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Rising stars in microbiome in health and disease 2022

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Rising stars in microbiome in health and disease: 2022

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In Vitro Hepatoprotective and Human Gut Microbiota Modulation of Polysaccharide-Peptides in *Pleurotus citrinopileatus*

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Pleurotus citrinopileatus, a golden oyster mushroom, is popular in Asia and has pharmacological functions. However, the effects of polysaccharide-peptides extracted from *Pleurotus citrinopileatus* and underlying mechanism on digestive systme have not yet been clarified. Here, we determined the composition of two polysaccharide-peptides (PSI and PSII) from *P. citrinopileatus* and investigated the protective effects of on hepatoprotective and gut microbiota. The results showed that PSI and PSII were made up of similar monosaccharide moieties, except for the varying ratios. Furthermore, PSI and PSII showed that they have the hepatoprotective effects and significantly increased the viabilities and cellular total superoxide dismutase activities increased significantly in HepG2 cells. Intracellular triglyceride content and extracellular alanine aminotransferase and aspartate transaminase contents markedly decreased following treatment with 40 and 50 μg/mL PSI and PSII, respectively. Moreover, PSI and PSII activated the adiponectin pathway and reduced lipid accumulation in liver cells. PSI and PSII elevated short-chain fatty acid concentrations, especially butyric and acetic acids. 16S rRNA gene sequencing analysis showed that PSI promoted the relative abundances of *Bifidobacteria*, *Lactobacillus*, *Faecalibacterium*, as well as *Prevotella* generas in the gut. PSII markedly suppressed the relative abundances of *Escherichia-Shigella* and *Bacteroides* generas. We speculate that the PSI and PSII play a role through liver-gut axis system. Polysaccharide-peptides metabolize by gut microbiota to produce short-chain fatty acids (SCFAs) and in turn influence liver functions.

Keywords: polysaccharide-peptide, *Pleurotus citrinopileatus*, hepatoprotection, gut microbiota, liver-gut axis

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is highly associated with chronic liver injury. In many countries, high-fat diets have increased the prevalence of NAFLD and lead to serious public health problems. NAFLD represents a spectrum of metabolic states that range from simple steatosis to non-alcoholic steatohepatitis, cirrhosis, hepatoma and fibrosis (Marchesini et al., 2001; Farrell and

Larter, 2006). As a disorder, NAFLD is characterized by hypertriglyceridemia and abnormal hepatic fat accumulations, which are linked to obesity and insulin resistance (Svegliati-Baroni et al., 2006; Utzschneider and Kahn, 2006). Diet, exercise and antioxidants are currently the most effective treatments for NAFLD.

As a functional food, edible mushrooms of *Pleurotus* spp. are a potential natural source for drug candidates. Polysaccharides and polysaccharide-peptide complexes from *Pleurotus* spp. have anti-obesity (Sheng et al., 2019), antioxidant (Wu and Chen, 2017), antibacterial (Li and Shah, 2014), and antitumor effects (Ren et al., 2015) and have been shown to exhibit immunomodulatory activities, inducing macrophages to produce interleukins, nitric oxide, interferon-γ, and tumor necrosis factor (Cui et al., 2015). Moreover, polysaccharides from *Pleurotus* have been shown to have heptoprotective effects, including antihyperlipidemic activities. Indeed, these polysaccharides have preventive effects on high-fat diet-induced hyperlipidemia in mice, indicating potential beneficial effects on liver function (Zhang et al., 2017). Intracellular mycelial polysaccharides from *Pleurotus geesteranus* exhibit hepatoprotective effects against alcohol-induced acute alcoholic liver diseases, suggesting potential curative effects in alcoholic hepatitis (Song et al., 2018). However, the heptaoprotective effects of polysaccharide-peptides from *P. citrinopileatus* have not been fully evaluated.

The hepatointestinal system mediated nutrient digestion and absorption. NAFLD is associated with gut dysbiosis and changes in its metabolic functions (Boursier et al., 2016). Polysaccharide-peptides could be digest by gut microbiota to produce short-chain fatty acids (SCFAs) and in turn affect the liver functions. The study of the gut-liver axis can help us to understand the basic biology of NAFLD and identify the mechanisms between gut microbiota and liver damage (Tripathi et al., 2018).

In this study, we evaluated the hepatoprotective effects of two polysaccharide-peptides (PSI and PSII) extracted from *P. citrinopileatus* in a hepatoma cell model (HepG2 cells) of NAFLD. Furthermore, to assess the impacts of PSI and PSII on human gut microbiota, 16S rRNA sequencing techniques were used to explore the effects of PSI and PSII on gut microbiota by adult fermentation models *in vitro*, which is an effective tool for evaluating the impact of prebiotics on gut microbiota. Our findings elucidated on the use of PSI and PSII in improving human gut microbiota and develop new protective agents for the treatment of fatty liver and gut disease.

MATERIALS AND METHODS

Materials and Regents

P. citrinopileatus fruiting bodies preserved at the Plant Protection Institute of Beijing Academy of Agricultural and Forestry Sciences (Beijing, China) were sun-dried and crushed to obtain fine powder. Standard monosaccharides (D-xylose, D-glucose, D-galacturonic acid, D-mannose, D-glucuronic acid, L-rhamnose, D-fructose, D-arabinose and D-galactose), oleic acid, DEAE-cellulose, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), as well as palmitate were purchased from Sigma-Aldrich (USA). Superdex-200 column was acquired from the General Electric Company (GE, USA), and HepG2 cells were bought from the American Type Tissue Culture Collection (Manassas, VA, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's minimum essential medium (DMEM), phosphate-buffered saline (PBS), penicillin, trypsin solution, and streptomycin were purchased from Invitrogen (USA). Protein, triglyceride (TG), alanine transaminase (ALT), aspartate transaminase (AST), and superoxide dismutase (SOD) assay kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu Province, China). The rest of the chemicals as well as solvents were of analytical reagent grades and were obtained from Peking Chemical Co. (Beijing, China).

Extraction and Purification of Polysaccharide-Peptides

The sun-dried fruiting bodies of *P. citrinopileatus* were placed in a high-speed universal crusher and repeatedly crushed four times for 20 s each. The crude polysaccharide-peptides were extracted thrice using the hot water method with a solid/liquid ratio of 1:50, at 90°C, and a 3 h extraction time. The obtained aqueous extracts were combined and concentrated using a rotary evaporator, and proteins in the concentrated solutions were removed by Sevag reagent (n-butanol and chloroform, 1:4 v:v ratio). Precipitation of the deproteinized solution was achieved by the addition of 100% ethanol (1:4 v:v ratio) at 25°C overnight, after which the polysaccharide-peptide extracts were acquired by centrifugation. Then, the polysaccharide-peptide extracts were dissolved in distilled water and applied to a DEAE-cellulose column (1 cm × 30 cm) that had been equilibrated with a 10 mM phosphate buffered solution (pH 7.0). Sequential elution of the column was done using 0, 0.2, and 1 M NaCl solution at a 1.5 mL/min flow rate. The adsorbed peak D2 and unadsorbed peak D1, with high carbohydrate levels as assessed by the phenol-sulfuric acid assay were collected. After being concentrated, to obtain bioactive PSI and PSII, the D1 and D2 fractions were applied to a Superdex-200 column equilibrated with ultrapure water using an AKTA Purifier (GE Healthcare).

Analysis of PSI and PSII Monosaccharide Compositions, Fourier-Transform Infrared (FT-IR) Spectra, and Molecular Weights

Monosaccharide contents of PSI and PSII were analyzed by gas chromatography-mass spectrometry (GC-MS) (Yu et al., 2015). The IR spectras of PSI as well as PSII were evaluated by FT-IR (iS5 FTIR Spectrometer; Nicolet, USA) at 4000 to 400 cm^{-1}. Molecular weights and homogeneity of PSI/PSII were determined by high-performance gel permeation chromatography (GPC) on TSK GMPWXL columns. Freeze-dried polysaccharide-peptides were analyzed by the Science Spectrum R&D Center (Shandong, China).

Analysis of N-Terminal and Inner Amino Acid Sequences of PSI and PSII

Polysaccharide-peptide bands excised from sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels were transferred to

polyvinylidenedifluoride membranes followed by Coomassie brilliant blue R-250 staining. Stained bands were analyzed by the automated Edman degradation assay (Wang et al., 2018). Polysaccharide bands on SDS-PAGE gels was obtained and subjected to partial amino acid sequence analysis at Tsinghua University (Beijing, China). Using known sequences, sequence homology was searched in the BLAST/NCBI database.

Analysis of the Cytotoxicity of Polysaccharides in Hepatocytes

Culture of HepG2 (hepatoma) cells was done in DMEM with 10% (v/v) FBS, 100 IU/mL penicillin and 100 mg/L streptomycin. Incubation at 37°C was done in a 5% (v/v) CO_2 humid environment. Then, cells were seeded onto 96-well plates at 8×10^3 cells/well followed by incubation for 12 h before the addition of PSI and PSII at 100, 200, 500, 800, or 1000 μg/mL concentrations. Incubation was then carried out for 72 more hours. Cytotoxicity was determined by MTT assays. Viability of PBS-treated control cells was set at 100%.

Preparation of Double Factor-Induced Hepatocyte Injury

Free fatty acids (FFAs) and ethanol can induce hepatocyte injury. Palmitic and oleic acids were mixed (1:2, respectively) and used as FFAs (Garcia et al., 2011). For the hepatocyte injury model, HepG2 cell seeding in 96-well plates was done at a density of 8×10^3 cells/well and subsequently incubated for 12 h after which FFAs and ethanol were added. Then, incubation was done for an additional 24 h, and MTT assays conducted to assess cell viabilities. PBS was used to replace FFAs and ethanol as the control.

Protective Effects of PSI and PSII on Hepatocytes

To determine the protective effects of the polysaccharide-peptides, injured HepG2 cells (as described above) were treated for 48 h using varying PSI and PSII concentrations. Cell viabilities were then measured by MTT assays. Injured HepG2 cells were used as the negative control, and PBS without FFAs or ethanol was used as the positive control.

Optimal concentrations of PSI and PSII were used to analyze the mechanisms of action. Seeding of HepG2 cells was done in 6-well plates at 3×10^4 cells/well, the injury model was induced, and cells were treated for 48 h using PSI and PSII. The cells as well as culture medium were then obtained to evaluate the protective and repairing abilities. The ALT as well as AST activities in the culture medium were measured using colorimetric assay kits. Quantification of cellular TG contents and SOD activities were done using commercial assay kits according to the manufacturers' protocols. ALT, AST, and SOD results were expressed as U/mg protein. Oil-red O staining was done for histological analyses of cellular lipids.

Gene Expression

Expressions of lipid metabolism-associated genes in the adiponectin pathway were assessed by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). The TRIzol reagent (Invitrogen) was used for total RNA extraction from each sample. Reverse transcription of the extracted RNA was done in the presence of oligo (dT) using EasyScript First-Strand cDNA Synthesis SuperMix (Transgen, China), as instructed by the manufacturer. qRT-PCR was conducted according to the Maxima SYBR Green/ROX qRT-PCR Master Mix (Fermentas, USA) protocol using an ABI 7500 (Applied Biosystems, USA). In these experiments, GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as the endogenous control. Gene-specific primer sets for mouse AdipoR2 (anti-adiponectin receptor 2), AMPK (AMP-activated protein kinase), CPTl (carnitine palmitoyltransferase 1), ACOX-1 (acyl-CoA Oxidase 1), PPARα (peroxisome proliferator–activated receptor α) were referred to previous studies (Spruiell et al., 2015; Han et al., 2019; Li et al., 2021). qRT-PCR conditions were: predenaturation for 5 min at 95°C and 40 cycles of 95°C for 30 s and for 1 min at 60°C. Relative expressions were calculated *via* the ΔΔCt method. Experiments were conducted in triplicates.

In Vitro Batch Culture Fermentation

Preparation of the *in vitro* fermentation medium was done in 10 mL vials with 5 mL of anaerobic YCFA medium. YCFA medium consisted of (per 100 ml): 1 g casitone, 0.25 g yeast extract, 0.4 g $NaHCO_3$, 0.1 g cysteine, 0.045 g K_2 HPO_4, 0.045 g KH_2 PO_4, 0.09 g NaCl, 0.009 g MgSO%.$7H_2$ O, 0.009 g $CaCl_2$, 0.1 mg resazurin, 1 mg haemin, 1 μg biotin, 1 μg cobalamin, 3 μg *p*-aminobenzoic acid, 5 μg folic acid and 15 μg pyridoxamine. Medium preparation was done in two different concentration gradients of PSI and PSII (40 μg/mL, 200 μg/mL) respectively, as the sole carbon source. Six healthy human volunteers (aged 22 - 42 years) from Hangzhou were enrolled in this study. They were fed on a normal Chinese diet, had no digestive ailments and had not been administered with any medications, including antibiotics, for >3 months before sample collections. Prior to inclusion in the study, volunteers were required to sign written informed consents. The Ethics Committee of Hangzhou center for diesease control and prevention (No. 202047) approved this study. The collection of fresh fecal samples was done in the morning. Then, preparation of fecal dilutions (10%) was done using the anaerobic phosphate buffer (PBS).

Suspension of 0.8 g Fresh fecal samples in 10 mL 0.1 mol/L anaerobic phosphate-buffered saline at pH 7.0 in an automatic fecal homogenizer was performed to obtain 10% (w/v) slurries. Then, 5 ml of the fecal suspensions were respectively inoculated into PSI and PSII medium as well as YCFA basal medium (control group). Batch fermentation was conducted *via* the inoculation of 1% fecal slurry into each vial followed by 24 h of incubation at 37°C.

SCFAs Quantification

Crotonic acid (0.6464 g) was added into 2.5% (W:V) metaphosphoric acid solution (100 ml) to prepare the crotonic acid metaphosphoric acid solution. Then, 0.5 mL of the fermentation broth was added to 0.1 mL of the crotonic acid metaphosphoric acid followed by acidification at -20°C for more than 24 h prior to gas chromatography (GC) assay. Centrifugation of the fermentation broth was done for 3 min at 12, 000 rpm. Subsequently, SCFAs were detected in the supernatant *via* GC (GC, Shi-madzu, GC-2010 Plus, Japan). GC assays were done using a DB-FFAP column (Agilent

Technologies, USA) and a H_2 flame ionization detector. Acetic, propionic, isobutyric, butyric, pentanoic, isopentanoic and caproic acids were obtained from Sigma.

16S rRNA Gene Sequencing

Bacterial 16S rRNA gene V3–V4 hypervariable regions were amplified using 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGG TWTCTAAT-3') primers. The sequencing was performed on an Illumina MiSeq 2500 platform, and analyzed OEbiotech Co. Ltd. (Shanghai, China) for microbial diversity analysis. Representatively, one sequence was obtained from every Operational taxonomic unit (OTU), which were clustered at a similarity of 97% using Mothur software system. Taxonomic annotation of the OTUs was done using the RDP Classifier against SILVA database v. 128, at a 0.7 confidence threshold. Communal structure was analyzed at the phylum level and genus level based on the taxonomic information. LEfSe evaluations of the various groups were conducted and thresholds on the logarithmic score of linear discriminant analysis (LDA) set at 2.0. Deposition of the 16S sequencing data in the NCBI Sequence Read Archive (SRA) database was done under the accession number PRJNA751711.

Statistical Analyses

Data are expressed as means ± SD of 3 replicates, and one-way ANOVA was used for statistical analyses by SPSS software. $p \leq 0.05$ denoted statistical significance.

RESULTS

Extraction and Purification of Polysaccharide-Peptides

The crude polysaccharide-peptides were acquired by water extraction and alcohol precipitation from *P. citrinopileatus* fruiting bodies. Following removal of free proteins, purification of the crude polysaccharide-peptides was done in a DEAE-cellulose column. Three fractions eluted by phosphate buffer (10 mM, pH 7.0), phosphate buffer (10 mM, pH 7.0) with NaCl (0.2 M), and phosphate buffer (10 mM, pH 7.0) with 1 M NaCl were obtained (**Figure 1A**). D1 and D2, which had high polysaccharide content detected by the phenol-sulfuric acid assay, were then obtained, concentrated, dialyzed, and subjected to additional purification. The results showed that both D1 as well as D2 generated a single peak each (PSI and PSII, respectively; **Figures 1B, C**).

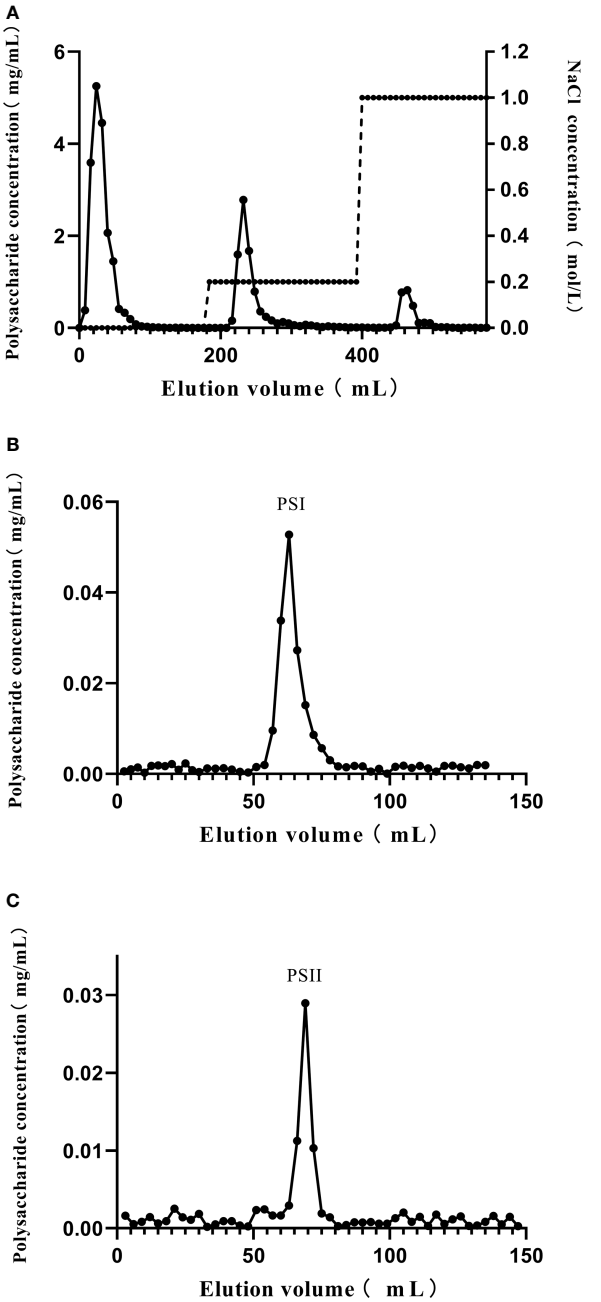

FIGURE 1 | Purification of polysaccharide-peptides from *P. citrinopileatus*. **(A)** The crude polysaccharide-peptides were separated by chromatography on DEAE-cellulose columns. **(B)** D1 was further subjected to Sephadex 200 column chromatography. **(C)** D2 was further separated by Sephadex 200 column chromatography.

Molecular Weight and Infrared Spectroscopy Analysis of PSI and PSII

The average molecular weight (Mw), number average molecular weight (Mn), and polydispersity (Mw/Mn) of PSI and PSII were evaluated by GPC. The Mw of PSI was 1.216×10^6 Da, while its Mw/Mn value was 1.06. The Mw of PSII was 1.608×10^4 Da, while its Mw/Mn value was 1.478 (**Table 1**).

Infrared absorption spectroscopy results are shown in **Figure 2**. PSI and PSII formed a broad peak about 3400 cm^{-1}, representing the stretching vibration absorption peak of hydroxyl groups (Wang et al., 2017), and an absorption peak near 2929 cm^{-1}, representing C-H (Dou et al., 2015), a characteristic peak of sugar. The peak near 1640 cm^{-1} indicated that both polysaccharides had C=O bonds (Liu et al., 2018), and the peak near 1017 cm^{-1} may be related to vibration of the ester carboxyl group (Lefsih et al., 2017). The absorption at 1411 cm^{-1} (**Figure 2A**) and 1418 cm^{-1} (**Figure 2B**) from O-H deformation indicated uronic acids presence. Regions at about 1078 cm^{-1} and 1047 cm^{-1} were representative of a galactan skeleton. Both

TABLE 1 | The average molecular weight (Mw), number average molecular weight (Mn), molecular weights, Z-average molecular weight (Mz) and the molecular weight of the highest peak (Mp) of PSI and PSII, as determined by GPC.

Molecular weight (Da)	PSI	PSII
Mn	1.147×10^6	1.088×10^4
Mw	1.216×10^6	1.608×10^4
Mz	1.231×10^6	2.135×10^4
Mp	4.308×10^5	1.601×10^4

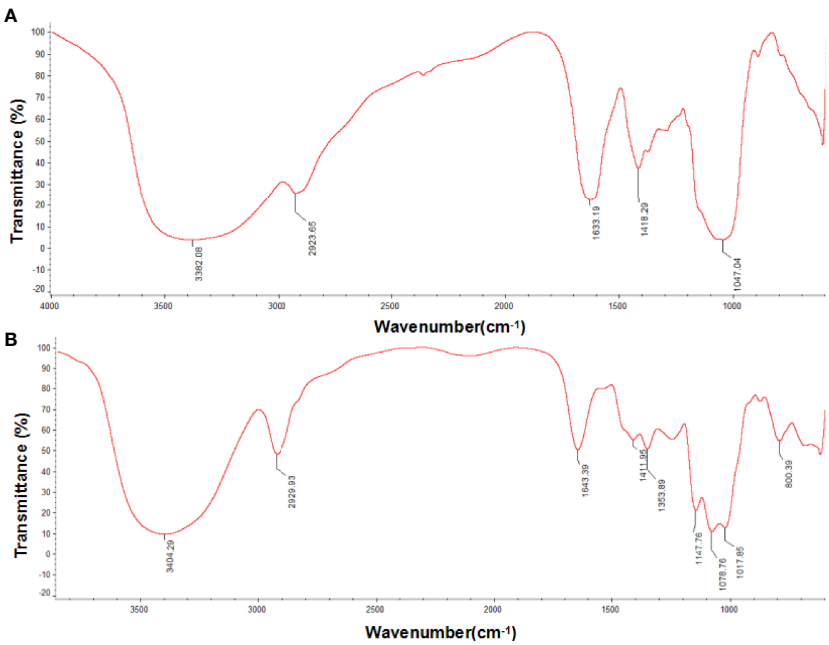

FIGURE 2 | Fourier transform infrared (FT-IR) spectra of *P. citrinopileatus* polysaccharide-peptides. **(A)** PSI, **(B)** PSII.

PSI and PSII exhibited typical polysaccharide absorption peaks with characteristic groups of sugars, but with differences in chemical structure. The peaks of PSI at 1353^{-1} cm and 1259 cm^{-1} indicated the presence of an S=O bond, corresponding to an ester sulfate group. PSI had a specific band in the region of 1200–1000 cm^{-1}, related to ring vibrations overlapping with stretching vibrations of the (C–OH) side groups as well as (C–O–C) glycosidic band vibrations.

Monosaccharide Component Analysis

PSI and PSII were subjected to acid hydrolysis and analyzed by GC-MS after hydrolysis and silylation. PSI was made up of arabinose, mannose, glucose, and galactose at a molar ratio of 1:6.2:6.3:67.2 (**Figure 3A**). PSII was a heteropolysaccharide made up of xylose, glucose, and galactose at a molar ratio of 1:83.9:4.2 (**Figure 3B**).

N-Terminal and Internal Amino Acid Sequences

N-terminal amino acid sequences of PSI and PSII were DLEQVVEGDW and KLSEGWERPP, respectively (**Supplemental Figure S1**). Evaluation of internal amino acid sequences revealed that the two peptide sequences of PSI, ITQSVLNIDR as well as VFQTNPNAFFR, were comparable to that of fruiting body lectin from *P. cornucopiae*. Moreover, the PSI sequences IQDKEGIPPDQQR, ISGLIYEETR, and KNGEILGGSWMVGAK were similar to those of ubiquitin, histone 4, and nucleoporin nup40 of *Lentinula edodes* (**Supplemental Table S1**). And three peptide sequences SSEREDLWQSTHVGHDEFSK, DGSLTGTYHSNV GEVPPTYHLSGR, and EDLWQSTHVGHDEFSK of PSII showed considerable homology with tamavidin-1 from *P. cornucopiae* (**Supplemental Table S2**). SYELPDGQVITIGNER and VAPEEHPVLLTEAPLNPK of PSII showed high similarity with actin-1 from *Hypsizygus marmoreus*.

Cytotoxicity of PSI and PSII in HepG2 Cells

As shown in **Table 2**, cell viability was maintained at a high level (more than 90%) for all concentrations of PSI and PSII. Thus, PSI and PSII were not cytotoxic towards HepG2 cells and could be used as potential hepatoprotective drugs.

Protective Roles of PSI and PSII *In Vitro*

Induction of HepG2 cell injury models reduced cell viability to approximately 55–60%. MTT assays showed that all

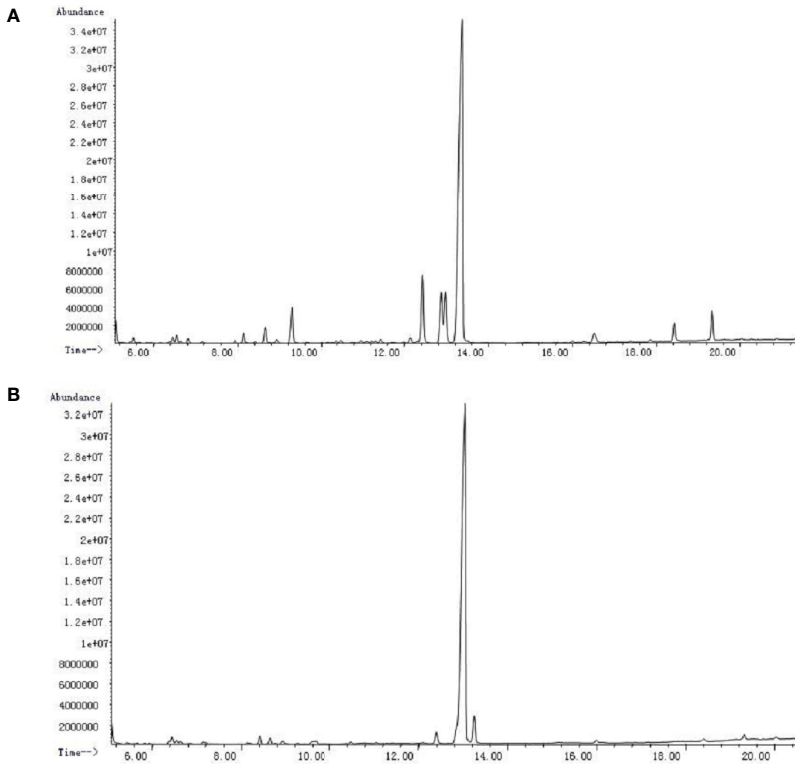

FIGURE 3 | GC-MS chromatograms of *P. citrinopileatus* polysaccharide-peptides. **(A)** PSI, **(B)** PSII.

TABLE 2 | The cytotoxicity of PSI and PSII in HepG2 cells.

Concentration (μg/mL)	Control	Cell viability (%)				
		100	200	500	800	1000
PSI	100	94.37 ± 3.62	100.40 ± 11.66	107.85 ± 5.28	108.71 ± 2.86	107.18 ± 7.24
PSII		95.18 ± 5.93	95.68 ± 2.87	106.05 ± 2.05	95.52 ± 4.25	94.93 ± 6.38

Data are presented as means ± SD (n = 3).

concentrations of PSI and PSII (30–80 μg/mL) increased the survival rates of injured cells. Compared with the model group, PSI and PSII increased cell viability up to 345.69% at 40 μg/mL and 96.15% at 50 μg/mL, respectively (**Figure 4**). The healing effects of PSI were better than those of PSII, and the survival rates of injured cells in the PSI and PSII treatment groups were both higher relative to the control group.

Protective Mechanisms of PSI and PSII on the Liver Cellular Index

Excessive alcohol and fat intake can disrupt TG metabolism in hepatocytes (Wang et al., 2015). When synthesis rate exceeds anabolism rate, TGs accumulate in the liver, representing the main pathogenic factor of fatty liver disease (Yin et al., 2017). Accordingly, we evaluated the effects of PSI and PSII on intracellular TG content, total SOD activity, and extracellular AST and ALT levels. Intracellular TGs were significantly decreased by PSI and PSII treatment relative to model group ($P < 0.05$; **Figure 5**), suggesting that PSI and PSII blocked cellular lipid accumulation. These findings were verified by Oil-red O staining (**Figure 6**).

Moreover, PSI and PSII enhanced SOD activities in the cells by 66.35% and 21.71% (**Table 3**), respectively, indicating that the polysaccharide-peptides could increase the antioxidant activity of the cells. Extracellular ALT and AST levels could serve as indicators of liver cell status, with high values indicating liver damage (Kew, 2000). Additionally, PSI and PSII significantly reduced extracellular ALT and AST activities ($P < 0.05$; **Table 3**), indicating that PSI and PSII could block extracellular transaminase release, maintain cell integrity, and protect the liver.

Therapeutic Mechanisms of PSI and PSII

We then analyzed changes in the adipogenic pathway in response to PSI and PSII by qRT-PCR (**Figure 7**). Notably,

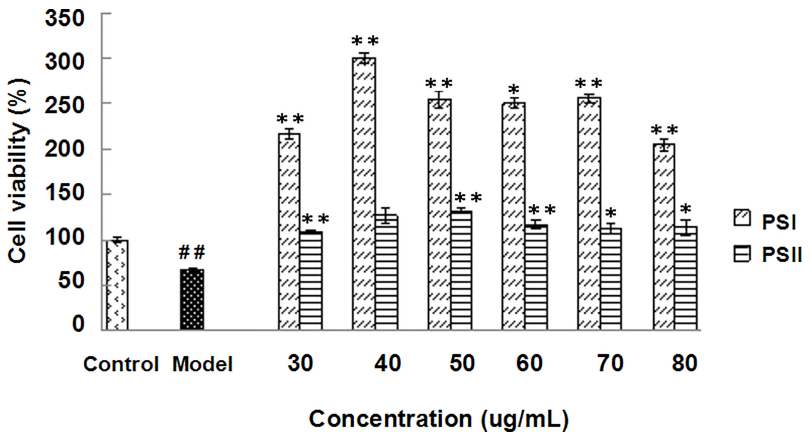

FIGURE 4 | Hepatoprotective effects of PSI and PSII *in vitro*. $^{\#\#}P < 0.01$ relative to the control group, $^{**}P < 0.01$ vs the model group, $^{*}P < 0.05$ compared to the model group.

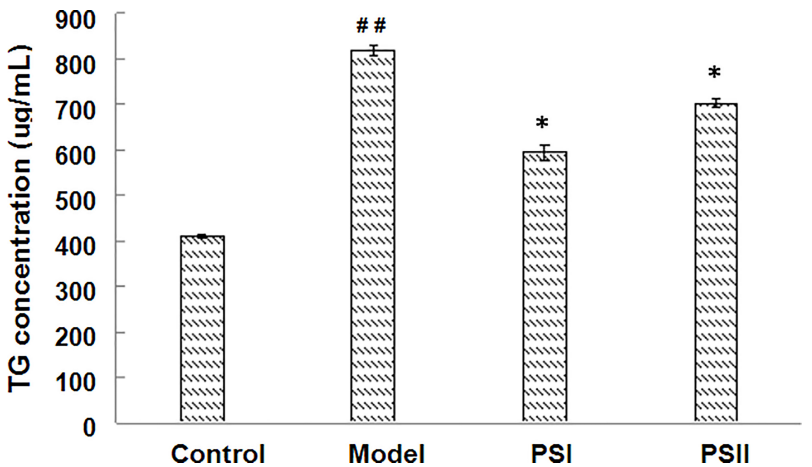

FIGURE 5 | Effects of PSI and PSII on intracellular TG contents. $^{\#\#}P < 0.01$ relative to the control group, $^{*}P < 0.05$ vs the model group.

expression levels of genes encoding AdipoR2, AMP-activated protein kinase (AMPK), peroxisome proliferator-activated receptor α (PPARα), carnitine palmitoyltransferase 1 (CPTI), and peroxisomal acyl-coenzyme A oxidase 1 (ACOX1) were markedly suppressed in the model group. Additionally, levels of TG in the model group were significantly increased. In contrast, treatment with PSI and PSII reduced hepatic lipogenesis by increasing *AdipoR2*, *AMPK*, *CPTl*, *PPARα*, and *ACOX1* expression in adipocytes to stimulate adiponectin secretion as well as activate the FFA metabolic pathway, thereby promoting triglyceride metabolism and reducing lipid accumulation.

Effects of PSI and PSII on SCFAs Production

Productions of SCFAs were achieved *via* fermentation of PSI and PSII in human fecal samples (**Figure 8**). Relative to the control group, propionate, acetate and butyrate concentrations were significantly higher in the PSI group. However, isobutyric acid and isopentanoic acid concentrations in the PSII group were low relative to the control group.

Effect of PSI and PSII on the Bacterial Community

Based on 16S rRNA sequencing, *Bacteroidetes*, *Proteobacteria*, *Firmicutes*, *Fusobacteriota* and *Actinobacteria* were found to be the abundant phyla in test samples (**Figure 9A**). After 24 h of fermentation with PSI and PSII, the increases in abundances of Bacteroidota and *Fusobacteriot*a for PSI and *Bacteroidota* and *Actinobacteria* and for PSII, respectively were significant. However, *Proteobacteria* enrichment in PSI and PSII groups were markedly suppressed relative to the control group.

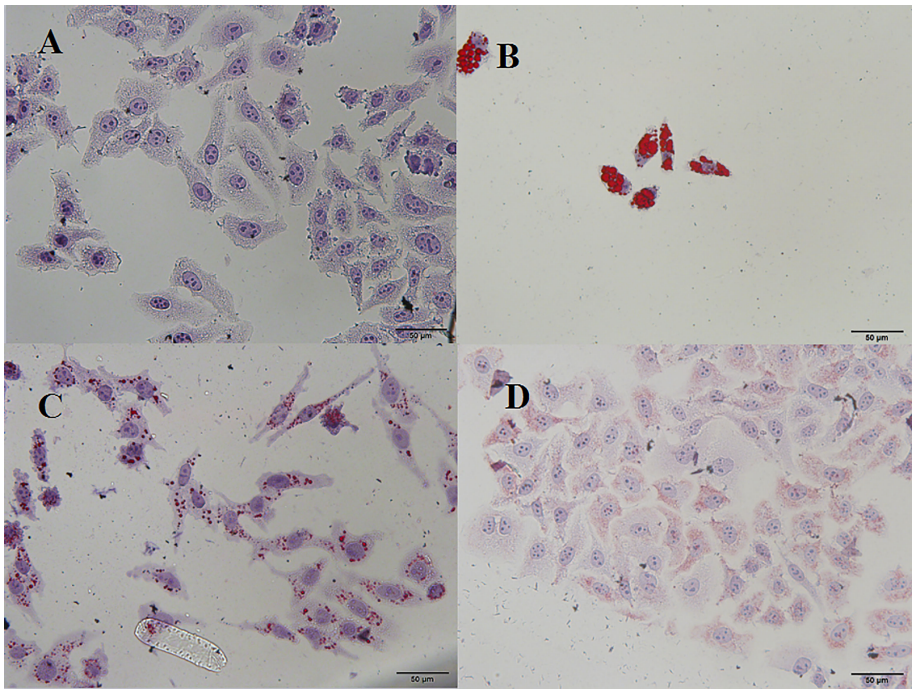

FIGURE 6 | Microscopic view of Oil-red O staining in HepG2 cells. **(A)** Control, **(B)** model, **(C)** PSI, **(D)** PSII.

The relative abundances of *Bacteroides, Fusobacterium, Faecalibacterium, Catenibacterium, Blautia* at the genus level were markedly higher in the PSI groups relative to the control group, and the abundance of *Escherichia-Shigella, Sutterella* and *Flavonifractor* were markedly low in the PSI group relative to the control (**Figure 9B**). In the PSII groups, the abundance of *Prevotella*, *Sutterella*, *Bifidobacterium*, *Lactococcus*, *Lactobacillus*, *Holdemanella, Catenibacterium* and *Blautia* were significantly high at genus levels, and the relative abundance of *Escherichia-Shigella, Faecalibacterium, Lachnospiraceae*_UCG 004, *Lachnoclostridium* and *Flavonifractor* was significantly lower relative to the control group.

Identification of bacterial taxa with significant differences in abundance between polysaccharide-peptides groups and the control groups was done by the linear discriminant analysis (LDA) effect size (LEfSe) method. Wilcoxon rank-sum test revealed that PSI enhanced the proliferation of *Bacteroides*, *Erysipelatoclostridiaceae*, *Hungatella*, *Carnobacterium*, *Acidaminococcaceae and Phascolarctobacterium*, while *Proteobacteria*, *Enterobacteriaceae, Oscillospiraceae*, *Desulfovibrionaceae*, *Lachnoclostridium*, *Flavonifractor*, *Odoribacter and Coriobacteriaceae* were markedly enriched in PSII group (**Figure 10A**). These results showed that PSI and PSII can regulate gut microbiota and promote probiotic proliferation.

Correlation Between Gut Microbiota and Metabolites Factors

Previous experiments showed that PSI had better effects on SCFAs production. Therefore, we studied the correlation between the production of SCFAs metabolites and the gut microbiota community of PSI. Acetic acid and propionic acid content were positively associated with the abundance *Bacteroidota* (**Figure 10B**). However, their contents were negatively associated with *Proteobacteri*a abundance. Butyric acid levels negatively correlated with *Campilobacterota* abundance.

DISCUSSION

NAFLD, a prevalent chronic liver disease, can cause several other diseases (Cohen et al., 2011). Some research groups have confirmed the protective effects of a polysaccharide-enriched fraction from *Pleurotus* sp. *in a* model of liver injury. Polysaccharides from *P. geesteranus* show antioxidant and hepatoprotective effects for preventing alcoholic liver diseases (Song et al., 2018). Moreover, mycelia zinc polysaccharides from *P. djamor* prevent CCl_4-induced acute liver damage, and alleviate liver as well as kidney injury in streptozocin-induced diabetic mice (Zhang et al., 2015).

The clinical diagnosis of NAFLD is based on elevation of serum ALT as well as AST, biochemical biomarkers of liver injury (Song et al., 2014; Liang et al., 2015). Herein, we investigated the antihyperlipidemic and hepatoprotective effects of PSI and PSII in a hepatocyte injury model. The results showed that PSI and PSII significantly reversed elevations in ALT/AST levels and increased cellular SOD activity and cell viability in HepG2 cells. SOD is a key player regulating cellular defense against reactive oxygen species. Moreover, PSI and PSII treatment markedly reduced TG contents, supporting the therapeutic effects of PSI and PSII in fatty liver.

TABLE 3 | Protective effects of PSI and PSII on cellular indexes.

	Control group	Model group	PSI	PSII
T-SOD (U/mg)	12.67 ± 0.54*	7.37 ± 1.09	12.26 ± 0.94*	8.97 ± 0.37*
ALT (U/mg)	19983 ± 580*	27871 ± 71	22086 ± 96*	24825 ± 367*
AST (U/mg)	4846 ± 16*	13880 ± 43	8953 ± 93*	11662 ± 180*

*Data are presented as means ± SD (n = 3). *P < 0.05 compared with the model group*

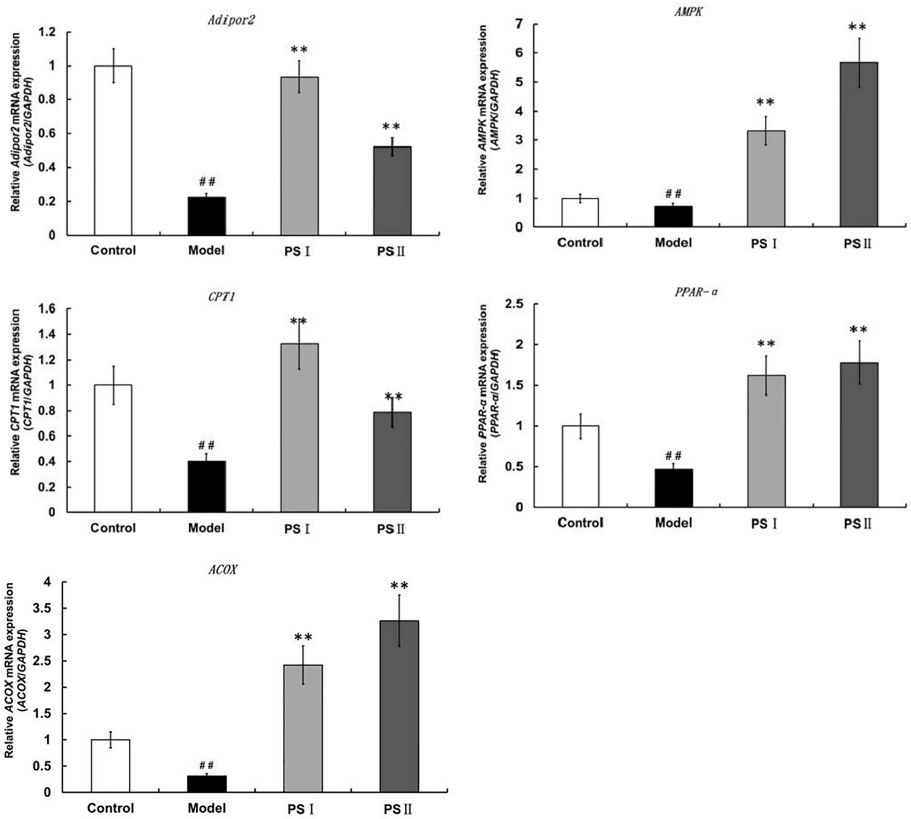

FIGURE 7 | qRT-PCR assessment of PSI and PSII on gene expressions in adiponectin pathways. Data are presented as means ± SD (n = 3). $^{\#\#}P < 0.01$ relative to the control group, $^{**}P < 0.01$ vs the model group.

Some groups reported the immune-promoting effects of polysaccharide-peptide and polysaccharide-protein complex obtained from mushrooms (Wang et al., 1996; Maruyama and Ikekawa, 2005). Li et al. isolated a polysaccharide-peptide complex from *Pleurotus abalonus* and found that it exhibited anti-proliferative, hypoglycaemic and antioxidant activities (Li et al., 2012). In this study, the IQDKEGIPPDQQR and KNGEILGGSWMVGAK sequences of PSI were similar to those of ubiquitin and nup40. nup40 has mitotic spindle checkpoint functions and inhibits cell cycle progression by binding to components of the ubiquitin-conjugating system (Chen et al., 2004). This may explain the effects of PSI on promoting cell viability.

FFAs stimulate hepatic TG synthesis and cause hepatic lipotoxicity as well as inflammation, thereby promoting NAFLD pathogenesis. Disruption of hepatocyte FFA metabolism and *de novo* FFA synthesis are involved in establishment of NAFLD. In hepatocytes, PPAR-α is a central regulator of TG and fatty acid metabolism (Aoyama et al., 1998; Kamijo et al., 2007) Additionally, ACOX1 catalyzes first as well as rate-limiting enzyme in fatty acid β-oxidation pathway of very-long-chain fatty acids in peroxisomes, which can be activated by PPARα to stimulate hepatic fatty acid oxidation (Fan et al., 1998). Gene expression assays revealed that PSI and PSII up-regulated *PPARα* and *ACOX1* as well as the downstream target gene *CPT1*, which is involved in peroxisomal as well as mitochondrial oxidation of fatty acids (Pathil et al., 2015). Thus, PSI and PSII stimulated FFA oxidation, which resulted in burning of excess energy in the liver.

Adiponectin, which is secreted by adipocytes, promotes the oxidation of fatty acids and modulates lipid metabolism by mediating the expressions of hepatic genes critical for lipid

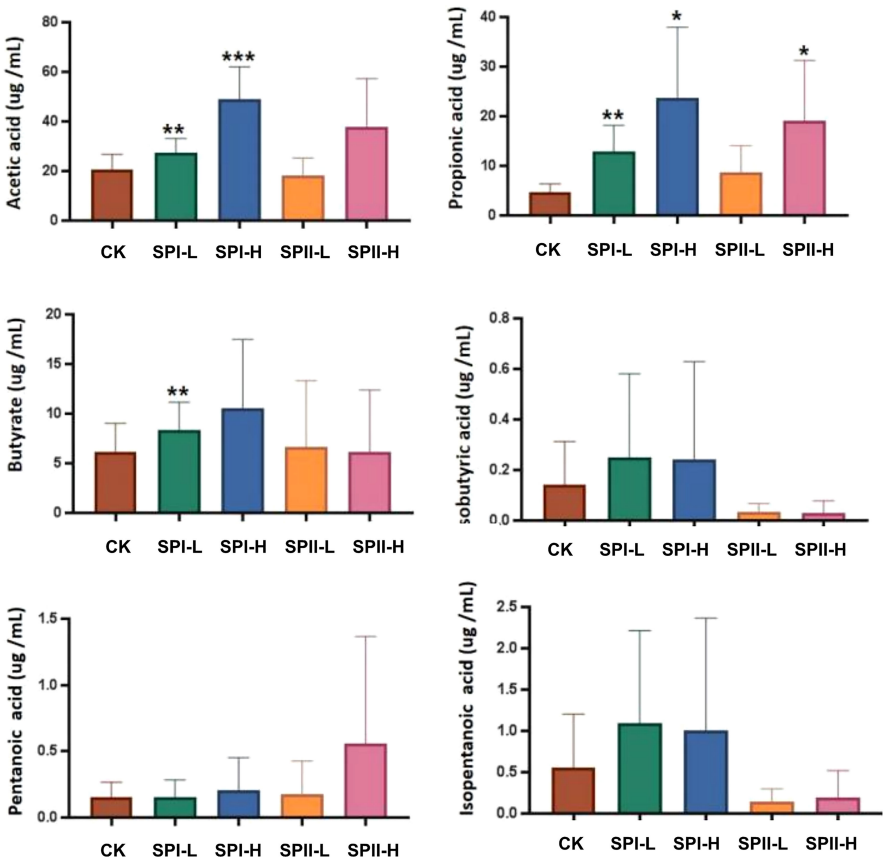

FIGURE 8 | Short-chain fatty acids (SCFAs) levels in fermentation broth after 24 h of fermentation. (G1) 40 μg/mL PSI, (G2) 200 μg/mL PSI, (H1) 40 ug/mL PSII, (H2) 200 ug/mL PSII. *$P < 0.05$, **$P < 0.01$ or ***$P < 0.001$ relative to control group.

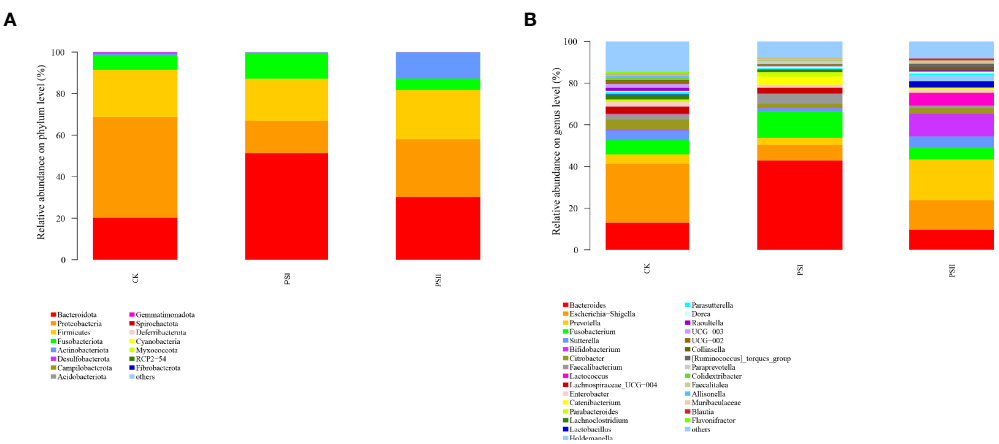

FIGURE 9 | Effects of PSI and PSII on the gut microbiota. **(A)** Bacterial phyla abundance; **(B)** Microbial communities on the genus level after various treatments. The ordinate denotes the species name, color gradient denotes species proportions, while the right side of the figure represents the value represented by the color gradient.

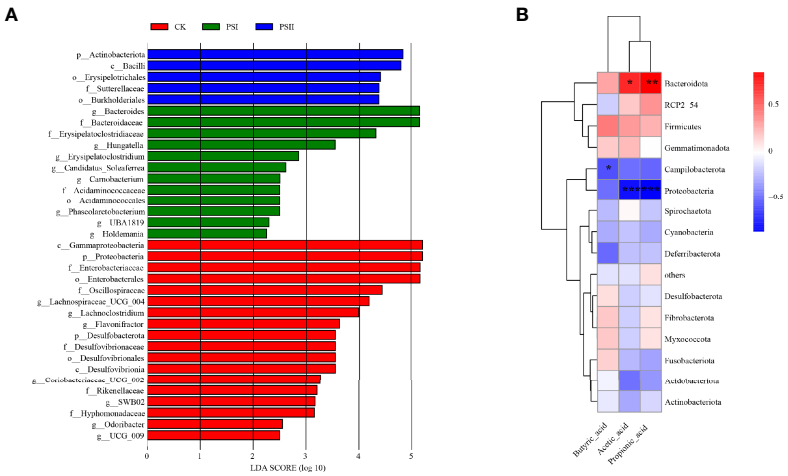

FIGURE 10 | Diagram of the linear discriminant (LDA) score and correlation analysis. **(A)** LDA score between the control and polysaccharide-peptieds groups (PSI, PSII), with a 2.0 score threshold. Diagram of the linear discriminant analysis (LDA) score between the control and polysaccharide-peptides groups (PSI, PSII), with a 2.0 score threshold. **(B)** Correlations between gut microbiota and metabolites SCFA factor fermented by PSI. The X axis represents SCFA species while the Y axis denotes the species. Color depth denotes R value size, while the legend denotes color intervals for various R values. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

metabolism (Liu et al., 2012). The AMPK pathway can also be activated adiponectin. AdipoR2 is an adiponectin receptor that regulates lipid metabolism, fatty-acid oxidation, and adiponectin-induced biological functions (Kadowaki and Yamauchi, 2005; Ghadge et al., 2018) Therefore, adiponectin and AdipoR2 are potential therapeutic targets to combat NAFLD. In this study, we found that that PSI and PSII increased *AMPK* and *AdipoR2* mRNA expression, implying that PSI and PSII activated adiponectin-related pathways and accelerated lipid metabolism.

