supports the association between dietary fat, increased bile acids secretion, and the expansion of microorganisms implicated in IBD. Furthermore, a reduction in the levels of Firmicutes, such as *Roseburia, Eubacterium rectale,* and *Ruminococcus bromii,* was associated with an animal-based diet (149). Analysis of fecal SCFAs suggested that changes in macronutrients in both diets resulted in a change in microbial metabolic activity. Animal-based diets demonstrated significantly high levels of amino acid fermentation and lower levels of products of carbohydrate fermentation (149). Branched chain SCFA that are products of amino acid fermentation were positively associated with putrefactive bacteria such as *Alistipes putredinis* and *Bacteroide*s spp. Meanwhile, products of carbohydrate fermentation, more abundant in the plant-based diet, were associated with clusters of saccharolytic microbes such as *Roseburia* spp., *E. rectale*, and *F. prausnitzii* (149).

Diet may be more relevant and have a stronger effect on CD than UC since most prior epidemiological studies on dietary risk factors have identified association with CD, but not UC (150–152). There also seems to be a slight difference in dysbiosis in different phenotypes of CD. A greater reduction in butyrate producers such as *F. prauznitzii* and increased abundance of *E. coli* is seen in ileal CD as compared to colonic CD, suggesting that there might be a stronger link between diet, microbiome, and CD phenotype (21). A plethora of studies have explored dietary patterns associated with CD. Many studies such as the Healthy Lifestyle in Europe by Nutrition in Adolescence Study and the prospective cohorts of the Nurses' Health Study (NHS) and Nurses' Health Study II (NHSII) have shown that a higher intake of fiber, specifically cruciferous vegetables and cereals, is associated with lower incidence of CD (150, 151). On the other hand, a prospective study of NHS I and NHSII demonstrated a reduction in CD risk with high dietary fiber, specifically derived from fruits (150).

The EPIC-IBD study found no evidence that dietary fiber was associated with CD or UC. However, a higher intake of cereal fiber in non-smokers was inversely associated with odds of developing CD (151). During analysis of 3 ongoing prospective cohort studies (NHS, NHSII, and The Health Professionals Follow-up Study (HPFS)), it was demonstrated that dietary patterns with high inflammatory potential were associated with increased risk for CD, but not UC. There seemed to be a dynamic risk with CD; not only was there a long-term pro-inflammatory diet associated with a higher risk, but also an increase in disease development from low to high inflammatory potential diet. In this study, the association between an inflammatory diet and the risk of CD remained unchanged for additional fiber intake. These results, confirmed by others (153), suggest that dietary fiber and low inflammatory potential foods are both important components of the diet to reduce CD risk (154). Examples of a high inflammatory potential diet is the Western diet, which is high in saturated fat, added sugar, and low in fiber (155). Khalili et al., showed in two large prospective studies in Sweden that poor adherence to the Mediterranean diet (a low-inflammatory potential diet) conferred a population attributable risk of 12% for later-onset Crohn's disease—suggesting that greater adherence to a Mediterranean diet is associated with a lower risk of later-onset CD (152). Moreover, Bolte et al., found that processed foods and animal-derived sources were associated with a higher abundance of microbes associated with inflammation such as Firmicutes, *Ruminococcus* spp., and endotoxin synthesis pathways (153). Meanwhile, plant-derived

TABLE 3 | Dietary patterns associated with inflammation or increased incidence of CD.

Study Type	Participants (*n*)	Duration	Outcome	Reference
Observational	170 776	26 years	Long-term intake of dietary fiber, especially fruit, is associated with lower risk of CD but not UC	(150)
Prospective cohort	401 326		No associations between fiber from specific sources and risk of UC/CD	(151)
Prospective cohort	83 147	17 years (SD±5)	Mediterranean Diet associated with lower risk of CD	(152)
Prospective cohort	208 834 (NHS, NHSII, HPFS)	-	High dietary inflammatory potential associated with 51% higher risk of CD	(154)
Cross-sectional	1425 (CD, UC, IBS, HC)	-	Processed foods and animal- based foods associated with increased abundances of Firmicutes, *Ruminococcus* spp. Plant-based foods and fish positively associated with short- chain fatty acid-producing commensal bacteria	(153)

CD, Crohn's disease; UC, Ulcerative colitis; SD, standard deviation; NHS, Nurses' Health Study (NHS); NHSII, Nurses' Health Study; HPFS, The Health Professionals Follow-up Study; IBS, Irritable Bowel Syndrome; HC, healthy controls.

sources were associated with SCFA-producing bacteria and improved metabolic pathways (153). **Table 3** includes various diets and their association with inflammation and risk of CD. These studies suggest that adherence to a low-inflammatory potential diet (i.e., Mediterranean diet) and the addition of fermentable dietary fiber may aid in reducing the risk of CD in those that are genetically predisposed.

Therapeutic diets have the potential to both induce and maintain remission. Exclusive Enteral Nutrition (EEN) is the use of a liquid elemental or polymeric formula consumed exclusively for up to 12 weeks (156). EEN has shown remarkable success in inducing remission (157, 158) and is especially recommended as first-line therapy to induce remission in pediatric patients with CD (159). The mechanisms of EEN are related to its role in reducing antigens in whole foods, improving micronutrient and macronutrient deficiencies, and improving gut dysbiosis (158, 160, 161). EEN reduces microbiota diversity and initiates modulation of intestinal bacterial communities (156, 162). Notably, EEN reduces inflammation through the modulation of *Bacteroides* species. A significant positive correlation between a change in *Bacteroides-Prevotella* groups and a reduction in pediatric Crohn's disease activity index (PCDAI) (156, 162). Following EEN's success, many dietary therapies have focused on eliminating certain dietary components to improve disease severity. The Specific Carbohydrate Diet (SCD) consists of eliminating all grains, sugars (except for honey), milk products (except for hard cheeses and fermented yogurt), and most processed foods (163). This diet has shown some efficacy in inducing remission; however, long-term adherence is difficult due to the limitations of this diet. Therefore, diets offering a greater variety of foods, such as the modified-Specific Carbohydrate Diet (mSCD) and Mediterranean diet (MD), have been explored as an alternative means of dietary intervention. Both the mSCD and MD show comparable results to the SCD but boast a more liberal dietary pattern that is customizable and easier to follow. Studies involving the Mediterranean diet have shown promise regarding inducing and maintaining remission, improving inflammatory biomarkers, and quality of life (QoL). The Crohn's Disease Exclusion Diet and Partial Enteral Nutrition (CDED + PEN) approach has been explored and has shown to induce and sustain remission in 75% of pediatric CD patients at 12 weeks (164, 165). The CDED consists of the removal of animal fat, wheat, dairy, red meat, emulsifier, maltodextrin, inulin, and carrageenan and the addition of fruits and vegetables. CDED + PEN was associated with a reduction in Proteobacteria and intestinal permeability, as measured by a Lactulose/Mannitol Test (165). In addition, this diet-induced a decrease in abundance in *Haemophilus*, *Veillonella*, *Anaerostipes*, and *Prevotella*, and an increase in *Roseburia* and *Oscillibacter* (165). As shown in **Table 4**, plant-based and low inflammatory diets contribute to an improved microbiome profile along with disease amelioration.

Fecal Microbiota Transplantation in CD

Fecal microbiota transplantation (FMT) is the transfer of fecal matter from a healthy donor to a person with dysbiotic gut microflora. The aim of FMT is the restoration of a healthy microbiota (173). FMT has been remarkably successful in the treatment of *Clostridioides difficle* infections (CDi) and is being explored as a therapeutic option for IBD; however, the results have not been as promising. Certain randomized control trials, such as a landmark FMT study by Moayeddi et al., have shown success in inducing clinical remission in patients with UC (174). In this study, success in remission induction was associated with a recent UC diagnosis, as the perturbation in microbial structure was easier to change in its early stages (174). The inconsistency in FMT results is likely due to the more complex pathogenesis of IBD as compared to CDi. Similar to probiotics and prebiotics, data regarding FMT and IBD is limited and heterogeneous, making it difficult to determine its absolute effect on IBD disease activity.

In a promising 5-year Chinese study by Xiang et al., a step-up FMT strategy was used in 174 patients with CD. This 3-step strategy used integrative treatment consisting of a single or multiple FMT in conjunction with steroids, immunomodulators, and exclusive enteral nutrition. Improvements in abdominal pain, hematochezia, fever, and diarrhea were seen from 1-month post-FMT to the end-of-study at 3 years post-FMT.

TABLE 4 | Dietary patterns to induce or maintain remission in Crohn's disease.