SCFAs, major by-products of microbial metabolism in the liver, play important roles in maintaining large intestine functions and colon epithelial cells. Many prebiotics can increase the content of SCFAs (Liu et al., 2020; Zhao et al., 2021), which could promote the human health *via* an indirect effects. SCFAs can mediated the gut microbiota modulation of host physiological as well as pathological processes (Koh et al., 2016). This study showed that PSI fermentation markedly elevated acetic, propionic, and butyric acids concentrations. Acetic acid, butyric acid and propionic acid are important mediators of fermented dietary fibers on metabolism (den Besten et al., 2013). Butyrate and propionate could suppress lipolysis as well as *de novo* lipogenesis, thereby protecting against obesity development (Lin et al., 2012; Heimann et al., 2015). Acetic acid can reduce appetite and intestinal inflammation, and inhibits human fat decomposition (Duseja and Chawla, 2014; Gangarapu et al., 2014). It indicates that PSI enhances the proliferation of bacteria that produce acetic acid, butyric acid and propionic acid. We speculate that the PSI and PSII play a role through liver-gut axis system. Intestinal allows microbial metabolites and microbial-associated molecular patterns to translocate to the liver (Tripathi et al., 2018; Ciaula et al., 2020). Polysaccharide-peptides were metabolized by gut microbiota to produce SCFAs and in turn influence liver functions. The study of the gut-liver axis can help us to understand the basic biology of NAFLD and identify the mechanisms between gut microbiota and liver damage, which offers an opportunity for interventions during liver disease.

The microbiota is required to maintain hepatic homeostasis. The changes in the gut microbiota have disclosed the interaction with the pathogenesis of NAFLD. The severity of NAFLD is associated with dysbiosis of the intestinal (Marra and Svegliati-Baroni, 2018). On account of gut microbiota is linked to NAFLD, we evaluated the effects of PSI and PSII on human intestinal microflora structure. Analysis of bacterial community composition showed that PSI and PSII promoted the proliferation of probiotics and inhibit the harmful bacteria, such as *Escherichia-Shigella*. There was a markedly elevated abundance of *Phascolarctobacterium*, *Bacteroides*, *Fusobacterium*, *Faecalibacterium*, *Catenibacterium*, *Blautia* at the genus level in the PSI groups. *Phascolarctobacterium* is a SCFAs producer, including acetic acid and propionic acid (Wu et al., 2017). Accumulated evidences showed that *Phascolarctobacterium faecium* has beneficial effects on the NAFLD rat model (Panasevich et al., 2016). *Fusobacteriota* metabolized carbohydrates into butyrate which has benefits to the host (Zhang et al., 2021). In the PSII groups, there were markedly higher abundances of *Bifidobacterium*, *Lactococcus*, *Lactobacillus*, *Desulfovibrionaceae*, *Lachnospiraceae*, *Odoribacter*, *Coriobacteriaceae* and *Blautia* at the genus level. *Bifidobacterium* and *Lactobacillus* are well-known probiotics. *Blautia* is also a potential probiotic (Liu et al., 2021). *Coriobacteriaceae* can metabolize cholesterol-derived metabolites (McGavigan et al., 2017). The family of *Lachnospiraceae* produces short-chain fatty acids, and previous study corroborated *Lachnospiraceae* in attenuating colitis and obesity (Guo et al., 2020). *Odoribacter splanchnicus* induced Th17 cell activated and protected mice from colitis and colorectal cancer (Xing et al., 2021). Evidence suggests that PSI and PSII can be used as potential prebiotics to regulate gut microbiota.

CONCLUSION

In this study, we purified and characterized PSI and PSII from *P. citrinopileatus*. These compounds exhibited hepatoprotective effects in injured HepG2 cells by increasing the survival rates of injured cells, reducing the accumulation of intracellular TGs, elevating the intracellular activity of SOD, decreasing extracellular transaminase release, and maintaining cell integrity. These results suggested that PSI and PSII exert potent antioxidant and hepatoprotective activities by regulating the expression of hepatic genes. On the other hand, PSI and PSII supplementation to an *in vitro* fermentation model affected human gut microbiota richness as well as diversity. PSII enhanced the abundance of *Oscillospiraceae*, *Lachnospiraceae*, *Lachnoclostridium*, *Flavonifractor*, *Desulfobacterota*, *Desulfovibrionaceae*, *Coriobacteriaceae*, *Rikenellaceae*, *Odoribacter*. PSI and PSII decrease the abundance of *Escherichia-Shigella* genera. Moreover, PSI enhanced the metabolism of acetic, propionic, as well as butyric acids in bacteria, which resulted in elevated concentrations of SCFAs. These SCFAs exert an indirect effect on intestinal microbiota and liver functions. PSI and PSII might play a role through liver-gut axis system. These findings provided important insights into the potential applications of PSI and PSII in ameliorating symptoms of liver disease and gut microbiota modulation.

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.ncbi.nlm.nih.gov/genbank/, PRJNA751711.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee of Hangzhou center for disease control and prevention. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

WL and SZ designed the study and all experiments. YH and YG carried out the assays. YG and XP analyzed the data. YH drafted this manuscript. WL and SZ revised the manuscript. All authors have read 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/fcimb.2022.892049/full#supplementary-material

REFERENCES

Aoyama, T., Peters, J. M., Iritani, N., Nakajima, T., Furihata, K., Hashimoto, T., et al. (1998). Altered Constitutive Expression of Fatty Acid-Metabolizing Enzymes in Mice Lacking the Peroxisome Proliferator-Activated Receptor Alpha (PPARalpha). *J. Biol. Chem.* 273 (10), 5678–5684. doi: 10.1074/jbc.273.10.5678

Boursier, J., Mueller, O., Barret, M., Machado, M., Fizanne, L., Araujo-Perez, F., et al. (2016). The Severity of Nonalcoholic Fatty Liver Disease Is Associated With Gut Dysbiosis and Shift in the Metabolic Function of the Gut Microbiota. *Hepatology* 63 (3), 764–775. doi: 10.1002/hep.28356

Chen, X. Q., Du, X., Liu, J., Balasubramanian, M. K., and Balasundaram, D. (2004). Identification of Genes Encoding Putative Nucleoporins and Transport Factors in the Fission Yeast Schizosaccharomyces Pombe: A Deletion Analysis. *Yeast* 21 (6), 495–509. doi: 10.1002/yea.1115

Ciaula, A. D., Baj, J., Garruti, G., Celano, G., Angelis, M. D., Wang, H. H., et al (2020). Liver Steatosis, Gut-Liver Axis, Microbiome and Environmental Factors. A Never-Ending Bidirectional Cross-Talk. *J. Clin. Med.* 9, 2648. doi: 10.3390/jcm9082648

Cohen, J. C., Horton, J. D., and Hobbs, H. H. (2011). Human Fatty Liver Disease: Old Questions and New Insights. *Science* 332 (6037), 1519–1523. doi: 10.1126/science.1204265

Cui, H.-Y., Wang, C.-L., Wang, Y.-R., Li, Z.-J., and Zhang, Y.-N. (2015). The Polysaccharide Isolated From Pleurotus Nebrodensis (PN-S) Shows Immune-Stimulating Activity in RAW264.7 Macrophages. *Chin. J. Nat. Med.* 13 (5), 355–360. doi: 10.1016/S1875-5364(15)30026-1

den Besten, G., van Eunen, K., Groen, A. K., Venema, K., Reijngoud, D. J., and Bakker, B. M. (2013). The Role of Short-Chain Fatty Acids in the Interplay Between Diet, Gut Microbiota, and Host Energy Metabolism. *J. Lipid Res.* 54 (9), 2325–2340. doi: 10.1194/jlr.R036012

Dou, J., Meng, Y., Liu, L., Li, J., Ren, D., and Guo, Y. (2015). Purification, Characterization and Antioxidant Activities of Polysaccharides From Thinned-Young Apple. *Int. J. Biol. Macromol.* 72, 31–40. doi: 10.1016/j.ijbiomac.2014.07.053

Duseja, A., and Chawla, Y. K. (2014). Obesity and NAFLD: The Role of Bacteria and Microbiota. *Clin. Liver Dis.* 18 (1), 59–71. doi: 10.1016/j.cld.2013.09.002

Fan, C. Y., Pan, J., Usuda, N., Yeldandi, A. V., Rao, M. S., and Reddy, J. K. (1998). Steatohepatitis, Spontaneous Peroxisome Proliferation and Liver Tumors in Mice Lacking Peroxisomal Fatty Acyl-CoA Oxidase. Implications for Peroxisome Proliferator-Activated Receptor Alpha Natural Ligand Metabolism. *J. Biol. Chem.* 273 (25), 15639–15645. doi: 10.1074/jbc.273.25.15639

Farrell, G. C., and Larter, C. Z. (2006). Nonalcoholic Fatty Liver Disease: From Steatosis to Cirrhosis. *Hepatology* 43 (2 Suppl 1), S99–S112. doi: 10.1002/hep.20973

Gangarapu, V., Yildiz, K., Ince, A. T., and Baysal, B. (2014). Role of gut microbiota: obesity and NAFLD. *Turk J. Gastroenterol.* 25 (2), 133–140. doi: 10.5152/tjg.2014.7886

Garcia, M. C., Amankwa-Sakyi, M., and Flynn, T. J. (2011). Cellular Glutathione in Fatty Liver *In Vitro* Models. *Toxicol. Vitro* 25 (7), 1501–1506. doi: 10.1016/j.tiv.2011.05.011

Ghadge, A. A., Khaire, A. A., and Kuvalekar, A. A. (2018). Adiponectin: A Potential Therapeutic Target for Metabolic Syndrome. *Cytokine Growth Factor Rev.* 39, 151–158. doi: 10.1016/j.cytogfr.2018.01.004

Guo, H., Chou, W. C., Lai, Y., Liang, K., Tam, J. W., Brickey, W. J., et al. (2020). Multi-Omics Analyses of Radiation Survivors Identify Radioprotective Microbes and Metabolites. *Science* 370 (6516), eaay9097. doi: 10.1126/science.aay9097

Han, Q., Fan, H., Peng, J., Zhou, L., and Gan, L. (2019). Pleiotropic Function of Vitamin C on Fatty Acids in Liver and Muscle of Juvenile Grass Carp (Ctenopharyngodon Idella). *Aquaculture* 512, 734352. doi: 10.1016/j.aquaculture.2019.734352

Heimann, E., Nyman, M., and Degerman, E. (2015). Propionic Acid and Butyric Acid Inhibit Lipolysis and *De Novo* Lipogenesis and Increase Insulin-Stimulated Glucose Uptake in Primary Rat Adipocytes. *Adipocyte* 4 (2), 81–88. doi: 10.4161/21623945.2014.960694

Kadowaki, T., and Yamauchi, T. (2005). Adiponectin and Adiponectin Receptors. *Endocr. Rev.* 26 (3), 439–451. doi: 10.1210/er.2005-0005

Kamijo, Y., Hora, K., Kono, K., Takahashi, K., Higuchi, M., Ehara, T., et al. (2007). PPARalpha Protects Proximal Tubular Cells From Acute Fatty Acid Toxicity. *J. Am. Soc. Nephrol.* 18 (12), 3089–3100. doi: 10.1681/ASN.2007020238

Kew, M. C. (2000). Serum Aminotransferase Concentration as Evidence of Hepatocellular Damage. *Lancet* 355 (9204), 591–592. doi: 10.1016/S0140-6736(99)00219-6

Koh, A., De Vadder, F., Kovatcheva-Datchary, P., and Backhed, F. (2016). From Dietary Fiber to Host Physiology: Short-Chain Fatty Acids as Key Bacterial Metabolites. *Cell* 165 (6), 1332–1345. doi: 10.1016/j.cell.2016.05.041

Lefsih, K., Giacomazza, D., Dahmoune, F., Mangione, M. R., Bulone, D., San Biagio, P. L., et al. (2017). Pectin From Opuntia Ficus Indica: Optimization of Microwave-Assisted Extraction and Preliminary Characterization. *Food Chem.* 221, 91–99. doi: 10.1016/j.foodchem.2016.10.073

Liang, B., Guo, X. L., Jin, J., Ma, Y. C., and Feng, Z. Q. (2015). Glycyrrhizic Acid Inhibits Apoptosis and Fibrosis in Carbon-Tetrachloride-Induced Rat Liver Injury. *World J. Gastroenterol.* 21 (17), 5271–5280. doi: 10.3748/wjg.v21.i17.5271

Li, N., Li, L., Fang, J. C., Wong, J. H., Ng, T. B., Jiang, Y., et al. (2012). Isolation and Identification of a Novel Polysaccharide-Peptide Complex With Antioxidant, Anti-Proliferative and Hypoglycaemic Activities From the Abalone Mushroom. *Biosci. Rep.* 32 (3), 221–228. doi: 10.1042/BSR20110012

Li, C., Li, Q., Li, J., Zhang, N., Li, Y., Li, Y., et al. (2021). Expression and Localization of Adiponectin and its Receptors (AdipoR1 and AdipoR2) in the Hypothalamic-Pituitary-Ovarian Axis of Laying Hens. *Theriogenology* 159, 35–44. doi: 10.1016/j.theriogenology.2020.10.020

Lin, H. V., Frassetto, A., Kowalik, E. J.Jr., Nawrocki, A. R., Lu, M. M., Kosinski, J. R., et al. (2012). Butyrate and Propionate Protect Against Diet-Induced Obesity and Regulate Gut Hormones *via* Free Fatty Acid Receptor 3-Independent Mechanisms. *PloS One* 7 (4), e35240. doi: 10.1371/journal.pone.0035240

Li, S., and Shah, N. P. (2014). Antioxidant and Antibacterial Activities of Sulphated Polysaccharides From Pleurotus Eryngii and Streptococcus Thermophilus ASCC 1275. *Food Chem.* 165, 262–270. doi: 10.1016/j.foodchem.2014.05.110

Liu, Q., Ge, X., Chen, L., Cheng, D., Yun, Z., Xu, W., et al. (2018). Purification and Analysis of the Composition and Antioxidant Activity of Polysaccharides From Helicteres Angustifolia L. *Int. J. Biol. Macromol.* 107, 2262–2268. doi: 10.1016/j.ijbiomac.2017.10.095

Liu, W., Li, X., Zhao, Z., Pi, X., Meng, Y., Fei, D., et al. (2020). Effect of Chitooligosaccharides on Human Gut Microbiota and Antiglycation. *Carbohydr. Polym.* 242, 116413. doi: 10.1016/j.carbpol.2020.116413

Liu, X., Mao, B., Gu, J., Wu, J., Cui, S., Wang, G., et al. (2021). Blautia-A New Functional Genus With Potential Probiotic Properties? *Gut Microbes* 13 (1), 1–21. doi: 10.1080/19490976.2021.1875796

Liu, Q., Yuan, B., Lo, K. A., Patterson, H. C., Sun, Y., and Lodish, H. F. (2012). Adiponectin Regulates Expression of Hepatic Genes Critical for Glucose and Lipid Metabolism. *Proc. Natl. Acad. Sci. U.S.A.* 109 (36), 14568–14573. doi: 10.1073/pnas.1211611109

Marchesini, G., Brizi, M., Bianchi, G., Tomassetti, S., Bugianesi, E., Lenzi, M., et al. (2001). Nonalcoholic Fatty Liver Disease: A Feature of the Metabolic Syndrome. *Diabetes* 50 (8), 1844–1850. doi: 10.2337/diabetes.50.8.1844

Marra, F., and Svegliati-Baroni, G. (2018). Lipotoxicity and the Gut-Liver Axis in NASH Pathogenesis. *J. Hepatol.* 68 (2), 280–295. doi: 10.1016/j.jhep.2017.11.014

Maruyama, H., and Ikekawa, T. (2005). Combination Therapy of Transplanted Meth-A Fibrosarcoma in BALB/c Mice With Protein-Bound Polysaccharide EA6 Isolated From Enokitake Mushroom Flammulina Velutipes (W.Curt.:Fr.) Singer and Surgical Excision. *Int. J. Med. Mushr.* 7(1&2): 213–220. doi: 10.1615/IntJMedMushr.v7.i12.200

McGavigan, A. K., Henseler, Z. M., Garibay, D., Butler, S. D., Jayasinghe, S., Ley, R. E., et al. (2017). Vertical Sleeve Gastrectomy Reduces Blood Pressure and Hypothalamic Endoplasmic Reticulum Stress in Mice. *Dis. Model Mech.* 10 (3), 235–243. doi: 10.1242/dmm.027474

Panasevich, M. R., Morris, E. M., Chintapalli, S. V., Wankhade, U. D., Shankar, K., Britton, S. L., et al. (2016). Gut Microbiota are Linked to Increased Susceptibility to Hepatic Steatosis in Low-Aerobic-Capacity Rats Fed an Acute High-Fat Diet. *Am. J. Physiol. Gastrointest Liver Physiol.* 311 (1), G166–G179. doi: 10.1152/ajpgi.00065.2016

Pathil, A., Liebisch, G., Okun, J. G., Chamulitrat, W., Schmitz, G., and Stremmel, W. (2015). Ursodeoxycholyl Lysophosphatidylethanolamide Modifies Aberrant Lipid Profiles in NAFLD. *Eur. J. Clin. Invest.* 45 (9), 925–931. doi: 10.1111/eci.12486

Ren, D., Jiao, Y., Yang, X., Yuan, L., Guo, J., and Zhao, Y. (2015). Antioxidant and Antitumor Effects of Polysaccharides From the Fungus Pleurotus Abalonus. *Chem. Biol. Interact.* 237, 166–174. doi: 10.1016/j.cbi.2015.06.017

Sheng, Y., Zhao, C., Zheng, S., Mei, X., Huang, K., Wang, G., et al. (2019). Anti-Obesity and Hypolipidemic Effect of Water Extract From Pleurotus Citrinopileatus in C57BL/6J Mice. *Food Sci. Nutr.* 7 (4), 1295–1301. doi: 10.1002/fsn3.962

Song, E., Fu, J., Xia, X., Su, C., and Song, Y. (2014). Bazhen Decoction Protects Against Acetaminophen Induced Acute Liver Injury by Inhibiting Oxidative Stress, Inflammation and Apoptosis in Mice. *PloS One* 9 (9), e107405. doi: 10.1371/journal.pone.0107405

Song, X., Shen, Q., Liu, M., Zhang, C., Zhang, L., Ren, Z., et al. (2018). Antioxidant and Hepatoprotective Effects of Intracellular Mycelium Polysaccharides From Pleurotus Geesteranus Against Alcoholic Liver Diseases. *Int. J. Biol. Macromol* 114, 979–988. doi: 10.1016/j.ijbiomac.2018.04.001

Spruiell, K., Gyamfi, A. A., Yeyeodu, S. T., Richardson, R. M., Gonzalez, F. J., and Gyamfi, M. A. (2015). Pregnane X Receptor-Humanized Mice Recapitulate Gender Differences in Ethanol Metabolism But Not Hepatotoxicity. *J. Pharmacol. Exp. Ther.* 354 (3), 459–470. doi: 10.1124/jpet.115.224295

Svegliati-Baroni, G., Candelaresi, C., Saccomanno, S., Ferretti, G., Bachetti, T., Marzioni, M., et al. (2006). A Model of Insulin Resistance and Nonalcoholic Steatohepatitis in Rats: Role of Peroxisome Proliferator-Activated Receptor-Alpha and N-3 Polyunsaturated Fatty Acid Treatment on Liver Injury. *Am. J. Pathol.* 169 (3), 846–860. doi: 10.2353/ajpath.2006.050953

Tripathi, A., Debelius, J., Brenner, D. A., Karin, M., Loomba, R., Schnabl, B., et al. (2018). The Gut-Liver Axis and the Intersection With the Microbiome. *Nat. Rev. Gastroenterol. Hepatol.* 15 (7), 397–411. doi: 10.1038/s41575-018-0011-z

Utzschneider, K. M., and Kahn, S. E. (2006). Review: The Role of Insulin Resistance in Nonalcoholic Fatty Liver Disease. *J. Clin. Endocrinol. Metab.* 91 (12), 4753–4761. doi: 10.1210/jc.2006-0587

Wang, S. N., Chen, Q. J., Zhu, M. J., Xue, F. Y., Li, W. C., Zhao, T. J., et al. (2018). An Extracellular Yellow Laccase From White Rot Fungus Trametes Sp. F1635 and Its Mediator Systems for Dye Decolorization. *Biochimie* 148, 46–54. doi: 10.1016/j.biochi.2018.02.015

Wang, L., Liu, F., Wang, A., Yu, Z., Xu, Y., and Yang, Y. (2017). Purification, Characterization and Bioactivity Determination of a Novel Polysaccharide From Pumpkin (Cucurbita Moschata) Seeds. *Food Hydrocolloids* 66, 357–364. doi: 10.1016/j.foodhyd.2016.12.003

Wang, X. Y., Luo, J. P., Chen, R., Zha, X. Q., and Pan, L. H. (2015). Dendrobium Huoshanense Polysaccharide Prevents Ethanol-Induced Liver Injury in Mice by Metabolomic Analysis. *Int. J. Biol. Macromol* 78, 354–362. doi: 10.1016/j.ijbiomac.2015.04.024

Wang, H. X., Ng, T. B., Ooi, V. E., Liu, W. K., and Chang, S. T. (1996). A Polysaccharide-Peptide Complex From Cultured Mycelia of the Mushroom Tricholoma Mongolicum With Immunoenhancing and Antitumor Activities. *Biochem. Cell Biol.* 74 (1), 95–100. doi: 10.1139/o96-010

Wu, S., and Chen, L. (2017). Preparation and Antioxidant Activities of Oligosaccharides Derived From Pleurotus Eryngii Polysaccharides. *J. Food Process. Preservation* 41 (4), e13007. doi: 10.1111/jfpp.13007

Wu, F., Guo, X., Zhang, J., Zhang, M., Ou, Z., and Peng, Y. (2017). Phascolarctobacterium Faecium Abundant Colonization in Human Gastrointestinal Tract. *Exp. Ther. Med.* 14 (4), 3122–3126. doi: 10.3892/etm.2017.4878

Xing, C., Wang, M., Ajibade, A. A., Tan, P., Fu, C., Chen, L., et al. (2021). Microbiota Regulate Innate Immune Signaling and Protective Immunity Against Cancer. *Cell Host Microbe* 29 (6), 959–974 e957. doi: 10.1016/j.chom.2021.03.016

Yin, Y., Gao, L., Lin, H., Wu, Y., Han, X., Zhu, Y., et al. (2017). Luteolin Improves Non-Alcoholic Fatty Liver Disease in Db/Db Mice by Inhibition of Liver X Receptor Activation to Down-Regulate Expression of Sterol Regulatory Element Binding Protein 1c. *Biochem. Biophys. Res. Commun.* 482 (4), 720–726. doi: 10.1016/j.bbrc.2016.11.101

Yu, Z., Liu, L., Xu, Y., Wang, L., Teng, X., Li, X., et al. (2015). Characterization and Biological Activities of a Novel Polysaccharide Isolated From Raspberry (Rubus Idaeus L.) Fruits. *Carbohydr. Polym.* 132, 180–186. doi: 10.1016/j.carbpol.2015.06.068

Zhang, C., Li, J., Wang, J., Song, X., Zhang, J., Wu, S., et al. (2017). Antihyperlipidaemic and Hepatoprotective Activities of Acidic and Enzymatic Hydrolysis Exopolysaccharides From Pleurotus Eryngii SI-04. *BMC Complement Altern. Med.* 17 (1), 403. doi: 10.1186/s12906-017-1892-z

Zhang, J., Meng, H., Kong, X., Cheng, X., Ma, T., He, H., et al. (2021). Combined Effects of Polyethylene and Organic Contaminant on Zebrafish (Danio Rerio): Accumulation of 9-Nitroanthracene, Biomarkers and Intestinal Microbiota. *Environ. Pollut.* 277, 116767. doi: 10.1016/j.envpol.2021.116767

Zhang, J., Meng, G., Zhang, C., Lin, L., Xu, N., Liu, M., et al. (2015). The Antioxidative Effects of Acidic-, Alkalic-, and Enzymatic-Extractable Mycelium Zinc Polysaccharides by Pleurotus Djamor on Liver and Kidney of Streptozocin-Induced Diabetic Mice. *BMC Complement Altern. Med.* 15, 440. doi: 10.1186/s12906-015-0964-1

Zhao, Z., Liu, W., and Pi, X. (2021). *In Vitro* Effects of Stachyose on the Human Gut Microbiota. *Starch Stärke* 73 (7-8), 2100029. doi: 10.1002/star.202100029

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Effect and Mechanism of *Bifidobacterium animalis* B94 in the Prevention and Treatment of Liver Injury in Rats

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Objective: To investigate the effect of *Bifidobacterium animalis* B94 on the prevention and treatment of liver injury in rats and to elucidate the underlying mechanism of this relationship.

Methods: Specific pathogen-free (SPF) rats were selected as the healthy control group, liver injury group and B94 treatment group, with 6 rats in each group. After the model was established, the experimental animals were tested for serum liver function indicators, gut microbiota composition, metabolite composition, and histopathology.

Results: The albumin/globulin ratio and serum TBA, alanine aminotransferase, aspartate aminotransferase, and indirect bilirubin levels in the B94 treatment group were significantly lower than those in the liver injury group. 16S rRNA analysis showed that the gut microbiota of the three groups of rats were significantly different. Metabolic profile analysis showed that there were significant differences in the gut metabolomes of the three groups. Haematoxylin–eosin staining of the intestinal mucosa and liver tissues showed that the degree of liver and intestinal tissue damage in the B94 treatment group was significantly lower than that in the liver injury group.

Conclusion: *Bifidobacterium animalis* B94 can affect the process of liver injury in rats by improving liver function, reducing intestinal damage, and regulating gut microbiota and metabolite production.

Keywords: liver injury, gut microbiota, metabolome, *Bifidobacterium animalis* B94, probiotics

Abbreviations: HC, Healthy control; GalN, Galactosamine; TBA, Total bile acid; TBil, Total bilirubin; DBil, Direct bilirubin; IBil, Indirect bilirubin; ALT, Alanine transaminase; AST, Aspartate transaminase; ALP, Alkaline phosphatase; GGT, γ-glutamyl transferase; GPDA, Glycylproline dipeptidyl aminopeptidase; MIP-1α, Macrophage inflammatory protein-1α; MCP-1, Macrophage chemoattractant protein-1.

INTRODUCTION

The liver has the functions of synthesis, detoxification, metabolism, secretion, biotransformation, and immune defence and is one of the important organs of the human body. When hepatocytes are severely damaged by viruses, alcohol, drugs, etc., they become necrotic in large numbers, resulting in serious impairment or failure of the abovementioned functions. Then, liver failure occurs and manifests as a group of clinical syndromes, including impaired coagulation and jaundice, hepatic encephalopathy, ascites, etc. Liver failure is a common clinical syndrome of severe liver disease, with rapid progression, a high mortality rate and a poor overall prognosis.

The intestine is the largest bacterial reservoir in the body, and it decomposes, utilizes, transforms and produces a large number of exogenous substances, participates in the enterohepatic circulation of bile acids and other substances, promotes tissue development, regulates immunity, and prevents invasion and infection by foreign microorganisms through nutritional competition and occupancy protection (Guarner and Malagelada, 2003; Backhed et al., 2005). Once microbiota dysbiosis occurs, the intestinal mucosal barrier can become disrupted, which will cause immune dysregulation of the host, and some pathogens can even translocate to other organs, such as the liver and induce disease (Yan et al., 2011; Dapito et al., 2012; Lv et al., 2014). Liver injury can cause intestinal motility, immune and metabolic changes and intestinal mucosal barrier damage, which can in turn lead to structural changes in the gut microbiota, endotoxaemia, and bacterial translocation (Acharya and Bajaj, 2017). Therefore, ameliorating gut microbiota dysbiosis has become an important element in the prevention and treatment of liver injury or liver failure. However, there is a general lack of corresponding products worldwide.

Studies have shown that *Bifidobacterium animalis* B94 can alleviate *Helicobacter pylori*-associated gastritis through the production of organic acids, autolysins, mucins and bacteriocins and immunomodulatory effects and that it promotes a high eradication rate for *Helicobacter pylori*-induced diarrhoea (Zhang et al., 2008; Donkor et al., 2012; Cekin et al., 2017). In regulating the immune system, *B. animalis* B94 can induce substantial secretion of pro- and anti-inflammatory cytokines from normal peripheral blood monocyte-derived monocytes and macrophages and effectively induce Th17 and Treg cell differentiation for immunomodulation (Donkor et al., 2012). B94 can also improve intestinal microecological dysregulation, inhibit the adhesion and colonization of intestinal pathogenic bacteria through multiple pathways, and be used for the treatment of irritable bowel syndrome (Basturk et al., 2016).

In this study, we investigated the effect of *B. animalis* B94 in the prevention and treatment of liver injury and the potential mechanism of action using a rat model of liver failure. The results of this study are important for research on the molecular mechanism and clinical application of B94.

MATERIALS AND METHODS

Microorganism Preparation

The *Bifidobacterium animalis* B94 strain was obtained from Lallemand (Ontario, Canada). B94 was inoculated into MRS liquid medium (Thermo Fisher, Shanghai, China) anaerobically at 37°C for 24 h to logarithmic growth phase. Bacteria were collected by centrifugation at 5000 ×g for 10 min, washed three times and resuspended in sterile saline (0.9% (w/v)) at a concentration of 3×10^9 CFU/mL.

Animal Experimental Design

Eighteen specific pathogen-free (SPF)-grade Sprague Dawley (SD) male rats (Shanghai SLAC Laboratory Animal Co., Ltd., China), weighing between 200-300 g, were housed at room temperature (22 ± 2°C) and given ad libitum access to food and water, with 12/12 h alternating between day and night (light and dark) each day. After a 2-week period of cohousing, the rats were randomly divided into three groups of six animals each, consisting of the healthy control group (HC): gavage with saline + intraperitoneal injection of saline; liver injury group (GalN): gavage with saline + intraperitoneal injection of D-galactosamine; and B94 treatment group (B94+GalN): gavage with B94 + intraperitoneal injection of D-galactosamine. All the experimental procedures followed the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. The study protocol was approved by the Animal Experimentation Ethics Committee of Zhejiang University.

For the first seven days, rats were gavaged with saline or 1 mL of a freshly prepared B94 solution (3×10^9 CFU/mL) once per day, according to the grouping described above. On day 7, the rat liver injury model was constructed by intraperitoneal injection of saline or 1.1 g/kg D-galactosamine (Sigma, Saint Louis, MO, USA), according to the grouping. At 24 h after D-galactosamine injection, the rats were anaesthetized with 400 mg/kg chloral hydrate (Sigma, Saint Louis, MO, USA) delivered intraperitoneally, and the rats were operated on with strict aseptic technique. The liver and colon tissues were fixed in 10% paraformaldehyde and embedded in paraffin for pathological specimen preparation. Finally, stool was taken from the rectum for 16S rRNA and metabolome assays.

Liver Function Assay

The serum was separated by centrifugation at 3000 rpm for 10 min at room temperature, and 400 μL of serum was collected and analysed by a fully automated biochemical analyser (Hitachi 7600-210; Tokyo, Japan) to determine alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein, globulin, albumin, total bile acid (TBA), total bilirubin (TBil), direct bilirubin (DBil), indirect bilirubin (IBil), γ-glutamyl transferase (GGT) and glycylproline dipeptidyl aminopeptidase (GPDA) levels.

Serum Cytokine Assay

We used a Bio-plex Pro™ rat cytokine 23-plex assay (Bio-Rad, Hercules, California, USA) kit to determine serum IL-1α, IL-1β,

IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, IL 17, IL-18, colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), growth-regulating α protein (GRO/KC), interferon-γ (IFN-γ), macrophage colony-stimulating factor (M-CSF), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1α (MIP-1α) MIP-3α, tumour necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF), and regulated on activation normal T-cell expressed and secreted (RANTES) levels.

16S rRNA Sequencing and Analysis

DNA was extracted from 0.2 g of stool using a QIAamp Fast DNA Stool Mini Kit (Qiagen, Valencia, USA). GGACTACHVGGGTWTCTAAT-3') for PCR amplification of the 16S rRNA V3+V4 region (Lv et al., 2021a). The PCR products were purified using AMPure XPbeeds (Agencourt, Beckman Coulter, USA), and the library was quantified by real-time quantitative PCR. Paired-end sequencing (2 × 300 bp) was performed using the Illumina MiSeq platform (Illumina, San Diego, CA). Raw reads were cleaned, filtered and then merged using FLASH (v1.2.11). Vsearch (v2.3.4) was used to select operational taxonomic units (OTUs) with sequence similarity greater than 97%. OTU clustering, identification based on the RDP database and the NCBI-16S database, and subsequent statistical analysis of microbial diversity and differential enrichment were analysed using QIIME (v1.9.1).

Detection and Analysis of Faecal Metabolites

Samples for metabolite assays were pretreated as described previously (Jiang et al., 2021; Lv et al., 2021b). Briefly, 20 mg of faeces was added to 800 μL of precooled chromatography grade methanol and then homogenized three times using a Precellys Evolution instrument (Bertin Technologies, USA) at 5,000 rpm for 30 s with 15 s intervals between the rounds for extraction. After centrifugation at 14,000 rpm for 15 min, the supernatant was filtered through a 0.22 μm membrane, and 20 μL of heptadecanoic acid (1 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) was added to the filtrate as an internal reference and then dried under nitrogen at room temperature. After drying, the samples were methoxymated with methoxypyridine (Sigma-Aldrich, St. Louis, MO, USA) and trimethylsilylated with N,O-bis(trimethylsilyl)acetamide containing 1% trimethylsilyl chloride. The pretreated samples were analysed with an Agilent 7890A-5975C GC-MS system (Agilent, USA). The downstream data were compared with the NIST 17 database programmatically to identify the corresponding metabolites (matching score ≥ 80%).

Histopathological Evaluation

The fixed and embedded liver and colon samples were sectioned (2 μm) and stained with haematoxylin-eosin (HE). Sequentially, the images were observed under a microscope to evaluate the liver damage according to the histopathological activity index (HAI) method (Knodell et al., 1981; Lv et al., 2021b) and the intestinal epithelium abnormalities according to the reference reported by C.J. Chiu et al. (1970).

Statistical Methods

For the comparison of liver function, liver and colon histopathology scores, gut bacterial α diversity and faecal metabolites among groups, the Shapiro-Wilk test was first used to determine whether the data of each group conformed to a normal distribution. One-way ANOVA followed by the Student-Newman-Keuls method was used to compare any two data sets that were normally distributed; otherwise, the Mann-Whitney U test was used. The Wilcoxon rank sum test combined with the Benjamini-Hochberg method was used to compare the relative abundances of each taxonomic level of gut bacteria between groups. Correlations between variables were analysed by Spearman's rank correlation test. $P < 0.05$ was considered statistically significant.

RESULTS

Bifidobacterium animalis B94 Alleviated D-Galactosamine-Induced Abnormal Liver Function and Immune Dysfunction

After D-galactosamine injection, the total protein, albumin and globulin levels were significantly lower in the GalN group rats than in the HC group rats, while the albumin/globulin ratio and ALT, AST, ALP, TBA, TBil, DBil, IBil, GGT and GPDA levels were significantly higher in the GalN group rats than in the HC group rats (**Figure 1**). B94 treatment significantly ameliorated the decrease in globulin levels induced by D-galactosamine, as well as the increase in the albumin/globulin ratio and ALT, AST, TBA, TBil and IBil levels.

To investigate the effect of B94 on immune function in rats, we examined the levels of 23 cytokines in rat serum. The results showed that D-galactosamine injection caused an upregulation of MCP-1 and MIP-1α levels in rat serum compared with that in healthy controls. B94 gavage attenuated the D-galactosamine-induced elevation of MCP-1 levels so that the difference with the HC group was no longer significant.

Bifidobacterium animalis B94 Ameliorated Liver and Intestinal Pathological Damage Caused by D-Galactosamine

The HE staining results of liver samples showed inflammatory cell infiltration and large areas of hepatocyte necrosis in liver sections of the GalN group rats, and liver injury was significantly reduced in liver sections of the B94+GalN group rats (**Figure 2A**). The HAI scores (**Figure 2B**) also showed that B94 treatment tended to alleviate the increase in the degree of liver damage caused by D-galactosamine ($P = 0.057$ in B94+GalN *vs.* GalN). The HE staining results of colon samples showed that the epithelium was damaged, with broken or missing villi and disintegrated crypts, in the GalN group (**Figure 2A**). In the B94 +GalN group, this damage was ameliorated, and significantly

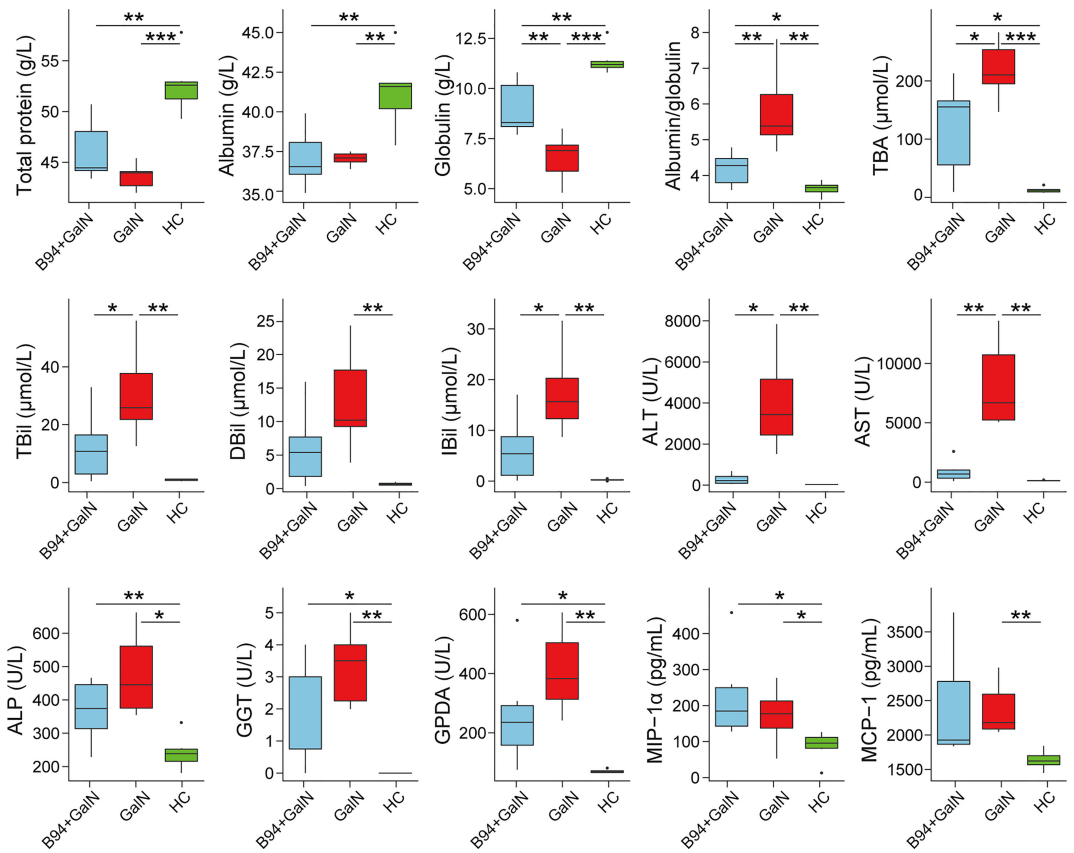

FIGURE 1 | B94 treatment alleviates D-galactosamine-induced abnormal liver function indicator levels and immune dysfunction. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

lower organ damage scores were also observed compared with the GalN group (**Figure 2B**).

Bifidobacterium animalis B94 Alleviates the Alteration of the Gut Microbiota Caused by D-Galactosamine

A total of 888,670 reads from 18 faecal samples were obtained by 16S rRNA sequencing. For alpha diversity, the community biodiversity and community richness, as indicated by the Shannon index and Chao1 index, were not significantly different among the HC, GalN and B94+GalN groups (**Figure 3A**). For beta diversity, the principle coordinate analysis (PCoA) showed that the microbiota profiles of the three groups could be clearly separated (**Figure 3B**), and the results of analysis of similarities (ANOSIM) confirmed that there was a significant intergroup difference in the bacterial composition among the three groups ($P = 0.001$).

We further performed a statistical analysis of the changes in the relative abundances of individual bacterial taxa among the HC, GalN and B94+GalN groups. Compared with those in the HC group, the phylum Acidobacteriota, the families *Caulobacteraceae* and *Desulfovibrionaceae* and the genus *Brevundimonas*, which belong to the phylum Pseudomonadota, were enriched in the GalN group. Conversely, the family *Erysipelotrichaceae* and the genera *Lachnoclostridium*, *Christensenellaceae* sp., uncultured *Ruminococcus* subsp. (GCA 900066225), *Oscillibacter*, *Ruminococcaceae* UCG-013 and Clostridiales Family XIII sp., which belong to the phylum Bacillota, and the family *Rikenellaceae* and genus *Alistipes*, which belong to the phylum Bacteroidota, were depleted in the GalN group (**Figure 3C**).

B94 treatment partially ameliorated the changes in the gut microbiota induced by D-galactosamine. First, compared to that in the GalN group, the genus *Paenalcaligenes*, which belongs to the phylum Pseudomonadota, was depleted in the B94+GalN group, and the genus GCA-900066225, which belongs to the phylum Bacillota, was enriched (**Figure 3C**). Second, B94 mitigated the changes in the relative abundances of the following taxa caused by D-galactosamine so that the differences with the HC group were no longer significant, including the phylum Acidobacteriota, the families *Caulobacteraceae* and *Desulfovibrionaceae* and genus *Brevundimonas*, which belong to phylum Pseudomonadota, the family *Erysipelotrichaceae* and genera *Christensenellaceae* sp., *Oscillibacter*, *Ruminococcaceae* UCG-013 and Clostridiales Family XIII sp., which belong to the phylum Bacillota, and the

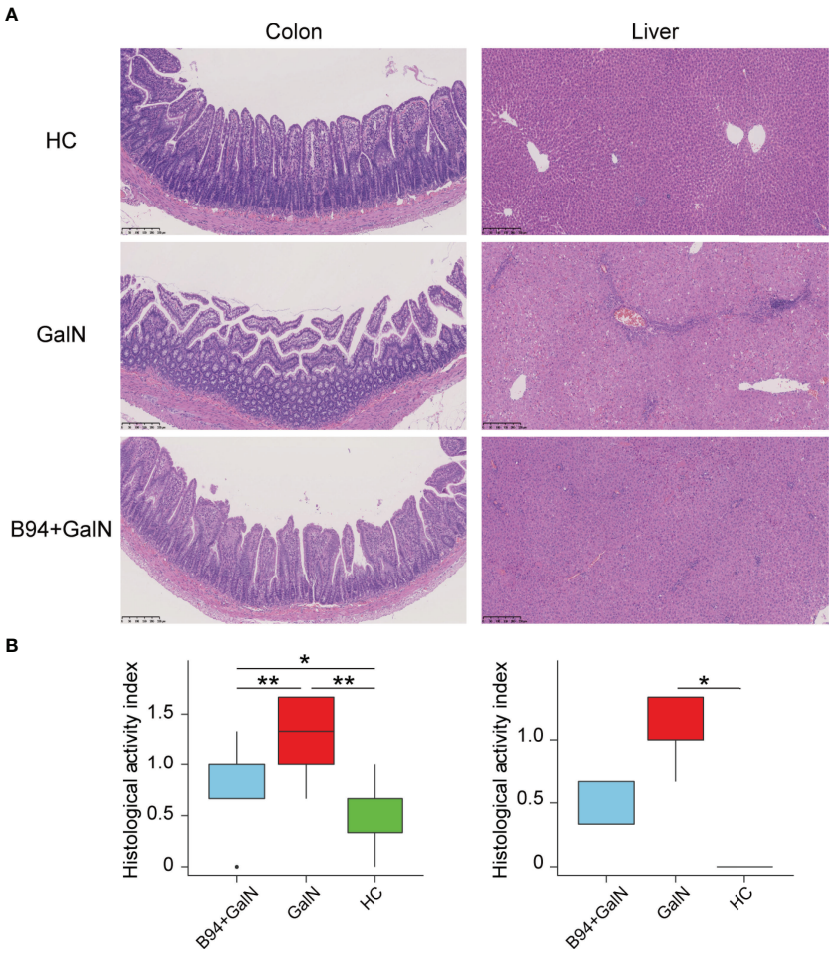

FIGURE 2 | Representative images of colon and liver samples stained with HE **(A)** and histological scores **(B)** indicating that B94 treatment partially alleviated D-galactosamine-induced organ damage. * $P < 0.05$; ** $P < 0.01$.

family *Rikenellaceae* and genus *Alistipes*, which belong to the phylum Bacteroidota (**Figure 3C**).

To study the microbial association network, the correlation of OTUs at the family and genus levels from the three groups was analysed using the Sparse Correlations for Compositional data (SparCC) method. The absolute value of the correlation coefficient R >0.2 and $P < 0.05$ were used as screening thresholds. As a result, bacterial taxa belonging to the phylum Bacteroidota and Bacillota, such as *Muribaculaceae*, *Ruminococcaceae*, *Rikenellaceae*, *Lachnospiraceae*, *Lactobacillaceae*, *Bacteroidaceae*, *Peptococcaceae*, and *Staphylococcaceae*, are located at the core of the network (**Figure 4**), suggesting their important role in the gut microbiota changes related to B94 treatment. Additionally, B94 treatment-ameliorated microbial taxa, such as the family *Rikenellaceae* and the genus *Alistipes*, were positively correlated with *Ruminococcaceae* (or *Ruminococcus*), Clostridiales sp. and *Lachnospiraceae*. This is in line with our finding that B94 mitigated the D-galactosamine-induced changes in the abundances of taxa belonging to the phyla Bacteroidota and Bacillota.

Bifidobacterium animalis B94 Improved Gut Metabolic Disorders

We investigated the effect of B94 on D-galactosamine-induced gut metabolic disorder based on gas chromatography-mass spectrometry (GC-MS) analysis. In total, 105 metabolites were identified from the three groups. The results of orthogonal projections to latent structures discriminant analysis (OPLS-DA) showed that the HC, GalN and B94+GalN groups were clearly separated (**Figure 5A**), indicating that their metabolome profiles were significantly different. The variable importance for projection (VIP) values of 13 metabolites (hypoxanthine, L-leucine, L-isoleucine tetracosanol, palmitelaidic acid, D-allose, L-threose, 2-aminobutanoic acid, malic acid, β-alanine, pentadecanoic acid, N-acetyl glucosamine and valeric acid) were greater than 1.5, suggesting their contributions to differentiating the metabolome profiles of the three groups in this OPLS-DA model (**Figure 5B**).

Next, we compared the levels of each faecal metabolite among the different groups. Compared to that in the HC group, 1-heptadecanol was significantly depleted in the GalN group,

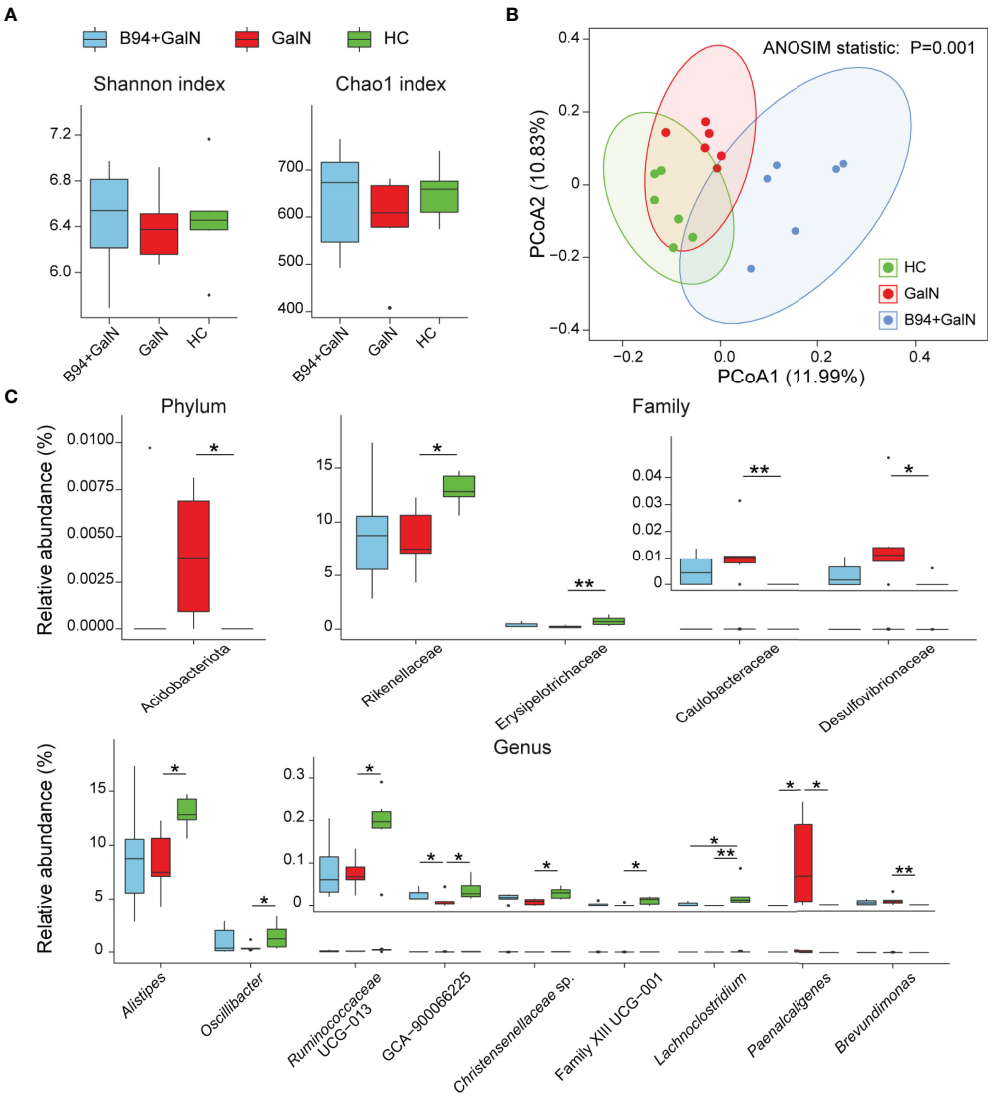

FIGURE 3 | B94 treatment alleviates D-galactosamine-induced dysbiosis of the gut microbiota. **(A)** Box plot of species richness and flora diversity estimated based on the Shannon indexes and Chao1 indexes. **(B)** Two-dimensional PCoA plot based on the unweighted UniFrac matrix confirmed by ANOSIM. **(C)** Alterations in the relative abundances of bacterial taxa in the GalN, B94+GalN, and HC groups at the phylum, family and genus levels. * $P < 0.05$; ** $P < 0.01$.

whereas 4-hydroxybenzene acetic acid, 5-aminovaleric acid, hypoxanthine, L-methionine and N-acetyl glucosamine were enriched. After B94 treatment, the D-galactosamine-induced changes in the levels of 1-heptadecanol, 5-aminovaleric acid, L-methionine and N-acetyl glucosamine were no longer significant (**Figure 5C**).

The Beneficial Effects of B94 Pretreatment on the Gut Microbiota, Metabolism, Serum Liver Function Indicators and Cytokines Were Closely Correlated

To explore the potential relationship between the gut microbiota and metabolism, we performed an association analysis using Spearman's rank correlation. Both the absolute value of the correlation coefficient R > 0.4 and $P < 0.05$ were used as the screening threshold to identify significant results. Among the metabolites, 1-heptadecanol was widely associated with microbes, such as positively correlated with *Alistipes*, *Christensenellaceae* sp., Clostridiales Family XIII sp., GCA-900066225, and *Erysipelotrichaceae*, and negatively correlated with *Caulobacteraceae* (**Figure 6A**). Moreover, *Alistipes* was negatively correlated with 5-aminovaleric acid (R = -0.72, P = 8.49E-04) and L-methionine (R = -0.63, P = 4.99E-03); *Erysipelotrichaceae* (R = -0.60, P = 8.98E-03) and *Ruminococcaceae* UCG-013 (R = -0.83, P = 1.78E-05) were negatively correlated with N-acetyl glucosamine; L-methionine was positively correlated with *Desulfovibrionaceae*; 5-aminovaleric acid was negatively correlated with *Christensenellaceae* sp. and *Ruminococcaceae* UCG-013 (**Figure 6A**).

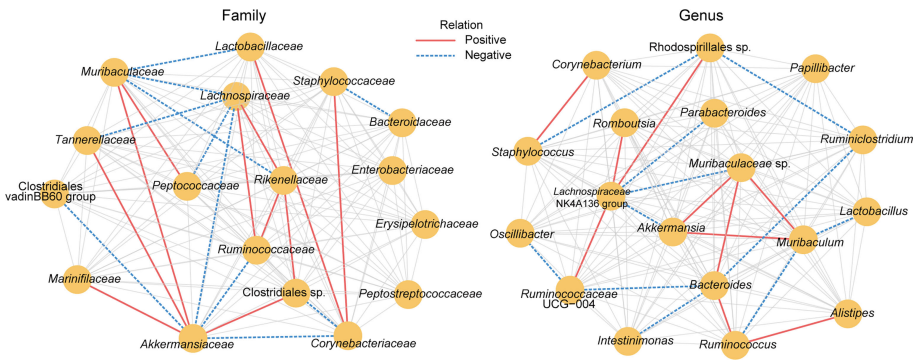

FIGURE 4 | The microbial association network influenced by B94 treatment was inferred by using the SparCC method. Significant correlations were screened (|R| >0.2 and $P < 0.05$) and are displayed with solid red lines (positive) and dashed blue lines (negative).

Next, we analysed the association of gut microbes and metabolites with liver function indicators and cytokines. First, the TBil, IBil, ALT, AST and TBA levels were positively correlated with the metabolite N-acetyl glucosamine and microbes *Caulobacteraceae*, *Desulfovibrionaceae*, *Brevundimonas* and *Paenalcaligenes*, which belong to the phylum Acidobacteriota or Pseudomonadota, and were negatively correlated with *Erysipelotrichaceae* and GCA-900066225, which belong to the phylum Bacillota (**Figure 6B**). However, globulin was correlated with these microbes and metabolites in the opposite way as TBA and the others mentioned above. Additionally, globulin was negatively correlated with 5-aminovaleric acid and positively correlated with *Alistipes* and *Christensenellaceae* sp.; ALT was negatively correlated with *Alistipes*, *Christensenellaceae* sp., and *Ruminococcaceae* UCG-013 but positively correlated with

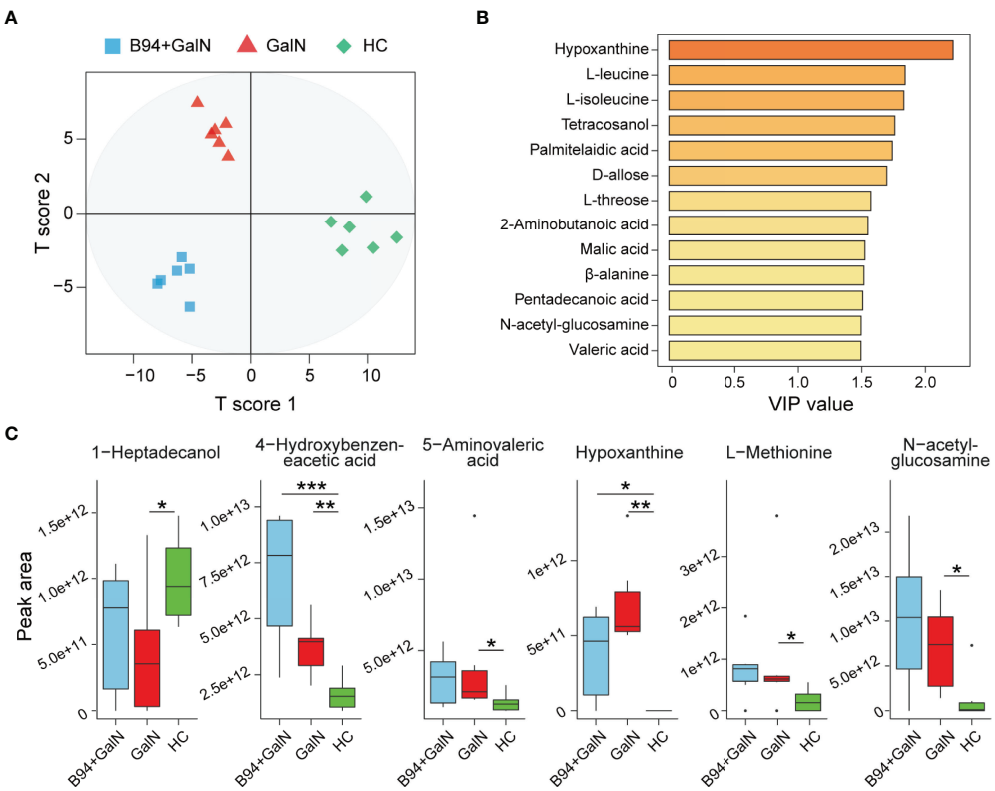

FIGURE 5 | B94 treatment alleviates D-galactosamine-induced gut metabolism disorder. **(A)** OPLS-DA plot illustrating clear separation of the gut metabolic profiles of the GalN, B94+GalN, and HC groups. **(B)** VIP values of 13 metabolites with the highest contribution to the separation of the three groups in the OPLS-DA model. **(C)** Levels of six differentially distributed metabolites in the three groups. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

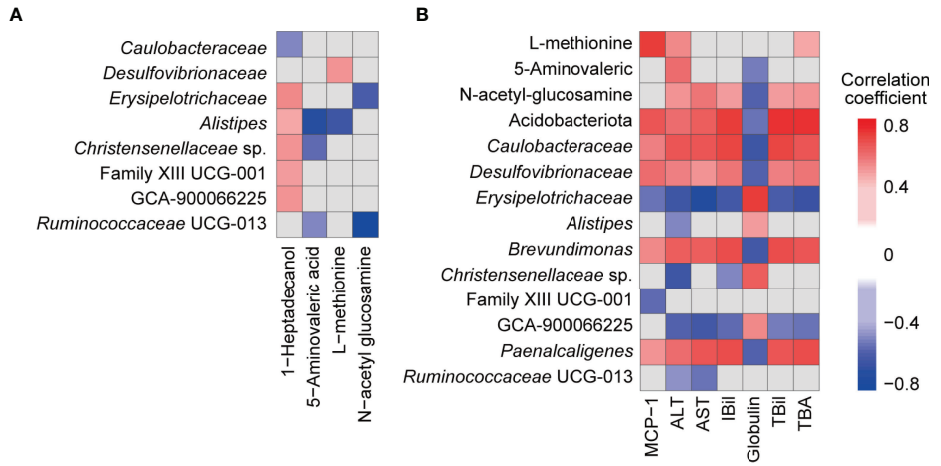

FIGURE 6 | Associations among faecal bacteria, faecal metabolites, and blood indicators influenced by B94 treatment (|R| >0.4 and $P < 0.05$). **(A)** Correlation of B94-influenced faecal bacteria with faecal metabolites. **(B)** Correlation of B94-influenced faecal bacteria and metabolites with liver function indicators and cytokines.

5-aminovaleric acid and L-methionine; AST was positively correlated with *Ruminococcaceae* UCG-013; IBil was negatively correlated with *Christensenellaceae* sp.; L-methionine was positively correlated with TBA. Second, the serum cytokine MCP-1 was positively correlated with Acidobacteriota, *Caulobacteraceae*, *Desulfovibrionaceae*, *Brevundimonas*, *Paenalcaligenes* and L-methionine but negatively correlated with *Erysipelotrichaceae* and Clostridiales Family XIII sp. (**Figure 6B**).

DISCUSSION

Liver failure refers to serious liver injury caused by various factors that is difficult to treat and has a poor prognosis. Liver injury is closely related to the gut microbiota. Alterations in gut microbiota have an important influence on the occurrence and development of liver failure. Probiotics have an important role in regulating the gut microbiota to prevent diseases; however, the role and mechanism of most probiotics in the prevention and treatment of liver failure are not clear. In this study, we found that B94 alleviated D-galactosamine-induced abnormal liver function, elevated MCP-1 levels in serum, and attenuated pathological damage to the colon and liver. B94 also ameliorated D-galactosamine-induced alterations in the gut microbiota and metabolome. These results provide a basis for the use of B94 in the prevention and treatment of liver injury.

Our results suggest that B94 extensively ameliorated D-galactosamine-induced liver function indicator abnormalities. The liver is the main site of globulin synthesis, and damage to hepatocytes decreases globulin levels. ALT and AST are important liver disease indicators that are mainly distributed in hepatocytes and will rapidly enter the bloodstream when hepatocytes are damaged. Total bile acids are a group of cholesterol metabolites in hepatic catabolism and intestinal-hepatic circulation, and bilirubin is the main metabolite of iron porphyrin compounds in the body. The serum TBA and bilirubin concentrations are elevated when liver disease or obstruction occurs, which can sensitively reflect liver function. B94 administration can significantly ameliorate the elevation in albumin/globulin ratio, ALT, AST, TBA, TBil and IBil, the decrease in globulin levels, and reduce liver and intestinal damage caused by D-galactosamine. This finding shows that B94 has a good ability to prevent liver injury.

Our results suggest that B94 ameliorated D-galactosamine-induced gut microbiota dysbiosis. First, B94 slowed the depletion of potentially beneficial gut bacteria. For example, the beneficial effects of *Ruminococcaceae* UCG-013 and uncultured *Ruminococcus* subsp. (GCA-900066225) on health include degradation of resistant starch and stabilization of the intestinal barrier (Cann et al., 2016). The relative abundance of *Christensenellaceae* in the human gut is inversely related to host body mass index (BMI), obesity and inflammatory bowel disease (Waters and Ley, 2019). *Odoribacteraceae* has been found in a group of Japanese people who have lived over a century and can produce isoallo-lithocholic acid, which is one of the most potent antimicrobial agents selectively against gram-positive microbes such as *Clostridium difficile* (Jiang et al., 2021). Our correlation analysis results are consistent with this finding; for example, *Erysipelotrichaceae* and GCA-900066225 were negatively correlated with serum TBil, IBil, ALT and TBA; *Alistipes*, *Christensenellaceae* sp. and *Ruminococcaceae* UCG-013 were negatively correlated with ALT; *Erysipelotrichaceae* and Family XIII UCG-001 were negatively correlated with MCP-1. Second, B94 slowed the increase in conditionally pathogenic bacteria. For example, *Paenalcaligenes hominis*, particularly its extracellular vesicles, is a risk factor for vagus nerve-mediated cognitive impairment (Lee et al., 2020); members of *Caulobacteraceae*, such as *Brevundimonas*, play a role in infections (Liu et al., 2021); and *Desulfovibrionaceae* are sulfate-reducing and endotoxin-producing bacteria (Liechty et al., 2020). In line with this finding, we found that serum TBil, IBil, TBA, ALT, AST and the cytokine MCP-1 were positively correlated with

Caulobacteraceae, *Desulfovibrionaceae*, *Brevundimonas*, and *Paenalcaligenes* in the gut. Moreover, the role of some of the B94-regulated gut microbes in health and disease needs to be further explored. For example, Acidobacteriota inhabit a wide variety of terrestrial and aquatic habitats and are particularly abundant in acidic soils, and Acidobacteriota has been reported to be depleted in patients with primary biliary cirrhosis (Lv et al., 2016). In this study, the B94-regulated taxon *Alistipes*, which belongs to the phylum Bacteroidota, has been reported to correlate not only with protection against diseases such as colitis, autism spectrum disorder, and various liver and cardiovascular fibrotic disorders but also with the development of diseases such as anxiety, myalgic encephalomyelitis/chronic fatigue syndrome, depression, pervasive developmental disorder-not otherwise specified (PDD-NOS) and colorectal cancer (CRC) (Parker et al., 2020). In summary, regulation of the gut microbiota is one of the important means by which B94 performs its function in the prevention and treatment of liver injury.

Stool contains products of cometabolism between the gut microbiota and the host, which act as a bridge for the interaction between the host and the microbes. We found that B94 pretreatment reduced the excretion of potentially beneficial metabolites in the faeces caused by D-galactosamine. For example, 5-aminovaleric acid is an analogue of γ-aminobutyric acid (GABA), which has been reported to inhibit GABA uptake and GABA aminotransferase activity (Lv et al., 2021c). L-methionine, an essential amino acid, is not only involved in protein synthesis but is also the main source of methyl groups in methyl transfer reactions and has important physiological functions, such as inhibition of fat accumulation and enhancement of the immune response (Shim et al., 2017). N-acetyl glucosamine is the basic unit of many important polysaccharides in biological cells and plays important roles in wound healing, antioxidant and immune-modulating effects, and regulation of liver function (Jiang et al., 2018). In addition, B94 pretreatment alleviated the reduction in the abundance of 1-heptadecanol in faeces caused by D-galactosamine. 1-heptadecanol is a long-chain primary alcohol with antimicrobial and anti-inflammatory potential observed in plants such as *Solena amplexicaulis* leaves (Lv et al., 2021b). In conclusion, B94 pretreatment slowed the disturbance of gut metabolism caused by D-galactosamine, which in turn was beneficial in reducing liver and intestinal damage.

Although there are many *Lactobacillus* and *Bifidobacterium* species, which are the major probiotics worldwide, only a small number of strains have been reported to have a role in preventing liver injury or failure. For example, in addition to the strain B94 that we found, *Lactobacillus salivarius* LI01, *Bifidobacterium adolescentis* CGMCC 15058, *Bifidobacterium longum* R0175, *Bifidobacterium pseudocatenulatum* LI09, *Bifidobacterium catenulatum* LI10, *Lactobacillus helveticus* R0052, *Lactobacillus reuteri* DSM 17938, *Lactobacillus acidophilus* LA14, and *Lactobacillus casei* Shirota have also been reported to have effects against liver injury (Osman et al., 2007; Wang et al., 2013; Lv et al., 2014; Fang et al., 2017; Li et al., 2019; Wang et al., 2019; Wang et al., 2020; Lv et al., 2021a; Jiang et al., 2021; Yan et al., 2021). There are certain commonalities in the effects of each strain on liver function, immunity and metabolism; for example, B94, LI01, DSM 17938, LA14, LI09 and LI10 lowered ALT and AST levels (Lv et al., 2014; Fang et al., 2017; Lv et al., 2021a; Jiang et al., 2021); B94, CGMCC 15058, R0175, Shirota; Li09, LI10, and LA14 lowered TBA levels (Fang et al., 2017; Li et al., 2019; Wang et al., 2020; Lv et al., 2021a; Yan et al., 2021); LI01 and LA14 lowered ALP levels (Lv et al., 2014; Lv et al., 2021a); and B94 and R0175 lowered TBil levels (Wang et al., 2020). Although these results regarding the prevention and treatment of liver injury are based on animal models and need further clinical validation, it is promising that most of these strains are commonly used probiotics, which have a good safety profile and are easily available to patients, thus providing a significant contribution to the prevention and treatment of liver injury.

In conclusion, this research examined the effect of *Bifidobacterium animalis* B94 on D-galactosamine-induced liver injury in rats. *Bifidobacterium animalis* B94 significantly reduced the elevated levels of ALT and AST induced by D-galactosamine, improved the gut microbiota as well as metabolic dysbiosis, reduced pathological abnormalities in liver and intestinal tissues, and has important potential applications for liver injury and liver failure.

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 in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Experimentation Ethics Committee of Zhejiang University.

AUTHOR CONTRIBUTIONS

TZ and LN conceived and designed the study. TZ, LN, JW, ZY, YZ, and SW performed the experiments and analysed the data. TZ, LN, and ZC 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/fcimb.2022.914684/full#supplementary-material

REFERENCES

Acharya, C., and Bajaj, J. S. (2017). Gut Microbiota and Complications of Liver Disease. *Gastroenterol. Clin. North Am.* 46 (1), 155–169. doi: 10.1016/j.gtc.2016.09.013

Backhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A., and Gordon, J. I.. (2005). Host-Bacterial Mutualism in the Human Intestine. *Science* 307 (5717), 1915–1920. doi: 10.1126/science.1104816

Basturk, A., Artan, R., and Yilmaz, A. (2016). Efficacy of Synbiotic, Probiotic, and Prebiotic Treatments for Irritable Bowel Syndrome in Children: A Randomized Controlled Trial. *Turk J. Gastroenterol.* 27 (5), 439–443. doi: 10.5152/tjg.2016.16301

Cann, I., Bernardi, R. C., and Mackie, R. I. (2016). Cellulose Degradation in the Human Gut: Ruminococcus Champanellensis Expands the Cellulosome Paradigm. *Environ. Microbiol.* 18 (2), 307–310. doi: 10.1111/1462-2920.13152

Cekin, A. H., Şahintürk, Y., Akbay Harmandar, F., Uyar, S., Yolcular, B. O., and Çekin, Y.. (2017). Use of Probiotics as an Adjuvant to Sequential H. Pylori Eradication Therapy: Impact on Eradication Rates, Treatment Resistance, Treatment-Related Side Effects, and Patient Compliance. *Turk J. Gastroenterol.* 28 (1), 3–11. doi: 10.5152/tjg.2016.0278

Chiu, C. J., McArdle, A. H., Brown, R., Scott, H. J., and Gurd, F. N.. (1970). Intestinal Mucosal Lesion in Low-Flow States. I. A Morphological, Hemodynamic, and Metabolic Reappraisal. *Arch. Surg.* 101 (4), 478–483. doi: 10.1001/archsurg.1970.01340280030009

Dapito, D. H., Mencin, A., Gwak, G. Y., Pradere, J. P., Jang, M. K., Mederacke, I., et al. (2012). Promotion of Hepatocellular Carcinoma by the Intestinal Microbiota and TLR4. *Cancer Cell* 21 (4), 504–516. doi: 10.1016/j.ccr.2012.02.007

Donkor, O. N., Ravikumar, M., Proudfoot, O., Day, S. L., Apostolopoulos, V., Paukovics, G., et al. (2012). Cytokine Profile and Induction of T Helper Type 17 and Regulatory T Cells by Human Peripheral Mononuclear Cells After Microbial Exposure. *Clin. Exp. Immunol.* 167 (2), 282–295. doi: 10.1111/j.1365-2249.2011.04496.x

Fang, D., Shi, D., Lv, L., Gu, S., Wu, W., Chen, Y., et al. (2017). Bifidobacterium Pseudocatenulatum LI09 and Bifidobacterium Catenulatum LI10 Attenuate D-Galactosamine-Induced Liver Injury by Modifying the Gut Microbiota. *Sci. Rep.* 7 (1), 8770.

Guarner, F., and Malagelada, J. R. (2003). Gut Flora in Health and Disease. *Lancet* 361 (9356), 512–519. doi: 10.1016/S0140-6736(03)12489-0

Jiang, Z., Li, Z., Zhang, W., Yang, Y., Han, B., Liu, W., and Peng, Y.. (2018). Dietary Natural N-Acetyl-D-Glucosamine Prevents Bone Loss in Ovariectomized Rat Model of Postmenopausal Osteoporosis. *Molecules* 23 (9):2302. doi: 10.3390/molecules23092302

Jiang, H., Yan, R., Wang, K., Wang, Q., Chen, X., Chen, L., et al. (2021). Lactobacillus Reuteri DSM 17938 Alleviates D-Galactosamine-Induced Liver Failure in Rats. *BioMed. Pharmacother.* 133, 111000. doi: 10.1016/j.biopha.2020.111000

Knodell, R. G., Ishack, K. G., Black, W. C., Chen, T. S., Craig, R., Kaplowitz, N., et al. (1981). Formulation and Application of a Numerical Scoringsystem for Assessing Histological Activity in Asymptomatic Chronic Active Hepatitis. *Hepatology* 1 (5), 431–435. doi: 10.1002/hep.1840010511

Lee, K. E., Kim, J. K., Han, S. K., Lee, D. Y., Lee, H. J., Yim, S. V., et al. (2020). The Extracellular Vesicle of Gut Microbial Paenalcaligenes Hominis is a Risk Factor for Vagus Nerve-Mediated Cognitive Impairment. *Microbiome* 8 (1), 107. doi: 10.1186/s40168-020-00881-2

Liechty, Z., Santos-Medellín, C., Edwards, J., Nguyen, B., Mikhail, D., Eason, S., et al. (2020). Comparative Analysis of Root Microbiomes of Rice Cultivars With High and Low Methane Emissions Reveals Differences in Abundance of Methanogenic Archaea and Putative Upstream Fermenters. *Msystems* 5 (1), e00897–e00819. doi: 10.1128/mSystems.00897-19

Li, Y., Lv, L., Ye, J., Fang, D., Shi, D., Wu, W., et al. (2019). Bifidobacterium Adolescentis CGMCC 15058 Alleviates Liver Injury, Enhances the Intestinal Barrier and Modifies the Gut Microbiota in D-Galactosamine-Treated Rats. *Appl. Microbiol. Biotechnol.* 103 (1), 375–393. doi: 10.1007/s00253-018-9454-y

Liu, L., Feng, Y., Wei, L., and Zong, Z.. (2021). Genome-Based Taxonomy of Brevundimonas With Reporting Brevundimonas Huaxiensis Sp. *Nov. Microbiol. Spectr.* 9 (1), e00111–e00121. doi: 10.1128/Spectrum.00111-21

Lv, L. X., Hu, X. J., Qian, G. R., Zhang, H., Lu, H. F., Zheng, B. W., et al. (2014). Administration of Lactobacillus Salivarius LI01 or Pediococcus Pentosaceus LI05 Improves Acute Liver Injury Induced by D-Galactosamine in Rats. *Appl. Microbiol. Biotechnol.* 98 (12), 5619–5632. doi: 10.1007/s00253-014-5638-2

Lv, L. X., Fang, D. Q., Shi, D., Chen, D. Y., Yan, R., Zhu, Y. X., et al. (2016). Alterations and Correlations of the Gut Microbiome, Metabolism and Immunity in Patients With Primary Biliary Cirrhosis. *Environ. Microbiol.* 18 (7), 2272–2286. doi: 10.1111/1462-2920.13401

Lv, L., Yao, C., Yan, R., Jiang, H., Wang, Q., Wang, K., et al. (2021a). Lactobacillus Acidophilus LA14 Alleviates Liver Injury. *mSystems* 6 (3), e0038421. doi: 10.1128/mSystems.00384-21

Lv, L., Mu, D., Du, Y., Yan, R., and Jiang, H.. (2021b). Mechanism of the Immunomodulatory Effect of the Combination of Live Bifidobacterium, Lactobacillus, Enterococcus, and Bacillus on Immunocompromised Rats. *Front. Immunol.* 12, 694344. doi: 10.3389/fimmu.2021.694344

Lv, L., Jiang, H., Yan, R., Xu, D., Wang, K., Wang, Q., et al. (2021c). The Salivary Microbiota, Cytokines, and Metabolome in Patients With Ankylosing Spondylitis Are Altered and More Proinflammatory Than Those in Healthy Controls. *mSystems* 6 (3), e01173-20. doi: 10.1128/mSystems.01173-20

Osman, N., Adawi, D., Ahrné, S., Jeppsson, B., and Molin, G.. (2007). Endotoxin- and D-Galactosamine-Induced Liver Injury Improved by the Administration of Lactobacillus, Bifidobacterium and Blueberry. *Dig. Liver Dis.* 39 (9), 849–856. doi: 10.1016/j.dld.2007.06.001

Parker, B. J., Wearsch, P. A., Veloo, A. C.M., and Rodriguez-Palacios, A.. (2020). The Genus Alistipes: Gut Bacteria With Emerging Implications to Inflammation, Cancer, and Mental Health. *Front. Immunol.* 11, 906. doi: 10.3389/fimmu.2020.00906

Shim, J., Shin, Y., Lee, I., and Kim, S. Y.. (2017). L-Methionine Production. *Adv. Biochem. Eng. Biotechnol.* 159, 153–177. doi: 10.1007/10_2016_30

Wang, Y., Li, Y., Xie, J., Zhang, Y., Wang, J., Sun, X., and Zhang, H.. (2013). Protective Effects of Probiotic Lactobacillus Casei Zhang Against Endotoxin- and D-Galactosamine-Induced Liver Injury in Rats via Anti-Oxidative and Anti-Inflammatory Capacities. *Int. Immunopharmacol.* 15 (1), 30–37. doi: 10.1016/j.intimp.2012.10.026

Wang, Q., Lv, L., Jiang, H., Wang, K., Yan, R., Li, Y., et al. (2019). Lactobacillus Helveticus R0052 Alleviates Liver Injury by Modulating Gut Microbiome and Metabolome in D-Galactosamine-Treated Rats. *Appl. Microbiol. Biotechnol.* 103 (23-24), 9673–9686. doi: 10.1007/s00253-019-10211-8

Wang, K., Lv, L., Yan, R., Wang, Q., Jiang, H., Wu, W., et al. (2020). Bifidobacterium Longum R0175 Protects Rats Against D-Galactosamine-Induced Acute Liver Failure. *mSphere* 5 (1), e00791-19. doi: 10.1128/mSphere.00791-19

Waters, J. L., and Ley, R. E. (2019). The Human Gut Bacteria Christensenellaceae are Widespread, Heritable, and Associated With Health. *BMC Biol.* 17 (1), 1–11. doi: 10.1186/s12915-019-0699-4

Yan, A. W., Fouts, D. E., Brandl, J., Stärkel, P., Torralba, M., Schott, E., Tsukamoto, H., et al. (2011). Enteric Dysbiosis Associated With a Mouse Model of Alcoholic Liver Disease. *Hepatology* 53 (1), 96–105. doi: 10.1002/hep.24018

Yan, R., Wang, K., Wang, Q., Jiang, H., Lu, Y., Chen, X., et al. (2021). Probiotic Lactobacillus Casei Shirota Prevents Acute Liver Injury by Reshaping the Gut Microbiota to Alleviate Excessive Inflammation and Metabolic Disorders. *Microb. Biotechnol.* 15 (1), 247–261. doi: 10.1111/1751-7915.13750

Zhang, L., Su, P., Henriksson, A., O'Rourke, J., and Mitchell, H.. (2008). Investigation of the Immunomodulatory Effects of Lactobacillus Casei and Bifidobacterium Lactis on Helicobacter Pylori Infection. *Helicobacter* 13 (3), 183–190. doi: 10.1111/j.1523-5378.2008.00595.x

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REVIEW
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The Gut Microbiota: A Novel Player in Autoimmune Hepatitis

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Autoimmune hepatitis (AIH) is a chronic immune-mediated liver disease distributed globally in all ethnicities with increasing prevalence. If left untreated, the disease will lead to cirrhosis, liver failure, or death. The intestinal microbiota is a complex ecosystem located in the human intestine, which extensively affects the human physiological and pathological processes. With more and more in-depth understandings of intestinal microbiota, a substantial body of studies have verified that the intestinal microbiota plays a crucial role in a variety of digestive system diseases, including alcohol-associated liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD). However, only a few studies have paid attention to evaluate the relationship between AIH and the intestinal microbiota. While AIH pathogenesis is not fully elucidated yet, some studies have indicated that intestinal microbiota putatively made significant contributions to the occurrence and the development of AIH by triggering several specific signaling pathways, altering the metabolism of intestinal microbiota, as well as modulating the immune response in the intestine and liver. By collecting the latest related literatures, this review summarized the increasing trend of the aerobic bacteria abundance in both AIH patients and AIH mice models. Moreover, the combination of specific bacteria species was found distinct to AIH patients, which could be a promising tool for diagnosing AIH. In addition, there were alterations of luminal metabolites and immune responses, including decreased short-chain fatty acids (SCFAs), increased pathogen associated molecular patterns (PAMPs), imbalanced regulatory T (Treg)/Th17 cells, follicular regulatory T (TFR)/follicular helper T (TFH) cells, and activated natural killer T (NKT) cells. These alterations participate in the onset and the progression of AIH *via* multiple mechanisms. Therefore, some therapeutic methods based on restoration of intestinal microbiota composition, including probiotics and fecal microbiota transplantation (FMT), as well as targeted intestinal microbiota-associated signaling pathways, confer novel insights into the treatment for AIH patients.

Keywords: intestinal microbiota, autoimmune hepatitis, gut-liver axis, probiotics, fecal microbiota transplantation

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INTRODUCTION

Autoimmune hepatitis (AIH) is a chronic progressive immune-mediated liver disease. It is predominantly characterized by the presence of autoantibodies, elevated levels of serum transaminase and immunoglobulin G, and liver histologic interface hepatitis (Cowling et al., 1956; Manns et al., 2010). AIH affects the health state of people in all age groups around the globe, with a female propensity (Cowling et al., 1956). The incidence of AIH is estimated at 4/100,000-24.5/100,000 per year in the Asia-pacific region, and it also increases by years in China (Lee et al., 2001; Jalihal et al., 2009; Haider et al., 2010; Ngu et al., 2010; Delgado et al., 2013; European Association for the Study of the Liver, 2015; Kim et al., 2017). AIH is a serious immune-mediated liver disease, which will lead to some detrimental consequences, including cirrhosis and liver failure (European Association for the Study of the Liver, 2015). Up to date, AIH pathogenesis is not completely elucidated. Genetic predisposition, environmental factors and immune tolerance breakdown are identified as significant contributors to the occurrence and the development of AIH (Wei et al., 2020). Notably, some studies have reported that intestinal microbiota dysbiosis had an intimate association with AIH (Yuksel et al., 2015; Liwinski et al., 2020; Wei et al., 2020).

The intestinal microbiota inhabits the human gut tract, mainly comprised of more than 100 trillion bacteria, and their genomes contain 150-fold more genes than humans (Qin et al., 2010).The intestinal microbiota is capable of coexisting with the host harmoniously and exerting significant influence on its pathological and physiological processes, such as assisting digestion and absorption of nutrients, preventing the colonization of pathobiont, and maintaining a steady immune system (Nicholson et al., 2012). A substantial body of studies have suggested that intestinal microbiota dysbiosis plays a significant role in immune-mediated disorders (Liwinski et al., 2020; Wang et al., 2021a), including AIH. It is commonly accepted that intestinal barrier destruction, intestinal microbiota translocation, as well as immune homeostasis breakdown contribute to the onset and progression of AIH (Yuksel et al., 2015; Wei et al., 2020; Yang et al., 2021).

Currently, the therapeutic methods for AIH are glucocorticoid or a combination with azathioprine (Mack et al., 2020). However, glucocorticoid and azathioprine have many negative effects, such as central obesity, osteoporosis, myelosuppression, and liver function damage (Lee et al., 2014; Mack et al., 2020). The therapeutic needs of patients not tolerating standard management or not achieving remission remain unmet (Mack et al., 2020). A number of researches associated with AIH animal models and AIH patients highlight the importance of "intestinal liver crosstalk" in AIH pathogenesis (Liang et al., 2021; Wang et al., 2021b), which offers a promise of novel diagnostic and therapeutic methods. Therefore, great significance should be attached to deeply explore the specific impact of intestinal microbiota on AIH and its associated mechanisms, and further discuss the efficacy and safety of several potential therapies, including probiotics, fecal microbiota transplantation (FMT), as well as some pharmacological agents which target intestinal microbiota-associated signaling pathways.

THE GUT-LIVER AXIS

The theory of the "the gut-liver axis" was initially raised by Marshall (Volta et al., 1987), which refers to an intimate anatomical, functional, and bidirectional interaction of the gut and the liver, predominantly *via* the portal circulation (Abdel-Misih and Bloomston, 2010). The gut-liver axis is identified as a pivotal contributor to the occurrence and development of multiple liver disease (Saffouri et al., 2019; Scorletti et al., 2020) and autoimmune disease (Allegretti et al., 2019; Fretheim et al., 2020; Wang et al., 2021a).

In healthy conditions, the intestinal epithelium constitutes a natural barrier to confer adequate protection against the intestinal microbiota as well as their metabolites through a tight junction, antibacterial molecules and mucus layer (Beyaz Coşkun and Sağdiçoğlu Celep, 2021). The liver is widely thought to be the first organ exposed to gut-derived harmful substances, including bacteria and bacterial metabolites (Seki and Brenner, 2008), but only a small quantity of them can move to the liver, where they are detoxified or diminished by the immune system in a healthy status (Lin et al., 2015). Microbiota dysbiosis leads to the destruction of intestinal barrier (Lin et al., 2015), which further results in the translocation of intestinal microbiota from the gut to the liver. The excessive gut-derived microbial toxins may destroy liver homeostasis by aberrantly activating the innate immune system and triggering signaling pathways related to liver inflammatory responses (Pradere et al., 2010). With compromised intestinal barrier and disrupted immune homeostasis, the intestinal microbiota, which can be regarded as a continuous source of antigens, initiates, maintains, and perpetuates the autoimmune responses in AIH (Sánchez et al., 2015; Van Praet et al., 2015; Ignacio et al., 2016).

Abbreviations: AIH, autoimmune hepatitis; FMT, fecal microbiota transplantation; Con A, concanavalin A; TCE, trichloroethene; AUC, area under curve; AST, aspartate aminotransferase; LPS, lipopolysaccharide; ALD, alcohol-associated liver disease; NAFLD, non-alcohol fatty liver disease; SCFA, short-chain fatty acid; B420, *Bifidobacterium* animal lactic acid 420; EAH, experimental autoimmune hepatitis; IECs, intestinal epithelial cells; TNF-α, tumor necrosis factor-α; IL, interleukin; GPBAR1, G-protein coupled bile acid receptor 1; NKT, natural killer T; GPR, G protein-coupled receptor; TLR, Toll-like receptor; FAK, adaptor protein focal adhesion kinase; MyD88, myeloid differentiation factor 88; IRAK4, IL-1R–associated kinase 4; ERK, extracellular signal-related kinase; MAPK, mitogen-activated protein kinase; AMPs, antimicrobial peptides; STAT3, signal transducers and activator of transcription 3; mTOR, mammalian target of rapamycin; NF-kB, nuclear factor kappa B; PPRs, pattern recognition receptors; PAMPs, pathogen associated molecular patterns; NLRs, NOD-like receptors; NLRP3, NOD-like receptor protein 3; TIR, Toll/interleukin-1 receptor; TRIF, TIR domain-containing adaptor inducing IFN-β; TRAM, TRIF-related adapter molecule; HSCs, hepatic stellate cells; TFR, follicular regulatory T; TFH, follicular helper T; Ah R, aryl hydrocarbon receptor; bHLH, basic region-helix-loop-helix; XRE, xenobiotic-response element; ARNT, Ah R nuclear translocator; CYP1A1, cytochrome P450 family 1A1; DCs, dendritic cells; PPs, Peyer patches; MAMPs, microbiome related molecular patterns; IBD, inflammatory bowel disease; UC, ulcerative colitis; ICAM1, intercellular adhesion molecule-1; Caco-2, colorectal adenocarcinoma; IFN-γ, interferon γ.

THE RELATIONSHIP BETWEEN AIH AND INTESTINAL MICROBIOTA

Intestinal microbiota makes great contributions to the onset and progression of AIH (Yuksel et al., 2015; Liwinski et al., 2020; Wei et al., 2020). Germ-free mice had a fair resistance to fulminant hepatitis induced by concanavalin A (Con A), which contrasted sharply with specific pathogen free mice (Wei et al., 2016). Moreover, gentamycin mitigated the liver injury induced by Con A through depleting gut-derived gram-negative bacteria, concomitantly with reduced liver immune cells infiltration, whereas administration of exogenous pathogenic bacteria aggravated Con A-induced acute hepatitis (Chen et al., 2014). Furthermore, in a recent study, compared to the controls, the antibiotic-treated mice exhibited AIH phenotypes after being transplanted with fecal microbiota from mice exposed to trichloroethene (TCE), accompanied by increased systematic autoantibodies and aggravated hepatic inflammation (Wang et al., 2021a). The afore-mentioned evidence supported an intimate linkage between the etiology of AIH and intestinal microbiota.

Besides, intestinal microbiota has changed significantly in AIH patients and animal models compared to the healthy group (**Table 1**) (Yuksel et al., 2015; Elsherbiny et al., 2020; Lou et al., 2020; Wei et al., 2020). Overall, the biodiversity of the intestinal microbiome has decreased remarkably, and the relative abundance of aerobic or facultative anaerobic bacteria increased (Lin et al., 2015; Yuksel et al., 2015; Elsherbiny et al., 2020; Wei et al., 2020). The taxonomic analysis of fecal microbiome from the controls as well as AIH patients showed that at the phylum level, Verrucomicrobia abundance remarkably increased while Synergistetes and Lentisphaerae abundance remarkably decreased in patients with AIH compared to healthy communities (Elsherbiny et al., 2020; Lou et al., 2020). Of note, Synergistetes and Lentisphaerae belong to anaerobic bacteria (Limam et al., 2010; Aoyagi et al., 2020), and Synergistetes have the capacity to participate in the anaerobic dissimilation of acetate (Aoyagi et al., 2020). However, the changes in Bacteroidetes, Firmicutes and Proteobacteria were controversial in different studies (Elsherbiny et al., 2020; Wei et al., 2020). At the genus level, compared to healthy group, *Veillonella*, *Streptococcus, Klebsiella, Akkermansia, Blautia, Eubacterium, Butyricicoccus* and *Haemophilus* were mainly enriched in AIH patients while *Bifidobacterium, Ruminococcus, Clostridiales, Rikenellaceae, Oscillospira, Sutterella, Parabacteriods* and *Coprococcus* were retracted in such patients (Lin et al., 2015; Elsherbiny et al., 2020; Liwinski et al., 2020; Lou et al., 2020; Wei et al., 2020). Furthermore, there were different outcomes concerning the abundance of *Lactobacillus, Faecali bacterium* and *Lachospiraceae* (Liwinski et al., 2020; Lou et al., 2020; Wei et al.,

TABLE 1 | Changes of intestinal microbiota associated with AIH in feces.

Participants	Comparison	Change of intestinal microbiota		Method	Ref
		Increased	Decreased		
AIH patients (n=24) Healthy individuals (n=8)	AIH vs Healthy		*Bifidobacterium; Lactobacillus*	16S rDNA quantitative PCR	Lin et al. (2015)
HLA-DR3 NOD mice WT NOD mice	AIH vs Healthy	Proteobacteria; Bacteriodetes		16S rRNA sequencing	Yuksel et al. (2015)
AIH patients (n=72) Healthy individuals (n=95)	AIH vs Healthy	*Streptococcus; Veillonella; Lactobacillus*	*Faecalibacterium; Bifidobacterium*	16S rRNA sequencing	Liwinski et al. (2020)
AIH patients (n=37) Healthy individuals (n=78)	AIH vs Healthy	Verrucomicrobia; *Veillonella; Faecalibacterium; Akkermansia*	Lentisphaerae; Synergistetes; *Pseudobutyrivibrio; Lachnospira; Ruminococcaceae*	16S rRNA sequencing	Lou et al. (2020)
AIH patients (n=15) Healthy individuals (n=10)	AIH vs Healthy	Firmicutes; Bacteroides; Proteobacteria; *Faecalibacterium; Blautia; Streptococcus; Haemophilus; Bacteroides; Veillonella; Eubacterium; Lachnospiraceae; Butyricicoccus*	*Prevotella; Parabacteroides; Dilaster*	16S rRNA sequencing	Elsherbiny et al. (2020)
AIH patients (n=91) Healthy individuals (n=98)	AIH vs Healthy	*Veillonella; Klebsiella; Streptococcus; Lactobacillus*	*Clostridiales; RF39; Ruminococcaceae; Rikenellaceae; Oscillospira; Parabacteroides; Coprococcus*	16S rRNA sequencing	Wei et al.[10]
TCE-treated mice Control mice	AIH vs Healthy	*Akkermansiaceae;Lachnospiraceae*	*Lactobacillaceae; Rikenellaceae; Bifidobacteriaceae*	16S rRNA sequencing	Wang et al[15]

Comparison of condition A vs condition B: ↑signifies an increase in condition A relative to condition B. ↓signifies a decrease in condition A relative to condition B.
AIH, autoimmune hepatitis; HLA, human leukocyte antigen; NOD, nonobese-diabetic; WT, wild type; TCE, Trichloroethene.

2020). Some researches established AIH mouse model that virtually mimicked the condition of AIH patients, and analyzed the fecal microbiome of these models. The results suggested that at the phylum level, compared to the controls, Proteobacteria and Bacteroidetes abundance were increased, and the increment of Proteobacteria (facultative anaerobic bacteria) was thought to correlate with inflammation, epithelial dysfunction, as well as the breakdown of host-microbiota homeostasis (Litvak et al., 2017). At the genus level, compared to healthy community, *Akkermansiaceae* and *Lachospiraceae* abundance were increased while *Lactobacillus, Bifidobacterium* and *Rikenellaceae* abundance were decreased (Yuksel et al., 2015; Wang et al., 2021a). Besides the aforementioned alterations of intestinal microbiota from fecal samples, *Enterococcus gallinarum* was remarkably enriched in the liver of AIH patients (Manfredo Vieira et al., 2018). The combination of *Lactobacillus, Veillonella, Clostridiales* and *Oscillospira* was regarded as a potent biomarker to make a distinction between healthy individuals and AIH patients with an area under curve (AUC) value of 78% (Wei et al., 2020), and another five genera including *Veillonella, Lachnospiraceae, Roseburia*, *Ruminococcaceae* and *Bacteroides* were able to discriminate AIH patients from healthy individuals, which were confirmed to achieve an AUC of 83.25% (Lou et al., 2020). These results suggested that the specific alterations of intestinal microbiota could be used as potent biomarkers to distinguish AIH patients from healthy communities.

Some specific microbiome is also confirmed to correlate with the severity of AIH (Lin et al., 2015), such as *Veillonella*, as it exhibits the strongest relativity to AIH (Wei et al., 2020). The abundance of *Veillonella* shows a positive correlation with the level of serum aspartate aminotransferase (AST), as well as the inflammation grades of the liver (Wei et al., 2020). A decline of *Bifidobacterium* is also related to the increased disease activity and failure to achieve remission (Liwinski et al., 2020). Moreover, the increment of plasma lipopolysaccharide (LPS) induced by dysbiosis in AIH is confirmed to correlate with advanced stages of the disease (Lin et al., 2015). These biomarkers can be used as noninvasive hallmarks to assist the diagnosis of AIH as well as the evaluation of the disease severity, which needs rigorous evaluation and further investigation.

Furthermore, with the increment of researches associated with non-bacterial communities in the gut, including fungi, viruses, and archaea, a number of literatures have reported the alterations of fungi, viruses, and archaea in some chronic liver diseases, including alcohol-associated liver disease (ALD) (Yang et al., 2017; Lang et al., 2020; Hartmann et al., 2021) and non-alcohol fatty liver disease (NAFLD) (You et al., 2021). It appears plausible to speculate that these non-bacterial communities also contribute to the progression of AIH despite the lack of reported relevant literatures, which warrants the emergence of new evidence.

In summary, intestinal microbiota in AIH patients and animal models has changed remarkably, with a decreased biodiversity and a conversion to aerobic or facultative anaerobic microorganisms (Lin et al., 2015; Yuksel et al., 2015; Elsherbiny et al., 2020; Wei et al., 2020). The specific alterations of the intestinal microbiota are conducive to making a distinction between AIH patients and healthy individuals (Lou et al., 2020; Wei et al., 2020). Moreover, some species of intestinal microbiota, such as *Veillonella* and *Bifidobacterium*, as well as bacterial products like LPS, are closely related to the disease severity (Lin et al., 2015; Liwinski et al., 2020; Wei et al., 2020), which are likely to be adjuvant to evaluate the progression of AIH. With a more in-depth understanding of non-bacterial communities, it is reasonable to speculate that they putatively take part in the progression of AIH, which warrants the emergence of new evidence.

THE INFLUENTIAL MECHANISMS OF ALTERED INTESTINAL MICROBIOTA IN AIH

Metabolite Pathway

The alterations of the intestinal microbiota in AIH disease model exert impact on the metabolism of luminal contents, including short-chain fatty acid (SCFA) (Liwinski et al., 2020; Lou et al., 2020), amino acid (Elsherbiny et al., 2020; Wei et al., 2020) as well as bile acid (Kayama et al., 2020; Wei et al., 2020), which affect the integrity and permeability of intestinal barrier and immune homeostasis.

SCFAs consist of acetic acids, propionic acids, and butyric acids, which belong to organic acids produced from undigested dietary fibers fermentation by intestinal bacteria (Bergman, 1990; Ríos-Covián et al., 2016; Martin-Gallausiaux et al., 2021). Their quantity and relative abundance are regarded as one of the biomarkers of health status (Ríos-Covián et al., 2016; Blaak et al., 2020). In the AIH disease model, the decrease of anaerobic bacteria, such as *Ruminococcus* (Lou et al., 2020), leads to the decrease of SCFAs (Liwinski et al., 2020; Lou et al., 2020), which exacerbates the inflammation response in AIH (Lou et al., 2020). Moreover, the administration of *Bifidobacterium* animal lactic acid 420 (B420) in experimental autoimmune hepatitis (EAH) mice increased the abundance of *Clostridium*, which had a correlation with the production of SCFAs, mitigating autoimmune hepatitis and intestinal barrier injury (Zhang et al., 2020). Therefore, it appears plausible that the altered intestinal microbiota in AIH causes the decrease of SCFAs, exacerbating the disease's progression. Putative mechanisms are described as follows. First of all, the production of SCFAs is accompanied by the decrease of luminal pH, which is not conducive to the growth of intestinal pathobiont, thus contributing to the restoration of altered intestinal microbiota in AIH (Perman et al., 1981). Moreover, several studies have shown that the administration of SCFAs alleviated inflammatory responses of systematic autoimmune diseases mediated by lymphocytes through increasing Tregs cells and reducing Th1 cells (Mizuno et al., 2017), which indicated that SCFAs might be able to mitigate the inflammatory injury in AIH. Furthermore, butyric acids, as the most significant constituent of SCFAs, can stimulate intestinal epithelial cells (IECs) so as to induce mucin expression, resulting

in the alteration of bacterial adhesion (Jung et al., 2015) and the improvement of the integrity of tight junction (Peng et al., 2009). In the meanwhile, the pretreatment of butyric acid can reduce the elevated level of proinflammatory factors induced by LPS, including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6, which also can stimulate anti-inflammatory factor secretion, like IL-10 (Wang et al., 2017). Therefore, it seems that the decrease of SCFAs is associated with intestinal barrier destruction and immune homeostasis breakdown, thus exacerbating the progression of AIH.

The altered arginine metabolism in AIH induced by the intestinal microbiota could decrease the serum polyamine level (Tabor and Tabor, 1985; Wei et al., 2020). Such a decrease is unfavorable for the differentiation and maturation of intestinal resident immune cells (Löser et al., 1999), thereby impacting the intestinal immune responses in AIH patients. Besides, the increase of branched-chain amino acids in AIH patients (Elsherbiny et al., 2020), including Leucine, Valine as well as Isoleucine, are conducive to upregulating innate and adaptive immune responses and modulating intestinal barrier function *via* multiple key signaling pathways (Negro et al., 2008; Nakamura, 2014)., thus participating in the development of the disease.

The secondary bile acid is thought to be a ligand for G-protein coupled bile acid receptor 1(GPBAR1) expressed on natural killer T (NKT) cell (Maruyama et al., 2002; Kawamata et al., 2003). The decreased abundance of *Clostridium* in AIH brings about the decrease of secondary bile acid (Kayama et al., 2020), which inhibits the polarization of NKT 10 cells from NKT cells and the secretion of anti-inflammatory cytokines IL-10 *via* the inactivation of GPBAR1, thereby alleviating liver injury in Con A-induced hepatitis (Biagioli et al., 2019).

In summary, dysbiosis in AIH disease models exerted influence on the metabolism of intestinal microbiota, and with it the altered concentrations of various intestinal metabolites, including the decrease of SCFAs, polyamine, and secondary bile acids, and the increase of branched-chain amino acids (Elsherbiny et al., 2020; Kayama et al., 2020; Liwinski et al., 2020; Lou et al., 2020; Wei et al., 2020). These alterations are identified as great contributors to intestinal barrier destruction, immune homeostasis breakdown, and inflammatory injury aggravation, thus giving impetus to the progression of AIH.

Receptor Pathway

Intestinal microbiota and their metabolites are identified as great contributors to the occurrence and the development of AIH by activating multiple signaling pathways through binding to different receptors distributed in the liver and the intestine. Principal receptors implicated in the associated signaling pathways in the intestine consist of Toll-like receptor 4 (TLR4) (Guo et al., 2015) and G protein-coupled receptors (GPR41/GPR43, GPR109a) (Thangaraju et al., 2009; Kim et al., 2013; Zhao et al., 2018) (**Figure 1**), while in the liver, NLRs (Luan et al., 2018), TLRs (TLR4, TLR9) (Zhang et al., 2018; Liu et al., 2021a), Ah R (Manfredo Vieira et al., 2018) and GPBAR1 (Biagioli et al., 2019) take part in the progression of AIH (**Figure 2**).

In the Intestine

TLR4 is able to sense various exogenous and endogenous ligands, including LPS, hyaluronic acid, fatty acid and so on (Pradere et al., 2010). Recent studies have indicated that TLR4 plays a significant role in liver diseases in both humans and animals (Seki and Brenner, 2008; Pradere et al., 2010; Yang and Seki, 2012). The altered intestinal microbiota in AIH leads to the increase of LPS, which activates TLR4 expressed on IECs. The activated TLR4 results in the phosphorylation and activation of adaptor protein focal adhesion kinase (FAK) in IECs, which then modulates the activation of myeloid differentiation factor 88 (MyD88) and IL-1R–associated kinase 4 (IRAK4), ultimately leading to intestinal barrier destruction and increased intestinal permeability (Guo et al., 2015). With a compromised intestinal barrier, gut-derived bacteria and metabolites are transferred from the intestine to mesenteric lymph nodes, systematic circulation, and the extraintestinal organs (Lin et al., 2015).