Diet	Study type	Participants (*n*)	Duration (months)	Outcome	Reference
Active Crohn's disease					
Mediterranean Diet	Open-label intervention	142 Adult	6	Improvement in BMI, waist circumference, liver steatosis, disease severity, inflammatory biomarkers, and quality of life	(166)
PREDIMED Mediterranean diet score	Observational	66 Adult	3	Daily intake of leafy green vegetables associated with FCP $\leq$ 100μg Higher omega 6:3 ratio associated with CRP $\leq$ 5 mg	(167)
Specific Carbohydrate Diet vs. Mediterranean Diet	RCT	194 Adult	3	Specific Carbohydrate Diet was not superior to the Mediterranean diet to achieve symptomatic remission, FCP response, and CRP response.	(168)
Specific Carbohydrate Diet vs. Modified Specific Carbohydrate Diet vs. Whole foods	RCT	18 Pediatric	3	All 3 diets were associated with high and comparable rates of clinical remission, and all had improvement in inflammation to differing degrees	(169)
Crohn's Disease Exclusion Diet and Partial Enteral Nutrition vs Exclusive Enteral Nutrition	RCT	74 Pediatric	3	CDED + PEN is better tolerated than EEN, both are effective at achieving remission in the short-term	(164, 165)
Inactive Crohn's disease					
Semi-vegetarian diet	Open-label intervention	22 Adult	24	SVD prevented relapse	(170)
High-meat vs. Low-meat	Observational	213 Adult	∼11	Red/processed meat is not associated with time to relapse	(171)
Low FODMAP	RCT	52 Adult	1	Low FODMAP diet reduced gut symptoms scores and significantly lower abundance of *Bifidobacterium adolescentis, B. longum,* and *Faecalibacterium. prausnitzii,* no change in microbiome diversity and markers of inflammation	(172)

RCT, randomized control trial; BMI, body mass index; FCP, fecal calprotectin; CRP, C-reactive protein; CDED + PEN, Crohn's Disease Exclusion Diet and Partial Enteral Nutrition; ENN, Exclusive Enteral Nutrition; SVD, Semi-vegetarian diet; Low FODMAP, Low fermentable oligosaccharides, disaccharides, monosaccharides, and polyols.

This study was the largest cohort of patients that underwent FMT and had a long-term follow-up. It is important to note that this study neither used a control group, nor assessed endoscopic biomarkers, quality of life, or microbial analysis (175). However, the optimal time between FMT doses was approximately 4 months, a similar time frame as in a previous study by Li et al. (176). However, it becomes evident that FMT on its own does not induce long-term remission. In a multivariate analysis, it was shown that degree of dysbiosis, longer-disease duration (>5 years), and severity (HBI > 8) was associated with poorer response to FMT (33, 176). FMT seems more likely to be successful in CD in early stages, in milder disease, when administered in multiple courses and conjunction to other treatment modalities. FMT has the potential to provide a profound improvement in specific patients with CD, but information regarding donor characteristics and time of administration (early vs. late disease course) has yet to be explored in a standardized and controlled manner. One major issue with the majority of these FMT studies is that they are underpowered, are often open-label, do not account for the healthy donor effect, and have a lack of reproducibility.

INTESTINAL PERMEABILITY AND CROHN'S DISEASE

Intestinal permeability (IP) refers to the functional property of the intestinal mucosal barrier that controls the interactions between the gut and gut microbes. Normal intestinal permeability allows for the coexistence of microbial symbionts of the host while preventing luminal penetration of macromolecules and pathogens (177). The purpose of the intestinal barrier is to reduce contact between luminal microbial contents and the mucosal immune system (178). In healthy humans, it acts as a semi-permeable physical barrier allowing selective movement of nutrients while protecting the body from pathogenic invasion (179). An impaired intestinal barrier and increased IP, also known as "leaky gut," has been the focus of research as it appears to be a defining factor in the pathogenesis of IBD (177, 179). Epithelial integrity is characterized by a 4–5-day turnover of cell shedding into the intestinal lumen at the surface and the proliferation of multipotent stem cells within the intestinal crypt to replace the loss of cells (180). Disruption of intestinal barrier turnover contributes to invasion of luminal antigens and

intestinal inflammation as seen in ulcerative colitis (UC) and Crohn's disease (CD) (181, 182).

Intestinal epithelial cells (IECs) are mechanically attached by the junctional complexes of tight junctions (TJs), adherence junctions, and desmosomes (183–185). These structures also control the paracellular transport of ions and small molecules between adjacent cells *via* passive transport. Patients with IBD display several TJ abnormalities leading to increased paracellular transport (186, 187). Patients with IBD display reduced expression and redistribution of TJs and their constituents such as occludins, claudins, and junctional adhesion molecules (JAM) (181, 182, 188, 189). Tumor-necrosis factor-α (TNF-α), a pro-inflammatory cytokine implicated in the progression of IBD, has been shown to modulate the transcription of TJ proteins (190, 191). Not only does TNF-α increase IP, but it also increases the rate of shedding of enterocytes *via* apoptosis which results in a lag of TJ redistribution to adhere cells together (181, 192).