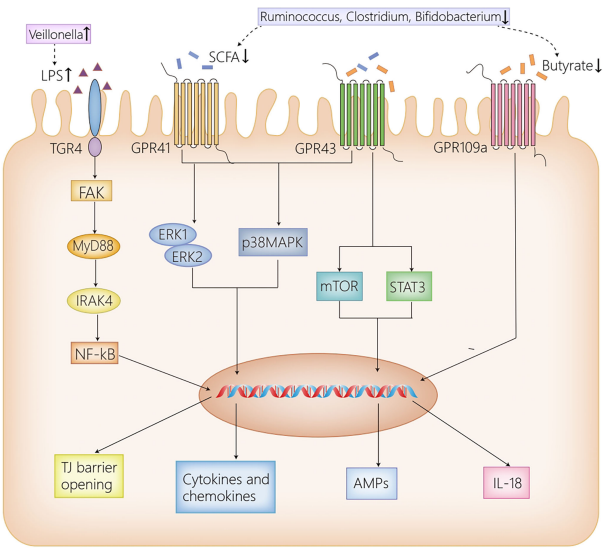

FIGURE 1 | The receptor pathways in the intestine. High dose LPS induced by the increased abundance of *Veillonella* in AIH activates TLR4 expressed on IECs, then leads to the phosphorylation and activation of FAK, which regulates the activation of MyD88 and IRAK4, ultimately disrupting the intestinal TJ barrier. GPR41/43 on IECs activates ERK1/2 and p38MPAK signaling pathways by SCFAs, contributing to cytokines and chemokines secretion, which mediate protective immunity. Moreover, the activation of GPR43 by butyrate induces AMPs production by activating mTOR and STAT3. Furthermore, GPR109a promotes the expression of IL-18, which confers protection against intestinal inflammation. Herein, the reduction of *Ruminococcus, Clostridium* and *Bifidobacterium* in AIH, which results in the decrease of SCFAs, inhibits the afore-mentioned signaling pathways activated by SCFAs, thus contributing to intestinal barrier disruption. AIH, autoimmune hepatitis; IECs, intestinal epithelial cells; LPS, lipopolysaccharide; TLR4, Toll-like receptor 4; FAK, focal adhesion kinase; MyD88, myeloid differentiation factor 88; IRAK4, IL-1R–associated kinase 4; NF-kB, nuclear factor kappa B; TJ, tight junction; SCFAs, short-chain fatty acids; GPR41/43/109a, G-protein-coupled receptors 41/43/109a; ERK1/2, extracellular signal-related kinase 1/2; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; STAT3, signal transducers and activator of transcription 3; AMPs, antimicrobial peptides; IL-18, interleukin 18.

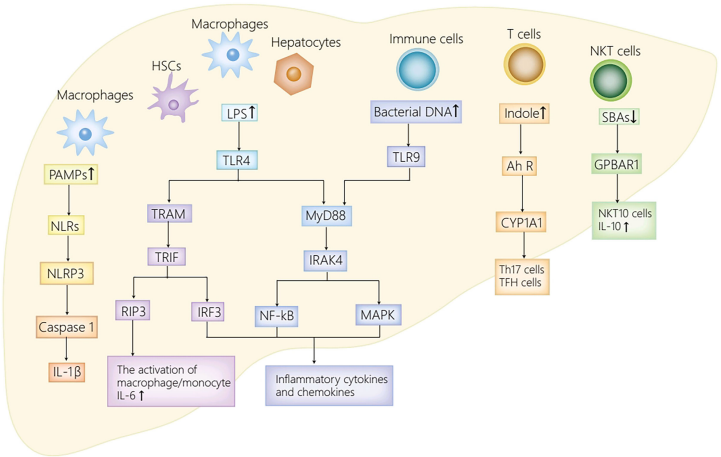

FIGURE 2 | The receptor pathways in the liver. The activation of NLRs by PAMPs activates NLRP3 inflammasome, thus promoting caspase-1 cleavage as well as IL-1β secretion. TLR4 expressed on HSCs, Kupffer cells and hepatocytes enhances inflammatory chemokines and cytokines secretion by activating IRF3, MAPK and NF-kB. Moreover, LPS also stimulates TLR4 on macrophages to initiate RIP3 signaling pathway, thus mediating macrophage/monocyte activation, and promoting the secretion of IL-6. Furthermore, the activation of TLR9 in immune cells by bacterial DNA activates NF-kB/MAPK signaling axis, and with it the secretion of IL-12 and TNF-α. Ah R ligand, derived from translocated *Enterococcus gallinarum*, activates AhR-CYP1A1 signaling pathway, thus inducing the production of Th17 cells and TFH cells. The decrease of secondary bile acids attenuates GPBAR1-IL10 axis, and with it, the decrease of NKT10 cells and IL10. PAMPs, pathogen associated molecular patterns; NLRs, NOD-like receptors; NLRP3, NOD-like receptor protein 3; IL-1β, interleukin-1β; LPS, lipopolysaccharide; TLR4/9, Toll-like receptor 4/9; MyD88, myeloid differentiation factor 88; IRAK4, IL-1R–associated kinase 4; NF-kB, nuclear factor kappa B; MAPK, mitogen-activated protein kinase; TRAM, TRIF-related adapter molecule; TRIF, TIR domain-containing adaptor inducing IFN-β; RIP3, receptor-interacting protein kinase 3; TNF-α, tumor necrosis factor-α; Ah R, aryl hydrocarbon receptor; CYP1A1, cytochrome P450 family 1A1; GPBAR1, G-protein coupled bile acid receptor 1; NKT cells, natural killer T cells; TFH, follicular helper T; HSC, hepatic stellate cell.

When the gut-derived bacteria and their metabolites are translocated to the liver, they initiate a host of inflammation and immune responses, participating in the occurrence and progression of AIH (Luan et al., 2018; Manfredo Vieira et al., 2018; Zhang et al., 2018; Biagioli et al., 2019; Liu et al., 2021a).

GPRs are identified as the most diverse and largest membrane protein families, suggesting a conserved mechanism for extracellular signal perception in eukaryotic organisms (Pierce et al., 2002). SCFAs act as ligands for GPRs distributed on IECs to maintain intestinal barrier as well as immune homeostasis *via* the activation of a variety of signaling pathways in healthy conditions, including GPR41, GPR43 and GPR109a (Thangaraju et al., 2009; Kim et al., 2013; Zhao et al., 2018). To be more specific, the SCFA-dependent activation of GPR41/GPR43 distributed on IECs promotes cytokines and chemokines production by activating the p38 mitogen-activated protein kinase (MAPK) signaling pathway and the extracellular signal-related kinase (ERK)1/2 signaling pathway, which mediated protective immunity in mice (Kim et al., 2013). Moreover, by activating the signal transducers and activator of transcription 3 (STAT3), and mammalian target of rapamycin (mTOR) in a GPR43-dependent way, butyrate upregulates antimicrobial peptides (AMPs) secretion in IECs and regulates the interaction of intestinal bacteria (Zhao et al., 2018). Butyrate is also regarded as a critical mediator in anti-inflammatory responses, which inhibits nuclear factor kappa B (NF-kB) activation induced by LPS, and induces IL-18 secretion in IECs *via* the activation of GPR109a, thus protecting the intestine against inflammation (Thangaraju et al., 2009; Dupaul-Chicoine et al., 2010). Herein, the decrease of SCFAs caused by dysbiosis in AIH exacerbates the disruption of intestinal barrier and inflammation responses in the intestine, contributing to the translocation of gut-derived bacteria and their metabolites from the intestine to the liver.

In summary, the increment of LPS, induced by dysbiosis in AIH, activates the TLR4/FAK/MyD88 signaling pathway in IECs, leads to intestinal barrier destruction, and increases intestinal permeability (Guo et al., 2015). Similarly, the reduction of SCFAs in AIH exacerbates the intestinal barrier disruption and inflammatory injury *via* the inactivation of GPR41/43 and GPR109a expressed on IECs (Thangaraju et al., 2009; Kim et al., 2013; Zhao et al., 2018). The compromised intestinal barrier results in the translocation of gut-derived bacteria and intestinal metabolites from the intestine to the liver, where the translocated material activate multiple inflammation and immune responses, thus taking part in the initiation and progression of AIH (Luan et al., 2018; Manfredo Vieira et al., 2018; Zhang et al., 2018; Biagioli et al., 2019; Liu et al., 2021a).

In the Liver

Pattern recognition receptors (PRRs) in the liver can sense translocated pathogen associated molecular patterns (PAMPs), including NOD-like receptors (NLRs) and TLRs. NLRs have the capacity to sense various ligands within the cytoplasm. In general, NLRs can upregulate inflammatory cytokines secretion

by activating MAPK, inflammasome or NF-kB by the recognition of PAMPs (Kumar et al., 2011). In an AIH mouse model induced by Con A, NOD-like receptor protein 3 (NLRP3) inflammasome activated by NLRs is identified as a critical mediator in the progression of Con A induced-hepatitis (Luan et al., 2018). To be more specific, the activated NLRP3 in macrophages contributes to the cleavage of caspase-1and the secretion of IL-1β, which then induces Th17 cells differentiation and recruits inflammatory cells. Therefore, the activated NLRP3 participates in various immune disease progression (Lasiglié et al., 2011; Pathak et al., 2011). Moreover, the secretion of IL-1β remarkably increased in AIH patients and was related to the aggravation of hepatitis in recent clinical studies (Longhi et al., 2012). To sum up, NLRs putatively exacerbate the progression of AIH mainly by activating NLRP3 inflammasome and its significant downstream molecules.

TLRs, the most extensively studied PRRs, recruit different adaptor molecules containing Toll/interleukin-1 receptor (TIR) domain, including TIR domain-containing adaptor inducing IFN-β (TRIF), TRIF-related adapter molecule (TRAM), and MyD88, to initiate different transcription factors including MAPK, IRF3/7 and NF-kB, thus inducing the secretion of proinflammatory cytokines (Kumar et al., 2011). In humans, ten TLR family members are identified, whereas there are twelve identified TLR family members that are extensively distributed in different cells in mice (Kumar et al., 2011). Dysbiosis in AIH patients leads to increment of PAMPs in the liver, such as LPS and bacterial DNA, which can bind to TLRs distributed in the liver and initiate a series of signaling pathways. Specifically, in hepatic stellate cells (HSCs), Kupffer cells, and hepatocytes, the activation of TLR4 by LPS contributes to proinflammatory chemokines and cytokines secretion by activating MAPK, IRF3, and NF-KB, which results in hepatic injury and fibrotic progression in AIH (Liu et al., 2021a). Moreover, the activation of TLR4 on macrophages initiates receptor-interacting protein kinase3 (RIP3) signaling pathway, thus mediating the activation of macrophage/monocyte in the liver, and promoting the secretion of IL-6 (Zhang et al., 2018). IL-6 can not only stimulate B cells to drive the production of IgG (Kishimoto, 1989), but also switch Tregs to Th17 cells in other autoimmune diseases (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006). Therefore, it appears plausible to speculate that IL-6 contributes significantly to the initiation and the progression of AIH. In addition, the activated TLR4/MyD88 signaling pathway in immune cells induced by LPS also contributes to the imbalance of follicular regulatory T (TFR)/ follicular helper T (TFH) cells (Levy et al., 2017). Such imbalance ultimately results in immune homeostasis breakdown and excessive autoantibodies production (Liang et al., 2020), thus playing a significant role in the pathogenesis of AIH (Liang et al., 2021). Furthermore, the activation of TLR9 by bacterial DNA initiates the NF-kB/MAPK signaling pathway in immune cells, and with it the secretion of IL-12 and TNF-α, which can exacerbate hepatic inflammatory injury in AIH (Liu et al., 2021a).

The aryl hydrocarbon receptor (Ah R) belongs to one kind of ligand-activated transcription factors and the basic region-helix-loop-helix (bHLH) superfamily of DNA binding proteins (Burbach et al., 1992), which is extensively distributed at barrier sites, including the skin, lung, gut and so on (Metidji et al., 2018). Substantial low-molecular-weight substances, such as tryptophan metabolites and indoles, act as ligands for Ah R (Denison and Nagy, 2003; Nguyen and Bradfield, 2008). Upon the recognition of Ah R ligands, Ah R is translocated from cytoplasmic to nucleus where it induces the transcription of target genes with the promoter. The promoter contains the xenobiotic-response element (XRE) sequence after dimerization with Ah R nuclear translocator (ARNT), including Ah R-cytochrome P450 family 1A1 (CYP1A1) (Sogawa and Fujii-Kuriyama, 1997). *Enterococcus gallinarum*, which is translocated to the liver from the intestine induced by disrupted intestinal barrier in AIH, encodes shikimic acid pathway, produces Ah R ligand, and activates AhR-CYP1A1 signaling pathway, ultimately promoting the transcription of CYP1A1 (Manfredo Vieira et al., 2018). The activated AhR-CYP1A1 pathway induces the production of TFH cells and Th17 cells (Veldhoen et al., 2008; Moura-Alves et al., 2014; Schiering et al., 2017), which is conducive to the secretion of systematic autoantibodies, thus putatively participating in the initiation of AIH (Manfredo Vieira et al., 2018).

GPBAR1 belongs to one kind of GPRs, which is extensively distributed in hepatic nonparenchymal cells, including cholangiocytes, activated HSCs, sinusoidal endothelial cells, as well as Kupffer cells (Keitel and Häussinger, 2012; Sawitza et al., 2015). Secondary bile acids are identified as ligands for GPBAR1 (Maruyama et al., 2002; Kawamata et al., 2003). In a recent study, GPBAR1-IL10 axis was reported to serve as a great contributor to the progression of Con A-induced hepatitis in a mouse model (Biagioli et al., 2019). To be more specific, GPBAR1 modulates the differentiation of type I and type II NKT cells in hepatic immune systems, and polarizes NKT cells to NKT 10 cells, which stimulates the secretion of anti-inflammatory cytokines IL10, thereby remarkably mitigating immune-mediated hepatitis induced by Con A (Biagioli et al., 2019). Therefore, the decrease of secondary bile acids owing to the reduced abundance of *Colstridium* in AIH (Kayama et al., 2020), exacerbates hepatic inflammatory injury *via* the inhibition of GPBAR1-IL10 axis.

In summary, dysbiosis in AIH results in the translocation of intestinal bacteria together with their products from the intestine to liver. NLRs recognize translocated PAMPs in the liver, which enhances the secretion of proinflammatory cytokines, thus aggravating hepatic inflammatory injury in AIH (Kumar et al., 2011; Luan et al., 2018). TLRs, specifically TLR4 and TLR9, are activated by LPS and bacterial DNA, respectively. The activated TLRs contribute to the breakdown of hepatic immune homeostasis, the excessive secretion of autoantibodies, as well as the production of proinflammatory chemokines and cytokines, thereby participating in the occurrence and development of AIH (Zhang et al., 2018; Liu et al., 2021a). Moreover, translocated *Enterococcus gallinarum* in the liver activates AhR-CYP1A1 signaling pathway to promote the production of systematic autoantibodies, which is likely to take part in the initiation of the disease (Manfredo Vieira et al., 2018). Additionally, the decrease of secondary bile acids represses GPBAR1-IL10 axis, thus aggravating hepatic inflammatory injury in

AIH (Biagioli et al., 2019). Herein, the afore-mentioned signaling pathways activated by different receptors distributed in the liver make great contributions to the onset and progression of AIH.

Immune Pathway

As a chronic immune-mediated inflammatory liver disorder, great significance should be attached to unravel the critical role of immune responses in AIH. Some studies have suggested that the dysregulation between Tregs and Th17 cells (Liu et al., 2021b), the activation of NKT cells (Diao et al., 2004; Liu et al., 2021a), and the imbalance of TFR/TFH cells induced by altered intestinal microbiota, presumably participated in the initiation and the progression of AIH (Liang et al., 2021).

The alterations of intestinal microbiota in AIH patients reduce the proportion of Tregs as well as raise the proportion of Th17 cells through exerting influence on the metabolism of luminal contents (Mizuno et al., 2017; Lou et al., 2020). Th17 cells promote proinflammatory cytokines secretion, including TNF-α, IL-22 and so on, which aggravates immune attack and inflammatory injury in the liver (de Oliveira et al., 2017). In contrast, Tregs release TGF-β and IL10 to repress the immune effector cells' activation, or restrain their function by interacting with dendritic cells (DCs), thereby regulating immune homeostasis (Chen et al., 2019; Wang et al., 2020). Herein, the increased Th17 cells and decreased Tregs induced by altered intestinal microbiota in AIH disrupt immune homeostasis and exacerbate inflammatory injury, putatively contributing to the progression of the disease. Moreover, the evidence that the Treg/Th17 cells ratio had an intimate association with the disease severity (Liu et al., 2021b) further supported the linkage between imbalanced Treg/Th17 cells and the progression of AIH.

In Con A-induced fulminant hepatitis, a condition similar to AIH patients, NKT cells in the liver can be activated by intestinal pathogens through two pathways. One pathway may be that intestinal pathogens initiate the activation of intestinal DCs. The intestinal DCs then migrate to the liver through Peyer patches (PPs), contributing to the activation of hepatic NKT cells. The other pathway is likely to be that a great host of translocated intestinal antigens first move to the liver, activate liver DCs, and subsequently activate NKT cells (Chen et al., 2014). The activation of NKT cells further activates Kupffer cells and recruits macrophages to secrete numerous inflammatory cytokines, which initiates the repairing responses including hepatocyte regeneration as well as fibrosis through activated HSCs (Diao et al., 2004; Liu et al., 2021a). Together, they contribute to the aggravation of hepatic inflammatory injury and fibrotic progression in AIH.

The elevated LPS in AIH disease model inhibited TFR cells and activated TFH cells by activating TLR4/MyD88 signaling pathway (Levy et al., 2017). The excessively activated TFH cells are intimately related to hypergammaglobulinemia, which accelerates the immunopathological process of AIH (Liang et al., 2020). TFR cell indirectly inhibits the activation of TFH cells upon the recognition of the coreceptor CLTA4, thus reducing the production of autoantibody (Liang et al., 2021). Therefore, the imbalance of TFR/TFH cells led to the destruction of immune homeostasis and the excess autoantibodies secretion, therefore taking part in the immunopathological process in AIH (Liang et al., 2021). Moreover, in a recent study, the dysregulation between TFH and TFR cells was augmented in EAH model mice after administration with broad-spectrum antibiotics (Liang et al., 2021), further supporting the intimate linkage between imbalanced TFR/TFH cells and AIH immunopathological process.

In summary, dysregulation between Tregs and Th17 cells leads to proinflammatory cytokines secretion and immune responses aggravation, and contributes to the progression of AIH (Liu et al., 2021b). The activated NKT cells are conducive to initiating repairing responses in the liver, as well as the production of proinflammatory cytokines (Diao et al., 2004; Chen et al., 2014; Liu et al., 2021a), which aggravate hepatic inflammatory injury and fibrotic progression in AIH. Moreover, the imbalance of TFR/TFH cells accelerates the pathological process of AIH by upregulating the secretion of autoantibodies (Liang et al., 2020; Liang et al., 2021). Therefore, the afore-mentioned alterations of immune cells or responses induced by dysbiosis in AIH give impetus to the progression of the disease.

NOVEL METHODS TARGETING FOR MICROBIOTA TO ATTENUATE AIH

Currently, the primary therapeutic methods for AIH are glucocorticoid or a combination with azathioprine, which effectively alleviate symptoms and prolong life in the majority of patients with AIH (Mack et al., 2020). However, some patients are still not tolerating standard management or not achieving remission. In addition, some detrimental effects of glucocorticoid and azathioprine cannot be ignored, such as central obesity, osteoporosis, myelosuppression, and liver function damage (Lee et al., 2014; Mack et al., 2020). Given that altered intestinal microbiota contributes greatly to the onset and progression of AIH, restoring intestinal microbiota putatively represents a new revenue for treating AIH. To date, probiotics (Lou et al., 2020; Zhang et al., 2020; Liu et al., 2021b), fecal microbiota transplantation (FMT) (Liang et al., 2021; Wang et al., 2021a), and some pharmacological agents targeted intestinal microbiota-associated signaling pathways (Frasca et al., 2012; Scaldaferri et al., 2014; Telesford et al., 2015; Hsu et al., 2017) have been confirmed to attenuate autoimmune hepatitis in AIH model mice, which putatively constitute a promising therapy for patients with AIH.

Probiotics and its Therapeutic Mechanisms

Probiotics

The international society of probiotics and prebiotics defines probiotics as "living microorganisms which are conducive to the health state of the host if administered in sufficient quantities" (Beyaz Coşkun and Sağdiçoğlu Celep, 2021). Currently, the most frequently used species are *Lactobacillus* and *Bifidobacterium*, and they have been evaluated as a useful therapy for the prevention or treatment of gastrointestinal infections, urogenital infections, periodontal diseases, as well as dental

caries (Caglar et al., 2005; White et al., 2017; Scorletti et al., 2020). In addition, probiotics are also conducive to treating immune-mediated diseases by regulating systematic immune responses (Vleggaar et al., 2008; De Filippis et al., 2021; Wang et al., 2022).

Liu et al. (2021b) have administered compound probiotics through gavage and dexamethasone through intraperitoneal injection to the AIH model mice for 42 days. The result suggested that the aforementioned interventions ameliorated liver inflammatory responses, and decreased the level of Th1 and Th17 cells, and serum aminotransferase. In addition, Tregs increased just in the probiotic group, indicating that compound probiotics have immunomodulatory effects. Zhang et al. (2020) found that B420 could revert the altered intestinal microbiota in the EAH model mice induced by S100 to normal, strengthen the function of the intestinal barrier, and alleviate inflammatory injury in the liver, thereby remarkably mitigating EAH induced by S100. Lou et al. (2020) illustrated that *Ruminococcus* decreased the frequency of Th1 and Th17 cells, inhibited the activation of effector T cells, and induced IL10 expression, further modulating intestinal homeostasis. These studies indicated that probiotics offered the promise of novel therapy in AIH.

The Associated Mechanisms of Probiotics in the Treatment of AIH

Probiotics make contributions to the treatment of AIH *via* multiple mechanisms. Firstly, probiotics compete with pathogenic bacteria for necessary nutrients and common adhesion receptors, thus affecting their survival and colonization (Bron et al., 2017). Herein, compound probiotic treatment is of great benefit to reduce harmful bacteria abundance and increase beneficial bacteria abundance in the intestine (Liu et al., 2021b). Secondly, microbiome related molecular patterns (MAMPs) from probiotics can activate PRRs expressed on the intestinal mucosa, thus enhancing intestinal barrier function mainly by upregulating the synthesis of tight junction proteins and enhancing their function (Bron et al., 2017). The integral intestinal barrier blocks the translocation of gut-derived pathogenic microorganisms as well as their metabolites. This blockage can inhibit the RIP3 signaling pathway in liver macrophages (Zhang et al., 2020), and repress TLR4/NF-kB signaling pathway in the liver and the intestine (Liu et al., 2021b), which conspicuously alleviates hepatitis induced by immune factors.

In addition, probiotics such as *Lactobacillus* can promote the production of SCFAs (Zhang et al., 2020), which are capable of triggering a variety of signaling pathways to modulate intestinal barrier function as well as immune homeostasis by binding to GPR41/43 and GPR109a (Thangaraju et al., 2009; Kim et al., 2013; Zhao et al., 2018). Besides SCFAs, lactic acid from probiotics also makes contributions to maintain intestinal barrier integrity. On the one hand, lactate induces Wnt3 expression in Paneth cells and stromal cells by binding to the lactate specific receptor GPR81, enhancing the proliferation of epithelial stem cells, thus preventing intestinal damage. On the other hand, lactic acid modulates immune responses by affecting CX3CR1+ phagocytes in the lamina propria, which enter the lumen to absorb luminal harmful bacteria by expanding dendrites (Kayama et al., 2020). Furthermore, the activation of NF-kB (MyD88) *via* TLR signaling pathway by probiotics triggers the expression of antimicrobial factors and intestinal epithelial defensins in Paneth cells, thus promoting the production of AMPs (Bron et al., 2017), and inhibiting the survival and colonization of intestinal pathobiont. In conclusion, probiotics can ameliorate the adverse condition of AIH by regulating intestinal microbiota composition and maintaining intestinal barrier and immune homeostasis.

Fecal Microbiota Transplantation

An introduction of the functional microbiota from the feces of healthy donors into the gut tract of patients is termed FMT (Milosevic et al., 2019), which aims at the reconstruction of new intestinal microbiota, and to exhibit potential efficacy against gastrointestinal and extra-gastrointestinal diseases (Beyaz Coşkun and Sağdiçoğlu Celep, 2021). Owing to the successful *C. difficile* fecal transplantation, more and more patients have registered for fecal transplantation, especially those with gastrointestinal diseases such as metabolic syndrome or inflammatory bowel disease (IBD) (Bron et al., 2017). Indeed, some studies with regard to metabolic syndrome (Vrieze et al., 2012) and ulcerative colitis (UC) (Borody et al., 2003) delineated that FMT ameliorated insulin resistance or prolonged the length of remission period, respectively, shedding light on the potential of FMT to treat microbiota-related diseases. Furthermore, some studies indicated that FMT had the capacity to effectively ameliorate hepatitis in EAH model mice putatively by restoring the composition of intestinal microbiota and rectifying the imbalance of TFR/TFH cells (Liang et al., 2021). Moreover, in a recent study, the antibiotic-treated mice exhibited AIH phenotypes after being transplanted with fecal microbiota from mice exposed to TCE, concomitantly with increased systematic autoantibodies and aggravated hepatic inflammation compared to the controls (Wang et al., 2021a). The aforementioned evidence verified the underlying therapeutic function of FMT in immune-mediated diseases.

Pharmacological Agents Targeted Intestinal Microbiota-Associated Pathways

Gelatin Tannate

The integrity of intestinal barrier is destructed by high dose LPS induced by the alterations of intestinal microbiota in AIH, and the resulting increased intestinal permeability (Guo et al., 2015; Lin et al., 2015). Restoring the compromised intestinal barrier blocks the translocation of intestinal bacteria and their metabolites from the gut to the liver (Lopetuso et al., 2015), thereby attenuating hepatic injury and fibrotic progression. Gelatin tannate constitutes a "mucus-like" shield for compromised intestinal mucosa, promoting the intestinal mucosal healing process and reducing intestinal leakage. The decreased blood LPS level in groups treated with gelatin tannate compared to controls further confirms its ability to restore shield activity of mucus layer (Scaldaferri et al., 2014). Moreover, gelatin tannate shows potent anti-inflammatory properties by inhibiting inflammatory biomarkers, such as TNF-α, IL-8, and intercellular adhesion molecule-1 (ICAM-1) in human epithelial colorectal adenocarcinoma (Caco-2) cells (Frasca et al., 2012).

JKB-122

TLR4, a significant cell surface receptor, takes part in the progression of AIH through activating multiple intracellular signaling pathways, which further bring about intestinal barrier destruction (Guo et al., 2015), promoting the production of proinflammatory cytokines (Liu et al., 2021a), and disrupting immune homeostasis (Zhang et al., 2018). JKB-122, as a TLR4 antagonist, conferred protection against Con A-induced hepatitis in mice, and exhibited anti-inflammatory properties mainly through suppressing proinflammatory cytokines production in both serum and liver, such as TNF-α, interferon γ (IFN-γ), IL5, IL6, as well as IL17 in a dose-dependent way (Hsu et al., 2017). Moreover, in a translational model of AIH, JKB-122 was proved to be effective alone or with prednisolone, which requires rigorous evaluation and further investigation (Hsu et al., 2017).

Polysaccharide A

The imbalance of Th17/Treg cells significantly contributes to the development of AIH (Liu et al., 2021b). Polysaccharide A, as a symbiosis factor derived from human commensal *Bacteroides fragilis*, could promote the immunologic development of mammalian hosts. It has been reported to induce $Foxp3^+$ Tregs production in mice, which inhibited the activity of Th17 cells, thereby rectifying imbalanced Th17/Treg cells (Telesford et al., 2015). Therefore, it is reasonable to speculate polysaccharide A offers the promise of a novel therapy for patients with AIH.

BAR 501

GPBAR1-IL10 axis has been confirmed to make contributions to the progression of AIH (Biagioli et al., 2019). BAR 501, as a potent agonist of GPBAR1, polarized NKT cells to NKT10 cells, and enhanced IL-10 secretion, which almost completely reversed inflammatory injury in the liver induced by Con A at a dose of 30-mg/kg in some preliminary experiments (Biagioli et al., 2019). Herein, BAR 501 putatively represents a novel therapy for patients with AIH.

CONCLUSION

AIH is a chronic immune-mediated inflammatory liver disease with obscure etiology (Cowling et al., 1956; Manns et al., 2010). An accumulating body of evidence highlights the importance of "intestinal liver crosstalk" in AIH pathogenesis (Lin et al., 2015). Many studies delineated the alterations of the intestinal microbiome in AIH disease model (Lin et al., 2015; Yuksel et al., 2015; Wei et al., 2020). The transformation of intestinal microbiota from anaerobic to aerobic (Wei et al., 2020) altered the immune responses and the metabolism of luminal contents (Lin et al., 2015), including the imbalance of Treg/Th17 cells and TFR/TFH cells, the activation of NKT cells, the increase of PAMPs, and the decrease of SCFAs and secondary bile acids, which subsequently lead to the destruction of the intestinal barrier, breakdown of immune homeostasis, augmentation of inflammatory injury and progression of fibrosis *via* multiple mechanisms, including receptor, immune and metabolites pathway. For patients not tolerating standard management or not achieving remission, probiotics, FMT, as well as some pharmacological agents targeted intestinal microbiota-associated pathways seem to represent new avenues for treatments for patients with AIH by restoring intestinal microbiota composition and modulating immune responses.

However, some knowledge gaps concerning AIH and intestinal microbiota still exist. For instance, predominant analyses of the composition and diversity of intestinal microbiota mainly depend on fecal samples from patients with AIH or AIH animal models, which are not able to completely reflect the abundance and composition of mucosal communities. Therefore, there is an urgent need to pay more attention to investigate mucosal microbiota, which is conducive to clarifying the alterations of intestinal microbiota in AIH. In addition, some studies have controversial results concerning the alterations of some specific species of intestinal microbiota, which warrant further investigation. Furthermore, with the complexity of microbial communities, the safety of FMT cannot be fully evaluated. Lack of evidence from clinical patients and a high rate of misdiagnosis and missed diagnosis in clinical practice are also problems to be solved. Herein, large scale patient follow-up and controlled prospective studies are still warranted to unravel the relationship between AIH and intestinal microbiota. More understanding regarding this relationship could provide direct evidence for the underlying mechanism of intestinal microbiota in AIH, offer favorable guidance for the treatment which targets intestinal microbiota, and supply the theoretical basis for the formulation of diagnosis and treatment guidelines for AIH.

AUTHOR CONTRIBUTIONS

ZC collected the literatures and drafted the manuscript. LY and HC critically revised the manuscript. All authors read and approved the final version of the manuscript.

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REFERENCES

Abdel-Misih, S. R., and Bloomston, M. (2010). Liver Anatomy. *Surg. Clin. North Am.* 90 (4), 643–653. doi: 10.1016/j.suc.2010.04.017

Allegretti, J. R., Kassam, Z., Carrellas, M., Mullish, B. H., Marchesi, J. R., Pechlivanis, A., et al (2019). Fecal Microbiota Transplantation in Patients With Primary Sclerosing Cholangitis: A Pilot Clinical Trial. *Am. J. Gastroenterol.* 114 (7), 1071–1079. doi: 10.14309/ajg.0000000000000115

Aoyagi, T., Inaba, T., Aizawa, H., Mayumi, D., Sakata, S., Charfi, A., et al (2020). Unexpected Diversity of Acetate Degraders in Anaerobic Membrane Bioreactor Treating Organic Solid Waste Revealed by High-Sensitivity Stable Isotope Probing. *Water Res.* 176, 115750. doi: 10.1016/j.watres.2020.115750

Bergman, E. N. (1990). Energy Contributions of Volatile Fatty Acids From the Gastrointestinal Tract in Various Species. *Physiol. Rev.* 70 (2), 567–590.

Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T. B., Oukka, M., et al (2006). Reciprocal Developmental Pathways for the Generation of Pathogenic Effector TH17 and Regulatory T Cells. *Nature* 441 (7090), 235–238. doi: 10.1038/nature04753

Beyaz Coşkun, A., and Sağdiçoğlu Celep, A. G. (2021). Therapeutic Modulation Methods of Gut Microbiota and Gut-Liver Axis. *Crit. Rev. Food Sci. Nutr* 22, 1–11. doi: 10.1080/10408398.2021.1902263

Biagioli, M., Carino, A., Fiorucci, C., Marchianò, S., Di Giorgio, C., Roselli, R., et al (2019). GPBAR1 Functions as Gatekeeper for Liver NKT Cells and Provides Counterregulatory Signals in Mouse Models of Immune-Mediated Hepatitis. *Cell. Mol. Gastroenterol. Hepatol.* 8 (3), 447–473. doi: 10.1016/j.jcmgh.2019.06.003

Blaak, E. E., Canfora, E. E., Theis, S., Frost, G., Groen, A. K., Mithieux, G., et al (2020). Short Chain Fatty Acids in Human Gut and Metabolic Health. *Benef. Microbes* 11 (5), 411–455. doi: 10.3920/BM2020.0057

Borody, T. J., Warren, E. F., Leis, S., Surace, R., and Ashman, O. (2003). Treatment of Ulcerative Colitis Using Fecal Bacteriotherapy. *J. Clin. Gastroenterol.* 37 (1), 42–47.

Bron, P. A., Kleerebezem, M., Brummer, R.-J., Cani, P. D., Mercenier, A., MacDonald, T. T., et al (2017). Can Probiotics Modulate Human Disease by Impacting Intestinal Barrier Function? *Br. J. Nutr.* 117 (1), 93–107. doi: 10.1017/S0007114516004037

Burbach, K. M., Poland, A., and Bradfield, C. A. (1992). Cloning of the Ah-Receptor cDNA Reveals a Distinctive Ligand-Activated Transcription Factor. *Proc. Natl. Acad. Sci. U. S. A.* 89 (17), 8185–8189.

Caglar, E., Sandalli, N., Twetman, S., Kavaloglu, S., Ergeneli, S., and Selvi, S. (2005). Effect of Yogurt With Bifidobacterium DN-173 010 on Salivary Mutans Streptococci and Lactobacilli in Young Adults. *Acta Odontol. Scand.* 63 (6), 317–320. doi: 10.1080/00016350510020070

Chen, J., Liu, W., and Zhu, W. (2019). Foxp3(+) Treg Cells Are Associated With Pathological Process of Autoimmune Hepatitis by Activating Methylation Modification in Autoimmune Hepatitis Patients. *Med. Sci. Monit.* 25, 6204–6212. doi: 10.12659/MSM.915408

Chen, J., Wei, Y., He, J., Cui, G., Zhu, Y., Lu, C., et al (2014). Natural Killer T Cells Play a Necessary Role in Modulating of Immune-Mediated Liver Injury by Gut Microbiota. *Sci. Rep.* 4, 7259. doi: 10.1038/srep07259

Cowling, D. C., Mackay, I. R., and Taft, L. I. (1956). Lupoid Hepatitis. *Lancet* 271 (6957), 1323–1326. doi: 10.1016/s0140-6736(56)91483-0

De Filippis, F., Paparo, L., Nocerino, R., Della Gatta, G., Carucci, L., Russo, R., et al (2021). Specific Gut Microbiome Signatures and the Associated Pro-Inflamatory Functions are Linked to Pediatric Allergy and Acquisition of Immune Tolerance. *Nat. Commun.* 12 (1), 5958. doi: 10.1038/s41467-021-26266-z

Delgado, J. S., Vodonos, A., Malnick, S., Kriger, O., Wilkof-Segev, R., Delgado, B., et al (2013). Autoimmune Hepatitis in Southern Israel: A 15-Year Multicenter Study. *J. Dig. Dis.* 14 (11), 611–618. doi: 10.1111/1751-2980.12085

Denison, M. S., and Nagy, S. R. (2003). Activation of the Aryl Hydrocarbon Receptor by Structurally Diverse Exogenous and Endogenous Chemicals. *Annu. Rev. Pharmacol. Toxicol.* 43, 309–334.

de Oliveira, G. L. V., Leite, A. Z., Higuchi, B. S., Gonzaga, M. I., and Mariano, V. S. (2017). Intestinal Dysbiosis and Probiotic Applications in Autoimmune Diseases. *Immunology* 152 (1), 1–22. doi: 10.1111/imm.12765

Diao, H., Kon, S., Iwabuchi, K., Kimura, C., Morimoto, J., Ito, D., et al (2004). Osteopontin as a Mediator of NKT Cell Function in T Cell-Mediated Liver Diseases. *Immunity* 21 (4), 539–550.

Dupaul-Chicoine, J., Yeretssian, G., Doiron, K., Bergstrom, K. S. B., McIntire, C. R., LeBlanc, P. M., et al (2010). Control of Intestinal Homeostasis, Colitis, and Colitis-Associated Colorectal Cancer by the Inflammatory Caspases. *Immunity* 32 (3), 367–378. doi: 10.1016/j.immuni.2010.02.012

Elsherbiny, N. M., Rammadan, M., Hassan, E. A., Ali, M. E., El-Rehim, A. S. A., Abbas, W. A., et al (2020). Autoimmune Hepatitis: Shifts in Gut Microbiota and Metabolic Pathways Among Egyptian Patients. *Microorganisms* 8 (7), 1011. doi: 10.3390/microorganisms8071011

European Association for the Study of the Liver (2015). EASL Clinical Practice Guidelines: Autoimmune Hepatitis. *J. Hepatol.* 63 (4), 971–1004. doi: 10.1016/j.jhep.2015.06.030

Frasca, G., Cardile, V., Puglia, C., Bonina, C., and Bonina, F. (2012). Gelatin Tannate Reduces the Proinflammatory Effects of Lipopolysaccharide in Human Intestinal Epithelial Cells. *Clin. Exp. Gastroenterol.* 5, 61–67. doi: 10.2147/CEG.S28792

Fretheim, H., Chung, B. K., Didriksen, H., Bækkevold, E. S., Midtvedt, Ø., Brunborg, C., et al (2020). Fecal Microbiota Transplantation in Systemic Sclerosis: A Double-Blind, Placebo-Controlled Randomized Pilot Trial. *PloS One* 15 (5), e0232739. doi: 10.1371/journal.pone.0232739

Guo, S., Nighot, M., Al-Sadi, R., Alhmoud, T., Nighot, P., and Ma, T. Y. (2015). Lipopolysaccharide Regulation of Intestinal Tight Junction Permeability Is Mediated by TLR4 Signal Transduction Pathway Activation of FAK and Myd88. *J. Immunol. (Baltimore Md. 1950)* 195 (10), 4999–5010. doi: 10.4049/jimmunol.1402598

Haider, A. S., Kaye, G., and Thomson, A. (2010). Autoimmune Hepatitis in a Demographically Isolated Area of Australia. *Intern. Med. J.* 40 (4), 281–285. doi: 10.1111/j.1445-5994.2009.02041.x

Hartmann, P., Lang, S., Zeng, S., Duan, Y., Zhang, X., Wang, Y., et al (2021). Dynamic Changes of the Fungal Microbiome in Alcohol Use Disorder. *Front. Physiol.* 12. doi: 10.3389/fphys.2021.699253

Hsu, M.-C., Liu, S.-H., Wang, C.-W., Hu, N.-Y., Wu, E. S. C., Shih, Y.-C., et al (2017). JKB-122 is Effective, Alone or in Combination With Prednisolone in Con A-Induced Hepatitis. *Eur. J. Pharmacol.* 812, 113–120. doi: 10.1016/j.ejphar.2017.07.012

Ignacio, A., Morales, C. I., Câmara, N. O. S., and Almeida, R. R. (2016). Innate Sensing of the Gut Microbiota: Modulation of Inflammatory and Autoimmune Diseases. *Front. Immunol.* 7. doi: 10.3389/fimmu.2016.00054

Jalihal, A., Telisinghe, P. U., and Chong, V. H. (2009). Profiles of Autoimmune Hepatitis in Brunei Darussalam. *Hepatobiliary Pancreat. Dis. Int.* 8 (6), 602–607.

Jung, T. H., Park, J. H., Jeon, W. M., and Han, K. S. (2015). Butyrate Modulates Bacterial Adherence on LS174T Human Colorectal Cells by Stimulating Mucin Secretion and MAPK Signaling Pathway. *Nutr. Res. Pract.* 9 (4), 343–349. doi: 10.4162/nrp.2015.9.4.343

Kawamata, Y., Fujii, R., Hosoya, M., Harada, M., Yoshida, H., Miwa, M., et al (2003). A G Protein-Coupled Receptor Responsive to Bile Acids. *J. Biol. Chem.* 278 (11), 9435–9440. doi: 10.1074/jbc.M209706200

Kayama, H., Okumura, R., and Takeda, K. (2020). Interaction Between the Microbiota, Epithelia, and Immune Cells in the Intestine. *Annu. Rev. Immunol.* 38, 23–48. doi: 10.1146/annurev-immunol-070119-115104

Keitel, V., and Häussinger, D. (2012). Perspective: TGR5 (Gpbar-1) in Liver Physiology and Disease. *Clinics Res. Hepatol. Gastroenterol.* 36 (5), 412–419. doi: 10.1016/j.clinre.2012.03.008

Kim, B. H., Choi, H. Y., Ki, M., Kim, K. A., Jang, E. S., and Jeong, S. H. (2017). Population-Based Prevalence, Incidence, and Disease Burden of Autoimmune Hepatitis in South Korea. *PloS One* 12 (8), e0182391. doi: 10.1371/journal.pone.0182391

Kim, M. H., Kang, S. G., Park, J. H., Yanagisawa, M., and Kim, C. H. (2013). Short-Chain Fatty Acids Activate GPR41 and GPR43 on Intestinal Epithelial Cells to Promote Inflammatory Responses in Mice. *Gastroenterology* 145 (2), 396–406.e1–10. doi: 10.1053/j.gastro.2013.04.056

Kishimoto, T. (1989). The Biology of Interleukin-6. *Blood* 74 (1), 1–10.

Kumar, H., Kawai, T., and Akira, S. (2011). Pathogen Recognition by the Innate Immune System. *Int. Rev. Immunol.* 30 (1), 16–34. doi: 10.3109/08830185.2010.529976

Lang, S., Duan, Y., Liu, J., Torralba, M. G., Kuelbs, C., Ventura-Cots, M., et al (2020). Intestinal Fungal Dysbiosis and Systemic Immune Response to Fungi in Patients With Alcoholic Hepatitis. *Hepatology (Baltimore Md.)* 71 (2), 522–538. doi: 10.1002/hep.30832

Lasigliè, D., Traggiai, E., Federici, S., Alessio, M., Buoncompagni, A., Accogli, A., et al (2011). Role of IL-1 Beta in the Development of Human T(H)17 Cells: Lesson From NLPR3 Mutated Patients. *PloS One* 6 (5), e20014. doi: 10.1371/journal.pone.0020014

Lee, Y. J., Choi, W. Y., Park, K. S., Kim, Y. J., Cho, K. B., Kim, E. S., et al (2014). Differences in the Adverse Effects of Azathioprine Between Inflammatory Bowel Disease and Autoimmune Hepatitis in Korean Patients. *Korean J. Gastroenterol.* 64 (6), 348–355.

Lee, Y. M., Teo, E. K., Ng, T. M., Khor, C., and Fock, K. M. (2001). Autoimmune Hepatitis in Singapore: A Rare Syndrome Affecting Middle-Aged Women. *J. Gastroenterol. Hepatol.* 16 (12), 1384–1389. doi: 10.1046/j.1440-1746.2001.02646.x

Levy, M., Kolodziejczyk, A. A., Thaiss, C. A., and Elinav, E. (2017). Dysbiosis and the Immune System. *Nat. Rev. Immunol.* 17 (4), 219–232. doi: 10.1038/nri.2017.7

Liang, M., Liwen, Z., Jianguo, S., Juan, D., Fei, D., Yin, Z., et al (2021). Fecal Microbiota Transplantation Controls Progression of Experimental Autoimmune Hepatitis in Mice by Modulating the TFR/TFH Immune Imbalance and Intestinal Microbiota Composition. *Front. Immunol.* 12. doi: 10.3389/fimmu.2021.728723

Liang, M., Liwen, Z., Juan, D., Yun, Z., Yanbo, D., and Jianping, C. (2020). Dysregulated TFR and TFH Cells Correlate With B-Cell Differentiation and Antibody Production in Autoimmune Hepatitis. *J. Cell. Mol. Med.* 24 (7), 3948–3957. doi: 10.1111/jcmm.14997

Limam, R. D., Bouchez, T., Chouari, R., Li, T., Barkallah, I., Landoulsi, A., et al (2010). Detection of WWE2-Related Lentisphaerae by 16S rRNA Gene Sequencing and Fluorescence *in Situ* Hybridization in Landfill Leachate. *Can. J. Microbiol.* 56 (10), 846–852. doi: 10.1139/w10-065

Lin, R., Zhou, L., Zhang, J., and Wang, B. (2015). Abnormal Intestinal Permeability and Microbiota in Patients With Autoimmune Hepatitis. *Int. J. Clin. Exp. Pathol.* 8 (5), 5153–5160.

Litvak, Y., Byndloss, M. X., Tsolis, R. M., and Bäumler, A. J. (2017). Dysbiotic Proteobacteria Expansion: A Microbial Signature of Epithelial Dysfunction. *Curr. Opin. Microbiol.* 39, 1–6. doi: 10.1016/j.mib.2017.07.003

Liu, Q., Tian, H., Kang, Y., Tian, Y., Li, L., Kang, X., et al (2021b). Probiotics Alleviate Autoimmune Hepatitis in Mice Through Modulation of Gut Microbiota and Intestinal Permeability. *J. Nutr. Biochem.* 98, 108863. doi: 10.1016/j.jnutbio.2021.108863

Liu, C., Wang, Y. L., Yang, Y. Y., Zhang, N. P., Niu, C., Shen, X. Z., et al (2021a). Novel Approaches to Intervene Gut Microbiota in the Treatment of Chronic Liver Diseases. *FASEB J.* 35 (10), e21871. doi: 10.1096/fj.202100939R

Liwinski, T., Casar, C., Ruehlemann, M. C., Bang, C., Sebode, M., Hohenester, S., et al (2020). A Disease-Specific Decline of the Relative Abundance of Bifidobacterium in Patients With Autoimmune Hepatitis. *Aliment. Pharmacol. Ther.* 51 (12), 1417–1428. doi: 10.1111/apt.15754

Longhi, M. S., Liberal, R., Holder, B., Robson, S. C., Ma, Y., Mieli-Vergani, G., et al (2012). Inhibition of Interleukin-17 Promotes Differentiation of $CD25^-$ Cells Into Stable T Regulatory Cells in Patients With Autoimmune Hepatitis. *Gastroenterology* 142 (7), 1526–1535.e6. doi: 10.1053/j.gastro.2012.02.041

Lopetuso, L. R., Scaldaferri, F., Bruno, G., Petito, V., Franceschi, F., and Gasbarrini, A. (2015). The Therapeutic Management of Gut Barrier Leaking: The Emerging Role for Mucosal Barrier Protectors. *Eur. Rev. Med. Pharmacol. Sci.* 19 (6), 1068–1076.

Löser, C., Eisel, A., Harms, D., and Fölsch, U. R. (1999). Dietary Polyamines are Essential Luminal Growth Factors for Small Intestinal and Colonic Mucosal Growth and Development. *Gut* 44 (1), 12–16.

Lou, J., Jiang, Y., Rao, B., Li, A., Ding, S., Yan, H., et al (2020). Fecal Microbiomes Distinguish Patients With Autoimmune Hepatitis From Healthy Individuals. *Front. Cell. Infect. Microbiol.* 10. doi: 10.3389/fcimb.2020.00342

Luan, J., Zhang, X., Wang, S., Li, Y., Fan, J., Chen, W., et al (2018). NOD-Like Receptor Protein 3 Inflammasome-Dependent IL-1β Accelerated ConA-Induced Hepatitis. *Front. Immunol.* 9. doi: 10.3389/fimmu.2018.00758

Mack, C. L., Adams, D., Assis, D. N., Kerkar, N., Manns, M. P., Mayo, M. J., et al (2020). Diagnosis and Management of Autoimmune Hepatitis in Adults and Children: 2019 Practice Guidance and Guidelines From the American Association for the Study of Liver Diseases. *Hepatology (Baltimore Md.)* 72 (2), 671–722. doi: 10.1002/hep.31065

Manfredo Vieira, S., Hiltensperger, M., Kumar, V., Zegarra-Ruiz, D., Dehner, C., Khan, N., et al (2018). Translocation of a Gut Pathobiont Drives Autoimmunity in Mice and Humans. *Science (New York N.Y.)* 359 (6380), 1156–1161. doi: 10.1126/science.aar7201

Mangan, P. R., Harrington, L. E., O'Quinn, D. B., Helms, W. S., Bullard, D. C., Elson, C. O., et al (2006). Transforming Growth Factor-Beta Induces Development of the T(H)17 Lineage. *Nature* 441 (7090), 231–234. doi: 10.1038/nature04754

Manns, M. P., Czaja, A. J., Gorham, J. D., Krawitt, E. L., Mieli-Vergani, G., Vergani, D., et al (2010). Diagnosis and Management of Autoimmune Hepatitis. *Hepatology* 51 (6), 2193–2213. doi: 10.1002/hep.23584

Martin-Gallausiaux, C., Marinelli, L., Blottière, H. M., Larraufie, P., and Lapaque, N. (2021). SCFA: Mechanisms and Functional Importance in the Gut. *Proc. Nutr. Soc.* 80 (1), 37–49. doi: 10.1017/S0029665120006916

Maruyama, T., Miyamoto, Y., Nakamura, T., Tamai, Y., Okada, H., Sugiyama, E., et al (2002). Identification of Membrane-Type Receptor for Bile Acids (M-BAR). *Biochem. Biophys. Res. Commun.* 298 (5), 714–719. doi: 10.1016/s0006-291x(02)02550-0

Metidji, A., Omenetti, S., Crotta, S., Li, Y., Nye, E., Ross, E., et al (2018). The Environmental Sensor AHR Protects From Inflammatory Damage by Maintaining Intestinal Stem Cell Homeostasis and Barrier Integrity. *Immunity* 49 (2), 353–362.e5. doi: 10.1016/j.immuni.2018.07.010

Milosevic, I., Vujovic, A., Barac, A., Djelic, M., Korac, M., Radovanovic Spurnic, A., et al (2019). Gut-Liver Axis, Gut Microbiota, and Its Modulation in the Management of Liver Diseases: A Review of the Literature. *Int. J. Mol. Sci.* 20 (2), 395. doi: 10.3390/ijms20020395

Mizuno, M., Noto, D., Kaga, N., Chiba, A., and Miyake, S. (2017). The Dual Role of Short Fatty Acid Chains in the Pathogenesis of Autoimmune Disease Models. *PloS One* 12 (2), e0173032. doi: 10.1371/journal.pone.0173032

Moura-Alves, P., Faé, K., Houthuys, E., Dorhoi, A., Kreuchwig, A., Furkert, J., et al (2014). AhR Sensing of Bacterial Pigments Regulates Antibacterial Defence. *Nature* 512 (7515), 387–392. doi: 10.1038/nature13684

Nakamura, I. (2014). Impairment of Innate Immune Responses in Cirrhotic Patients and Treatment by Branched-Chain Amino Acids. *World J. Gastroenterol.* 20 (23), 7298–7305. doi: 10.3748/wjg.v20.i23.7298

Negro, M., Giardina, S., Marzani, B., and Marzatico, F. (2008). Branched-Chain Amino Acid Supplementation Does Not Enhance Athletic Performance But Affects Muscle Recovery and the Immune System. *J. Sports Med. Phys. Fitness* 48 (3), 347–351.

Ngu, J. H., Bechly, K., Chapman, B. A., Burt, M. J., Barclay, M. L., Gearry, R. B., et al (2010). Population-Based Epidemiology Study of Autoimmune Hepatitis: A Disease of Older Women? *J. Gastroenterol. Hepatol.* 25 (10), 1681–1686. doi: 10.1111/j.1440-1746.2010.06384.x

Nguyen, L. P., and Bradfield, C. A. (2008). The Search for Endogenous Activators of the Aryl Hydrocarbon Receptor. *Chem. Res. Toxicol.* 21 (1), 102–116.

Nicholson, J. K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., et al (2012). Host-Gut Microbiota Metabolic Interactions. *Science (New York N.Y.)* 336 (6086), 1262–1267. doi: 10.1126/science.1223813

Pathak, S., Goldofsky, E., Vivas, E. X., Bonagura, V. R., and Vambutas, A. (2011). IL-1β is Overexpressed and Aberrantly Regulated in Corticosteroid Nonresponders With Autoimmune Inner Ear Disease. *J. Immunol.* 186 (3), 1870–1879. doi: 10.4049/jimmunol.1002275

Peng, L., Li, Z. R., Green, R. S., Holzman, I. R., and Lin, J. (2009). Butyrate Enhances the Intestinal Barrier by Facilitating Tight Junction Assembly *via* Activation of AMP-Activated Protein Kinase in Caco-2 Cell Monolayers. *J. Nutr.* 139 (9), 1619–1625. doi: 10.3945/jn.109.104638

Perman, J. A., Modler, S., and Olson, A. C. (1981). Role of pH in Production of Hydrogen From Carbohydrates by Colonic Bacterial Flora. Studies *In Vivo* and *In Vitro*. *J. Clin. Invest.* 67 (3), 643–650. doi: 10.1172/jci110079

Pierce, K. L., Premont, R. T., and Lefkowitz, R. J. (2002). Seven-Transmembrane Receptors. *Nat. Rev. Mol. Cell Biol.* 3 (9), 639–650.

Pradere, J.-P., Troeger, J. S., Dapito, D. H., Mencin, A. A., and Schwabe, R. F. (2010). Toll-Like Receptor 4 and Hepatic Fibrogenesis. *Semin. Liver Dis.* 30 (3), 232–244. doi: 10.1055/s-0030-1255353

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., et al (2010). A Human Gut Microbial Gene Catalogue Established by Metagenomic Sequencing. *Nature* 464 (7285), 59–65. doi: 10.1038/nature08821

Ríos-Covián, D., Ruas-Madiedo, P., Margolles, A., Gueimonde, M., de Los Reyes-Gavilán, C. G., and Salazar, N. (2016). Intestinal Short Chain Fatty Acids and Their Link With Diet and Human Health. *Front. Microbiol.* 7. doi: 10.3389/fmicb.2016.00185

Saffouri, G. B., Shields-Cutler, R. R., Chen, J., Yang, Y., Lekatz, H. R., Hale, V. L., et al (2019). Small Intestinal Microbial Dysbiosis Underlies Symptoms Associated With Functional Gastrointestinal Disorders. *Nat. Commun.* 10 (1), 2012. doi: 10.1038/s41467-019-09964-7

Sánchez, B., Hevia, A., González, S., and Margolles, A. (2015). Interaction of Intestinal Microorganisms With the Human Host in the Framework of Autoimmune Diseases. *Front. Immunol.* 6. doi: 10.3389/fimmu.2015.00594

Sawitza, I., Kordes, C., Götze, S., Herebian, D., and Häussinger, D. (2015). Bile Acids Induce Hepatic Differentiation of Mesenchymal Stem Cells. *Sci. Rep.* 5, 13320. doi: 10.1038/srep13320

Scaldaferri, F., Lopetuso, L. R., Petito, V., Cufino, V., Bilotta, M., Arena, V., et al (2014). Gelatin Tannate Ameliorates Acute Colitis in Mice by Reinforcing Mucus Layer and Modulating Gut Microbiota Composition: Emerging Role for 'Gut Barrier Protectors' in IBD? *United European Gastroenterol. J.* 2 (2), 113–122. doi: 10.1177/2050640614520867

Schiering, C., Wincent, E., Metidji, A., Iseppon, A., Li, Y., Potocnik, A. J., et al (2017). Feedback Control of AHR Signalling Regulates Intestinal Immunity. *Nature* 542 (7640), 242–245. doi: 10.1038/nature21080

Scorletti, E., Afolabi, P. R., Miles, E. A., Smith, D. E., Almehmadi, A., Alshathry, A., et al (2020). Synbiotics Alter Fecal Microbiomes, But Not Liver Fat or Fibrosis, in a Randomized Trial of Patients With Nonalcoholic Fatty Liver Disease. *Gastroenterology* 158 (6), 1597–1610.e1597. doi: 10.1053/j.gastro.2020.01.031

Seki, E., and Brenner, D. A. (2008). Toll-Like Receptors and Adaptor Molecules in Liver Disease: Update. *Hepatology* 48 (1), 322–335. doi: 10.1002/hep.22306

Sogawa, K., and Fujii-Kuriyama, Y. (1997). Ah Receptor, a Novel Ligand-Activated Transcription Factor. *J. Biochem.* 122 (6), 1075–1079.

Tabor, C. W., and Tabor, H. (1985). Polyamines in Microorganisms. *Microbiol. Rev.* 49 (1), 81–99. doi: 10.1128/mr.49.1.81-99.1985

Telesford, K. M., Yan, W., Ochoa-Reparaz, J., Pant, A., Kircher, C., Christy, M. A., et al (2015). A Commensal Symbiotic Factor Derived From Bacteroides Fragilis Promotes Human CD39(+)Foxp3(+) T Cells and Treg Function. *Gut Microbes* 6 (4), 234–242. doi: 10.1080/19490976.2015.1056973

Thangaraju, M., Cresci, G. A., Liu, K., Ananth, S., Gnanaprakasam, J. P., Browning, D. D., et al (2009). GPR109A is a G-Protein-Coupled Receptor for the Bacterial Fermentation Product Butyrate and Functions as a Tumor Suppressor in Colon. *Cancer Res.* 69 (7), 2826–2832. doi: 10.1158/0008-5472.CAN-08-4466

Van Praet, J. T., Donovan, E., Vanassche, I., Drennan, M. B., Windels, F., Dendooven, A., et al (2015). Commensal Microbiota Influence Systemic Autoimmune Responses. *EMBO J.* 34 (4), 466–474. doi: 10.15252/embj.201489966

Veldhoen, M., Hirota, K., Westendorf, A. M., Buer, J., Dumoutier, L., Renauld, J.-C., et al (2008). The Aryl Hydrocarbon Receptor Links TH17-Cell-Mediated Autoimmunity to Environmental Toxins. *Nature* 453 (7191), 106–109. doi: 10.1038/nature06881

Veldhoen, M., Hocking, R. J., Atkins, C. J., Locksley, R. M., and Stockinger, B. (2006). TGFbeta in the Context of an Inflammatory Cytokine Milieu Supports *De Novo* Differentiation of IL-17-Producing T Cells. *Immunity* 24 (2), 179–189. doi: 10.1016/j.immuni.2006.01.001

Vleggaar, F. P., Monkelbaan, J. F., and van Erpecum, K. J. (2008). Probiotics in Primary Sclerosing Cholangitis: A Randomized Placebo-Controlled Crossover Pilot Study. *Eur. J. Gastroenterol. Hepatol.* 20 (7), 688–692. doi: 10.1097/MEG.0b013e3282f5197e

Volta, U., Bonazzi, C., Bianchi, F. B., Baldoni, A. M., Zoli, M., and Pisi, E. (1987). IgA Antibodies to Dietary Antigens in Liver Cirrhosis. *Ric. Clin. Lab.* 17 (3), 235–242.

Vrieze, A., Van Nood, E., Holleman, F., Salojärvi, J., Kootte, R. S., Bartelsman, J. F. W. M., et al (2012). Transfer of Intestinal Microbiota From Lean Donors Increases Insulin Sensitivity in Individuals With Metabolic Syndrome. *Gastroenterology* 143 (4), 913–916.e7. doi: 10.1053/j.gastro.2012.06.031

Wang, H., Banerjee, N., Liang, Y., Wang, G., Hoffman, K. L., and Khan, M. F. (2021a). Gut Microbiome-Host Interactions in Driving Environmental Pollutant Trichloroethene-Mediated Autoimmunity. *Toxicol. Appl. Pharmacol.* 424, 115597. doi: 10.1016/j.taap.2021.115597

Wang, H., Feng, X., Yan, W., and Tian, D. (2020). Regulatory T Cells in Autoimmune Hepatitis: Unveiling Their Roles in Mouse Models and Patients. *Front. Immunol.* 11. doi: 10.3389/fimmu.2020.575572

Wang, F., Liu, J., Weng, T., Shen, K., Chen, Z., Yu, Y., et al (2017). The Inflammation Induced by Lipopolysaccharide can be Mitigated by Short-Chain Fatty Acid, Butyrate, Through Upregulation of IL-10 in Septic Shock. *Scand. J. Immunol.* 85 (4), 258–263. doi: 10.1111/sji.12515

Wang, H., Wang, G., Banerjee, N., Liang, Y., Du, X., Boor, P. J., et al (2021b). Aberrant Gut Microbiome Contributes to Intestinal Oxidative Stress, Barrier Dysfunction, Inflammation and Systemic Autoimmune Responses in MRL/lpr Mice. *Front. Immunol.* 12. doi: 10.3389/fimmu.2021.651191

Wang, C. H., Yen, H. R., Lu, W. L., Ho, H. H., Lin, W. Y., Kuo, Y. W., et al (2022). Adjuvant Probiotics of Lactobacillus Salivarius Subsp. Salicinius AP-32, L. Johnsonii MH-68, and Bifidobacterium Animalis Subsp. Lactis CP-9 Attenuate Glycemic Levels and Inflammatory Cytokines in Patients With Type 1 Diabetes Mellitus. *Front. Endocrinol. (Lausanne)* 13. doi: 10.3389/fendo.2022.754401

Wei, Y., Li, Y., Yan, L., Sun, C., Miao, Q., Wang, Q., et al (2020). Alterations of Gut Microbiome in Autoimmune Hepatitis. *Gut* 69 (3), 569–577. doi: 10.1136/gutjnl-2018-317836

Wei, Y., Zeng, B., Chen, J., Cui, G., Lu, C., Wu, W., et al (2016). Enterogenous Bacterial Glycolipids are Required for the Generation of Natural Killer T Cells Mediated Liver Injury. *Sci. Rep.* 6, 36365. doi: 10.1038/srep36365

White, R., Atherly, T., Guard, B., Rossi, G., Wang, C., Mosher, C., et al (2017). Randomized, Controlled Trial Evaluating the Effect of Multi-Strain Probiotic on the Mucosal Microbiota in Canine Idiopathic Inflammatory Bowel Disease. *Gut Microbes* 8 (5), 451–466. doi: 10.1080/19490976.2017.1334754

Yang, F., Fan, X., Liu, Y., Shen, Y., Zhao, S., Zheng, Y., et al (2021). Long Noncoding RNA and Circular RNA Expression Profiles of Monocyte-Derived Dendritic Cells in Autoimmune Hepatitis. *Front. Pharmacol.* 12. doi: 10.3389/fphar.2021.792138

Yang, A.-M., Inamine, T., Hochrath, K., Chen, P., Wang, L., Llorente, C., et al (2017). Intestinal Fungi Contribute to Development of Alcoholic Liver Disease. *J. Clin. Invest.* 127 (7), 2829–2841. doi: 10.1172/JCI90562

Yang, L., and Seki, E. (2012). Toll-Like Receptors in Liver Fibrosis: Cellular Crosstalk and Mechanisms. *Front. Physiol.* 3. doi: 10.3389/fphys.2012.00138

You, N., Xu, J., Wang, L., Zhuo, L., Zhou, J., Song, Y., et al (2021). Fecal Fungi Dysbiosis in Nonalcoholic Fatty Liver Disease. *Obesity (Silver Spring Md.)* 29 (2), 350–358. doi: 10.1002/oby.23073

Yuksel, M., Wang, Y., Tai, N., Peng, J., Guo, J., Beland, K., et al (2015). A Novel "Humanized Mouse" Model for Autoimmune Hepatitis and the Association of Gut Microbiota With Liver Inflammation. *Hepatology* 62 (5), 1536–1550. doi: 10.1002/hep.27998

Zhang, J., Guo, L., Liu, M., Jing, Y., Zhou, S., Li, H., et al (2018). Receptor-Interacting Protein Kinase 3 Mediates Macrophage/Monocyte Activation in Autoimmune Hepatitis and Regulates Interleukin-6 Production. *United European Gastroenterol. J.* 6 (5), 719–728. doi: 10.1177/2050640618756124

Zhang, H., Liu, M., Liu, X., Zhong, W., Li, Y., Ran, Y., et al (2020). Ssp. Lactis 420 Mitigates Autoimmune Hepatitis Through Regulating Intestinal Barrier and Liver Immune Cells. *Front. Immunol.* 11. doi: 10.3389/fimmu.2020.569104

Zhao, Y., Chen, F., Wu, W., Sun, M., Bilotta, A. J., Yao, S., et al (2018). GPR43 Mediates Microbiota Metabolite SCFA Regulation of Antimicrobial Peptide Expression in Intestinal Epithelial Cells *via* Activation of mTOR and STAT3. *Mucosal Immunol.* 11 (3), 752–762. doi: 10.1038/mi.2017.118

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Potential Implications of the Lung Microbiota in Patients with Chronic Obstruction Pulmonary Disease and Non-Small Cell Lung Cancer

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Recently, chronic obstructive pulmonary disease (COPD) has been considered as a common risk factor of non-small cell lung cancer (NSCLC). However, very few studies have been conducted on the effects of COPD on the lung microbiota in patients with NSCLC. To identify the lung microbiota in patients with COPD and NSCLC (CN), the microbiome of the induced sputa of 90 patients was analyzed using 16S rDNA sequencing. The results showed no significant differences in the bacterial diversities of induced sputa among patients with COPD, NSCLC, and CN and no intrinsic differences among patients with different pathological types of lung cancer. After surgical operation, the diversities of the induced sputa in patients with CN significantly decreased. More remarkably, both the microbial community phenotypes and the components of the induced sputa in patients with CN obviously differed from those in patients with COPD or NSCLC. The relative abundances of *Streptococcus*, *Veillonella*, *Moraxella*, and *Actinomyces* significantly decreased, but those of *Neisseria* and *Acinetobacter* significantly increased in patients with CN compared with those in patients with COPD or NSCLC alone, resulting in increased Gram-negative microbiota and, therefore, in potential pathogenicity and stress tolerance, as well as in enhancement of microbial glycolipid metabolism, amino acid metabolism, and oxidative stress. Although COPD did not affect the number of pulmonary flora species in patients with NSCLC, these significant alterations in the microbial populations, phenotypes, and functions of induced sputa due to COPD would contribute to inflammation-derived cancer progression in patients with CN.

Keywords: COPD, NSCLC, lung dysbiosis, complication, clinical trial

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is currently the fourth leading cause of death in the world, and the burden from COPD is expected to increase over the next few decades (Lange et al., 2021). According to the reports of the Pulmonary Health Observational Study in China, there are nearly 100 million COPD patients in the country, which is a heavy burden to the society (Guo et al., 2020; Dong et al., 2021). In addition to irreversible obstructive ventilatory disorders, COPD is also a systemic inflammatory disease, which can also increase the risk of lung cancer. Some studies have shown that the risk of lung cancer in patients with COPD is two to five times higher than that in non-smokers, and the link between the two is not significantly related to age or tobacco exposure (Hou et al., 2019; Parris et al., 2019; Zheng Y. et al., 2021). Moreover, the 5-year overall survival rate of patients with COPD is significantly lower than that of patients without COPD, especially in men and patients with squamous cell carcinoma (Parris et al., 2019; Ahn et al., 2020; Kang et al., 2020). However, a recent study has shown that, compared with never smokers without COPD, the lung cancer incidence rates in never smokers with COPD, ever smokers without COPD, and ever smokers with COPD were increased by 97%, 167%, and 519%, respectively. This indicated that COPD is a strong independent risk factor of lung cancer, irrespective of smoking status (Park et al., 2020).

Growing evidence has demonstrated that the overall changes in pulmonary flora are associated with COPD status (Hou et al., 2019). Through microbiota analysis in pulmonary tissues, bronchoalveolar lavage fluid (BALF), and sputa, the greatest differences in flora between patients with COPD and healthy controls were found to be in *Pseudomonas*, *Streptococcus*, *Prevotella*, and *Haemophilus*, whose relative abundances were significantly elevated with COPD aggravation (Houghton, 2013; Bozinovski et al., 2016; Park et al., 2020). Notably, an increased abundance of TM7 had been found in both COPD and lung cancer patients, indicating that TM7 plays a potential role in the progression of COPD into lung cancer (Cheng et al., 2020; Wang et al., 2021). However, previous reports presented diverse results, and the global perspective of macrobiotic changes from COPD to lung cancer remains to be elucidated.

To date, our understanding of the microbiome in patients with lung cancer is still in its nascent stage. Respiratory samples, such as saliva, sputa, bronchoscopy samples (e.g., bronchial aspirated fluid, BALF, and bronchial mucosa), and lung biopsies, have been widely used in the field of lung microecological research. Given that lung biopsy is invasive and difficult to perform in patients without clinical biopsy indications, induced sputum and BALF samples are easier to implement as noninvasive procedures for dynamically observing the airway microbiome in patients with lung diseases. However, there were obvious differences in the microbiota composition among the above specimens. The microbiota in the upper airway tract differs from that in alveolar tissues, which is partly due to the parenchymal components of the airway and vascular tissues mainly contained in lung tissue samples (Lee et al., 2016; Yang et al., 2021; Zheng Y. et al., 2021; Zitvogel and Kroemer, 2021). The induced sputa obtained from patients with COPD had the most similar compositions to bronchoalveolar aspirated sputa, while BALF had the closest results to the upper bronchial mucosa flora rather than the lower respiratory flora, indicating that induced sputa could be a better representative of lower bronchial dysbiosis in lung diseases (Sze et al., 2012; Huang et al., 2014; Tiew et al., 2021). Therefore, in this study, to investigate the characteristics of the microbiome in patients with COPD and non-small cell lung cancer (NSCLC), 16S ribosomal DNA (rDNA) sequencing was performed to compare the microbiome diversities and differences among the induced sputum samples and to estimate its application value in NSCLC and lung precancerous lesion screening.

MATERIALS AND METHODS

Ethics Approval and Inclusion Criteria

This research was approved by the ethics committees of the participating institution: The First Affiliated Hospital of Zhejiang Chinese Medical University. Informed consent was obtained from all patients. The samples and data were completely anonymized. Basic information was collected on patients' age, sex, weight, type of pathology, and treatment modality on admission. The study was conducted in accordance with the ethical guidelines and regulations for human research and the Helsinki Declaration.

The inclusion criteria were as follows: 1) lung cancer patients with a clear pathological diagnosis of lung squamous carcinoma or lung adenocarcinoma; benign lung lesions with a clear pathological diagnosis, combined with computed tomography (CT) findings and clinical features; and COPD diagnosis meeting the diagnostic criteria of GOLD 2017 or confirmed diagnosis in the past; 2) age 18–90 years; 3) absence of other types of respiratory infections, such as community-acquired pneumonia, upper respiratory tract infection, acute bronchitis, bronchiectasis with infection, asthma, and acute exacerbation of COPD; and 4) no ongoing antibiotic treatment, immunotherapy, radiotherapy, targeted therapy, or other interventions for tumors.

The exclusion criteria were as follows: 1) patients without clear pathological diagnosis; 2) patients with COPD that could not be clarified through clinical data; 3) patients with lung cancer suspected or clearly combined with lung infection; 4) presence of yellow pus or dark sputum; 5) unknown antibiotic use status prior to specimen collection; 6) patients who received interventions such as immunotherapy, radiotherapy, and targeted therapy prior to specimen collection; and 7) sequencing results that presented insufficient absolute abundance or a homogeneous composition of flora.

After the application of the inclusion and exclusion criteria, a total of 90 patients were eligible for this study, including 67 patients with NSCLC and COPD [18 samples were collected after surgical treatment (CLA group) and 49 samples were obtained from patients not undergoing surgical treatment (CLB group)], 9 patients with NSCLC only (LC), and 14 patients with COPD only. Patients were aged 28–88 years, and the mean age was 64.4

years. There was only one female COPD patient; all other patients in this study were men.

Collection of BALF Samples

The induced sputum samples from each patient were collected after waking up in the morning. The patients repeatedly gargled three times with normal saline to remove oral bacteria and foreign bodies, and then ultrasonic nebulized 3% saline was inhaled for 15 min. Sputum (2 ml) was expectorated from the deep part of the trachea and collected into sterile Eppendorf tubes. The sputum samples were immediately sent to the laboratory for bacterial smear and sample culture.

Each sputum sample was mixed with two volumes of 0.1% dithiothreitol solution (Merck, Darmstadt, Germany) and then centrifuged at 1,300 rpm for 5 min. Subsequently, two volumes of phosphate-buffered saline (PBS) solution were added to the supernatant and centrifuged again (800 rpm for 5 min). The supernatant was collected and stored at −80°CC. The eligibility criteria for induced sputum were determined as follows: the volume of each sample was >2 ml; the number of epithelial cells was <50% of the total cells; and the number of non-epithelial cells exceeded 200. All of the above processes were performed under sterile conditions.

DNA Extraction

The sputum was centrifuged at 14,000 rpm for 10 min, the supernatant was discarded, and the pellet was resuspended in 200 μl of sterile PBS and applied to DNA extraction using Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

16S rDNA Sequencing

The DNA concentration and purity were examined using NanoDrop2000 (Thermo Scientific, Waltham, MA, USA) and the DNA quality tested with 1% agarose gel electrophoresis. Primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) were used for PCR amplification of the V3–V4 variable regions of 16S rDNA. PCR products were recovered with 2% agarose gel, purified with AMPure XT beads (Beckman, Brea, CA, USA), eluted with Tris–HCl, and detected with 2% agarose electrophoresis. The Quantifluor TM-ST system was used for quantitative detection. Qualified samples were used to construct a PE 2*300 library and sequenced following the standard operating procedures of the Illumina MiSeq Platform.

Data Analysis

The Trimmomatic software was used for quality control in the original sequencing, splicing was performed with FLASH, and UPARSE (version 7.1) was applied for the operational taxonomic unit (OTU) clustering of the sequences based on 97% similarity. Chimeras were culled with UCHIME. Each sequence was annotated for species classification with the RDP classifier and aligned with the Silva 128/16S bacteria database. The alignment threshold was set to 0.8. The indices S_{obs} (observed accumulated richness), Chao, and ACE were adopted to evaluate the richness of the microbial community. The Shannon and Simpson indices were used to assess community diversity. The diversity index was used to comprehensively evaluate the community richness and evenness of the samples. The larger the Shannon value, the greater the community diversity. The smaller the Simpson value, the lower the community diversity. Species composition analysis was performed using I-Sanger.

Statistical Methods

The paired signed-rank test was used to assess intergroup differences for the α-diversity index and species difference analysis, and statistical analysis was performed using SPSS version 23.0. Principal component analysis (PCA) was applied for the β-diversity analysis to evaluate the similarity and difference between different samples, and statistical analysis was performed using R language. All statistical tests were two-tailed, with $p < 0.05$ considered statistically significant.

RESULTS

Analysis of Microbiota Diversity

A total of 1,500 OTUs were obtained using Illumina high-throughput sequencing analysis. To distinguish the shared and unique OTUs in the test groups, a Venn diagram was drawn to visualize the number of OTUs and overlaps between each group. As shown in **Figure 1**, 187 OTUs were shared among the four groups, accounting for 12.5% of the total, whereas 533 OTUs were shared between the lung cancer (LC) and COPD groups and 198 among the LC, CLA, and CLB groups. The OTUs were identified as belonging to 22 phyla and 364 genera.

The Chao1 and Observed_otu indices were positively correlated with the number of species contained in the community, whereas the Simpson and Shannon values indicated the richness and evenness of species, respectively. As shown in **Figure 2**, the values of Chao1 and Observed_otu in the LC group were significantly increased compared with the other three groups ($p < 0.05$). The values in the COPD and CLB groups were very close ($p > 0.05$), but were much higher than those in the CLA group, indicating that the LC group had the highest species diversity, while the CLA group had the lowest. Moreover, there were no significant differences in the Simpson and Shannon values among the four groups, indicating the non-significant difference in the richness and evenness of the lung microbiota among the groups.

In addition, a principal coordinate analysis (PCoA) graph was obtained by calculating the weighted UniFrac distance (**Figure 3**), which displayed the similarity and difference between the lung microbiome in different environments. Each point in **Figure 2** represents an individual sample, and the distance between points represents the similarity between the samples. In other words, the smaller the distance, the more similar the property. The contributions of the principal components PC1 and PC2 were 40.33% and 31.75%, respectively, based on the weighted principal coordinates of the UniFrac distance, which reflected the overall status of the samples well. Furthermore, samples from the same group

presented remarkable discrete states under the same clinicopathological characteristics, indicating that there were individual differences in the microbiome among samples from the same group. Notably, there was a huge overlap between the CLA and CLB groups and between the COPD and LC groups, indicating the similarity of the microbiota composition between the two groups. However, both the COPD and LC groups showed partial overlaps with the combined COPD and LC groups (i.e., CLA and CLB), indicating marked differences among the CLA, CLB, COPD, and LC groups.

Alterations in the Components of the Lung Microbiome

To visualize the differences in species richness among the groups, we compared the relative abundance of the top 30 species using clustering stacked bar charts (**Figure 4A**). For this purpose, clusters were dynamically generated by merging four groups into two clusters. Interestingly, at both the genus and phylum levels of taxonomic criteria, CLA and CLB were grouped into one cluster, whereas COPD and LC comprise another cluster. These results demonstrate that the relative abundances of the top 30 microbial compositions at the genus and phylum levels were not significantly altered by surgical operation. More importantly, the microbial compositions of the COPD and NSCLC combination groups differed from those of the COPD and LC groups, indicating the high specificity of COPD patients with lung cancer. The dominant phyla were Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, and Fusobacteria; especially, the levels of the first two accounted for >60% of the total. On the other hand, the dominant genera in these four groups were *Streptococcus*, *Neisseria*, *Veillonella*, *Prevotella_7*, *Actinomyces*, *Moraxella*, *Acinetobacter*, *Corynebacterium_1*, *Haemophilus*, *Gemella*, *Alloprevotella*, *Porphyromonas*, *Rothia*, *Fusobacterium*, and *Leptotrichia*, all of which accounted for more than nearly 70% of the total abundances. Remarkably, the relative abundances of *Veillonella*, *Haemophilus*, *Alloprevotella*, and *Acinetobacter* in the CLA group were significantly elevated compared with the other three groups, indicating obvious alterations in the Gram-negative microbial compositions, which was found to be in agreement with the results of BugBase (**Figure 5**).

To further display the specific flora visually in the different groups of patients, a Sankey diagram was plotted to demonstrate the taxonomic abundance of the main lung microbiota. As shown in **Figure 4B**, *Streptococcus*, *Neisseria*, *Veillonella*, and *Prevotella_7* were the main species in the induced sputa of patients. Compared with the other groups, the relative abundances of *Streptococcus* and *Actinomyces* were significantly elevated in the LC group. *Moraxella*, *Corynebacterium_1*, and *Gemella* had high expressions in the COPD group, whereas these had little or no expression in the LC, CLA, and CLB groups, whose data were consistent with those of previous reports (Leung et al., 2017; Beech et al., 2020). Compared with the other groups, *Haemophilus* and *Neisseria* showed the highest levels in the CLA group, whereas *Acinetobacter* was only observed in the CLB group. These results revealed that induced sputum from different types of patients had different microbiota profiles, which could be used as special diagnostic markers and therapeutic targets for the prevention of COPD and lung cancer.

However, there were no significant differences in the relative abundances of *Streptococcus*, *Neisseria*, *Veillonella*, and *Prevotella_7* among patients with lung adenocarcinoma,

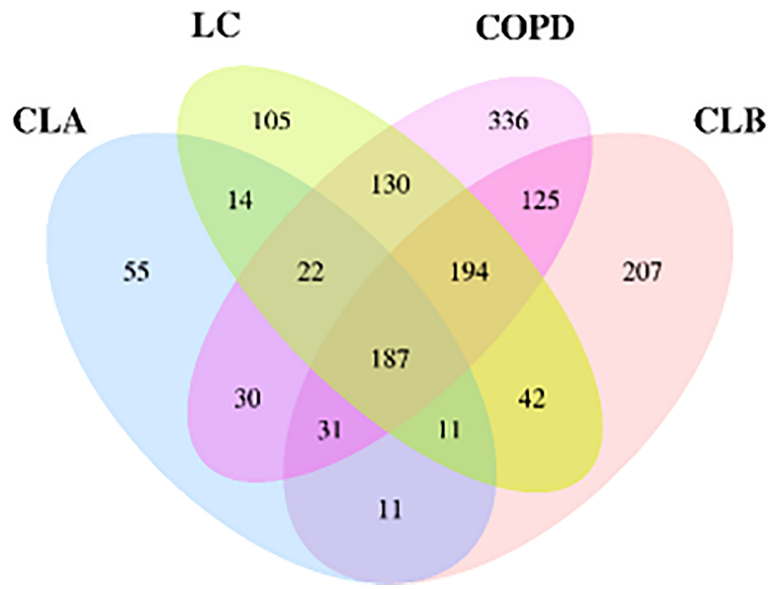

FIGURE 1 | Veen diagram of the number of shared and unique operational taxonomic units (OTUs) among the CLA (surgical treatment), CLB (non-surgical treatment), LC (lung cancer), and COPD (chronic obstructive pulmonary disease) groups.

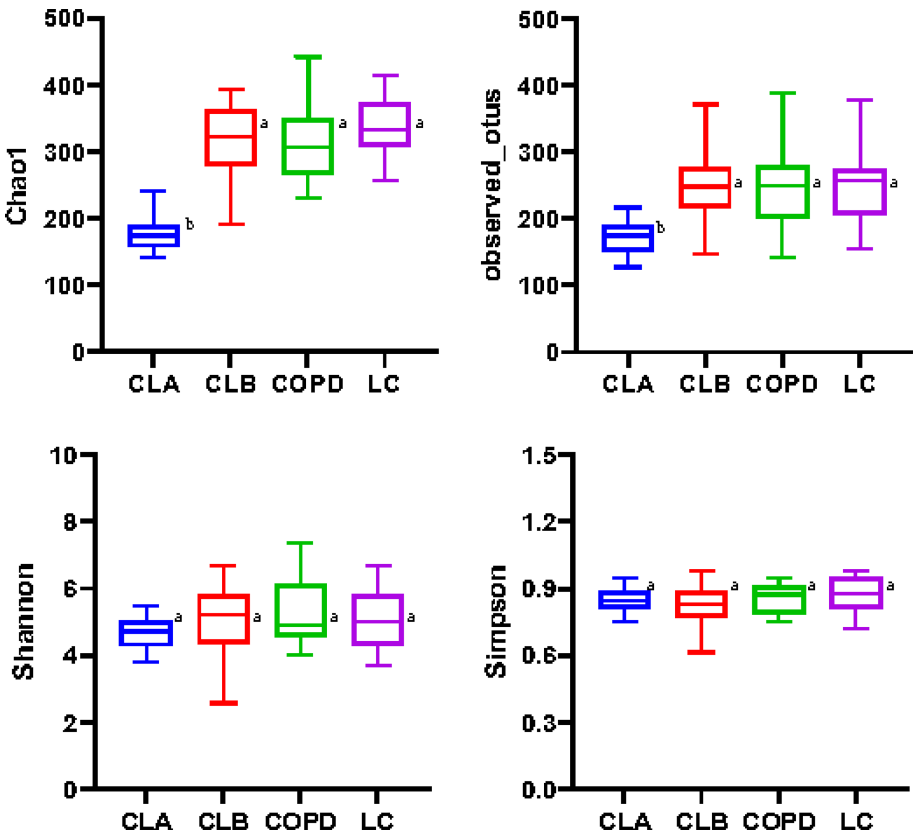

FIGURE 2 | Alpha diversities of the induced sputum samples from different groups. Different indices (Chao1, Observed_otus, Shannon, and Simpson) were used to estimate the number of microbiota in samples. Data shown are the median ± quartile. *Different letters* indicate statistically significant differences. $p < 0.05$.

squamous cell carcinoma, and other subtypes of NSCLC (**Figure 6**). The limited number of samples collected in the study may have affected the accuracy and representation of the outcome, and further studies need to be developed in the future.

Alterations in the Function of the Lung Microbiome

As shown in **Figure 7**, the functions of the differently expressed lung microbiome were analyzed using the PICRUSt2 algorithm, and their biological annotations were referenced from the KEGG database. Compared with those of the LC group, the rates of L-glutamate and L-glutamine biosynthesis, superpathway of L-threonine biosynthesis, superpathway of polyamine biosynthesis, superpathway of *S*-adenosyl-L-methionine biosynthesis, and of arginine, ornithine, and proline interconversion significantly decreased in the CLB group ($p < 0.05$), but the rates of superpathway of fatty acid biosynthesis initiation, superpathway of L-methionine biosynthesis, TCA cycle, glycolysis, stearate biosynthesis, ppGpp biosynthesis, and nitrate biosynthesis significantly increased in the CLB group ($p < 0.05$). This indicates that the abnormal alterations in glycolipid metabolism, amino acid metabolism, and oxidative stress, which are mediated by lung microbes, would contribute to inflammation-driven lung cancer.

DISCUSSION

On the surface, COPD and lung cancer are two distinct diseases. COPD is characterized by chronic lung injury with two main manifestations—airflow limitation and parenchymal destruction—which are often accompanied by increased apoptosis, autophagy, and senescence caused by smoking. In contrast, lung cancer is characterized by abnormal DNA damage and genomic instability, leading to tumor angiogenesis and immune escape. However, a lot of studies now suggest that the pathogenesis between the two diseases has some commonalities, tobacco smoke inhalation being the most common trigger (Szalontai et al., 2021). In addition, immune dysfunction, lung microbiota dysbiosis and inflammatory infections, oxidative stress, and DNA damage play a role in the development of COPD and lung cancer (Caramori et al., 2019; Hou et al., 2019; Parris et al., 2019; Sears, 2019), all of which may be potential drivers of the progression of COPD into lung cancer.

Clinical and animal studies have revealed tumor-associated dysregulation of the local microbiome in the lung, which in turn impacted cancer progression through systemic inflammatory response (Weinberg et al., 2020; Zitvogel and Kroemer, 2021). In addition, epidemiological evidence revealed that the repeated use of antibiotics would induce an increased risk of lung cancer,

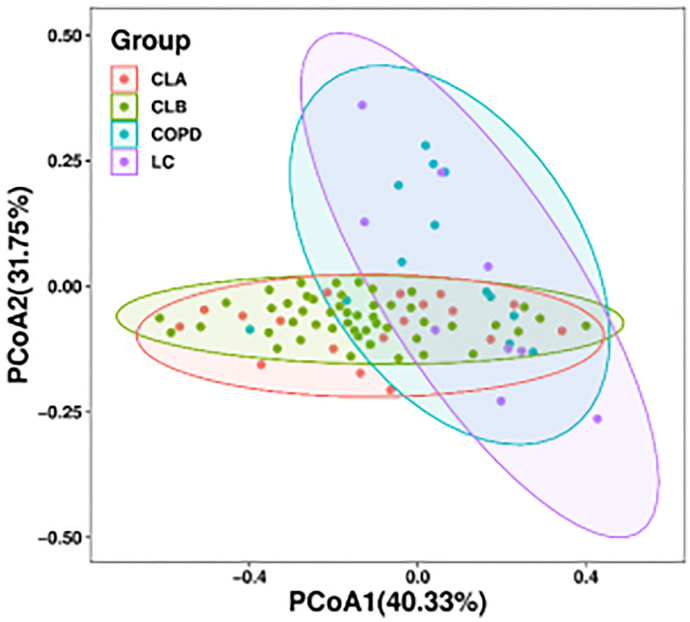

FIGURE 3 | Bacterial diversity clustering by weighted UniFrac principal coordinate analysis (PCoA) of the lung microbiota.

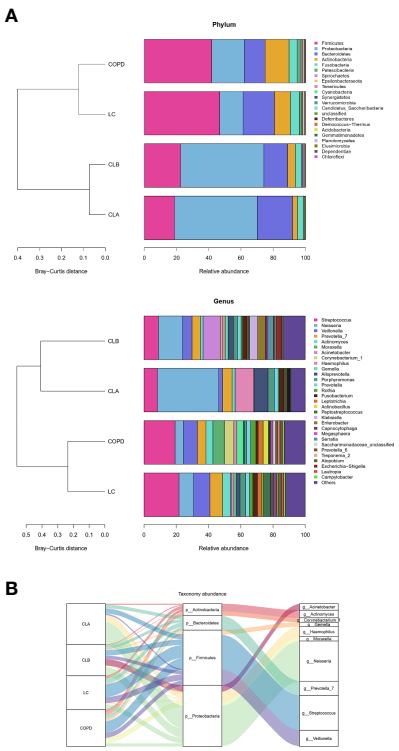

FIGURE 4 | Comparison of the phyla and genera of the microbiomes among the CLA (surgical treatment), CLB (non-surgical treatment), COPD (chronic obstructive pulmonary disease), and lung cancer (LC) groups. **(A)** Comparison of the abundances of the bacterial phyla (*left*) and genera (*right*) of each group. **(B)** Sankey plots of the relative abundances of the discriminatory bacterial phyla and genera among the groups.

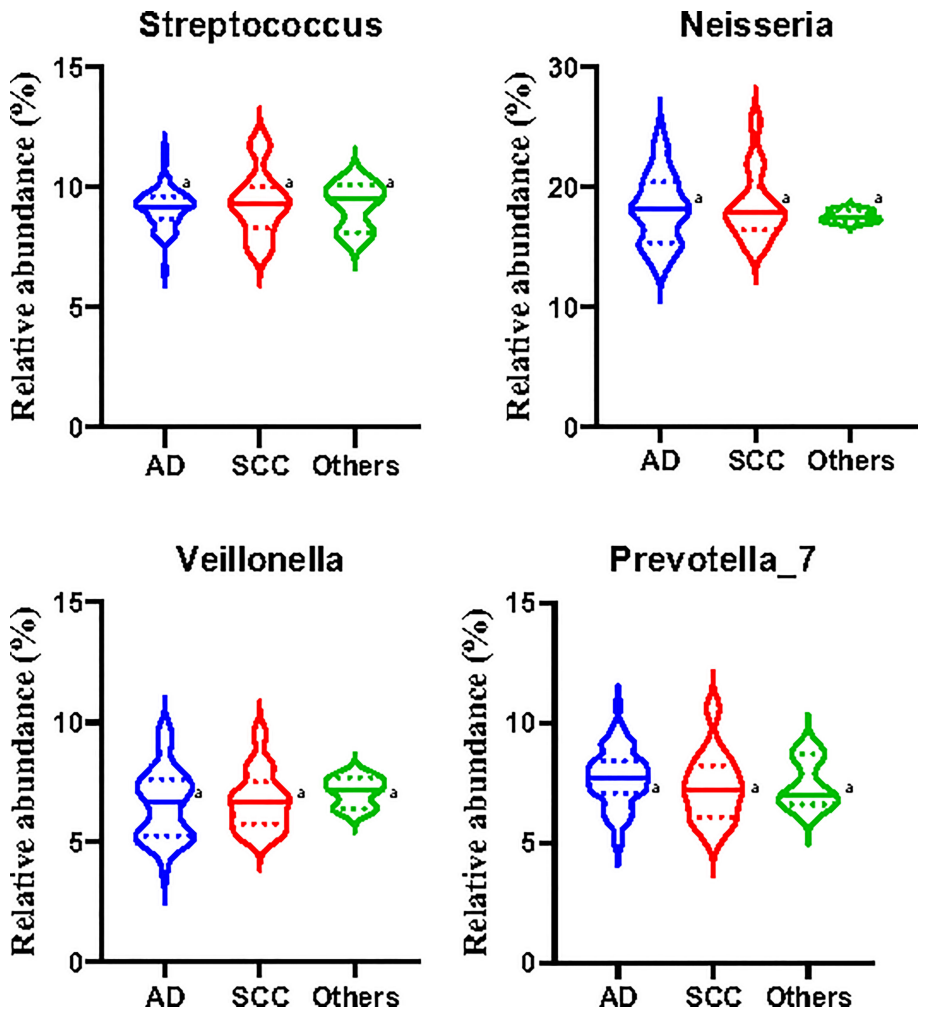

FIGURE 5 | Microbial community phenotypes in the induced sputa of patients with chronic obstructive pulmonary disease (COPD) and/or lung cancer. This community function was predicted using the BugBase web server for quantifying the relative abundance of the microbiome in nine categories. Data shown are the median ± quartile. *Different letters* indicate statistically significant differences. $p < 0.05$.

suggesting that abnormal pulmonary microbial communities could play a part in the occurrence of lung cancer. However, the association between pulmonary flora and lung cancer remains unclear. As yet, systematic and large-scale clinical observation of lung dysbiosis in patients with COPD and NSCLC has been lacking. In this study, the microbial α-diversity in the induced sputa of CLB patients was not significantly higher than those in patients with COPD and NSCLC ($p > 0.05$), but the diversity in CLA patients was obviously decreased compared with that of the other three groups, indicating that the microbial species were reduced after surgical operation. Furthermore, *Streptococcus*, *Neisseria*, *Veillonella*, *Prevotella_7*, *Actinomyces*, *Moraxella*, *Acinetobacter*, *Haemophilus*, and *Gemella* were the dominant bacteria in the induced sputa of all these patients, whose populations varied with the disease type. Compared with those in patients with COPD and LC, the relative abundances of *Streptococcus*, *Veillonella*, *Actinomyces*, *Corynebacterium_1*, *Rothia*, and *Leptotrichia* significantly decreased in the CLB group, whereas the abundances of *Neisseria*, *Haemophilus*, and *Alloprevotella* significantly increased. After tumor resection, the levels of *Haemophilus* and *Neisseria* were drastically elevated in the CLA group. Particularly, a high level of *Acinetobacter* was only observed in the CLB group, indicating its specificity in the progression of COPD-related lung cancer.

The lung microbiome regulates inflammatory factors in lung tissue by producing oncogenic metabolites and toxins, which can bind to Toll-like receptors on antigen-presenting cells such as monocytes and dendritic cells, inducing chronic inflammation, which in turn disrupts the cell cycle, leading to the upregulation of oncogene signaling pathways and promoting lung carcinogenesis (Nowrin et al., 2014; Mendez et al., 2019;

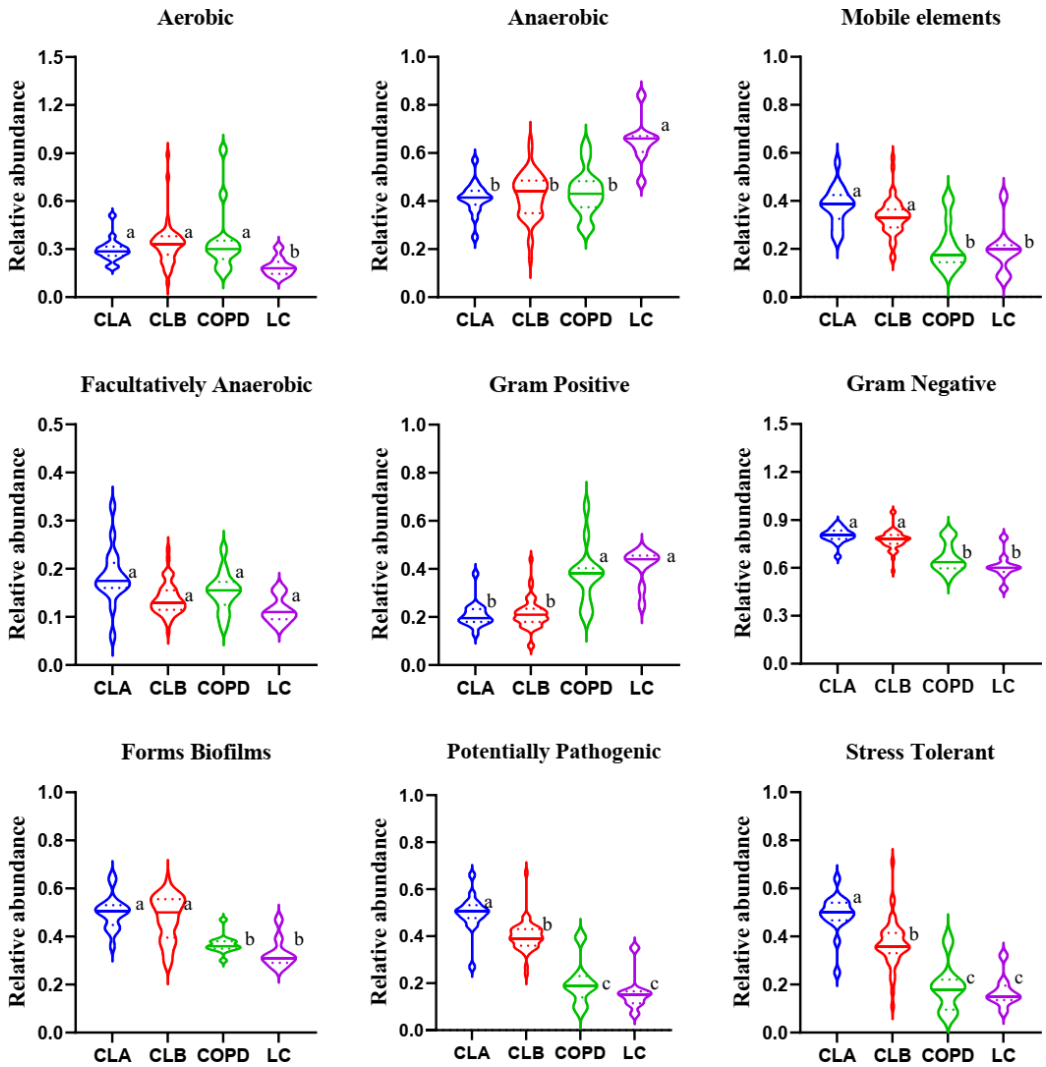

FIGURE 6 | Relative abundances of the main microbiota in the induced sputa of patients with non-small cell lung cancer (NSCLC). *AD*, patients with lung adenocarcinoma; *SCC*, patients with squamous cell carcinoma; *Other*, except AD and SCC, patients with other subtypes of NSCLC. Data shown are the median ± quartile. *Different letters* indicate statistically significant differences. $p < 0.05$.

Weinberg et al., 2020; Zitvogel and Kroemer, 2021). Through analysis with the BugBase and PICRUSt2 algorithms, the phenotypes and the functions of the mainly differentially expressed microbiota among patients with different pathological types of lung diseases were predicted, respectively. The results demonstrated that the Gram-negative microbiota, potential pathogenicity, and the stress tolerance of the microbiota in the COPD-combined NSCLC patients significantly increased compared to those in patients with COPD or NSCLC only. Moreover, the microbial glycolipid metabolism, amino acid metabolism, and oxidative stress of such perturbed lung microbial community were also enhanced. During inflammation and cancer, host immune cells or tumor cells need energy to support their physiological function, leading to increased anaerobic glycolysis and amino acid consumption (Fahrmann et al., 2020; Schiliro and Firestein, 2021; Stepka et al., 2021; Tomé, 2021), which was consistent with our findings on the metabolic alteration of the lung microbiome. Thus, these changes may be associated with specific metabolic characteristics of COPD-related lung cancer.

Unlike in Western countries, most smokers in China are men (Yang et al., 2020). Thus, it was difficult to collect samples from the same number of male and female patients, resulting in an imbalance in this study. Similarly, the chances of developing cancer increase with age (Toumazis et al., 2020). Most patients with NSCLC in this study were retired. Recently, sex and age differences have been demonstrated to exert a direct influence on oncological treatments, specifically immunotherapy, with documented distinctions between men and women (Vavalà et al., 2021). Consequently, to correctly assess cancer

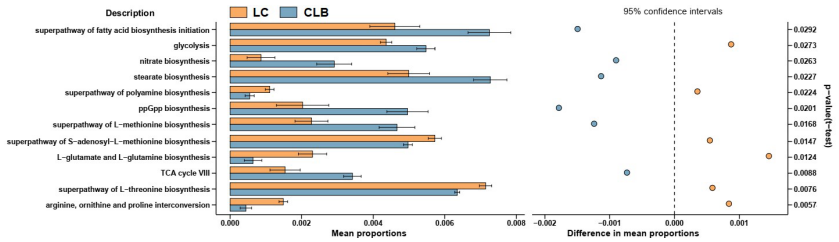

FIGURE 7 | Function alterations of the lung microbiota between patients with lung cancer only (LC) and patients with both COPD and lung cancer (CLB). Microbial gene functions were predicted by using the PICRUSt2 algorithm.

outcomes, a multicenter study with greater population is required in the future.

In conclusion, the microbial populations, phenotypes, and functions of induced sputa exhibited intrinsic differences among patients with COPD, NSCLC, and CN. Compared with patients with COPD and NSCLC alone, the relative abundances of *Streptococcus*, *Veillonella*, *Moraxella*, and *Actinomyces* in patients with CN were significantly reduced, but those of *Neisseria* and *Acinetobacter* were significantly elevated, resulting in increased potential microbial pathogenicity and energy metabolism. The results showed that COPD may affect the populations of pulmonary microbiota in patients with lung cancer, and drastic alterations in the phenotypes and functions of induced sputa among the different pathological types of lung cancer were also related to the presence of COPD.

DATA AVAILABILITY STATEMENT

The datasets analyzed for this study can be found in the repository Jinguoyun https://www.jianguoyun.com/p/Dft7UUEQ5e3kChiNzs4EIAA.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the First Affiliated Hospital of Zhejiang Chinese Medical University. The patients/participants provided written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

J-QH, QC, ZW, and C-HY conceived and directed the study. J-QH, QC, S-ZZ, S-YZ, and J-FW did the analysis and visualization. S-JW, QC, D-QW prepared the sample, and R-LC prepared the samples. J-QH and C-HY drafted the manuscript. ZW and CH-Y revised the manuscript. All authors agree to be accountable for the content of the work. All authors contributed to the article and approved the submitted version.