Paracellular movement of molecules is limited by the function of TJs between IECs. Therefore, regulation of TJ function is essential for the normal movement of solutes between cells. Another factor affecting paracellular transport is epithelial damage through erosion or ulceration (193). Zonulin is a protein in humans that has been identified as a reversible regulator of TJ function. Zonulin modulates permeability by TJs disassembly leading to increased intestinal permeability (194). In autoimmune conditions, such as IBD, Celiac Disease, and Type 1 Diabetes, enhanced expression of zonulin has been observed, making it a biomarker of impaired gut function along with a potential target for therapy. Other biomarkers of intestinal permeability include glucagon-like peptide-2 (GLP-2). GLP-2 is involved in intestinal cell proliferation in the crypts of IECs, therefore beneficial in reducing the permeability of the gut (193, 194).

ROLE OF MICROFLORA-ALTERING THERAPY TO PREVENT CROHN'S DISEASE

It is well-documented that there is a genetic component to CD susceptibility with up to 12% of patients having a family history of IBD. Genome-wide association studies (GWAS) have identified 240 single nucleotide polymorphisms (SNPs) in IBD (195). However, genetics alone do not explain the onset of CD, as many people with the identified alleles do not develop the disease (196). In a prospective study, Turpin et al. measured intestinal permeability by the urinary fractional excretion of lactulose-to-mannitol ratio (LMR) in 1,420 asymptomatic first-degree relatives (FDR) of patients with CD with a median follow-up of 7.8 years. An abnormal LMR (>0.3) was associated with a diagnosis of CD onset during the follow-up period, whereas the test was performed more than 3 years before the diagnosis of CD. Not only did these results demonstrate that increased intestinal permeability is associated with the risk of development of CD, but they also support the hypothesis that abnormal intestinal barrier function can contribute to the pathogenesis of CD and can serve as a biomarker for the risk of CD onset in healthy asymptomatic FDR (197). Another analysis by Turpin et al. of a GWAS of LMR within the same study population showed that host genetics provide only a small contribution to an abnormal LMR in FDR of patients with CD, suggesting that an abnormal LMR may be more likely a result of environmental triggers or insult (198). Morkl et al. found that intestinal permeability in women, as measured by serum zonulin, was reflective of diet composition including calories, protein, carbohydrate, sodium, and vitamin B_{12} intake. It also was associated with the composition of the gut microbiota. Specifically, butyrate-producing *Faecalibacterium* and *Ruminococcaceae* were significantly more abundant in the low-zonulin group (199). These results suggest that controlling environmental factors using diet and dietary supplements may affect abnormal LMR to improve intestinal permeability. Currently, there are no therapies approved by US Food and Drug Administration or Health Canada to directly target and restore the abnormal intestinal barrier. The use of prebiotics and a low-inflammatory diet could be promising as therapeutic agents to restore a defective mucosal barrier and reduce intestinal permeability, either directly and/or by restoring gut dysbiosis (177, 200).

CONCLUSION

Approaches to modulate the gut microbiota in CD toward a healthier state are a topic of great interest. Despite the great advances in the gut microbiota field, we are far from fully uncovering the interrelations between the different bacteria, fungi, archaea, and viruses and how this is translated into host health. Current findings imply environmental factors, such as the Western diet, and some medications that can modulate directly or indirectly (through increased intestinal permeability) the intestinal microbiome, hence enabling the initiation of a cascade of pathophysiological changes. Concurrently, medicine regulatory authorities and drug developers should extend their pharmacodynamics and pharmacovigilance guidelines to incorporate possible drug-microbiota interactions with respect to safety and mode of action. In contrast, some diets, specifically Mediterranean-like diets, are suggested to restore eubiosis of the gut microbiome. Complementing a diet with other modulatory approaches, such as the addition of immune-modulating probiotics and prebiotics may improve the clinical efficacy and suppress chronic inflammation. Similarly, FMT has the potential to induce profound changes in the global gut community, thus rigorous safety and technical implementations are a prerequisite for a successful application in CD. Given the complexity of CD, it is crucial that future research focuses on the nuances of personalized medicine to recommend individually tailored care plans regarding therapeutics and nutraceuticals to prevent or delay the onset of Crohn's disease or reduce disease severity. Such knowledge in this rapidly evolving field is also important for clinicians and translational scientists to incorporate these microbiota-altering adjunct therapies into CD treatment options, along with awareness of the limitations of these therapies at this time, until more research is performed.

AUTHOR CONTRIBUTIONS

RR, RV, and LD conceptualized the manuscript. RR and RV wrote the manuscript. LD edited the manuscript. All authors contributed to the article and approved the submitted version.

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