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REFERENCES

Ahn, S. V., Lee, E., Park, B., Jung, J. H., Park, J. E., Sheen, S. S., et al. (2020). Cancer Development in Patients With COPD: A Retrospective Analysis of the National Health Insurance Service-National Sample Cohort in Korea. *BMC Pulm. Med.* 20 (1), 170. doi: 10.1186/s12890-020-01194-8

Beech, A. S., Lea, S., Kolsum, U., Wang, Z., Miller, B. E., Donaldson, G. C., et al. (2020). Bacteria and Sputum Inflammatory Cell Counts; a COPD Cohort Analysis. *Respir. Res.* 21 (1), 289. doi: 10.1186/s12931-020-01552-4

Bozinovski, S., Vlahos, R., Anthony, D., McQualter, J., Anderson, G., Irving, L., et al. (2016). COPD and Squamous Cell Lung Cancer: Aberrant Inflammation and Immunity is the Common Link. *Br. J. Pharmacol.* 173 (4), 635–648. doi: 10.1111/bph.13198

Caramori, G., Ruggeri, P., Mumby, S., Ieni, A., Bello, F., Chimankar, V., et al. (2019). Molecular Links Between COPD and Lung Cancer: New Targets for Drug Discovery? *Expert Opin. Ther. Targets* 23 (6), 539–553. doi: 10.1080/14728222.2019.1615884

Cheng, C., Wang, Z., Wang, J., Ding, C., Sun, C., Liu, P., et al. (2020). Characterization of the Lung Microbiome and Exploration of Potential Bacterial Biomarkers for Lung Cancer. *Transl. Lung Cancer Res.* 9 (3), 693–704. doi: 10.21037/tlcr-19-590

Dong, F., Huang, K., Ren, X., Qumu, S., Niu, H., Wang, Y., et al. (2021). Factors Associated With Inpatient Length of Stay Among Hospitalised Patients With Chronic Obstructive Pulmonary Disease, China, 2016-2017: A Retrospective Study. *BMJ Open* 11 (2), e040560. doi: 10.1136/bmjopen-2020-040560

Fahrmann, J. F., Vykoukal, J. V., and Ostrin, E. J. (2020). Amino Acid Oncometabolism and Immunomodulation of the Tumor Microenvironment in Lung Cancer. *Front. Oncol.* 10. doi: 10.3389/fonc.2020.00276

Guo, Y., Xing, Z., Shan, G., Janssens, J. P., Sun, T., Chai, D., et al. (2020). Prevalence and Risk Factors for COPD at High Altitude: A Large Cross-Sectional Survey of Subjects Living Between 2,100-4,700 M Above Sea Level. *Front. Med. (Lausanne)* 7. doi: 10.3389/fmed.2020.581763

Houghton, A. M. (2013). Mechanistic Links Between COPD and Lung Cancer. *Nat. Rev. Cancer* 13 (4), 233–245. doi: 10.1038/nrc3477

Hou, W., Hu, S., Li, C., Ma, H., Wang, Q., Meng, G., et al. (2019). Cigarette Smoke Induced Lung Barrier Dysfunction, EMT, and Tissue Remodeling: A Possible Link Between COPD and Lung Cancer. *BioMed. Res. Int.* 2019, 2025636. doi: 10.1155/2019/2025636

Huang, Y. J., Sethi, S., Murphy, T., Nariya, S., Boushey, H. A., and Lynch, S. V. (2014). Airway Microbiome Dynamics in Exacerbations of Chronic Obstructive Pulmonary Disease. *J. Clin. Microbiol.* 52 (8), 2813–2823. doi: 10.1128/JCM.00035-14

Kang, N., Shin, S. H., Noh, J. M., Kang, D., Kim, H., Kwon, O. J., et al. (2020). Treatment Modality and Outcomes Among Early-Stage non-Small Cell Lung Cancer Patients With COPD: A Cohort Study. *J. Thorac. Dis.* 12 (9), 4651–4660. doi: 10.21037/jtd-20-667

Lange, P., Ahmed, E., Lahmar, Z. M., Martinez, F. J., and Bourdin, A. (2021). Natural History and Mechanisms of COPD. *Respirology* 26 (4), 298–321. doi: 10.1111/resp.14007

Lee, S. H., Sung, J. Y., Yong, D., Chun, J., Kim, S. Y., Song, J. H., et al. (2016). Characterization of Microbiome in Bronchoalveolar Lavage Fluid of Patients With Lung Cancer Comparing With Benign Mass Like Lesions. *Lung Cancer* 102, 89–95. doi: 10.1016/j.lungcan.2016.10.016

Leung, J. M., Tiew, P. Y., Aogáin, M. M., Budden, K. F., Yong, V. F., Thomas, S. S., et al. (2017). The Role of Acute and Chronic Respiratory Colonization and Infections in the Pathogenesis of COPD. *Respirology* 22 (4), 634–650. doi: 10.1111/resp.13032

Mendez, R., Banerjee, S., Bhattacharya, S. K., and Banerjee, S. (2019). Lung Inflammation and Disease: A Perspective on Microbial Homeostasis and Metabolism. *IUBMB Life.* 71 (2), 152–165. doi: 10.1002/iub.1969

Nowrin, K., Sohal, S. S., Peterson, G., Patel, R., and Walters, E. H. (2014). Epithelial-Mesenchymal Transition as a Fundamental Underlying Pathogenic Process in COPD Airways: Fibrosis, Remodeling and Cancer. *Expert Rev. Respir. Med.* 8 (5), 547–559. doi: 10.1586/17476348.2014.948853

Park, H. Y., Kang, D., Shin, S. H., Yoo, K. H., Rhee, C. K., Suh, G. Y., et al. (2020). Chronic Obstructive Pulmonary Disease and Lung Cancer Incidence in Never Smokers: A Cohort Study. *Thorax* 75 (6), 506–509. doi: 10.1136/thoraxjnl-2019-213732

Parris, B. A., O'Farrell, H. E., Fong, K. M., and Yang, I. A. (2019). Chronic Obstructive Pulmonary Disease (COPD) and Lung Cancer: Common Pathways for Pathogenesis. *J. Thorac. Dis.* 11 (Suppl 17), S2155–S2172. doi: 10.21037/jtd.2019.10.54

Schiliro, C., and Firestein, B. L. (2021). Mechanisms of Metabolic Reprogramming in Cancer Cells Supporting Enhanced Growth and Proliferation. *Cells* 10 (5), 1056. doi: 10.3390/cells10051056

Sears, C. R. (2019). DNA Repair as an Emerging Target for COPD-Lung Cancer Overlap. *Respir. Investig.* 57 (2), 111–121. doi: 10.1016/j.resinv.2018.11.005

Stepka, P., Vsiansky, V., Raudenska, M., Gumulec, J., Adam, V., and Masarik, M. (2021). Metabolic and Amino Acid Alterations of the Tumor Microenvironment. *Curr. Med. Chem.* 28 (7), 1270–1289. doi: 10.2174/0929867327666200207114658

Szalontai, K., Gémes, N., Furák, J., Varga, T., Neuperger, P., Balog, J. Á., et al. (2021). Chronic Obstructive Pulmonary Disease: Epidemiology, Biomarkers, and Paving the Way to Lung Cancer. *J. Clin. Med.* 10 (13), 2889. doi: 10.3390/jcm10132889

Sze, M. A., Dimitriu, P. A., Hayashi, S., Elliott, W. M., McDonough, J. E., Gosselink, J. V., et al. (2012). The Lung Tissue Microbiome in Chronic Obstructive Pulmonary Disease. *Am. J. Respir. Crit. Care Med.* 185 (10), 1073–1080. doi: 10.1164/rccm.201111-2075OC

Tiew, P. Y., Jaggi, T. K., Chan, L. L. Y., and Chotirmall, S. H. (2021). The Airway Microbiome in COPD, Bronchiectasis and Bronchiectasis-COPD Overlap. *Clin. Respir. J.* 15 (2), 123–133. doi: 10.1111/crj.13294

Tomé, D. (2021). Amino Acid Metabolism and Signalling Pathways: Potential Targets in the Control of Infection and Immunity. *Eur. J. Clin. Nutr.* 75 (9), 1319–1327. doi: 10.1038/s41430-021-00943-0

Toumazis, I., Bastani, M., Han, S. S., and Plevritis, S. K. (2020). Risk-Based Lung Cancer Screening: A Systematic Review. *Lung Cancer* 147, 154–186. doi: 10.1016/j.lungcan.2020.07.007

Vavalà, T., Catino, A., Pizzutilo, P., Longo, V., and Galetta, D. (2021). Gender Differences and Immunotherapy Outcome in Advanced Lung Cancer. *Int. J. Mol. Sci.* 22 (21), 11942. doi: 10.3390/ijms222111942

Wang, Y. J., Wu, S. S., Chu, J., and Kong, X. Y. (2021). Lung Microbiome Mediates the Progression From Chronic Obstructive Pulmonary Disease to Lung Cancer Through Inflammation. *Yi Chuan* 43 (1), 30–39. doi: 10.16288/j.yczz.20-315

Weinberg, F., Dickson, R. P., Nagrath, D., and Ramnath, N. (2020). The Lung Microbiome: A Central Mediator of Host Inflammation and Metabolism in Lung Cancer Patients? *Cancers (Basel)* 13 (1), 13. doi: 10.3390/cancers13010013

Yang, C. Y., Li, S. W., Chin, C. Y., Hsu, C. W., Lee, C. C., Yeh, Y. M., et al. (2021). Association of Exacerbation Phenotype With the Sputum Microbiome in Chronic Obstructive Pulmonary Disease Patients During the Clinically Stable State. *J. Transl. Med.* 19 (1), 121. doi: 10.1186/s12967-021-02788-4

Yang, D., Liu, Y., Bai, C., Wang, X., and Powell, C. A. (2020). Epidemiology of Lung Cancer and Lung Cancer Screening Programs in China and the United States. *Cancer Lett.* 468, 82–87. doi: 10.1016/j.canlet.2019.10.009

Zheng, Y., Huang, Y., Zheng, X., Peng, J., Chen, Y., Yu, K., et al. (2021). Deaths From COPD in Patients With Cancer: A Population-Based Study. *Aging (Albany NY)* 13 (9), 12641–12659. doi: 10.18632/aging.202939

Zitvogel, L., and Kroemer, G. (2021). Lower Airway Dysbiosis Exacerbates Lung Cancer. *Cancer Discovery* 11 (2), 224–226. doi: 10.1158/2159-8290.CD-20-1641

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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Gut microbiota: An emerging therapeutic approach of herbal medicine for prevention of colorectal cancer

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The gut dysbiosis has emerged as a prominent player in the pathogenesis and development of colorectal cancer (CRC), which in turn intensifies dysregulated gut microbiota composition and inflammation. Since most drugs are given orally, this dysbiosis directly and indirectly impinges the absorption and metabolism of drugs in the gastrointestinal tract, and subsequently affects the clinical outcome of patients with CRC. Herbal medicine, including the natural bioactive products, have been used traditionally for centuries and can be considered as novel medicinal sources for anticancer drug discovery. Due to their various structures and pharmacological effects, natural products have been found to improve microbiota composition, repair intestinal barrier and reduce inflammation in human and animal models of CRC. This review summarizes the chemo-preventive effects of extracts and/or compounds derived from natural herbs as the promising antineoplastic agents against CRC, and will provide innovative strategies to counteract dysregulated microbiota and improve the lives of CRC patients.

KEYWORDS

intestinal homeostasis, natural products, chronic inflammation, probiotic, immunoenhancement, tumor microenvironment

Abbreviations: AMPK, adenosine 5'-monophosphate-activated protein kinase; APC, adenomatosis polyposis coli; AOM, azoxymethane; CAC, colitis associated cancer; COX2, cyclo-oxygenase-2; CRC, colorectal cancer; DSS, dextran sodium sulfate; FOXO3, forkhead box O3; IL-17, interleukin 17; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappa-B; PI3K, phosphoinositide 3-kinase; TGF-β1, transforming growth factor-β1; ROS, reactive oxygen species; SCFAs, short chain fatty acids; STAT3, signal transducer and activator of transcription 3.

1 Introduction

The incidence of colorectal cancer (CRC) has boosted greatly in the last decades and has become the third leading cause of cancer death, which now accounts for approximately 10% of cancer-related mortality in the world (Bray et al., 2018). The high incidence of CRC has been attributed to the increasingly aging population, unfavorable dietary habits, low physical exercise and excessive obesity (Lund et al., 2011). With the in-depth application of high-throughput sequencing technologies, such as 16S rRNA, metagenomics and metatranscriptomics, in human gut microbiota, emerging evidence has implicated the microbiota in the pathogenesis and prognosis of CRC (Watanabe et al., 2020). Some bacterial species including *Fusobacterium* spp., *Enterococcus* spp., *Escherichia coli*, and *Bacteroides* spp. are most commonly associated with the onset and progression of CRC (Lennard et al., 2016). Changes in microbiota composition (dysbiosis) impair the gut barrier function of epithelial tight junctions and the mucus layer. Consequently, it increases the exposure of the epithelium to bacteria and their toxic metabolites, which may have carcinogenic potential to interfere with cell cycle regulation or directly damage DNA. Bacterial translocation also induces chronic inflammation and triggers a cascade of suppressive immune responses associated with the production of procarcinogens or chemicals such as reactive oxygen species (ROS), bacterial genotoxins (colibactin), and hydrogen sulfide (H_2S) (Gagnière et al., 2016). In turn, the excessive oxidative stress aggravates colitis and neoplastic processes. Thus, targeting and improving gut microbial dysbiosis could be plausible therapeutic strategies for the prevention and treatment of CRC.

Herbal medicines have been used to prevent and treat diseases for thousands of years which are being developed into decoction and liquid extract for clinical application. When herbal medicines enter the digestive system, they will inevitably come into contact with gut microbes, which could limit excessive inflammatory response and maintain intestinal homeostasis (Chen et al., 2017). Some prodrugs derived from herbal medicines are produced under the metabolism of gut microbes, and subsequently display their antitumor effects to reduce tumor mass and prevent tumorigenesis through several mechanism (Meng et al., 2013; Chen et al., 2016). In addition, bioactive ingredients in herbal medicines may stimulate microorganisms to secrete certain endogenous substances which can enhance barrier stabilization and immune surveillance (Vivarelli et al., 2019). Despite these advances, the underlying molecular mechanism of herbal medicines and their bioactive compounds on microbe-mediated CRC remains extremely deficient. In this review, we highlight the importance of herbal medicine intervention on the intestinal microbiota as an instrument for dysbacteriosis, and consequently, for the prevention of colorectal cancer, suggesting anti-inflammatory, antioxidant and anticarcinogenic properties.

2 Gut microbiota and CRC: Potential disease mechanism

The gut microbiota constitutes a natural defensive barrier to infection. Growing evidence has demonstrated the role of gut microbes in promoting inflammatory responses, creating a suitable microenvironment for the development of skewed interactions between the gut microbiota and cancer initiation (Perillo et al., 2020). Thus, the gut microbiota has been proposed as a novel therapeutic target in light of recent promising data in which it seems to modulate the response to cancer immunotherapy. Moreover, the microbiota involves in numerous protective, structural and metabolic roles in the intestinal epithelium to maintain gut homeostasis. The human intestinal mucosal surface area is more than 200 m^2. There are about 10^3 different microorganisms, which are 10-fold more than the total number of human cells (Sekirov et al., 2010). More than 3×10^7 genes in the gut microbiota are considered as the second genome of humans, and approximately 10% of the metabolite cycles occur in the human intestinal micro-ecological environment (Gill et al., 2006; Wu et al., 2013). CRC is frequently associated with dramatic alterations in the microbial composition of the tumor and adjacent mucosa (Figure 1). Clinical trials proved that the abundance of *Fusobacterium nucleatum* started at stage 0 and increased as the CRC progressed, while *Atopobium parvulum* and *Actinomyces odontolyticus* were significantly increased in patients with multiple polypoid adenomas and/or stage 0 but no longer increased in more advanced stages, *Peptostreptococcus anaerobius*, *Peptostreptococcus stomatis*, and *Parvimonas micra* increased at stage I~IV (Sobhani et al., 2011). In addition, the number of beneficial bacteria such as *Bifidobacterium*, *Helicobacter oxysporum*, and *Haemophilus* was reduced from the polyps to CRC stage 0 (Mizutani et al., 2020). Although the causal relationship between gut dysbiosis and CRC remains unclear, gut dysbiosis exacerbates the development of colorectal cancer mainly *via* intestinal inflammation, immunotolerance, and oxidative stress (Figure 2).

2.1 Inhibition of intestinal inflammation

Inflammations caused by gut microbes are the main mechanisms to induce tumorigenesis (Konstantinov et al., 2013). The disorder of gut microbiota induces the hyperpermeability of the intestinal wall, and then helps the pathogenic bacteria and its endotoxin to break out into the bloodstream, resulting in chronic inflammatory response and immuno-suppression (Avril and DePaolo, 2021). Chronic inflammation reshapes the tumor microenvironment, and promote tumorigenesis and even metastasis *via* activating

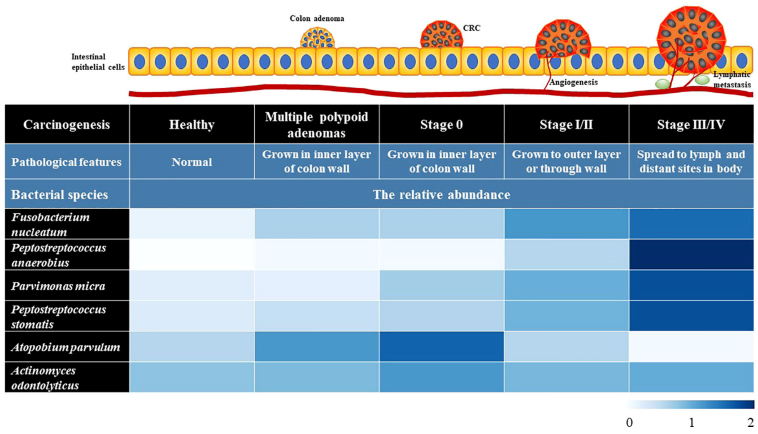

FIGURE 1
The profiles of gut microbiota in the intestinal tissues of healthy people and the CRC patients at different stages. The data in heatmap were obtained from the reference (Sobhani et al., 2011). The microbiome signature potentially can be used as auxiliary diagnostic biomarkers.

numerous exogenous and endogenous signaling pathways (Yu and Fang, 2015). Pathogen recognition receptors (PPRs) are a series of innate immune receptors mainly including toll-like receptors (TLRs) and Nod-like receptors (NLRs). Once the intestinal epithelial barrier is breached, PPRs rapidly sense nucleic acids and antigen components from bacteria and subsequently induce the secretion of type I interferon and antimicrobial peptides to defense against intestinal pathogens. However, inappropriately vigorous innate immune responses can also activate cell survival signaling mainly *via* NF-κB, STAT3 and MAPK pathways, which elevate the transcription of pro-inflammatory cytokines. Then secondary enteric inflammatory challenges prolong systemic inflammation and expedite proliferation and metastasis of tumor cells (Fukata and Abreu, 2009). Indeed, the patients with inflammatory bowel disease (IBD) presented an increased risk of developing CRC (Feagins et al., 2009; Rogler, 2014). In those patients, the number of probiotics such as *Lactobacillus* and *Bifidobacteria* is reduced, while *Parvimonas micra*, Phascolarctobacterium, *Streptococcus bovis* and *S. gallolyticus* are increased (Uronis et al., 2009; Richard et al., 2018). Oral administration of antibiotics, probiotic preparation or antioxidants significantly decrease the number of mucosal nodules and suppress colon tumorigenesis in the azoxymethane (AOM) and dextran sulfate sodium (DSS)-induced CRC mouse model (Hattori et al., 2019; Luo et al., 2019), suggesting that altering mucosa-associated bacterial microbiota and chronic inflammation in the IBD patients may be beneficial for CRC prevention.

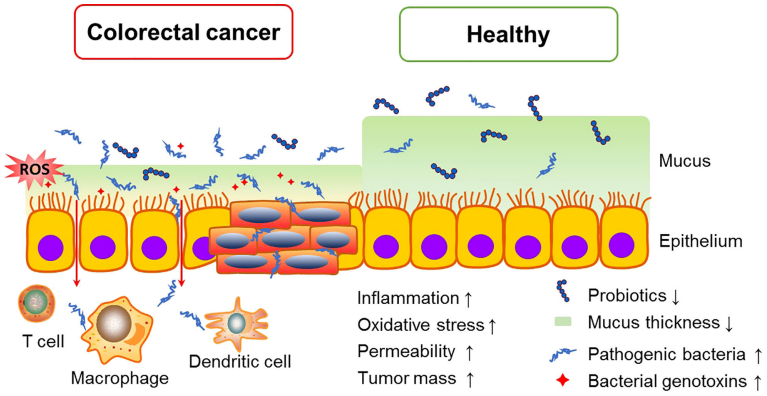

FIGURE 2
Intestinal dysbiosis accelerate CRC tumorigenesis. Overgrowth of pathogenic bacteria produce toxic metabolites, which can interfere with the cell cycle regulation and directly damage DNA, and also induce chronic inflammation and oxidative stress, consequently promoting CRC growth and spread.

2.2 Regulation of bacterial enzymes and metabolites

Gut microbiota is also involved in the production of various enzymes and metabolites. During gastrointestinal tumorigenesis, the physiological capacities of several bacteria are changed, resulting in the odd levels of bacterial enzymes and their metabolites. Bacterial enzymes including β-glucuronidase, nitroso-reductase, nitrate reductase, β-glucosidase, azo-reductase and 7α-dehydrooxygenase from gut microbiota disorders can induce the alteration of intestinal metabolites (such as secondary bile acids and H_2S), thereby producing various carcinogens and promoting the occurrence of colorectal cancer (Azcárate-Peril et al., 2011). Primary bile acids excreted into the gut are converted into secondary bile acids which can increase reactive oxygen species through microbial derived-metabolism, such as hydrolase, leading to DNA damage and genomic instability, and finally induce the growth of tumors (Saracut et al., 2015). Clostridium converts primary bile acid into deoxycholic acid, increasing free radicals and ROS to induce chronic inflammation and colorectal cancer. H_2S is a metabolite produced by sulfate-reducing bacteria in the gut tract which can cause DNA damage, free radical release, colonic mucosal inflammation and hyperplasia, suppress cytochrome oxidase and DNA methylation, and ultimately contribute to tumor initiation (Wang et al., 2021).

2.3 Reduction of oxidative stress

ROS is also blamed as being a driving force behind CRC initiation. *Enterococcus faecalis* releases extracellular superoxide, and after transformed by hydrogen peroxide, these free radicals as powerful mutagens can cause DNA breakage, and local genomic instability in CRC patients with colorectal cancer (Kabwe et al., 2021; Rivas-Domínguez et al., 2021). Similarly, *Helicobacter pylori* promotes the development of gastrointestinal inflammation and carcinogenesis *via* elevating ROS and reactive nitrogen species (RNS) to upregulate oncogenic pathways such as HIF-1α, NF-κB and PI3K/AKT (Liu et al., 2019; Lu et al., 2020). Therefore, targeting those pathogenic bacteria or counteract their deleterious effects (reducing ROS generation) have been considered as potential strategies for preventing CRC.

3 Herbal medicine, gut microbiota and colorectal cancer

Herbal medicines including herbal formulas, extracts, and compounds have been studied for many years in the treatment of gut-related diseases *via* regulating gut micro-ecosystem. It can apparently alter the composition and metabolism of gut microbiota, and dramatically affect the number and function of intestinal epithelial cells to achieve the rebalancing of gut microecology (Figure 3) (Li et al., 2021).

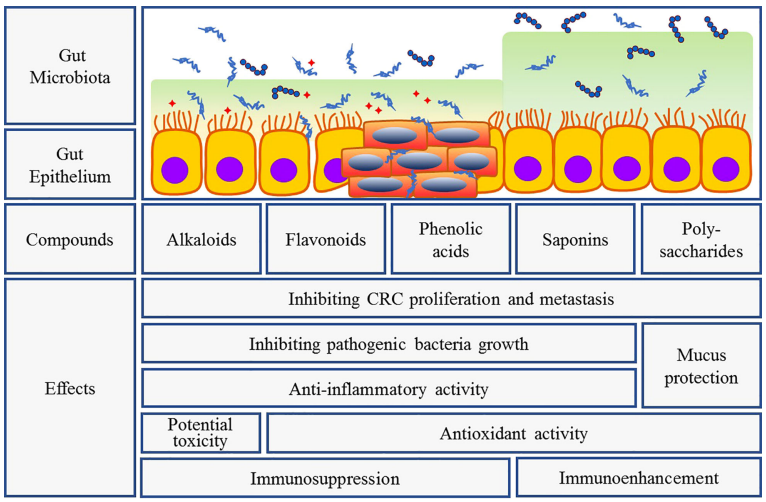

FIGURE 3
Intervention of herbal bioactive components on gut bacteria and CRC. Interaction between herbal medicine and gut microbiota can fight tumor growth and prevent tumorigenesis through several mechanisms: (1) inhibiting pathogenic bacteria overgrowth and promoting probiotics growth; (2) anti-inflammatory and antioxidant activities as well as intestinal mucosal protection and immune regulation; (3) direct anti-tumor activity. But different components exert their respective features.

3.1 Regulation of gut microbiota and metabolites by herbal medicine

Herbal medicines are rich in chemical constituents which contains not only bioactive ingredients such as glycosides, flavonoids, alkaloids, quinones and steroids, but also nutrients such as protein and vitamins, leading to the variety of their pharmacodynamic effects. The mechanism of herbal medicine in improving gut microbiota imbalance includes two aspects: inhibiting pathogenic bacterial overgrowth and promoting probiotics growth. At present, CRC-associated probiotics are mainly divided into three categories: *Lactobacillus*, *Bifidobacteriums*, and Gram-positive cocci. On the one hand, probiotics indirectly inhibit the growth and invasion of pathogenic bacteria through strengthening the barrier function of intestinal epithelial cells and producing beneficial metabolites (Kaur et al., 2021). On the other hand, probiotics have physiologically positive effects on the host (animals or human) through regulating the immune function of the host mucosa and system, or improving the balance of gut nutrition and microbiota composition. What's more, probiotics also play a role in inhibiting allergies, controlling serum cholesterol level, and regulating immune function such as metabolic transformation and metabolic detoxification for preventing the gastrointestinal carcinogenesis (Paveljšek et al., 2021).

Many herbal medicines, such as *Ganoderma Lucidum*, American Ginseng, Red Ginseng, *Gynostemma Pentaphyllum*, and Curcumin, significantly inhibit the growth of pathogenic bacteria such as *Clostridium*, *Escherichia*, *Staphylicus*, *Verrucomicrobia* but increase the number of probiotic *Bifidobacteria* and *lactobacilli*, resulting in the increased diversity of the gut microbiota in DSS-induced CRC mouse model (Guo et al., 2015; McFadden et al., 2015; Yu et al., 2015; Chen et al., 2016; Wang et al., 2016; Gong et al., 2019; Lv et al., 2019 ;Jiang et al., 2020; Shen et al., 2020; Sui et al., 2020; Sun et al., 2020 ;Zhang et al., 2020; Hao et al., 2021; Wang et al., 2021; Zhang et al., 2021; Zhu et al., 2021). These anti-CRC profiles of herbal medicine on gut microbiota are summarized as shown in Table 1.

3.2 Anti-inflammatory and intestinal mucosal immunity of herbal bioactive ingredients

It has been known that chronic intestinal inflammation is closely related to gut microenvironment, and gut inflammation mostly leads to colorectal cancer (Konstantinov et al., 2013; Avril and DePaolo, 2021). Under physiological conditions, both gut bacteria and viruses can't transmit through the mucosa. However, when inflammatory and neoplastic intestinal disorders exist, the permeability of intestinal barrier will increase, causing higher translocation of bacteria and viruses into the bloodstream. The active ingredients of herbal medicines can alleviate the stimulating effect of gut microbiota on tumors by improving the tumor microenvironment, such as inflammation and immunosuppression, thus inhibiting the development of tumors and even the metastasis and recurrence after operation.

Coptidis Rhizoma (also called as Huanglian), is derived from the rhizome of Coptis chinensis Franch., Coptis deltoidea C.F. Cheng et Hisao or Coptis teeta Wall. Berberine is a main activealkaloid isolated and identified from this herb. Evidences support the folkloric medicinal properties of *Coptidis Rhizoma*, in particular berberine, as a promising anticancer candidate in CRC *via* inducing AMPK activation and autophagic cell death (Huang et al., 2017; La et al., 2017). Berberine mitigates intestinal inflammation and oxidant stress through blocking the IL-6/STAT3, Nrf2 and PPAR pathways on colitis-associated tumorigenesis in mice (Li et al., 2017; Zhu et al., 2019). Moreover, it can not only significantly increase the abundance of Brucella, Bacteroides, Clostridium butyricum and Helicobacter in gut tract, but also protects intestinal epithelial cells against CRC-induced intestinal barrier dysfunction (Zhu et al., 2019).

Genistein is the predominant isoflavone found in Leguminous plants (such as *Sophora japonica L.* and *Glycine max*) and acts as the strong tyrosine kinase inhibitor, topoisomerase inhibitor and PPARγ agonist. It exerts phytoestrogenic, antioxidant and anti-inflammatory effects on gut microenvironment *via* regulating COX-2-related signaling pathway in CRC mice (He et al., 2016; Song et al., 2018). Crocin, a natural carotenoid from saffron (*Croci stigma*) and gardenia (*Gardeniae fructus*), can inhibit the expression of pro-inflammatory cytokines and inducible inflammatory enzymes in AOM/DSS-induced colorectal inflammation model, and significantly reduce inflammation and mucosal ulcer (Kawabata et al., 2012). The flavanol-rich foods as well as red wine polyphenolics inhibited ROS generation and NF-κB activation in colon cells by inducing miR-126 and miR-146a (Noratto et al., 2011; Angel-Morales et al., 2012).

FCT, a synbiotic combination of probiotic Lactobacillus gasseri 505 (LG) and Cudrania tricuspidata leaf extract (CT), reduced the risk of colitis-associated colon cancer *via* regulating inflammation, carcinogenesis, and gut microbiota composition. Compared with CT and LG, FCT significantly down-regulated pro-inflammatory mediators (TNF-α, IFN-γ, IL-1β, IL-6, iNOS and COX-2), and up-regulated anti-inflammatory cytokines (IL-4 and IL-10). In addition, FTC enhanced gut barrier function *via* up-regulating mucus layer markers (MUC-2 and TFF3) and tight junction (occludin and ZO-1), decreasing *Staphylococcus* and increasing *Lactobacillus*, *Bifidobacterium*, and *Akkermansia*, resulting in the increased production of short-chain fatty acids (SCFAs) (Oh et al., 2020).

Table 1 Regulation of herbal medicine on gut microbiota changes in CRC-related animal models.

Herbal name	Animal model	Gut Microbiota change	Anti-CRC mechanism	Reference(s)
Red Ginseng (Radix Et Rhizoma Ginseng)	Trinitro–Benzene–Sulfonic acid induced ulcerative colitis Wistar rats	Bifidobacteriu↑ Lactobacillu↑ E.coli↓	Promotes probiotic growth; inhibits pathogenic bacteria growth	(Zhu et al., 2021)
American Ginseng (Radix Panacis Quinquefolii)	AOM/DSS-induced colitis and colon carcinogenesis A/J mice	Firmicutes↑ Verrucomicrobia ↓	Inhibits inflammatory cytokines; inhibits pathogenic bacteria growth	(Wang et al., 2021)
Gynostemma Leaf (Gynostemmatis Pentaphylli Folium)	C57BL/6J-$Apc^{Min/+}$ mice	Sulfate-reducing bacteria↓	Inhibits pathogenic bacteria growth; modulates inflammatory intestinal microenvironment.	(Zhang et al., 2021)
Curcumin	AOM-induced colitis and colon cancer $IL10^{-/-}$ mice	Lactobacillus↑ Coriobacterales↓	Promotes probiotic growth; inhibits pathogenic bacteria growth	(Hao et al., 2021)
Berberine	DMH-induced colon cancer mice	Fusobacterium nucleatum↓ Tenericutes↓ Verrucomicrobia↓	Inhibits pathogenic bacteria growth; increases the secretion of IL-21/22/31 and CD40L; up-regulates the expression of p-STAT3, p-STAT5 and p-ERK1/2.	(Zhang et al., 2020)
Yi-Yi-Fu-Zi-Bai-Jiang-San (YYFZBJS)	C57BL/6J-$Apc^{Min/+}$ mice	Bacteroides fragilis Lachnospiraceae	Reduces Intestinal lymphatic, and mesenteric lymph nodes, accumulated $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells, along with reduction of the phosphorylation of β-catenin.	(Shen et al., 2020)
Wu Mei Wan (WMW)	AOM/DSS-induced CAC mouse	Bacteroidetes↓ Bacteroidales_s24-7_group↓ Firmicutes↑ Lachnospiraceae↑	Regulates the balance between "tumor-promoting bacteria" and "tumor-suppressing bacteria" and inactivated the NF-κB/IL-6/STAT3 pathway.	(Sun et al., 2020)
Gegen Qinlian decoction (GOD)	Patients with CRC	Megamonas↓ Veillonella↓ Bacteroides↑ Akkermansia↑ Prevotella↑	Promotes probiotic growth; inhibits pathogenic bacteria growth.	(La et al., 2017)
Neohesperidin (NHP)	$Apc^{Min/+}$ mouse	Bacteroidetes↓ Firmicutes↑ Proteobacteria↑	Promotes probiotic growth; inhibits pathogenic bacteria growth.	(Huang et al., 2017)
Evodiamine (EVO)	AOM/DSS-induced CAC mouse	Enterococcus faecalis↓ Escherichia col↓ Bifidobacterium↑ Campylobacter↑ Lactobacillus↑	Promotes probiotic growth; inhibits pathogenic bacteria growth; inhibits the IL6/STAT3/P65 signaling pathway.	(Li et al., 2017; Zhu et al., 2019)
Pai-Nong-San (PNS)	AOM/DSS-induced CAC mouse	Firmicutes↑ Bacteroidetes↓ Proteobacteria↑ Lactobacillus↑	Regulates the expression of CD4+ and CD8+ T cells; inhibits the production of HIF-α, IL-6, and TNF-α; promotes the expression of IL-4 and IFN-γ in colon tissues; improves gut microbiota; inhibits the Wnt signaling pathway.	(He et al., 2016)

(Continued)

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Continued

Herbal name	Animal model	Gut Microbiota change	Anti-CRC mechanism	Reference(s)
Xiaoyaosan (XYS)	CRC xenografts mice	Bacteroides Lactobacillus Desulfovibrio Rikenellaceae.	Promotes probiotic growth; inhibits pathogenic bacteria growth.	(Kawabata et al., 2012; Song et al., 2018)
Huangqin-tea (HQT)	Pseudo-germ-free rat model	Lachnoclostridium↑ Alistipes↑ Roseburia↑ Lactococcus↑ Bacteroides↓ Parasutterella ↓ Clostridiales↓.	Decreases IL-1β, IL-6, IL-10, and TNF-α expression; elevates IFN-γ production; Promotes probiotic growth; inhibits pathogenic bacteria growth.	(Noratto et al., 2011)
Quxie capsule	Patients with metastatic colorectal cancer	g_Bifidobacterium ↓ Collinsella ↓ Ruminiclostridium_9 ↓	Enhances CD4+ cells among mCRC patients; increase the abundance of gut anticancer bacteria Actinobacteria and butyrate-producing bacteria Lachnospiraceae.	(Angel-Morales et al., 2012)

AOM, azoxymethane; DSS, dextran sulfate sodium; DMH, 1,2-dimethylhydrazine; SCFAs, short-chain fatty acids; TCM, traditional Chinese medicine; APC, adenomatosis polyposis coli; ↑, upregulate. ↓downregulate.

Most notably, clinical responses to immune checkpoint inhibitors are closely associated with the abnormal gut microbiome composition, especially the relative abundance of *Akkermansia muciniphila* (Routy et al., 2018). Although several herbal medicines had been demonstrated to prevent the CRC growth, reduce side effects of chemotherapy and enhance the efficacy of PD-1 inhibitors *via* modulating the gut microbiota composition and CD4+ T cell proportion in tumor beds (Lv et al., 2019; Zhang et al., 2021; Huang et al., 2022; Messaoudene et al., 2022), the key orchestrators responsible for the primary resistance to PD-1 blockers remain unclear.

3.3 Improving bioavailability of herbal medicine by gut microbiota

Gut microbiota modifies the chemical composition of herbal medicine through their own enzymatic system. Intestinal cells also influence the metabolism and absorption of herbal medicine through transporter proteins and metabolic enzymes. After underwent by gut microbiota biotransformation (including hydrolysis, oxidation and reduction reaction), the chemical composition, pharmacological activity and toxicity of the herbal medicines will be changed, and it will form new active metabolites. Therefore, the biotransformation induced by gut microbiota has a central impact on exerting the efficiency of herbal medicine (Shen et al., 2013).

3.3.1 Glycosides

Most glycosides, including saponins, flavonoids and anthraquinones, will be hydrolyzed by gut microbiota to remove glycosyl groups and form aglycones, which reduces polarity, increases lipo-solubility and facilitates absorption into the blood. Both licorice and ginseng contain saponins. Glycyrrhetinic acid could be detected in normal rats after oral administration of glycyrrhizin, but not in sterile rats, indicating that gut microbiota could be converted into glycyrrhetinic acid and then absorbed by organisms (Takeda et al., 1996). Gut microbiota can promote the absorption and metabolic transformation of ginsenoside Rb1 and ginsenoside Rd, which can promote the biosynthesis of RNA and protein, regulate body metabolism and enhance immune function (Kim et al., 2014). Compound K, the main metabolite of ginseng saponin, induces apoptosis of colorectal cancer cells by inhibiting histone deacetylase activity (Kang et al., 2013). Most flavonoids, such as baicalin and isoquercitrin, are α-glucosylated by gut microbiota, but those enzymatical modification enhances their intestinal absorption and pharmacological action (Wang et al., 2015; Lee et al., 2017; Terao, 2017). More notably, degraded by *Clostridium orbiscindens*, diet flavonoids were cracked and converted into desaminotyrosine, which can up-regulate the signal pathway of type I interferon and enhance the host antiviral immune response (Steed et al., 2017).

3.3.2 Alkaloids

Alkaloids are a class of nitrogen-containing organic compounds, which have strong pharmacological activities in the central nervous system, cardiovascular system, immune function, anti-bacterial, anti-inflammatory and anti-cancer. As like as flavonoids, many alkaloids, such as berberine, aconitine and scopolamine, are usually characterized by small molecules, or by ether bonds and coordination bond which are prone to hydrolysis and dehydration under the action of gut microbiota. Under the action of gut bacteria, aconitine, a poisonous alkaloid mainly obtained from *Aconitum carmichaeli Debxwhich* shows chondroprotective activity can produce new monoester, diester and lipid alkaloids through deacetylation, demethylation, dehydroxylation and esterification, which greatly reduces the toxicity of aconitine and alleviates intestinal irritation (Tong et al., 2014). α-Chaconine, a potato glycoside alkaloid, induces apoptosis of HT-29 colon cancer cells by activating caspase-3 and inhibiting ERK 1/2 phosphorylation, and inhibits *Enterobacter aerogenes*, *Escherichia coli* and *Staphylococcus aureus* (Yang et al., 2006).

3.3.3 Phenylpropanoids

Phenylpropanoids generally have lactone structure, including phenylpropionic acid, coumarin and lignans. After the catalysis of gut microbiota, lactone structure can be broken or demethylated. The metabolic transformation of silymarin in Eubacterium limosum produced demethylsilybin A, demethylsilybin B, demethylisosilybin A and demethylisosilybin B which had stronger inhibitory effects on Alzheimer's amyloid protein-β42 (Zhang et al., 2014). Proteasome degraded flaxseed lignans in human intestinal tract bacteria. Hydrolysis and deglycosylation removed two sugars to form isopine resin diol diester (SECO). Pine resin diol diester was produced by digestive Streptococcus *Petostreptococcus productus*, *Eubacterium limosum*, *Clodium methoxybenzo-vorans* and *Eggelentala tarda* under the action of digestive *peptococcus*, *Eubacterium limosum*, *Clodium methoxybenzo-vorans* and *Eggetala tarda*. They were demethylated and dehydroxylated to form enterediol and enterolactone (Eeckhaut et al., 2008; Woting et al., 2010; Mabrok et al., 2012). A study of the metabolic mechanism of lignans in flaxseed, demonstrated that Ruminal Prevotella was the main microbial group for lignans metabolism (Schogor et al., 2014).

4 Conclusion and perspectives

Herbal medicines contain a variety of bioactive compounds and have unique advantages on maintenance of intestinal homeostasis and regulation of host immune. It precisely regulates the microbiota composition to indirectly prevent the CRC occurrence and development. On the other hand, the active ingredients in herbal medicine can directly inhibit the growth of colon cancer cells. Gut microorganisms produce many metabolic enzymes during their growth and reproduction, such as hydrolase, lyase, transferase and redox enzymes, which improve the bioavailability of the effective components of herbal medicine by biotransformation. Many active ingredients of Chinese herbal medicines can be transformed by gut microorganisms to produce metabolites with strong pharmacological effects, which can be easily absorbed by the body and exert anticancer activity. Thus, herbal medicine has the promise of preventing or delaying CRC progression *via* maintenance of intestinal homeostasis.

However, it should be pointed out that, in terms of current research, there is no evidence that herbal medicine can cure tumors only by improving gut microbiota, or more by exerting direct effects on cancer cells. Secondly, it is difficult to determine the sequence of the effects of herbal medicine and gut microbiota, just like the problem of "eggs and chickens" which one appears first. Although some specific bacteria that cause a precancerous phenotype *in vivo* have been identified, whether gut dysbiosis is the culprit behind CRC rather than a result of inflammation is still in dispute. Thus, deep signal regulation pathways and key targets of gut microbiota as a potent herbal medicine intervention in colorectal cancer need to be further explored.

Author contributions

H-ZY, WX, and M-CW searched the articles and drafted the manuscript. J-QH and H-HZ checked the contents. H-ZY and C-HY revised the manuscript. C-HY was responsible for the project administration and funding acquisition. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

References

Angel-Morales, G., Noratto, G., and Mertens-Talcott, S. (2012). Red wine polyphenolics reduce the expression of inflammation markers in human colon-derived CCD-18Co myofibroblast cells: Potential role of microRNA-126. *Food Funct.* 3 (7), 745–752. doi: 10.1039/c2fo10271d

Avril, M., and DePaolo, R. W. (2021). "Driver-passenger" bacteria and their metabolites in the pathogenesis of colorectal cancer. *Gut Microbes* 13 (1), 1941710. doi: 10.1080/19490976.2021.1941710

Azcárate-Peril, M. A., Sikes, M., and Bruno-Bárcena, J. M. (2011). The intestinal microbiota, gastrointestinal environment and colorectal cancer: A putative role for probiotics in prevention of colorectal cancer. *Am. J. Physiol. Gastrointest Liver Physiol.* 301 (3), G401–G424. doi: 10.1152/ajpgi.00110.2011

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., and Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* 68 (6), 394–424. doi: 10.3322/caac.21492

Chen, L., Brar, M. S., Leung, F. C., and Hsiao, W. L. (2016). Triterpenoid herbal saponins enhance beneficial bacteria, decrease sulfate-reducing bacteria, modulate inflammatory intestinal microenvironment and exert cancer preventive effects in ApcMin/+ mice. *Oncotarget* 7 (21), 31226–31242. doi: 10.18632/oncotarget.8886

Chen, F., Jiang, J., Tian, D. D., Wen, Q., Li, Y. H., Zhang, J. Q., et al. (2017). Targeting obesity for the prevention of chronic cardiovascular disease through gut microbiota-herb interactions: An opportunity for traditional herbs. *Curr. Pharm. Des.* 23 (8), 1142–1152. doi: 10.2174/1381612822666161014115724

Chen, F., Wen, Q., Jiang, J., Li, H. L., Tan, Y. F., Li, Y. H., et al. (2016). Could the gut microbiota reconcile the oral bioavailability conundrum of traditional herbs. *J. Ethnopharmacol.* 179, 253–264. doi: 10.1016/j.jep.2015.12.031

Eeckhaut, E., Struijs, K., Possemiers, S., Vincken, J. P., Keukeleire, D. D., and Verstraete, W. (2008). Metabolism of the lignan macromolecule into enterolignans in the gastrointestinal lumen as determined in the simulator of the human intestinal microbial ecosystem. *J. Agric. Food Chem.* 56 (12), 4806–4812. doi: 10.1021/jf800101s

Feagins, L. A., Souza, R. F., and Spechler, S. J. (2009). Carcinogenesis in IBD: potential targets for the prevention of colorectal cancer. *Nat. Rev. Gastroenterol. Hepatol.* 6 (5), 297–305. doi: 10.1038/nrgastro.2009.44

Fukata, M., and Abreu, M. T. (2009). Pathogen recognition receptors, cancer and inflammation in the gut. *Curr. Opin. Pharmacol.* 9 (6), 680–687. doi: 10.1016/j.coph.2009.09.006

Gagnière, J., Raisch, J., Veziant, J., Barnich, N., Bonnet, R., Buc, E., et al. (2016). Gut microbiota imbalance and colorectal cancer. *World J. Gastroenterol.* 22 (2), 501–518. doi: 10.3748/wjg.v22.i2.501

Gill, S. R., Pop, M., Deboy, R. T., Eckburg, P. B., Turnbaugh, P. J., Samuel, B. S., et al. (2006). Metagenomic analysis of the human distal gut microbiome. *Science* 312 (5778), 1355–1359. doi: 10.1126/science.1124234

Gong, Y., Dong, R., Gao, X., Li, J., Jiang, L., Zheng, J., et al. (2019). Neohesperidin prevents colorectal tumorigenesis by altering the gut microbiota. *Pharmacol. Res.* 148, 104460. doi: 10.1016/j.phrs.2019.104460

Guo, M., Ding, S., Zhao, C., Gu, X., He, X., Huang, K., et al. (2015). Red ginseng and semen coicis can improve the structure of gut microbiota and relieve the symptoms of ulcerative colitis. *J. Ethnopharmacol.* 162, 7–13. doi: 10.1016/j.jep.2014.12.029

Hao, W., Wu, J., Yuan, N., Gong, L., Huang, J., Ma, Q., et al. (2021). Xiaoyaosan improves antibiotic-induced depressive-like and anxiety-like behavior in mice through modulating the gut microbiota and regulating the NLRP3 inflammasome in the colon. *Front. Pharmacol.* 12. doi: 10.3389/fphar.2021.619103

Hattori, N., Niwa, T., Ishida, T., Kobayashi, K., Imai, T., Mori, A., et al. (2019). Antibiotics suppress colon tumorigenesis through inhibition of aberrant DNA methylation in an azoxymethane and dextran sulfate sodium colitis model. *Cancer Sci.* 110 (1), 147–156. doi: 10.1111/cas.13880

He, X., Bai, Y., Zhao, Z., Wang, X., Fang, J., Huang, L., et al. (2016). Local and traditional uses, phytochemistry, and pharmacology of sophora japonica l.: A review. *J. Ethnopharmacol.* 187, 160–182. doi: 10.1016/j.jep.2016.04.014

Huang, J., Liu, D., Wang, Y., Liu, L., Li, J., Yuan, J., et al. (2022). Ginseng polysaccharides alter the gut microbiota and kynurenine/tryptophan ratio, potentiating the antitumour effect of antiprogrammed cell death 1/programmed cell death ligand 1 (anti-PD-1/PD-L1) immunotherapy. *Gut* 71 (4), 734–745. doi: 10.1136/gutjnl-2020-321031

Huang, T., Xiao, Y., Yi, L., Li, L., Wang, M., Tian, C., et al. (2017). Coptisine from rhizoma coptidis suppresses HCT-116 cells-related tumor growth *in vitro* and *in vivo*. *Sci. Rep.* 7, 38524. doi: 10.1038/srep38524

Jiang, F., Liu, M., Wang, H., Shi, G., Chen, B., Chen, T., et al. (2020). Wu Mei wan attenuates CAC by regulating gut microbiota and the NF-kB/IL6-STAT3 signaling pathway. *BioMed. Pharmacother.* 125, 109982. doi: 10.1016/j.biopha.2020.109982

Kabwe, M., Meehan-Andrews, T., Ku, H., Petrovski, S., Batinovic, S., Chan, H. T., et al. (2021). Lytic bacteriophage EFA1 modulates HCT116 colon cancer cell growth and upregulates ROS production in an enterococcus faecalis Co-culture system. *Front. Microbiol.* 12. doi: 10.3389/fmicb.2021.650849

Kang, K. A., Piao, M. J., Kim, K. C., Zheng, J., Yao, C. W., Cha, J. W., et al. (2013). Compound K, a metabolite of ginseng saponin, inhibits colorectal cancer cell growth and induces apoptosis through inhibition of histone deacetylase activity. *Int. J. Oncol.* 43 (6), 1907–1914. doi: 10.3892/ijo.2013.2129

Kaur, H., Gupta, T., Kapila, S., and Kapila, R. (2021). Protective effects of potential probiotic lactobacillus rhamnosus (MTCC-5897) fermented whey on reinforcement of intestinal epithelial barrier function in a colitis-induced murine model. *Food Funct.* 12 (13), 6102–6116. doi: 10.1039/d0fo02641g

Kawabata, K., Tung, N. H., Shoyama, Y., Sugie, S., Mori, T., and Tanaka, T. (2012). Dietary crocin inhibits colitis and colitis-associated colorectal carcinogenesis in Male ICR mice. *Evid. Based. Complement Alternat. Med.* 2012, 820415. doi: 10.1155/2012/820415

Kim, K. A., Yoo, H. H., Gu, W., Yu, D. H., Jin, M. J., Choi, H. L., et al. (2014). Effect of a soluble prebiotic fiber, NUTRIOSE, on the absorption of ginsenoside Rd in rats orally administered ginseng. *J. Ginseng Res.* 38 (3), 203–207. doi: 10.1016/j.jgr.2014.03.003

Konstantinov, S. R., Kuipers, E. J., and Peppelenbosch, M. P. (2013). Functional genomic analyses of the gut microbiota for CRC screening. *Nat. Rev. Gastroenterol. Hepatol.* 10 (12), 741–745. doi: 10.1038/nrgastro.2013.178

La, X., Zhang, L., Li, Z., Yang, P., and Wang, Y. (2017). Berberine-induced autophagic cell death by elevating GRP78 levels in cancer cells. *Oncotarget* 8 (13), 20909–20924. doi: 10.18632/oncotarget.14959

Lee, Y. S., Woo, J. B., Ryu, S. I., Moon, S. K., Han, N. S., and Lee, S. B. (2017). Glucosylation of flavonol and flavanones by bacillus cyclodextrin glucosyltransferase to enhance their solubility and stability. *Food Chem.* 229, 75–83. doi: 10.1016/j.foodchem.2017.02.057

Lennard, K. S., Goosen, R. W., and Blackburn, J. M. (2016). Bacterially-associated transcriptional remodelling in a distinct genomic subtype of colorectal cancer provides a plausible molecular basis for disease development. *PloS One* 11 (11), e0166282. doi: 10.1371/journal.pone.0166282

Liu, I. L., Tsai, C. H., Hsu, C. H., Hu, J. M., Chen, Y. C., Tian, Y. F., et al. (2019). Helicobacter pylori infection and the risk of colorectal cancer: A nationwide population-based cohort study. *QJM* 112 (10), 787–792. doi: 10.1093/qjmed/hcz157

Li, X., Wu, D., Niu, J., Sun, Y., Wang, Q., Yang, B., et al. (2021). Intestinal flora: A pivotal role in investigation of traditional Chinese medicine. *Am. J. Chin. Med.* 49 (2), 237–268. doi: 10.1142/S0192415X21500130

Li, D., Zhang, Y., Liu, K., Zhao, Y., Xu, B., Xu, L., et al. (2017). Berberine inhibits colitis-associated tumorigenesis *via* suppressing inflammatory responses and the consequent EGFR signaling-involved tumor cell growth. *Lab. Invest.* 97 (11), 1343–1353. doi: 10.1038/labinvest.2017.71

Lund, E. K., Belshaw, N. J., Elliott, G. O., and Johnson, I. T. (2011). Recent advances in understanding the role of diet and obesity in the development of colorectal cancer. *Proc. Nutr. Soc.* 70 (2), 194–204. doi: 10.1017/S0029665111000073

Luo, S., Wen, R., Wang, Q., Zhao, Z., Nong, F., Fu, Y., et al. (2019). Rhubarb peony decoction ameliorates ulcerative colitis in mice by regulating gut microbiota to restoring Th17/Treg balance. *J. Ethnopharmacol.* 231, 39–49. doi: 10.1016/j.jep.2018.08.033

Lu, Y., Rong, J., Lai, Y., Tao, L., Yuan, X., and Shu, X. (2020). The degree of helicobacter pylori infection affects the state of macrophage polarization through crosstalk between ROS and HIF-1α. *Oxid. Med. Cell Longev.* 2020, 5281795. doi: 10.1155/2020/5281795

Lv, J., Jia, Y., Li, J., Kuai, W., Li, Y., Guo, F., et al. (2019). Gegen qinlian decoction enhances the effect of PD-1 blockade in colorectal cancer with microsatellite stability by remodelling the gut microbiota and the tumour microenvironment. *Cell Death Dis.* 10 (6), 415. doi: 10.1038/s41419-019-1638-6

Mabrok, H. B., Klopfleisch, R., Ghanem, K. Z., Clavel, T., Blaut, M., and Loh, G. (2012). Lignan transformation by gut bacteria lowers tumor burden in a gnotobiotic rat model of breast cancer. *Carcinogenesis* 33 (1), 203–208. doi: 10.1093/carcin/bgr256

McFadden, R. M., Larmonier, C. B., Shehab, K. W., Midura-Kiela, M., Ramalingam, R., Harrison, C. A., et al. (2015). The role of curcumin in modulating colonic microbiota during colitis and colon cancer prevention. *Inflamm Bowel Dis.* 21 (11), 2483–2494. doi: 10.1097/MIB.0000000000000522

Meng, Q. X., Roubin, R. H., and Hanrahan, J. R. (2013). Ethnopharmacological and bioactivity guided investigation of five TCM anticancer herbs. *J. Ethnopharmacol.* 148 (1), 229–238. doi: 10.1016/j.jep.2013.04.014

Messaoudene, M., Pidgeon, R., Richard, C., Ponce, M., Diop, K., Benlaifaoui, M., et al. (2022). A natural polyphenol exerts antitumor activity and circumvents anti-PD-1 resistance through effects on the gut microbiota. *Cancer Discov* 12 (4), 1070–1087. doi: 10.1158/2159-8290.CD-21-0808

Mizutani, S., Yamada, T., and Yachida, S. (2020). Significance of the gut microbiome in multistep colorectal carcinogenesis. *Cancer Sci.* 111 (3), 766–773. doi: 10.1111/cas.14298

Noratto, G. D., Kim, Y., Talcott, S. T., and Mertens-Talcott, S. U. (2011). Flavonol-rich fractions of yaupon holly leaves (Ilex vomitoria, aquifoliaceae) induce microRNA-146a and have anti-inflammatory and chemopreventive effects in intestinal myofibroblast CCD-18Co cells. *Fitoterapia* 82 (4), 557–569. doi: 10.1016/j.fitote.2011.01.013

Oh, N. S., Lee, J. Y., Kim, Y. T., Kim, S. H., and Lee, J. H. (2020). Cancer-protective effect of a synbiotic combination between lactobacillus gasseri 505 and a cudrania tricuspidata leaf extract on colitis-associated colorectal cancer. *Gut Microbes* 12 (1), 1785803. doi: 10.1080/19490976.2020.1785803

Paveljšek, D., Ivičak-Kocjan, K., Treven, P., Benčina, M., Jerala, R., and Rogelj, I. (2021). Distinctive probiotic features share common TLR2-dependent signalling in intestinal epithelial cells. *Cell Microbiol.* 23 (1), e13264. doi: 10.1111/cmi.13264

Perillo, F., Amoroso, C., Strati, F., Giuffrè, M. R., Díaz-Basabe, A., Lattanzi, G., et al. (2020). Gut microbiota manipulation as a tool for colorectal cancer management: Recent advances in its use for therapeutic purposes. *Int. J. Sci.* 21 (15), 5389. doi: 10.3390/ijms21155389

Richard, M. L., Liguori, G., Lamas, B., Brandi, G., da Costa, G., Hoffmann, T. W., et al. (2018). Mucosa-associated microbiota dysbiosis in colitis associated cancer. *Gut Microbes* 9 (2), 131–142. doi: 10.1080/19490976.2017.1379637

Rivas-Domínguez, A., Pastor, N., Martínez-López, L., Colón-Pérez, J., Bermúdez, B., and Orta, M. L. (2021). The role of DNA damage response in dysbiosis-induced colorectal cancer. *Cells* 10 (8), 1934. doi: 10.3390/cells10081934

Rogler, G. (2014). Chronic ulcerative colitis and colorectal cancer. *Cancer Lett.* 345 (2), 235–241. doi: 10.1016/j.canlet.2013.07.032

Routy, B., Le Chatelier, E., Derosa, L., Duong, C. P. M., Alou, M. T., Daillère, R., et al. (2018). Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors. *Science* 359 (6371), 91–97. doi: 10.1126/science.aan3706

Saracut, C., Molnar, C., Russu, C., Todoran, N., Vlase, L., Turdean, S., et al. (2015). Secondary bile acids effects in colon pathology. *Exp. Mice Study Acta Cir. Bras.* 30 (9), 624–631. doi: 10.1590/S0102-865020150090000007

Schogor, A. L., Huws, S. A., Santos, G. T., Scollan, N. D., Hauck, B. D., Winters, A. L., et al. (2014). Ruminal prevotella spp. may play an important role in the conversion of plant lignans into human health beneficial antioxidants. *PloS One* 9 (4), e87949. doi: 10.1371/journal.pone.0087949

Sekirov, I., Russell, S. L., Antunes, L. C., and Finlay, B. B. (2010). Gut microbiota in health and disease. *Physiol. Rev.* 90 (3), 859–904. doi: 10.1152/physrev.00045.2009

Shen, H., Leung, W. I., Ruan, J. Q., Li, S. L., Lei, J. P., Wang, Y. T., et al. (2013). Biotransformation of ginsenoside Rb1 *via* the gypenoside pathway by human gut bacteria. *Chin. Med.* 8 (1), 22. doi: 10.1186/1749-8546-8-22

Shen, J., Li, P., Liu, S., Liu, Q., Li, Y., Zhang, Z., et al. (2020). The chemopreventive effects of huangqin-tea against AOM-induced preneoplastic colonic aberrant crypt foci in rats and omics analysis. *Food Funct.* 11 (11), 9634–9650. doi: 10.1039/d0fo01731k

Sobhani, I., Tap, J., Roudot-Thoraval, F., Roperch, J. P., Letulle, S., Langella, P., et al. (2011). Microbial dysbiosis in colorectal cancer (CRC) patients. *PloS One* 6 (1), e16393. doi: 10.1371/journal.pone.0016393

Song, S., Cheng, D., Wei, S., Wang, X., Niu, Y., Qi, W., et al. (2018). Preventive effect of genistein on AOM/DSS-induced colonic neoplasm by modulating the PI3K/AKT/FOXO3 signaling pathway in mice fed a high-fat diet. *J. Funct. Foods* 46, 237–242. doi: 10.1016/j.jff.2018.05.006

Steed, A. L., Christophi, G. P., Kaiko, G. E., Sun, L., Goodwin, V. M., Jain, U., et al. (2017). The microbial metabolite desaminotyrosine protects from influenza through type I interferon. *Science* 357 (6350), 498–502. doi: 10.1126/science.aam5336

Sui, H., Zhang, L., Gu, K., Chai, N., Ji, Q., Zhou, L., et al. (2020). YYFZBJS ameliorates colorectal cancer progression in ApcMin/+ mice by remodeling gut microbiota and inhibiting regulatory T-cell generation. *Cell Commun. Signal.* 18 (1), 113. doi: 10.1186/s12964-020-00596-9

Sun, L., Yan, Y., Chen, D., and Yang, Y. (2020). Quxie capsule modulating gut microbiome and its association with T cell regulation in patients with metastatic colorectal cancer: Result from a randomized controlled clinical trial. *Integr. Cancer Ther.* 19, 1534735420969820. doi: 10.1177/1534735420969820

Takeda, S., Ishthara, K., Wakui, Y., Amagaya, S., Maruno, M., Akao, T., et al. (1996). Bioavailability study of glycyrrhetic acid after oral administration of glycyrrhizin in rats; relevance to the intestinal bacterial hydrolysis. *J. Pharm. Pharmacol.* 48 (9), 902–905. doi: 10.1111/j.2042-7158.1996.tb05998.x

Terao, J. (2017). Factors modulating bioavailability of quercetin-related flavonoids and the consequences of their vascular function. *Biochem. Pharmacol.* 139, 15–23. doi: 10.1016/j.bcp.2017.03.021

Tong, P., Xu, S., Cao, G., Jin, W., Guo, Y., Cheng, Y., et al. (2014). Chondroprotective activity of a detoxicated traditional Chinese medicine (Fuzi) of aconitum carmichaeli debx against severe-stage osteoarthritis model induced by mono-iodoacetate. *J. Ethnopharmacol.* 151 (1), 740–744. doi: 10.1016/j.jep.2013.11.048

Uronis, J. M., Mühlbauer, M., Herfarth, H. H., Rubinas, T. C., Jones, G. S., and Jobin, C. (2009). Modulation of the intestinal microbiota alters colitis-associated colorectal cancer susceptibility. *PloS One* 4 (6), e6026. doi: 10.1371/journal.pone.0006026

Vivarelli, S., Salemi, R., Candido, S., Falzone, L., Santagati, M., Stefani, S., et al. (2019). Gut microbiota and cancer: From pathogenesis to therapy. *Cancers (Basel)* 11 (1), 38. doi: 10.3390/cancers11010038

Wang, Y., Nguyen, L. H., Mehta, R. S., Song, M., Huttenhower, C., and Chan, A. T. (2021). Association between the sulfur microbial diet and risk of colorectal cancer. *JAMA Netw. Open* 4 (11), e2134308. doi: 10.1001/jamanetworkopen.2021.34308

Wang, C. Z., Yu, C., Wen, X. D., Chen, L., Zhang, C. F., Calway, T., et al. (2016). American Ginseng attenuates colitis-associated colon carcinogenesis in mice: Impact on gut microbiota and metabolomics. *Cancer Prev. Res. (Phila)* 9 (10), 803–811. doi: 10.1158/1940-6207.CAPR-15-0372

Wang, C. Z., Zhang, C. F., Chen, L., Anderson, S., Lu, F., and Yuan, C. S. (2015). Colon cancer chemopreventive effects of baicalein, an active enteric microbiome metabolite from baicalin. *Int. J. Oncol.* 47 (5), 1749–1758. doi: 10.3892/ijo.2015.3173

Wang, M., Zhou, B., Cong, W., Zhang, M., Li, Z., Li, Y., et al. (2021). Amelioration of AOM/DSS-induced murine colitis-associated cancer by evodiamine intervention is primarily associated with gut microbiota-Metabolism-Inflammatory signaling axis. *Front. Pharmacol.* 12. doi: 10.3389/fphar.2021.797605

Watanabe, D., Murakami, H., Ohno, H., Tanisawa, K., Konishi, K., Tsunematsu, Y., et al. (2020). Association between dietary intake and the prevalence of tumourigenic bacteria in the gut microbiota of middle-aged Japanese adults. *Sci. Rep.* 10 (1), 15221.doi: 10.1038/s41598-020-72245-7

Woting, A., Clavel, T., Loh, G., and Blaut, M. (2010). Bacterial transformation of dietary lignans in gnotobiotic rats. *FEMS Microbiol. Ecol.* 72 (3), 507–514. doi: 10.1111/j.1574-6941.2010.00863.x

Wu, N., Yang, X., Zhang, R., Li, J., Xiao, X., Hu, Y., et al. (2013). Dysbiosis signature of fecal microbiota in colorectal cancer patients. *Microb. Eco.* 66 (2), 462–470. doi: 10.1007/s00248-013-0245-9

Yang, S. A., Paek, S. H., Kozukue, N., Lee, K. R., and Kim, J. A. (2006). Alpha-chaconine, a potato glycoalkaloid, induces apoptosis of HT-29 human colon cancer cells through caspase-3 activation and inhibition of ERK 1/2 phosphorylation. *Food Chem. Toxicol.* 44 (6), 839–846. doi: 10.1016/j.fct.2005.11.007

Yu, Y. N., and Fang, J. Y. (2015). Gut microbiota and colorectal cancer. *Gastrointest. Tumors* 2 (1), 26–32. doi: 10.1159/000380892

Yu, Y. N., Yu, T. C., Zhao, H. J., Sun, T. T., Chen, H. M., Chen, H. Y., et al. (2015). Berberine may rescue fusobacterium nucleatum-induced colorectal tumorigenesis by modulating the tumor microenvironment. *Oncotarget* 6 (31), 32013–32026. doi: 10.18632/oncotarget.5166

Zhang, S. L., Mao, Y. Q., Zhang, Z. Y., Li, Z. M., Kong, C. Y., Chen, H. L., et al. (2021). Pectin supplement significantly enhanced the anti-PD-1 efficacy in tumor-bearing mice humanized with gut microbiota from patients with colorectal cancer. *Theranostics* 11 (9), 4155–4170. doi: 10.7150/thno.54476

Zhang, Z., Shao, S., Zhang, Y., Jia, R., Hu, X., Liu, H., et al. (2020). Xiaoyaosan slows cancer progression and ameliorates gut dysbiosis in mice with chronic restraint stress and colorectal cancer xenografts. *BioMed. Pharmacother.* 132, 110916. doi: 10.1016/j.biopha.2020.110916

Zhang, Y., Yang, D. H., Zhang, Y. T., Chen, X. M., Li, L. L., and Cai, S. Q. (2014). Biotransformation on the flavonolignan constituents of silybi fructus by an intestinal bacterial strain eubacterium limosum ZL-II. *Fitoterapia* 92, 61–71. doi: 10.1016/j.fitote.2013.10.001

Zhang, M. M., Yin, D. K., Rui, X. L., Shao, F. P., Li, J. C., Xu, L., et al. (2021). Protective effect of pai-Nong-San against AOM/DSS-induced CAC in mice through inhibiting the wnt signaling pathway. *Chin. J. Nat. Med.* 19 (12), 912–920. doi: 10.1016/S1875-5364(22)60143-2

Zhu, L., Gu, P., and Shen, H. (2019). Protective effects of berberine hydrochloride on DSS-induced ulcerative colitis in rats. *Int. Immunopharmacol.* 68, 242–251. doi: 10.1016/j.intimp.2018.12.036

Zhu, L. Q., Zhang, L., Zhang, J., Chang, G. L., Liu, G., Yu, D. D., et al. (2021). Evodiamine inhibits high-fat diet-induced colitis-associated cancer in mice through regulating the gut microbiota. *J. Integr. Med.* 19 (1), 56–65. doi: 10.1016/j.joim.2020.11.001

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Cephalosporins-induced intestinal dysbiosis exacerbated pulmonary endothelial barrier disruption in streptococcus pneumoniae-infected mice

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Antibiotic abuse is growing more severe in clinic, and even short-term antibiotic treatment can cause long-term gut dysbiosis, which may promote the development and aggravation of diseases. Cephalosporins as the broad-spectrum antibiotics are widely used for prevention and treatment of community-acquired respiratory tract infection in children. However, their potential consequences in health and disease have not been fully elaborated. In this study, the effects of cefaclor, cefdinir and cefixime on intestinal microbiota and lung injury were investigated in *Streptococcus pneumoniae* (Spn)-infected mice. The results showed that the proportion of coccus and bacillus in intestinal microbiota were changed after oral administration with cefaclor, cefdinir and cefixime twice for 10 days, respectively. Compared with the Spn-infected group, the proportion of *Bifidobacterium* and *Lactobacillus* in intestine were significantly reduced, while *Enterococcus* and *Candida* was increased after cephalosporin treatment. Furthermore, 3 cephalosporins could obviously increase the number of total cells, neutrophils and lymphocytes in BALF as well as the serum levels of endotoxin, IL-2, IL-1β, IL-6 and TNF-α. Mechanically, cephalosporins accelerated Spn-induced pulmonary barrier dysfunction *via* mediating the mRNA expressions of endothelial barrier-related proteins (Claudin 5, Occludin, and ZO-1) and inflammation-related proteins (TLR4, p38 and NF-κB). However, all of those consequences could be partly reversed by *Bifidobacterium bifidum* treatment, which was closely related to the elevated acetate production, indicating the protective effects of probiotic against antibiotic-induced intestinal dysbiosis. Therefore, the present study demonstrated that oral administration with cephalosporins not only disrupted intestinal microecological homeostasis, but also increased the risk of Spn infection, resulting in severer respiratory inflammation and higher bacterial loads in mice.

KEYWORDS

community-acquired infection, endothelial barrier, endotoxin, probiotic, butyrate

Introduction

Streptococcus pneumoniae (Spn) is an important pathogen of community-acquired infections and a common cause of respiratory infections with high rates of death, which can cause otitis media, sinusitis, meningitis, bacteremia and sepsis in children, the elderly and immunodeficient patients (Engholm et al., 2017; Lanks et al., 2019). According to WHO data, approximately 1.6 million people die from Spn infection worldwide each year, including 1 million children under 5 years of age, and more than 90% of deaths occurring in developing countries (Mathur et al., 2018). Oral cephalosporins are commonly used in pediatric clinics to treat Spn infections, but emerging evidence display that the intervention with antibiotics during Spn infection can interfere with the colonization of the intestinal microbiota (Diallo et al., 2020; Larsson et al., 2021; von Specht et al., 2021). Even short-term antibiotic treatment can cause long-term gut dysbiotic states, which may cause endothelial barrier disruption of both intestinal and lung tissues (Trivedi and Barve, 2020; Andremont et al., 2021).

Gut microbiota is composed of various microorganisms, such as bacteria, viruses and fungi in the intestine, and their living environment, which affect the physiological functions and pathological changes of host cells (Chen et al., 2021). The intestinal microbiota has been taken attention increasingly because of its crucial roles in human immunity, metabolism, endocrine, neurodegeneration and other physiological processes (Michaudel and Sokol, 2020; Jia et al., 2021; Murciano-Brea et al., 2021; Xu et al., 2020). Patients with respiratory diseases, including allergic asthma, acute lung injury, pulmonary fibrosis, and bacterial infection, often have drastic changes of the proportion and function of intestinal and lung microbiota (Zhou et al., 2021). Intestinal dysbiosis affects respiratory immunity and barrier function through the microbiota-gut-lung axis, resulting in the reduction of the pulmonary resistance to Spn; in turn, pulmonary barrier disruption aggravates the development of acute lung injury and causes intestinal dysfunction through excessive production of cytokine storms (Schuijt et al., 2016; Wang et al., 2018). Under normal conditions, the intestinal microbiota is relatively constant, but it is susceptible to be changed by various environmental factors, especially the antibiotic abuse (Ianiro et al., 2016). Oral cephalosporins as the most widely used antibiotics for treating Spn infection will inevitably lead to the dysbiosis in gut-lung axis and alter host immune status (Becker et al., 2016). However, whether the aggravation of bacterial pneumonia is the primary cause or precipitating cause induced by increased pathogen acquisition have not been well clarified. Therefore, in this study, the intestinal microbiota was depleted by using 3 different cephalosporins cefaclor, cefdinir and cefixime, respectively, and then the mice were infected intranasally with Spn to investigate the host inflammatory response and pulmonary hyperpermeability.

Materials and methods

Reagents

Cefaclor tablets, cefdinir powers and cefixime tablets were purchased from Yabang Pharmaceutical Co. Ltd., Shanghai Aladdin Biochemical Technology Co. Ltd., and Tianjin Pharmaceutical Co. Ltd., respectively. *Bifidobacterium bifidum* (FXJCJ9M10) was purchased from Xi'an Victory Biochemical Technology Co. Ltd., China.

Spn (ATCC 49619) was obtained from Peking BeNa Biotech Co. Ltd., China. It was inoculated in Muller-Hinton agar (MH) culture mediums at 37°C for days until the bacteria presented logarithmic growth. The MH mediums was centrifuged (12,000 r/min for 2 min) and then the bacterial suspension was diluted by sterile normal saline and the final concentration of the bacterial suspension was adjusted to 1.5×10^9 by using a turbidimeter (Dichen Biotech Co. Ltd., China).

Spn-infected model and antibiotic treatment

The male ICR mice (body weight 16-18 g) were obtained from Zhejiang Laboratory Animal Center. The experiment project was approbated by the ethics committee of Hangzhou Medical College, and the approval number was 2021R10-003. The mice bred in a close system and individually ventilated cages for 1 week to adapt themselves to the new situations.

The mice were randomly divided into 8 groups and the method of Spn-infected modeling was described as shown in Figure 1. The doses of cephalosporins used in the study were established according to their clinical doses in the manufacturer's instructions. Each group had 20 mice. Except the mice in the control group and model group, others were pre-administrated orally with corresponding cephalosporins twice for 10 days. After anaesthetized by inhalation of ether in operation, except the mice in the control group, other mice were challenged intranasally with 50 µl of Spn suspension (6×10^8 CFU/ml) as previous studies reported (Kim et al., 2019; Hu et al., 2021). And the mice in cephalosporin and *B. bifidum*-co-treated groups were administrated alone with *B. bifidum* suspension (2.5×10^8 CFU) for 7 days (Lu et al., 2021). Twelve hours after the last probiotic administration, ten mice were anesthetized by isoflurane and killed. The lung tissues and blood were collected for the detection of related indicators.

Groups	Treatment	Spn infection
Control group	Sterile saline (0.2ml/d/mouse)	-
Model group	Sterile saline (0.2ml/d/mouse)	+
Cefaclor group	Cefaclor (100 mg/kg)	+
Cefdinir group	Cefdinir (30 mg/kg)	+
Cefixime group	Cefixime (50 mg/kg)	+
Cefaclor + Probiotic group	Cefaclor (100 mg/kg) + *B. bifidum* (10^9 CFU/d/mouse)	+
Cefdinir + Probiotic group	Cefdinir (30 mg/kg) + *B. bifidum* (10^9 CFU/d/mouse)	+
Cefixime + Probiotic group	Cefixime (50 mg/kg) + *B. bifidum* (10^9 CFU/d/mouse)	+

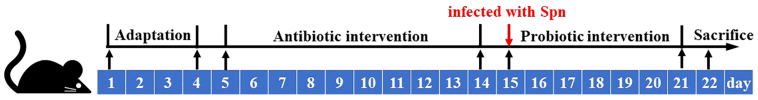

FIGURE 1
The flow of animal experiment.

Observation on the pathological changes of lung tissues in mice

The upper lobe of the right lung tissues of mice in each group was fixed with 10% formaldehyde for 24 hours, soaked in 75% ethanol for 24 hours, embedded in paraffin, and sectioned at 4 μm. The lung tissue was stained with htoxylin-eosin dye and the pathological changes were observed under the microscopes and imaging systems (Leica, Germany). The pathological changes were assessed by using the grading system of 1 (degree, 0), 2 (degree, 1%-25%), 3 (degree, 26%-50%), 4 (degree, 51%-75%) and 5 (degree, 76%-100%) as previous study (Takenaka et al., 2006).

In addition, the expression of TLR4 in lung tissues was detected by the immunohistochemical method, and the processed were operated according to the instructions of the kits. Three high-power visual fields were randomly selected from each slice and calculated by the microscopes and imaging systems (Leica, Germany), and the mean value was taken for further analysis.

Determination of pulmonary hyperpermeability

Two hours after the last probiotic administration, 5 mice in each group were randomly selected, weighed and injected with 2% Evan Blue (20 mg/kg body weight) in the tail vein. About 30 min after anesthetized by isoflurane, the mice were killed, the whole lung tissues were obtained and washed with PBS to remove blood around the lungs. Then, 100 mg of lung tissues was taken and mixed with 3 ml of acetone-saline solution (volume 7:3) in a 37°C incubator for 24 hours. Three milliliters of supernatant were added into the cuvette and the absorbance values of the samples at 620 nm were detected by using an ultraviolet spectrophotometer (PerkinElmer, USA) to reflect the pulmonary hyperpermeability as previously reported (Yu et al., 2020).

Cell count in bronchoalveolar lavage fluid

At the day 3rd after Spn infection, five mice in each group were executed. About 0.3 ml of sterile PBS was aspirated from the trachea into the lungs with a syringe each time, and BALF was collected by gently drawing back and repeated three times. The collected BALF was centrifuged at 4°C, 3000 r/min for 10 min, and the upper layer was transferred to a clean plastic pipe and stored at -20°C. The lower layer of cell precipitate was added with erythrocyte lysate, centrifuged at 4°C, 3000 r/min for 10 min, and resuspended with 200 μl of PBS for cell count. Under the light microscope, the number of cells in different colors was counted, and the changes in the ratio of macrophages, neutrophils and lymphocytes were calculated.

Determination of pro-inflammatory cytokines in serum

Two hours after the last probiotic administration, ten mice were anesthetized by isoflurane. The blood was obtained from abdominal aorta and kept in tubes at 4°C for 4 hours. After the clotted blood was centrifuged, 3000 r/min for 10 min, the supernatant was collected. The levels of pro-inflammatory cytokines (IL-2, IL-1β, IL-6 and TNF-α) and endotoxin in serum were detected by using commercialized ELISA kits (Lioke Biotech Co. Ltd., China) according to the manufacturer's instructions.

Real-time quantitative PCR assay

Total RNA was extracted from right lung tissue by using RNA extraction kit (Beyotime Biotech Co. Ltd., Shanghai, China), and its concentration and purity were measured by UV spectrophotometer (PerkinElmer, USA). cDNA was obtained by reverse transcription and stored at -20°C. The fragments of ZO-1, Claudin 5, Occludin, TLR4, p38 and NF-κB mRNA were amplified by real-time quantitative PCR (RT-qPCR). The sequences of the primers used in the study were shown in Supplementary Table S1. The reaction system was employed as follows: 10.0 μl, ROX Reference Dye II (50×); 0.4 μl, cDNA (50 ng/μl); 2.0 μl, upstream primers (10 μmol/L); 0.8 μl, downstream primers (10 μmol/L); 6.0 μl, ddH_2O. The specific operation was carried out strictly according to the kit instructions. Reaction conditions was employed as follows: 95°C for 30 s; 95°C for 5 s, 60°C for 34 s, 40 cycles. The relative expression levels were quantified by the $2^{-\Delta\Delta Ct}$ method.

Determination of bacterial loads in lung tissues

About 0.1 g of lung tissues was added into 9 ml of sterile saline and ground well by using a homogenizer. One milliliter of homogenate was taken and then mixed with 9 ml of sterile saline as the test sample. Subsequently, 1 ml of the diluted homogenate was added into a sterile flat dish which contained sterile MH mediums with 5% defibrinated sheep blood. Each sample was measured three times. The dishes were kept in an incubator (Thermo, USA) at 37°C with 5% CO_2 and the number of colonies was counted after 24 h of incubation.

Determination of intestinal microbiota

After anesthetized by isoflurane, the contents of the intestine were taken as 0.5 g under aseptic conditions. The contents were diluted 10 times with saline as the test solution. Each dilution (100 μl) was daubed with a glass applicator on the surface of different culture mediums, including blood agar plates, MacConkey plates, Enterococcus plates and Candida color plates. Blood agar plates are used to culture Campylobacter and Helicobacter pylori for 24 h; MacConkey plates culture Gram-negative enterobacteria for 24 h; Enterococcus plates culture Enterococcus for 24 h; and Candida plates culture fungi for 48 h. Three plates were made for each concentration of each specimen and incubated in a CO_2 incubator at 37°C. The number of colonies on each plate was counted and the average number of colonies on each gradient was calculated. Bacterial quantification was performed according to the following formula: tissue bacterial content (CFU/g) = number of colonies × dilution times/tissue weight. Each type of bacteria is initially identified according to Gram staining and colony morphology, and then identified by a bacterial auto-analyzer (Hangzhou ZEXI Biotech Co. Ltd., China).

The levels of short chain fatty acids in intestinal contents

The levels of SCFAs in intestinal contents were detected by GC-MS assay as previous study (Chen et al., 2019). The appropriate amounts of acetic acid, propionic acid and butyric acid were weighed and mixed with ultrapure water in a 10 ml flask as the standard solution. The standard curves were calculated and plotted according to the chromatograms of the corresponding concentrations. The mouse feces (1.0 g) were weighed and added to 2 ml of ultrapure water. The mixture was mixed thoroughly and centrifuged for 10 min at 1 000 r/min. The supernatant was passed through a 0.22 μm filter membrane for GC-MS analysis, and the contents of ethylene, propionic and butyric acids in mouse feces were calculated.

The levels of SCFAs were detected on an Agilent DB-FFAP125-3232 column (30 m × 250 μm × 0.25 μm) by using the Thermo Trace1310 GC-MS system (Thermo, USA) as follows: carrier gas was He. Flow rate was 1 mL/min. The column temperature was first set at 80°C for 1 min and then speeded at 10°C/min to 200°C. The detector temperature was controlled at 230°C, and the inlet temperature was 200°C. The flow rates of hydrogen, air and nitrogen were set at 30, 300 and 20 ml/min, respectively. The injection volume was 1 μl and the analysis time was 13.5 min for each sample.

Statistical analysis

Statistical analysis was performed by using GraphPad Prism 8.0 software. Data were expressed as mean ± standard deviation (SD). One-way ANOVA and Turkey tests were used for comparison between groups. $P<0.05$ indicated that there was statistically significant difference.

Results

Cephalosporins exacerbated Spn-induced acute lung injury in mice

Previous reports had demonstrated that there was no susceptibility difference to S. pneumonia upon the C3H/HeN, C57BL/6, and ICR mice (Takashima et al., 1996; Kim et al., 2019). Thus, ICR mice were used in this study for investigation of Spn infection. As shown in Figure 2, the alveolar structure of lung tissues in the control group was observed to be intact under the microscope, while the model group showed marked

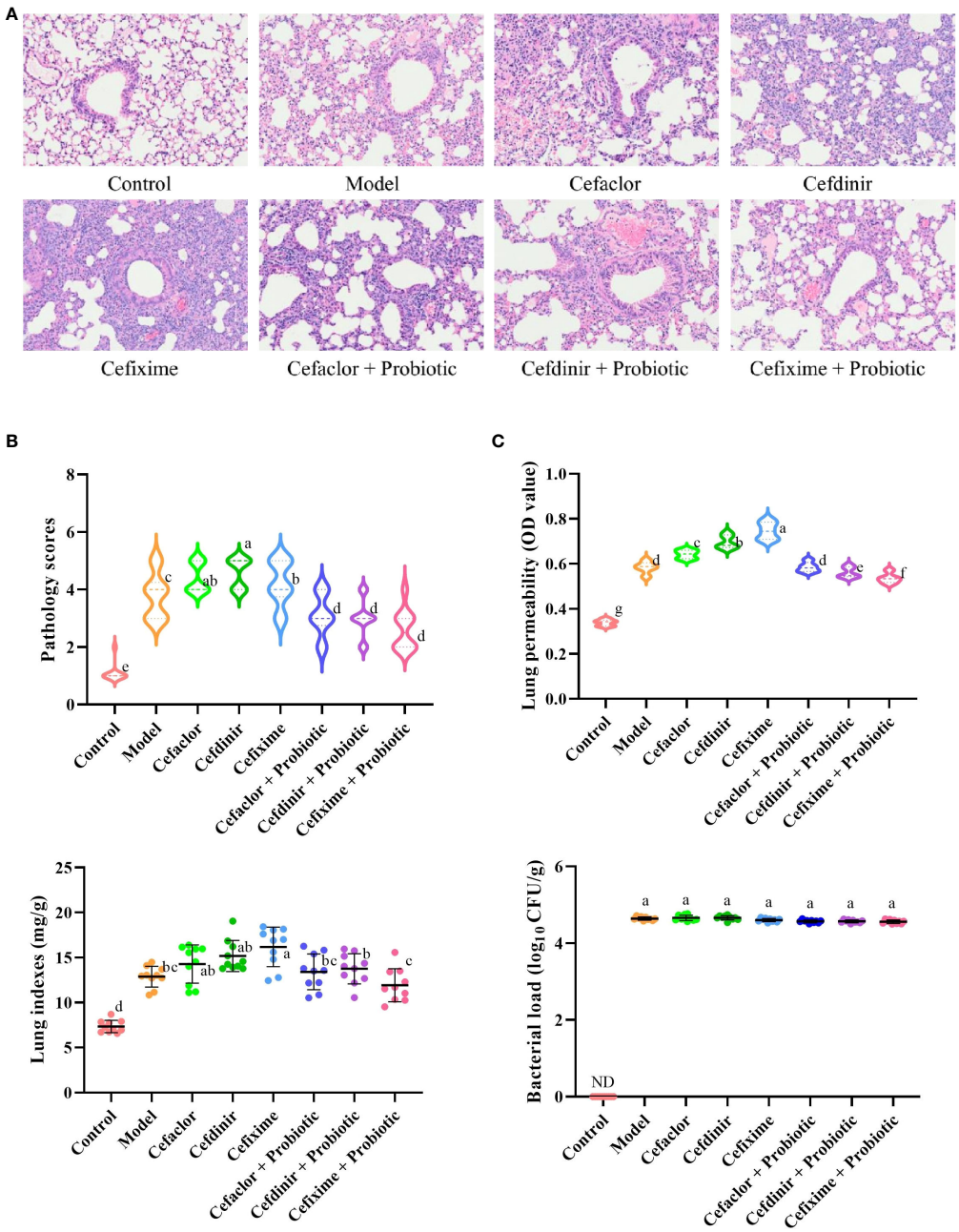

FIGURE 2
Effects of cephalosporins on Spn-induced acute lung injury in mice. **(A)** The histopathological features of the lung tissues in Spn-infected mice. **(B)** The levels of lung injury were estimated by pathological scores. **(C)** Effects of cephalosporins on pulmonary hyperpermeability, lung indexes and bacterial loads in infected mice. ND, not detected. Data were showed as mean ± SD. Different letters indicated statistically significant differences, P <0.05.

exudation of inflammatory cells from the alveolar lumen, interstitial edema, ruptured capillary walls and thickened alveolar walls. However, inflammatory responses in the lung tissues of the cephalosporins-treated groups were more serious, and also the pathology scores, lung permeabilities and lung indexes were dramatically increased (P <0.05) compared with those in model group. In the probiotic-treated groups, the alveolar structure was relatively intact, the alveolar lumen exudate was significantly reduced, the degree of interstitial oedema was decreased and the permeability was lightened (P <0.05), indicating that probiotic could alleviate the acute lung damage caused by Spn infection.

In addition, the bacterial loads in the model group were sharply elevated after Spn infection, while it could not be detected in the control group. However, neither cephalosporin pre-treatment nor probiotic intervention could reduce the bacterial loads in the lung tissue of Spn-infected mice (P >0.05). It indicated that long-term pre-treatment with cephalosporin could induce intrinsic drug resistance and probiotic intervention could not inhibit the growth of Spn in the lung tissue of infected mice.

Cephalosporins exacerbated Spn-induced inflammatory responses in mice

As shown in Figures 3 and 4, the number of total cells, Neutrophils, macrophages, and lymphocytes in BALF as well as the levels of pro-inflammatory cytokines (IL-2, IL-1β, IL-6 and TNF-α) and endotoxin in serum were significantly increased in the model group (P <0.05), compared with these in the control group. However, the levels of those indexes in cephalosporins-treated groups were much higher than those in the model group (P <0.05), indicating that long-term antibiotic intervention could induce gut dysbiosis and thereby aggravate Spn-induced inflammatory responses in mice. However, treatment with probiotic could variably decline the levels of IL-2, IL-1β, IL-6, TNF-α and endotoxin in serum. It also inhibited the exudation of total cells, Neutrophils, macrophages, and lymphocytes in BALF. These results indicated that probiotic intervention weakened antibiotic-exacerbated inflammatory responses induced by Spn.

Cephalosporins exacerbated intestinal dysbiosis in mice

The results in Figure 5 showed that after Spn infection, the proportions of G^+ bacilli and G^- cocci to total intestinal bacteria was significantly lower than those in the normal control group (P<0.05), while the proportions of G^- bacilli and G^+ cocci were significantly increased. However, those abnormal intestinal microbiota alteration could be completely reversed after

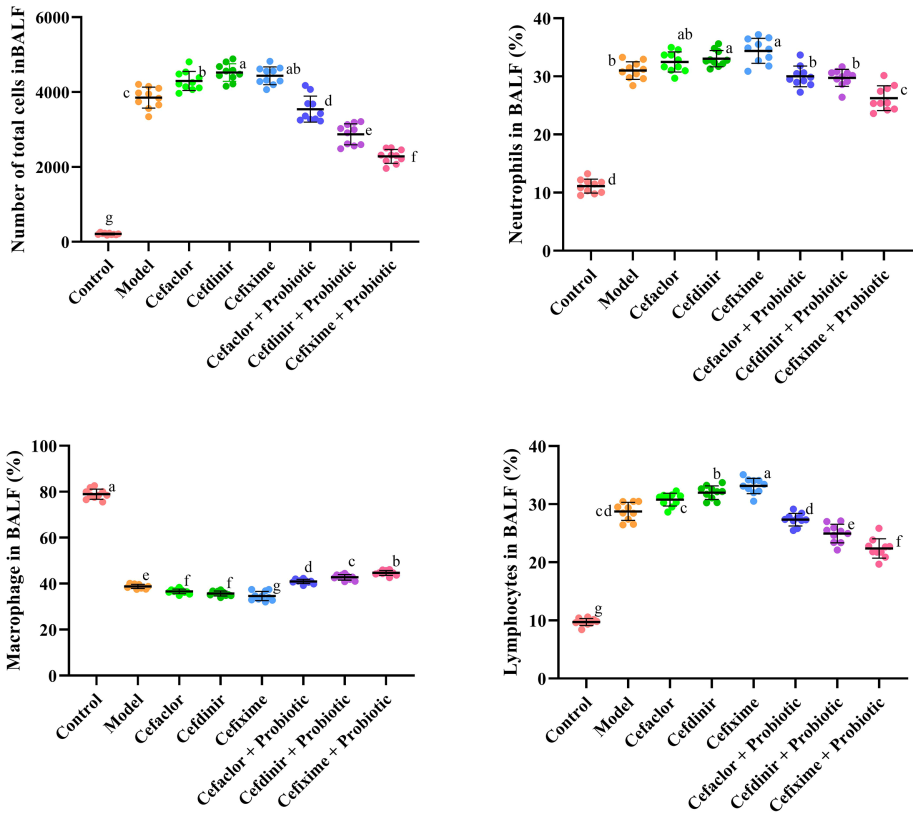

FIGURE 3
Effects of cephalosporins on the recruitment of inflammatory cells in the BALF of Spn-infected mice. Data were showed as mean ± SD. Different letters indicated statistically significant differences, P <0.05.

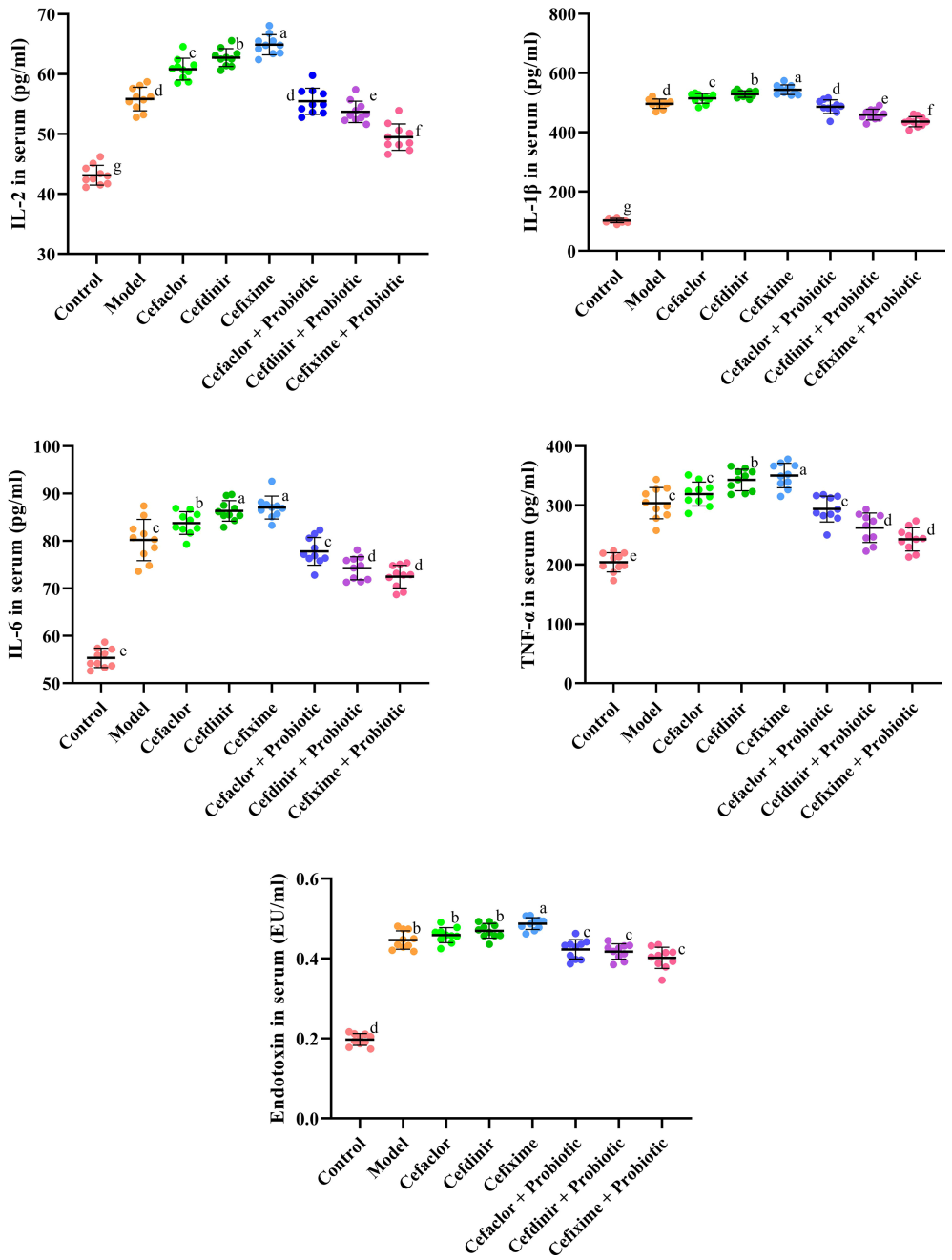

FIGURE 4
Effects of cephalosporins on the production of pro-inflammatory cytokines and endotoxin in the serum of Spn-infected mice. Data were showed as mean ± SD. Different letters indicated statistically significant differences, P <0.05.

cephalosporin treatment. Notably, probiotic intervention dramatically elevated the proportions of G^+ bacilli to total intestinal bacteria, which was even higher than that in the normal control group. In addition, the numbers of *Enterococcus*, *Bifidobacterium*, *Lactobacillus* and *Candida* were significantly higher after Spn infection compared with those in the normal control group ($P<0.05$). The number of *Enterococcus* was significantly increased after 10 days of cefaclor and cefdinir treatment, but decreased after cefixime treatment compared with the model control group ($P<0.05$). Both the number of *Bifidobacterium* and *Lactobacillus* was remarkably elevated after the application of cefixime compared to the normal control group,

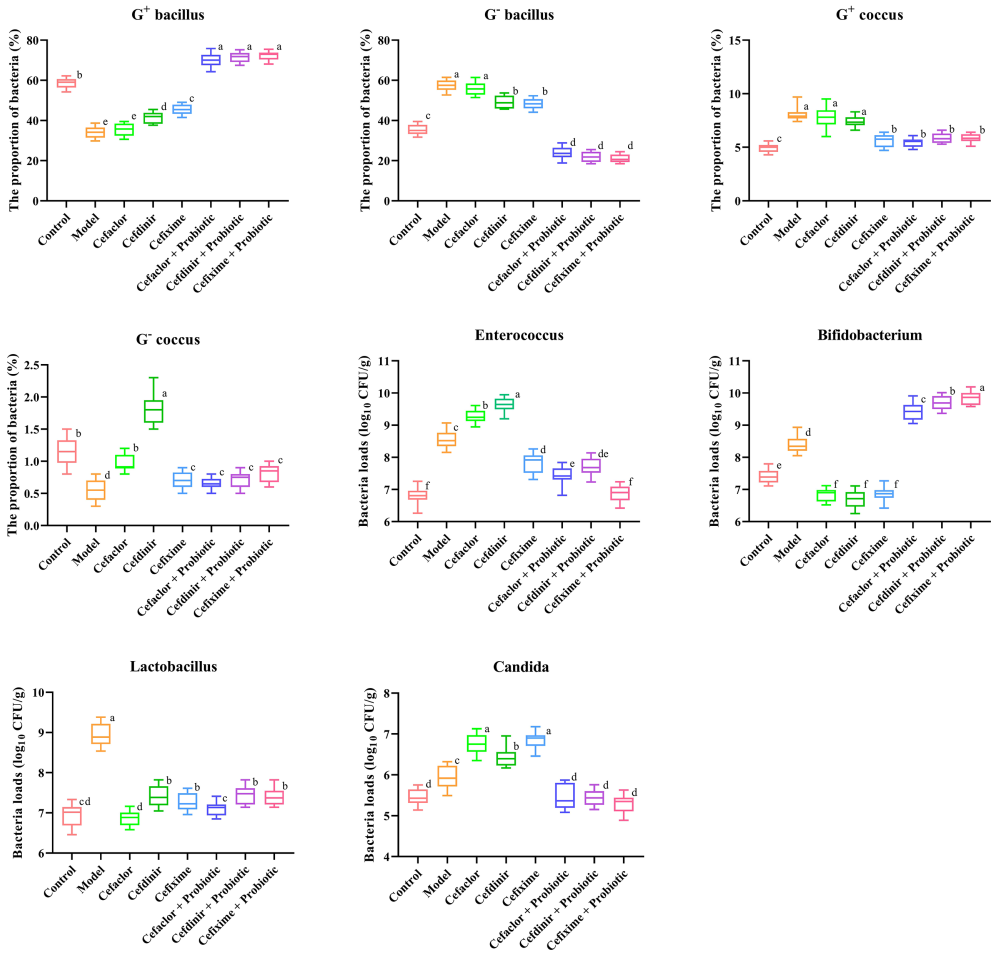

FIGURE 5
Effects of cephalosporins on the microbial proportions and compositions in the intestinal contents of Spn-infected mice. Data were showed as mean ± SD. Different letters indicated statistically significant differences, P <0.05.

but the difference was not statistically significant (P>0.05); the number of *enterococci* was significantly reduced after the intervention with cephalosporins compared with the model group (P<0.05). However, the number of Candida was significantly increased after 10 days of treatment with cefaclor, cefdinir and cefixime compared with the that in the intestinal contents of mice after Spn infection (P<0.05). Compared with those in the cephalosporins-treated groups, except the levels of Bifidobacterium were increased, other 3 bacteria were significantly decreased in the intestine of probiotic-treated groups.

To further investigate the changes of intestinal microbiota after antibiotic treatment, the composition of resistant bacteria and opportunistic pathogens was detected by using bacterial auto-analyzer. *Escherichia coli* and *Enterococcus faecalis* were detected in the normal control group, but *Klebsiella pneumoniae*, *Proteus bacilii*, *Pseudomonas aeruginosa* and *Enterococcus faecium* could not be found. After Spn infection, the detection of both *K. pneumoniae* and *P. bacilii* in the model group was observed in 2 out of 10 cases. Therefore, *E. coli* and *E. faecalis* were the dominant bacteria in the intestine of all the groups. Compared with the model group, the number of *E. coli* detected after 10 days of treatment with cefaclor, cefdinir and cefixime were 8, 7 and 8, respectively, and the detection was decreased to 80%, 70% and 80% respectively. The detection rate of *E. faecalis* also showed most significant reduction after cefdinir treatment, which fell by half. More importantly, the detection of *K. pneumoniae*, *P. bacilii*, *P. aeruginosa* and *E. faecium* in cephalosporin-treated groups was sharply increased compared with those in the model group, indicating the exacerbation of intestinal dysbiosis. However, probiotic intervention could obviously reduce the detection of those pathogenic bacteria (Supplementary Table S2).

Cephalosporins promoted pulmonary endothelial barrier disruption in streptococcus pneumoniae-infected mice

After Spn infection, the expressions of endothelial barrier-related genes (ZO-1, Claudin 5 and Occludin) in the lung tissues of the mice were remarkably reduced whereas the Pathogen-associated molecular pattern (PAMP) genes (TLR4, p38 and NF-κB) were remarkably elevated ($P<0.05$) compared with those in the control mice, indicating the pulmonary endothelial barrier disruption induced by Spn (Figure 6). Cephalosporin treatment intensified the trend of those gene expression gaps. But intervention with probiotic could reverse those trends. The immunohistochemical results of TLR4 and ZO-1 protein expressions in the lung tissues of Spn-infected mice were also consistent with the abnormal profiles of those 6 mRNAs (Figure 7). Therefore, those results indicated inhibition of probiotic on cephalosporin-deteriorated pulmonary endothelial barrier disruption.

Cephalosporins altered the intestinal microbial metabolites

After 7 days of Spn infection, the concentrations of microbial metabolites (acetate, propionate and butyrate) in the intestinal contents of the mice were significantly decreased, while cephalosporin treatment accelerated those declining trends (Figure 8). But intervention with probiotic significantly increased the concentrations of acetate, propionate and butyrate in the intestinal contents of the mice. Interestingly, by Pearson correlation analysis, the concentrations of intestinal acetate seemed to be closely related to the serum pro-inflammatory cytokines, indicating that antibiotic and probiotic might impact the lung

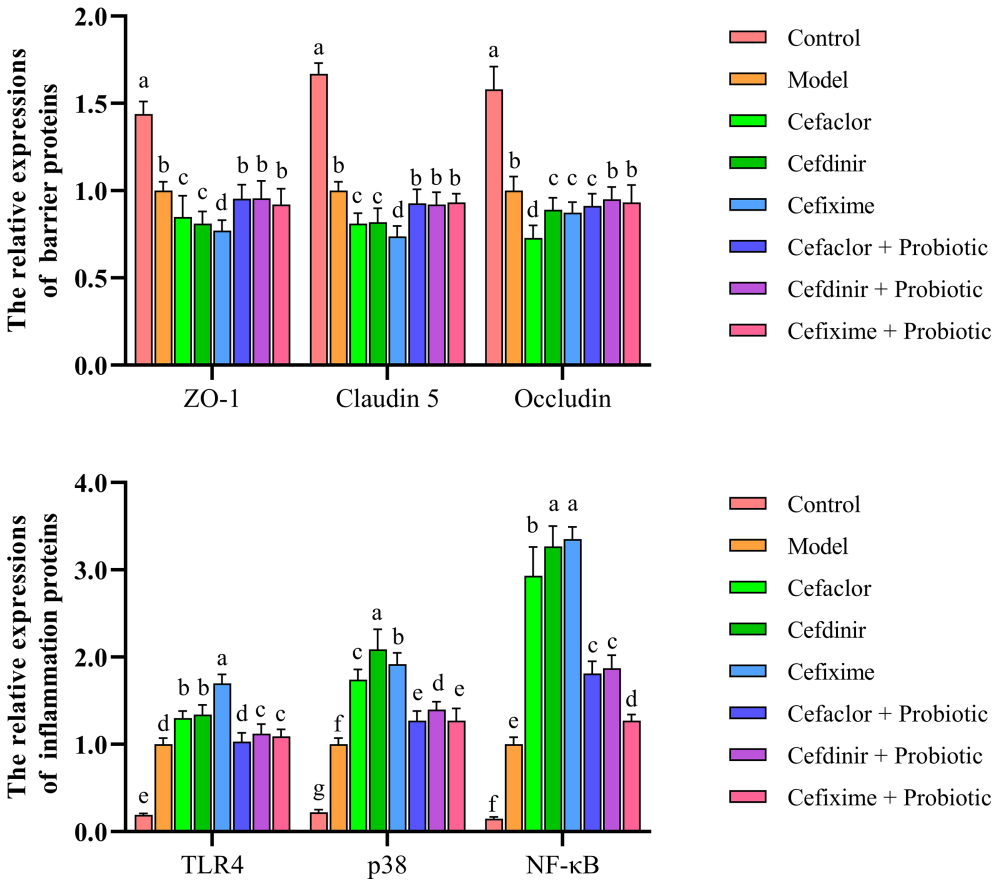

FIGURE 6
Effects of cephalosporins on expressions of ZO-1, Claudin 5, Occludin, TLR4, p38 and NF-κB mRNAs in the lung tissues of Spn-infected mice. Data were showed as mean ± SD. Different letters indicated statistically significant differences, P <0.05.

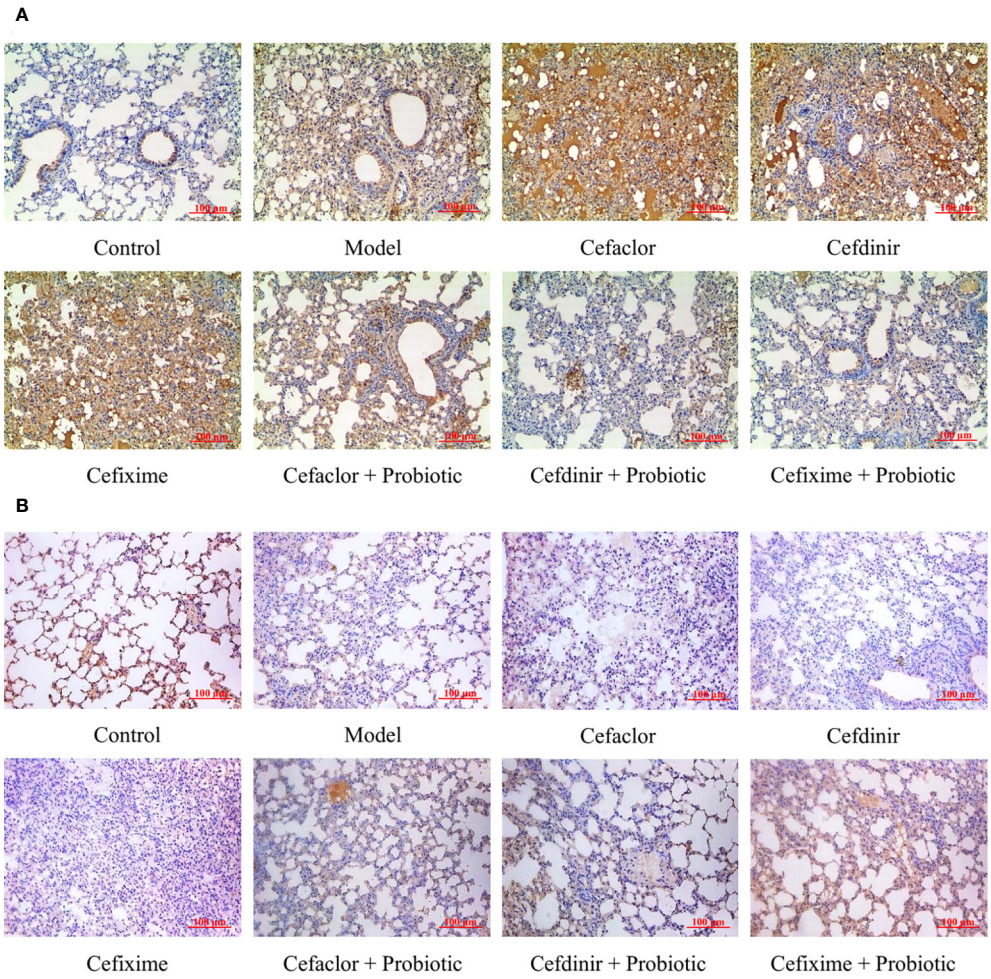

FIGURE 7
Effects of cephalosporins on **(A)** TLR4 and **(B)** ZO-1 expressions in the lung tissues of Spn-infected mice.

inflammation in Spn-infected mice partly *via* mediating the production of intestinal acetate.

Discussion

The development and application of antibiotics has led to a significant reduction in mortality from infection-related diseases, but the widespread use of broad-spectrum antibiotics has also led to increasing bacterial resistance to antibiotics. The problem of bacterial resistance has become an increasingly serious global public health issue (Mc Carlie et al., 2020). The intestinal microbiota is an important component of the intestinal environment, regulating intestinal homeostasis through interactions with the host, and has important immunomodulatory, endocrine and metabolic functions. The disruption of the intestinal micro-ecological barrier under the pressure of broad-spectrum antibiotics may not only allow exogenous conditioned pathogenic bacteria to colonize the intestinal tract, but may also induce intestinal origin of conditioned pathogenic bacteria to cause intestinal-derived infections (Kim et al., 2017; Dhar and Mohanty, 2020; Yang et al., 2021).

Spn is an important pathogen of community-acquired infections in children and a common cause of respiratory infections in children (Leung et al., 2018). Oral cephalosporins are commonly used in the clinical management of Spn infections in children. Data from clinical trials have shown that cephalosporins has a significant impact on the proportion and composition of human intestinal microbiota. In addition, early application of broad-spectrum antibiotics can interfere with the colonization of the normal intestinal microbiota in infants and young children, disrupting the balance of the microbiota established early in life and making it easier for a few conditional pathogenic bacteria to translocate and develop drug resistance, the younger the age, the more pronounced the

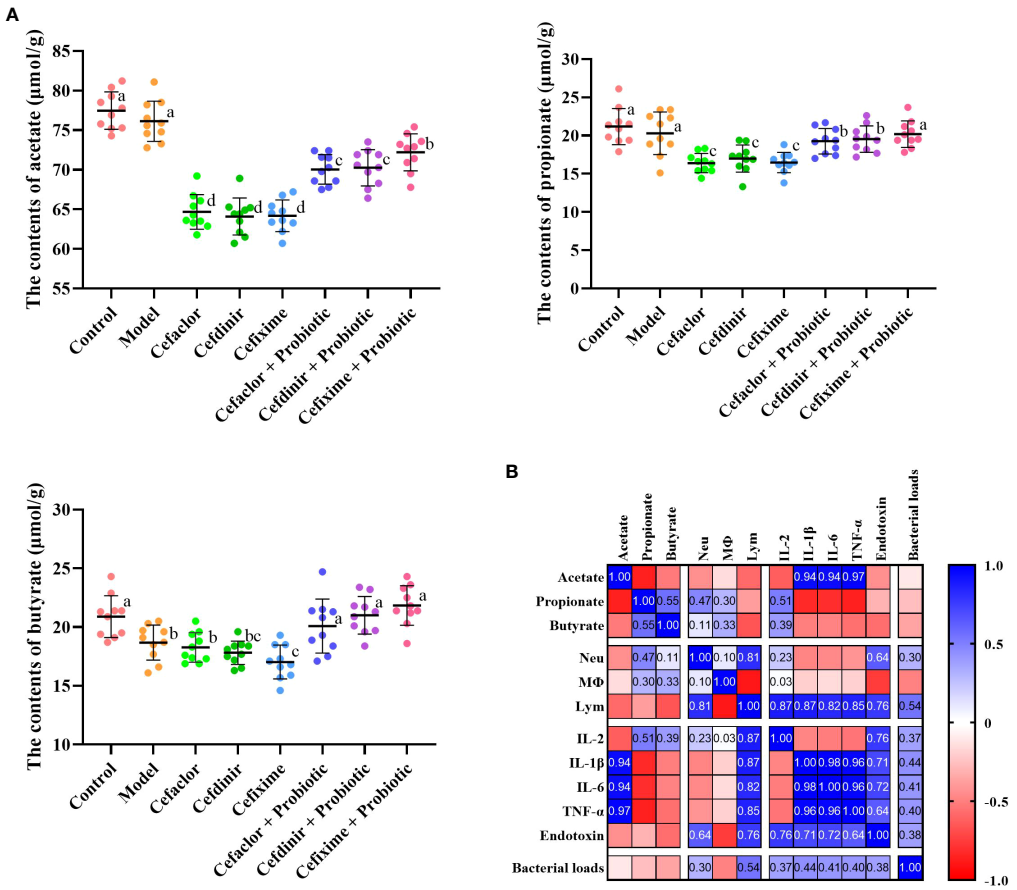

FIGURE 8
Short-chain fatty acids (SCFA) production and the correlation with disease indicators. **(A)** The concentrations of SCFA in intestinal contents of infected mice. **(B)** The correlation between SCFA and disease indicators. Neu, neutrophils; Mφ, macrophages; Lym, lymphocytes. Data were showed as mean ± SD. Different letters indicated statistically significant differences, P <0.05.

effects (Zimmermann and Curtis, 2019; Gamage et al., 2021). Also, the use of broad-spectrum antibiotics in infancy leads to an increased incidence of allergic disease, and that the high incidence of allergic disease in children is associated with an imbalance in the intestinal microbiota due to changes in the intestinal microbiota caused by the use of antibiotics (Fernandez et al., 2014; Esposito et al., 2016). Therefore, it is important to investigate the effect of cephalosporins on the proportion and composition of intestinal microbiota during the treatment of common infectious diseases in children, in order to guide the rational use of drugs in clinical practice.

In this study, the traditional isolation and detection method was still adopted, in which the feces and intestinal contents of mice were collected for smear observation and bacterial culture counting. The number of cocci and bacilli in the fecal smear is a basic indicator of the balance of the intestinal microbiota. In the pneumonia model of Spn infection, continuous application of cefaclor, cefdinir or cefixime for 10 days could affect the proportion of cocci and bacilli in the intestinal microbiota. It is well known that the normal intestinal microbiota in the human body includes aerobic, parthenogenic and anaerobic bacteria, of which, anaerobic bacteria are absolutely dominant. *Lactobacillus*, *Bifidobacterium* and other specific anaerobic bacteria in the intestine are planted on the epithelial surface of the intestinal mucosa, and are physiological probiotics of the body (Maldonado Galdeano et al., 2019; Rose et al., 2021). In a variety of disease states or with the use of antibiotics, the intestinal probiotic bacteria are reduced, and the intestinal aerobic and partly aerobic bacteria proliferate and become the dominant microbiota, resulting in the intestinal dysbiosis. Given the fact that the intestinal microbiota is complex and diverse in terms of species and numbers, in this study, common parthenogenic aerobic bacteria, *Enterococci*, *Bifidobacteria*, *Lactobacilli* and fungi were selected as subjects. It was shown that the application of cephalosporins in Spn-induced pneumonia model caused a significant decrease in the relative abundance of *Bifidobacterium* and *Lactobacillus*, but a significant increase in *Enterococci* and *Candida*. This finding

was consistent with the previous reports that long-term use of ceftriaxone might lead to the production of drug-resistant and conditionally pathogenic bacteria and replace beneficial bacteria as the dominant microbiota (Harris et al., 2019; Yuan et al., 2019). In the present study, we also observed the changes in intestinal parthenogenic aerobic bacteria, enterococci and fungal species. Our study demonstrated that E. coli and E. faecalis were the dominant bacteria in the intestine, and other conditional pathogens were hardly detected by conventional plate culture methods since their numbers were below the sensitivity of detection. However, after the application of cephalosporins, conditional pathogens such as *K. pneumoniae*, *P. bacilii*, *P. aeruginosa* and *E. faecium* were presented to varying degrees, but *B. bifidum* intervention could partly reverse those alterations. It might be related to a reduction in the number of *Lactobacillus*, resulting in reduced resistance to their colonization. It indicated that the common pathogens of community-acquired infections, such as *K. pneumoniae*, *P. bacilii*, *P. aeruginosa* and *E. faecium*, might in part be of enteric origin, and maintaining intestinal microbiological homeostasis could provide a new strategy to control community-acquired infections in clinic.

The blood-gas barrier is composed of alveolar epithelial cells and pulmonary microvascular endothelial cells. The integrity of the barrier function presents the indispensable ability for maintaining the gas exchange of the lung. During sepsis-induced damage to the blood-gas barrier, there is obvious attenuation and translocation of the tight junction proteins (including ZO-1, Claudin 5 and Occludin) in the alveolar epithelial cell membrane, demonstrating that the tight junctions are disrupted (Shirvaliloo, 2021; Pang et al., 2022). These can cause alveolar oedema, persistent hypoxia and a deteriorating prognosis (Mittal et al., 2014). Therefore, maintaining the integrity of the tight junctions in the alveolar epithelium could be a potential strategy for the treatment of respiratory tract infection (Abedi et al., 2020). Toll-like receptors are considered to be the key interface among the intestinal epithelial barrier, the microbiota and the immune system. TLR4, as the only receptor that recognizes pathogen-associated molecular patterns, acts as a 'switch' in this process and is one of the main receptors that activate intrinsic immunity. In normal lung tissue, low TLR4 expression ensures a controlled state of inflammation. In Contrast, in Spn-infected patients, increased expression of TLR4 is sustained, resulting in inappropriate signaling by lung epithelial cells to LPS, through activating various molecules including MyD88, which ultimately activates the NF-κB signaling pathway and boosts the excessive release of TNF-α and other pro-inflammatory cytokines, resulting in the blood-gas barrier damage and lung epithelial cell apoptosis (Aboudounya and Heads, 2021; Tang et al., 2021; Hao et al., 2022). In this study, as the serum levels of pro-inflammatory cytokines and endotoxin were increased, the expressions of ZO-1, Claudin 5 and Occludin in the lung tissues of Spn-infected mice were significantly decreased while TLR4, p38 and NF-κB were increased. Pre-treatment with cephalosporins not only promoted the growth of conditional pathogenic bacteria, but also remarkably disrupted pulmonary endothelial barrier function. However, probiotic treatment protected against cephalosporin-induced lung endothelial barrier dysfunction and intestinal dysbiosis.

In summary, long-term oral administration with cephalosporins could aggravate Spn-induced lung injury in mice *via* disrupting the intestinal microbiological homeostasis and triggering inflammatory processes. The use of antibiotics in clinics should be rational for different indications, and the duration of antibiotic use must be strictly controlled to avoid allergic and immune disorders caused by intestinal dysbiosis. Probiotic supplementation could be considered to reduce the adverse effects of intestinal dysbiosis and prevent bacterial pulmonary inflammation. Therefore, this study also provides new ideas for the prevention and treatment of clinical bacterial infectious diseases.

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.jianguoyun.com/p/DS4TYOgQ4qnjChifiMwEIAA.

Ethics statement

The animal study was reviewed and approved by Hangzhou Medical College.

Author contributions

H-ZY conceived and directed the study. C-YS, and J-FW did the analysis and visualization. C-YS, and J-FW drafted the manuscript. H-ZY revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.997368/full#supplementary-material

References

Abedi, F., Hayes, A. W., Reiter, R., and Karimi, G. (2020). Acute lung injury: The therapeutic role of rho kinase inhibitors. *Pharmacol. Res.* 155, 104736. doi: 10.1016/j.phrs.2020.104736

Aboudounya, M. M., and Heads, R. J. (2021). COVID-19 and toll-like receptor 4 (TLR4): SARS-CoV-2 may bind and activate TLR4 to increase ACE2 expression, facilitating entry and causing hyperinflammation. *Mediators Inflamm.* 2021, 8874339. doi: 10.1155/2021/8874339

Andremont, A., Cervesi, J., Bandinelli, P. A., Vitry, F., and de Gunzburg, J. (2021). Spare and repair the gut microbiota from antibiotic-induced dysbiosis: state-of-the-art. *Drug Discovery Today* 26 (9), 2159–2163. doi: 10.1016/j.drudis.2021.02.022

Becker, K. J., Zierath, D., Kunze, A., Fecteau, L., Lee, B., and Skerrett, S. (2016). The contribution of antibiotics, pneumonia and the immune response to stroke outcome. *J. Neuroimmunol.* 295-296, 68–74. doi: 10.1016/j.jneuroim.2016.04.005

Chen, R., Xu, Y., Wu, P., Zhou, H., Lasanajak, Y., Fang, Y., et al. (2019). Transplantation of fecal microbiota rich in short chain fatty acids and butyric acid treat cerebral ischemic stroke by regulating gut microbiota. *Pharmacol. Res.* 148, 104403. doi: 10.1016/j.phrs.2019.104403

Chen, Y., Zhou, J., and Wang, L. (2021). Role and mechanism of gut microbiota in human disease. *Front. Cell Infect. Microbiol.* 11, 625913. doi: 10.3389/fcimb.2021.625913

Dhar, D., and Mohanty, A. (2020). Gut microbiota and covid-19- possible link and implications. *Virus Res.* 285, 198018. doi: 10.1016/j.virusres.2020.198018

Diallo, O. O., Baron, S. A., Abat, C., Colson, P., Chaudet, H., and Rolain, J. M. (2020). Antibiotic resistance surveillance systems: A review. *J. Glob. Antimicrob. Resist.* 23, 430–438. doi: 10.1016/j.jgar.2020.10.009

Engholm, D. H., Kilian, M., Goodsell, D. S., Andersen, E. S., and Kjærgaard, R. S. (2017). A visual review of the human pathogen streptococcus pneumoniae. *FEMS Microbiol. Rev.* 41 (6), 854–879. doi: 10.1093/femsre/fux037

Esposito, S., Castellazzi, L., Tagliabue, C., and Principi, N. (2016). Allergy to antibiotics in children: an overestimated problem. *Int. J. Antimicrob. Agents.* 48 (4), 361–366. doi: 10.1016/j.ijantimicag.2016.08.001

Fernandez, T. D., Mayorga, C., Ariza, A., Corzo, J. L., and Torres, M. J. (2014). Allergic reactions to antibiotics in children. *Curr. Opin. Allergy Clin. Immunol.* 14 (4), 278–285. doi: 10.1097/ACI.0000000000000069

Gamage, H. K. A. H., Venturini, C., Tetu, S. G., Kabir, M., Nayyar, V., Ginn, A. N., et al. (2021). Third generation cephalosporins and piperacillin/tazobactam have distinct impacts on the microbiota of critically ill patients. *Sci. Rep.* 11 (1), 7252. doi: 10.1038/s41598-021-85946-4

Hao, Q., Shetty, S., Tucker, T. A., Idell, S., and Tang, H. (2022). Interferon-γ preferentially promotes necroptosis of lung epithelial cells by upregulating MLKL. *Cells.* 11 (3), 563. doi: 10.3390/cells11030563

Harris, P. N. A., Tambyah, P., and Paterson, D. L. (2019). Antibiotics for ceftriaxone resistant gram-negative bacterial bloodstream infections-reply. *JAMA.* 321 (6), 613. doi: 10.1001/jama.2018.19353

Hu, Y., Liu, Y., Yin, Y., and Zhang, X. (2021). Protective efficacy of mucosal and subcutaneous immunization with DnaJ-ΔA146Ply against influenza and streptococcus pneumoniae co-infection in mice. *Microbes Infect.* 23 (8), 104813. doi: 10.1016/j.micinf.2021.104813

Ianiro, G., Tilg, H., and Gasbarrini, A. (2016). Antibiotics as deep modulators of gut microbiota: between good and evil. *Gut.* 65 (11), 1906–1915. doi: 10.1136/gutjnl-2016-312297

Jia, X., Xu, W., Zhang, L., Li, X., Wang, R., and Wu, S. (2021). Impact of gut microbiota and microbiota-related metabolites on hyperlipidemia. *Front. Cell Infect. Microbiol.* 11, 634780. doi: 10.3389/fcimb.2021.634780

Kim, S., Covington, A., and Pamer, E. G. (2017). The intestinal microbiota: Antibiotics, colonization resistance, and enteric pathogens. *Immunol. Rev.* 279 (1), 90–105. doi: 10.1111/imr.12563

Kim, J. Y., Seo, S. M., Lee, H. K., Kim, H. W., and Choi, Y. K. (2019). Comparison of the virulence of streptococcus pneumoniae in ICR mouse stocks of three different origins. *Lab. Anim. Res.* 35, 5. doi: 10.1186/s42826-019-0002-4

Lanks, C. W., Musani, A. I., and Hsia, D. W. (2019). Community-acquired pneumonia and hospital-acquired pneumonia. *Med. Clin. North Am.* 103 (3), 487–501. doi: 10.1016/j.mcna.2018.12.008

Larsson, M., Nguyen, H. Q., Olson, L., Tran, T. K., Nguyen, T. V., and Nguyen, C. T. K. (2021). Multi-drug resistance in streptococcus pneumoniae among children in rural Vietnam more than doubled from 1999 to 2014. *Acta Paediatr.* 110 (6), 1916–1923. doi: 10.1111/apa.15795

Leung, A. K. C., Wong, A. H. C., and Hon, K. L. (2018). Community-acquired pneumonia in children. *Recent Pat. Inflammation Allergy Drug Discovery* 12 (2), 136–144. doi: 10.2174/1872213X12666180621163821

Lu, W., Fang, Z., Liu, X., Li, L., Zhang, P., Zhao, J., et al. (2021). The potential role of probiotics in protection against influenza a virus infection in mice. *Foods.* 10 (4), 902. doi: 10.3390/foods10040902

Maldonado Galdeano, C., Cazorla, S. I., Lemme Dumit, J. M., Vélez, E., and Perdigón, G. (2019). Beneficial effects of probiotic consumption on the immune system. *Ann. Nutr. Metab.* 74 (2), 115–124. doi: 10.1159/000496426

Mathur, S., Fuchs, A., Bielicki, J., Van Den Anker, J., and Sharland, M. (2018). Antibiotic use for community-acquired pneumonia in neonates and children: WHO evidence review. *Paediatr. Int. Child Health* 38 (sup1), S66–S75. doi: 10.1080/20469047.2017.1409455

Mc Carlie, S., Boucher, C. E., and Bragg, R. R. (2020). Molecular basis of bacterial disinfectant resistance. *Drug Resist. Updat.* 48, 100672. doi: 10.1016/j.drup.2019.100672

Michaudel, C., and Sokol, H. (2020). The gut microbiota at the service of immunometabolism. *Cell Metab.* 32 (4), 514–523. doi: 10.1016/j.cmet.2020.09.004

Mittal, M., Siddiqui, M. R., Tran, K., Reddy, S. P., and Malik, A. B. (2014). Reactive oxygen species in inflammation and tissue injury. *Antioxid. Redox Signal.* 20 (7), 1126–1167. doi: 10.1089/ars.2012.5149

Murciano-Brea, J., Garcia-Montes, M., Geuna, S., and Herrera-Rincon, C. (2021). Gut microbiota and neuroplasticity. *Cells.* 10 (8), 2084. doi: 10.3390/cells10082084

Pang, Y. L., Fang, S. Y., Cheng, T. T., Huang, C. C., Lin, M. W., Lam, C. F., et al. (2022). Viable allogeneic mitochondria transplantation improves gas exchange and alveolar-capillary permeability in rats with endotoxin-induced acute lung injuries. *Int. J. Med. Sci.* 19 (6), 1036–1046. doi: 10.7150/ijms.73151

Rose, E. C., Odle, J., Blikslager, A. T., and Ziegler, A. L. (2021). Probiotics, prebiotics and epithelial tight junctions: A promising approach to modulate intestinal barrier function. *Int. J. Mol. Sci.* 22 (13), 6729. doi: 10.3390/ijms22136729

Schuijt, T. J., Lankelma, J. M., Scicluna, B. P., de Sousa e Melo, F., Roelofs, J. J., de Boer, J. D., et al. (2016). The gut microbiota plays a protective role in the host defence against pneumococcal pneumonia. *Gut.* 65 (4), 575–583. doi: 10.1136/gutjnl-2015-309728

Shirvaliloo, M. (2021). The blood-gas barrier in COVID-19: an overview of the effects of SARS-CoV-2 infection on the alveolar epithelial and endothelial cells of the lung. *Tissue Barriers.* 9 (4), 1937013. doi: 10.1080/21688370.2021.1937013

Takashima, K., Tateda, K., Matsumoto, T., Ito, T., Ilzawa, Y., Nakao, M., et al. (1996). Establishment of a model of penicillin-resistant streptococcus pneumoniae pneumonia in health CBA/J mice. *J. Med. Microbiol.* 45, 319–322. doi: 10.1099/00222615-45-5-319

Takenaka, K., Nishimura, Y., Nishiuma, T., Sakashita, A., Yamashita, T., Kobayashi, K., et al. (2006). Ventilator-induced lung injury is reduced in transgenic mice that overexpress endothelial nitric oxide synthase. *Am. J. Physiol. Lung Cell Mol. Physiol.* 290 (6), L1078–L1086. doi: 10.1152/ajplung.00239.2005

Tang, J., Xu, L., Zeng, Y., and Gong, F. (2021). Effect of gut microbiota on LPS-induced acute lung injury by regulating the TLR4/NF-kB signaling pathway. *Int. Immunopharmacol.* 91, 107272. doi: 10.1016/j.intimp.2020.107272

Trivedi, R., and Barve, K. (2020). Gut microbiome a promising target for management of respiratory diseases. *Biochem. J.* 477 (14), 2679–2696. doi: 10.1042/BCJ20200426

von Specht, M., García Gabarrot, G., Mollerach, M., Bonofiglio, L., Gagetti, P., Kaufman, S., et al. (2021). Resistance to β-lactams in streptococcus pneumoniae. *Rev. Argent Microbiol.* 53 (3), 266–271. doi: 10.1016/j.ram.2021.02.007

Wang, H., Lian, P., Niu, X., Zhao, L., Mu, X., Feng, B., et al. (2018). TLR4 deficiency reduces pulmonary resistance to streptococcus pneumoniae in gut microbiota-disrupted mice. *PloS One* 13 (12), e0209183. doi: 10.1371/journal.pone.0209183

Xu, H., Wang, X., Feng, W., Liu, Q., Zhou, S., Liu, Q., et al. (2020). The gut microbiota and its interactions with cardiovascular disease. *Microb. Biotechnol.* 13 (3), 637–656. doi: 10.1111/1751-7915.13524

Yang, M., Yang, Y., He, Q., Zhu, P., Liu, M., Xu, J., et al. (2021). Intestinal microbiota-a promising target for antiviral therapy? *Front. Immunol.* 12, 676232. doi: 10.3389/fimmu.2021.676232

Yuan, Q., Li, Y., Xiu, L., Zhang, C., Fu, Y., Jiang, C., et al. (2019). Identification of multidrug-resistant neisseria gonorrhoeae isolates with combined resistance to both ceftriaxone and azithromycin, china 2017-2018. *Emerg. Microbes Infect.* 8 (1), 1546–1549. doi: 10.1080/22221751.2019.1681242

Yu, W. Y., Li, L., Wu, F., Zhang, H. H., Fang, J., Zhong, Y. S., et al. (2020). Moslea herba flavonoids alleviated influenza a virus-induced pulmonary endothelial barrier disruption *via* suppressing NOX4/NF-κB/MLCK pathway. *J. Ethnopharmacol.* 253, 112641. doi: 10.1016/j.jep.2020.112641

Zhou, A., Lei, Y., Tang, L., Hu, S., Yang, M., Wu, L., et al. (2021). Gut microbiota: the emerging link to lung homeostasis and disease. *J. Bacteriol.* 203 (4), e00454–e00420. doi: 10.1128/JB.00454-20

Zimmermann, P., and Curtis, N. (2019). The effect of antibiotics on the composition of the intestinal microbiota - a systematic review. *J. Infect.* 79 (6), 471–489. doi: 10.1016/j.jinf.2019.10.008

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Implications of m6A methylation and microbiota interaction in non-small cell lung cancer: From basics to therapeutics

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N6-methyladenine (m6A) is one of the most common RNA epigenetic modifications in all higher eukaryotes. Increasing evidence demonstrated that m6A-related proteins, acted as oncogenes or tumor suppressors, are abnormally expressed in the cell lines and tissues of non-small cell lung cancer (NSCLC). In addition, lung as the special immune organ contacts with the outer environments and thereby inevitably suffers from different types of microbial pathogen attack. Those microbial pathogens affect the development, progression, and clinical outcomes of NSCLC *via* altering host m6A modification to disrupt pulmonary immune homeostasis and increase the susceptibility; conversely, host cells modulate m6A modification to repress bacterial colonization. Therefore, m6A harbors the potential to be the novel biomarkers and targets for predicting poor prognosis and chemotherapy sensitivity of patients with lung cancer. In this paper, we provided an overview of the biological properties of m6A-modifying enzymes, and the mechanistic links among lung microbiota, m6A modification and NSCLC. Although the flood of novel m6A-related inhibitors represents many dramatic improvements in NSCLC therapy, their efficacy and toxicity in NSCLC are explored to address these pivotal gaps in the field.

KEYWORDS

epigenetics, biomarker, METTL3, FTO, inhibitor, m6A

Introduction

Epigenetic modifications are heritable changes in gene expression caused without altering the DNA nucleotide sequence and the development of tumors. In recent years, with the continuous development of tumor epigenetic research, especially following the in-depth study of abnormal DNA methylation, microRNA and non-coding RNA dysregulation and histone modification, N6-methyladenosine (m6A) has not only ushered in a new era of post-transcriptional gene regulation in eukaryotes, but also rapidly become a hot spot in the field of RNA methylation modification (Zhang et al., 2020). It is a hot topic of research in RNA methylation modification. As the most prevalent internal mRNA modification in all higher eukaryotes, m6A has been shown to be aberrantly expressed in a variety of tumors and plays an important role in the regulation of cell proliferation, invasion, metastasis and other malignant biological behaviors (Chen et al., 2021; Xu et al., 2021; Zhang et al., 2021; Deng et al., 2022).

Microbiota disorders or microbial pathogens in the lungs have been identified to affect the occurrence, development, and prognosis of non-small cell lung cancer (NSCLC) through different means, such as infllammation, metabolism, and cell signaling transduction (Shi et al., 2021). Moreover, some common risk factors like COPD may play a significant role on the populations of pulmonary microbiota in patients with NSCLC (He et al., 2022). However, the molecular mechanism of microbiota in the prognosis of NSCLC remains poorly elucidated.

This paper reviews the research progress on m6A and non-small cell lung cancer induced by microbiota, aiming to provide a theoretical basis for a profound understanding of non-small cell lung carcinogenesis and the search for tumor predictive biomarkers and therapeutic targets.

Biological properties of m6A

m6A, a methylation occurring at the adenosine N6 position, was first detected in poly(A) RNA in 1974 and was thought to be a potentially broad modification with the potential to selectively control gene expression (Desrosiers et al., 1974). However, the technical means of detection at that time could not meet the requirements for the study of the biological significance of m6A modifications. With the development of immunoprecipitation techniques as well as high-throughput sequencing, which only enabled researchers to target m6A modifications with greater precision, m6A regained the focus of attention and opened a new chapter in RNA epigenetic modifications. Recent studies have revealed that m6A is a very common modification in mRNA and ncRNA that affects RNA shear, translation, stability, and the epigenetic effects of certain ncRNAs, containing on average 1 to 2 m6A residues per 1000 nucleotides. m6A occurs mainly in the 3′ noncoding region, the stop codon, and in the RRACH sequence near the long exon (where R=A or G and H=A, C, or U) (Tang et al., 2021; Deng et al., 2022). There is growing evidence that m6A affects almost all stages of mRNA metabolism, from processing in the nucleus to translation and decay in the cytoplasm, which in turn affects circadian rhythms, regulates the cell cycle, accelerates cell state changes, regulates cell differentiation and reprogramming, and ultimately affects homeostasis of the body, causing a variety of diseases, including tumors (Zaccara et al., 2019; Gao et al., 2021; Kumari et al., 2021).

Similar to DNA and histone methylation, RNA m6A methylation modification is a dynamic and reversible epistatic regulatory process, mainly regulated by methylesterases (writers), demethylases (erasers) and recognition proteins (readers). The m6A methyltransferases, such as methyltransferase like 3/14 (METTL3/14), Wilm's tumor 1-associated protein (WTAP), and the demethylases FTO and AlkB homolog 5 (AlkB homolog 5, ALKBH5), are responsible for the methylation and demethylation of intracellular mRNAs, respectively. In contrast, recognition proteins such as YT521-B homology (YTH) family proteins YTHDF1-3, nuclear members YTHDC1-2, and heterogeneous nuclear ribonucleoproteins (hnRNPs), recognize m6A modifications of mRNAs and thus regulate mRNA biological behaviors (Figure 1).

The roles of m6A-RNA methylation in NSCLC

The RNA epigenetic modification system is complex, and m6A modifying enzymes can recognize both oncogenes and anti-oncogenes. Previous studies have shown that m6A-RNA methylation plays an important role in the development of NSCLC, and its methylation-related factors can act as oncogenes (e.g., ALKBH5) or anti-oncogenes (e.g., METTL3, FTO, YTHDF1, HNRNPA2B1, HNRNPC and eIF3, etc.) which play roles in NSCLC cell proliferation, migration, invasion, apoptosis and cell cycle, and may be potential new targets for NSCLC therapy.

m6A methylation transferases in NSCLC

The m6A modification is catalyzed by the methyltransferase complex (Writers) consisting of methyltransferase-like 3 (METTL3) and methyltransferase-like 14 (METTL14) and its WTAP, RBM15, KIAA1429, ZC3H13, and METTL16. Among them, METTL3 is the catalytic subunit active, METTL14 can form a dimer with METTL3, and WTAP recruits METTL3-METTL14 dimer to nucleosites for methylation modification of the substrate by binding to RBM15, KIAA1429, and ZC3H13 to form a complex (Wang et al., 2016). METTL3 is the major

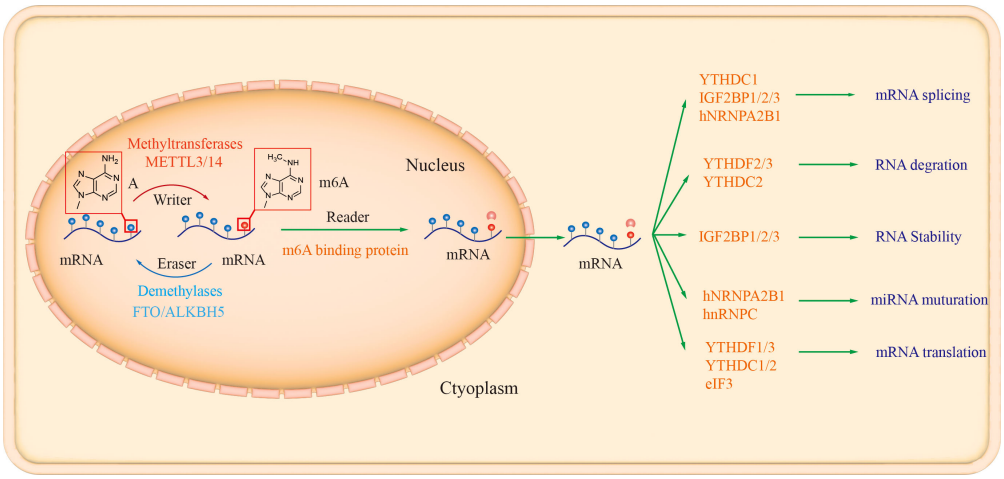

FIGURE 1
The biology functions of m^{6}A enzymes. There are three different types of m6A enzymes, namely "Writer" (such as METTL3, METTL14, WTAP, and KIAA1429), "Eraser" (ALKBH5 and FTO), and "Reader" (YTHDF1/2/3, YTHDC1/2, YTHDC2 HNRNPA2B1, HNRNPC and IGF2BP1/2/3, which involved in mRNA degradation, translation, stability, and non-coding RNA processing (Tang et al., 2021; Deng et al., 2022).

catalase in the m6A methyltransferase system, which can act as a proto-oncogene or anti-oncogene to participate in biological processes such as tumorigenesis, proliferation, invasion, migration, cell cycle, and differentiation (Lin et al., 2016; Zeng et al., 2020; Shi et al., 2022). Currently, the role of m6A methyltransferases in NSCLC is mainly focused on METTL3-related studies. It has been shown that knockdown of METTL3 can inhibit the proliferation, drug resistance and metastasis of NSCLC cells and induce apoptosis, as well as alter the phosphorylation of PI3K/AKT signaling pathway members, thereby exerting oncogenic effects (Jin et al., 2021; Zhang et al., 2021). The alternation of m6A in mRNAs mediated by SUMO-ization modification of METTL3 and the subsequent alteration of gene expression profiles may directly affect lung cancer H1299 cell growth (Du et al., 2018). In addition, METTL3 may also affect NSCLC progression in a non-m6A-dependent manner. METTL3 enhances translation efficiency and promotes NSCLC metastasis by interacting with eIF3h, which cyclizes mRNA and increases the efficiency of ribosome recycling and reuse. Inhibition of METTL3 may not only be a target for the treatment of NSCLC, but also enhance chemotherapy sensitivity (Jin et al., 2021). Those findings suggested that METTL3 could play a predominantly pro-cancer role in NSCLC cells and be a potentially diagnostic and therapeutic target for NSCLC.

m6A demethylases in NSCLC

The main m6A demethylases are fass obesity-associated protein (FTO) and alkane hydroxylase homolog 5 (ALKBH5), both of which belong to the ALKB homolog family and are classified as 2-oxoglutarate and iron-dependent nucleic acid oxygenases (Wang et al., 2020). FTO and ALKBH5 primarily demethylate m6A- modified bases, with FTO being the first m6A demethylase identified, showing high demethylation activity for m6A, and ALKBH5 catalyzing the removal of m6A modifications from nuclear RNAs (mainly mRNA), thereby affecting metabolic disease and human obesity. FTO was the first m6A demethylase to be identified, showing high demethylation activity against m6A, which can affect metabolic diseases and the development of obesity in humans, while ALKBH5 can catalyze the removal of m6A modifications from nuclear RNA (mainly mRNA), which in turn can affect nuclear RNA export, metabolism and gene expression, and even fertility in mice (Zheng et al., 2013; Huang et al., 2020; Prakash et al., 2021). However, the current studies showed that although ALKBH5 and FTO are both m6A demethylases, they play different roles in the pathological development of NSCLC. Among them, ALKBH5 mainly plays an oncogenic role in NSCLC, while FTO may play a pro-cancer role in NSCLC. It was found that aberrantly expressed ALKBH5 down-regulated Yes-associated protein (YAP) expression mediated by YTHDFs and inhibited miR-107/LATS2-mediated YAP activity, thereby inhibiting NSCLC cell proliferation, invasion, migration and epithelial mesenchymal transition (Jin et al., 2020). Knockdown of the FTO gene in lung squamous cell carcinoma cells was found to effectively inhibit cell proliferation and promote apoptosis, while overexpression of FTO promoted their malignant progression (Liu et al., 2018). Overexpression of FTO in lung adenocarcinoma cells also showed enhanced proliferation, migration and invasion, and downregulation of m6A-RNA expression, indicating that FTO may promote lung

adenocarcinoma progression through m6A demethylation leading to cell viability, migration, and invasion *in vitro* (Wang et al., 2021). Moreover, FTO was overexpressed and m6A content was sharply reduced in NSCLC tissues and cell lines. Further knockdown of FTO expression in cells was found to inhibit NSCLC cell proliferation, and the mechanism of action may be related to the demethylation enzyme activity of FTO (Li et al., 2019). Meanwhile, it was found that FTO overexpression did not promote NSCLC cell proliferation and invasion after mutation, suggesting that the oncogenic effect of FTO on NSCLC may mainly depend on its catalytic activity (Liu et al., 2018). The above findings suggest that FTO plays a pro-carcinogenic role in NSCLC and inhibits FTO control may slow the progression of NSCLC.

m6A methylated reading protein in NSCLC

Emerging studies have shown that m6A methylated reading proteins mainly act as pro-cancer factors affecting NSCLC proliferation, migration and invasion. There are m6A reading proteins in organisms that specifically recognize and bind to m6A and mediate its exercise of biological functions, including YTHDF1, YTHDF2, YTHDC1, hnRNPA2B1 and hnRNPC, eIF3, IGF2BP1, etc. These proteins bind specifically to m6A to mediate selective shearing, intracellular localization and translational control of RNA metabolic processes (Huang et al., 2018; Shi et al., 2019; Hu et al., 2021).

YTHDF1 deficiency could affect the translation efficiency of CDK2, CDK4 and cyclin D1 and inhibit NSCLC cell proliferation and lung squamous cell carcinoma progression, while high YTHDF1 expression was associated with better clinical outcome (Shi et al., 2019). Compared with para-cancerous tissues, hnRNPA2B1 was highly expressed in NSCLC and could bind to ERK/p53/HDM2 signaling pathway, suggesting that hnRNPA2B1 may be involved in NSCLC pathogenesis (Kim et al., 2021; Jin et al., 2022). In addition, hnRNPC overexpression is associated with later clinical stage and lymph node and distant metastasis, and promotes NSCLC cell proliferation, migration and invasion, possibly through activation of the IFN-α-JAK-STAT1 signaling pathway (Yan et al., 2019).

eIF3 is the largest and most complex translation initiation factor. eIF3 is one of the largest and most complex translation initiation factors, consisting of 13 subunits, such as eIF3a, eIF3b, eIF3d and eIF3h. Among them, eIF3a is the housekeeping gene, which is closely associated with lung carcinogenesis and drug resistance (Yin et al., 2018). In NSCLC cells, eIF3b was highly expressed and was associated with disease progression and poor prognosis, and also promoted NSCLC cell proliferation and inhibited apoptosis. Knockdown of eIF3d in NSCLC cells was also found to significantly inhibit cell proliferation and colony formation, and to block the cell cycle in G2/M phase. This effect may be achieved by inhibiting integrin α 5 and TNFRSF21 expression or activating the β-linked protein signaling pathway (Desnoyers et al., 2015; Lin et al., 2015). In addition, direct physical and functional interactions between eIF3h and METTL3 have been reported to be required for enhanced translation of oncogenic mRNAs, formation of densely packed polyribosomes and oncogenic transformation of lung adenocarcinoma (Choe et al., 2018). In contrast, one study reported that eIF3h protein was highly expressed in lung adenocarcinoma tissues and that eIF3h overexpression promoted lung adenocarcinoma cell migration and invasion, which further confirmed that eIF3h is an oncogenic factor in lung adenocarcinoma (Esteves et al., 2020). Thus, it can be seen that eIF3 subunits in NSCLC mainly exhibit proliferation-promoting, migration, invasion and apoptosis-inhibiting effects on cancer cells, and are expected to be potential targets for NSCLC therapy (Figure 2).

m6A enzymes have potential to be new diagnostic biomarkers and therapeutic target for NSCLC

It was well known that m6A is one of the most common internal mRNA modifications in higher eukaryotic. Notably, accumulating evidences have demonstrated that m6A enzymes are widely involved in various biological process of NSCLC, including proliferation, metastasis as well as inflammatory response (Table 1). Thus, m6A enzymes may provide us effectively diagnostic biomarkers for molecular diagnosis of NSCLC and efficient therapeutic intervention targets for NSCLC treatment. It has been reported that METTL3 with high expression level was closely associated with shorter overall survival, which indicated that METTL3 may have potential to be a prognostic biomarker in NSCLC (Shi et al., 2021). Additionally, leukocyte m6A was not only positively related to the number of lymphocytes but also negatively correlated with monocytes in NSCLC, which was mainly caused by upregulated METTL3 and downregulated FTO and ALKBH5. Therefore, leukocyte m6A and m6A enzymes may be novel noninvasive biomarkers for NSCLC diagnosis (Pei et al., 2020). Moreover, Shen et al. have suggested that eIF3a played an essential role in radically resected NSCLC patients and it was of prognostic value for aberrant eIF3a expression to independently predict prognosis of NSCLC (Shen et al., 2014). Consequently, these findings indicated that m6A enzymes, as m6A RNA methylation regulators, have potential to be used as diagnostic and prognostic biomarkers, suggesting great clinical value.

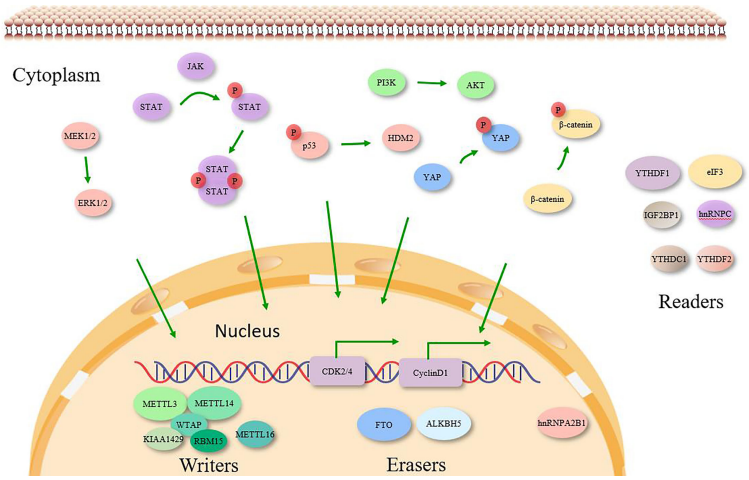

FIGURE 2
Molecular targets of m6A enzymes in various signaling pathways. m6A 'Writers', 'Erasers' and 'Readers' play significant role in cancer by targeting specific RNA transcripts of cell signaling molecules causing activation/inactivation of various intracellular signaling pathways, which are indicated by the colors corresponding to the regulators.

m6A inhibitors

The main representative drugs are azacitidine, vorinostat and other specific inhibitors of epigenetic- related proteins, which play a significant role in clinical practice. Given that epigenetic inheritance is based on the molecular mechanism of regulation of transcription and post-transcriptional products, and that the results of previous studies have shown that m6A plays an important role in the malignant biological behavior of tumors, the development of specific inhibitors of m6A-related proteins is of great scientific significance and clinical value (Table 2).

FTO inhibitors

The development of inhibitors based on m6A-related enzymes has focused on the first discovered RNA demethylase, FTO, a conserved class of 2OG oxygenases, whose activity can be inhibited by the universal inhibitor NOG. Rhein, a natural anthraquinone in the Rhubarb, can inhibit FTO by competitively binding to single- stranded RNA (ssRNA) substrate in the catalytic domain, which can inhibit the demethylation activity of FTO on m6A on mRNA *in vitro* and *in vivo*, thereby increasing the level of m6A in cells (Chen et al., 2021). Meclofenamic acid (MA), a selective FTO inhibitor, could bind to and stabilize FTO without affecting the demethylation enzymatic activity of ALKBH5, and could significantly increase the level of cellular m6A, but had no significant effect on the level of m6A in FTO-deficient cells, indicating that the crystal structure of the FTO/MA complex clearly demonstrates that β3i and β4i in FTO form hydrophobic pockets that specifically recognize MA, while ALKBH5 lacks the corresponding structure, providing a chemical basis for the development of specific FTO inhibitors (Huang et al., 2015).

Aik et al. identified a series of compounds that inhibit FTO activity by competing with 2OG through metal ion chelating groups, but such inhibitors need to be developed to avoid inhibition of other 2OG oxygenases and to improve specificity.the compounds identified by Zheng et al. effectively

TABLE 1 The roles of m6A enzymes in NSCLC progression.

Type	m6A enzymes	Regulation	Role in NSCLC	Reference
Writers	METTL3	Up-regulation	Induces NSCLC drug resistance and metastasis	Jin et al., 2019
	METTL14	Down-regulation	Suppresses the malignant progression of NSCLC	Yang et al., 2021
Erasers	FTO	Up-regulation	Accelerates tumor growth and metastasis	Wang et al., 2021
	ALKBH5	Up-regulation	Inhibits tumor growth and metastasis	Jin et al., 2020
Readers	YTHDF1	Down-regulation	represses NSCLC cell proliferation, invasion and migration abilities, whereas enhances apoptosis	Zhou et al., 2020
	eIF3	Up-regulation	inhibits proliferation and cisplatin sensibility in NSCLC	Fang et al., 2017

TABLE 2 The inhibitors of m6A-related enzymes and their biological function.

Inhibitor	IC50 (mM)	Target	Biological function	Reference
Rhein	21	FTO	Inhibit FTO by competitively binding the catalytic domain against ssRNA substrate, also effectively inhibit m6A demethylation *in vitro* and increase cellular levels of m6A	Chen et al., 2021
MA	17.4	FTO	Bind and stabilize FTO but had minimal influence on ALKBH5	Huang et al., 2015
Radicicol	16.0	FTO	Radicicol, as an FTO inhibitor *in vitro*, provided new information on designing more potent compounds to inhibit the activity of the enzyme	Wang et al., 2018
N-CDPCB	4.95	FTO	Inhibitory activity on FTO demethylation of the 15-mer ssRNA, significantly decreased the level of m6A of mRNA in preadipocytes	He et al., 2015
CHTB	39.2	FTO	CHTB complexed with human FTO reveals that the novel small molecule binds to FTO in a specific manner, regulation of mRNA splicing and adipogenesis by modulating m6A levels	He et al., 2015
Entacapone	3.5	FTO	Entacapone as a chemical inhibitor of FTO mediating metabolic regulation through FOXO1	Peng et al., 2019
IOX3	2.8	FTO/ ALKBH5	An inhibitor of the HIF prolyl hydroxylases, decreased cellular protein expression of FTO, failed to alter the m6A level inside of cells. IOX3 also could bind to ALKBH5 in a covalent attachment	Han et al., 2019
FMN	Unknown	Nucleoside	Combined with blue-light irradiation substantially decreases m6A levels in cells by directly targeting the nucleoside modification	Xie et al., 2018
Clausine E	Unknown	FTO	Bound by positive entropy and negative enthalpy changes.	Wang et al., 2019
6-chloro-2-phenyl-1H-benzimidazole(CPBZD)	24.65	FTO	Bound by positive entropy.	Li et al., 2019

inhibited FTO of their 2OG oxygenase family and had IC50 values close to those of the broad- spectrum 2OG enzyme inhibitor NOG (Aik et al., 2013). Based on the molecular mechanism of FTO recognition of m6A modified substrates and other characteristics, Huang et al. applied crystal structure-based compound design and synthesis optimization to obtain FTO small molecule inhibitors. The compounds selectively inhibited the demethylation of FTO in AML cells, upregulated the m6A modification on the mRNA of key AML genes, increased the abundance of oncogenic proteins such as ASB2 and RARA, and decreased the abundance of oncogenic proteins such as MYC and CEBPA, thereby inhibiting the proliferation of AML cells, and demonstrated the anti-leukemia therapeutic effect in a PDX mouse model. This study indicates a new direction for molecularly targeted intervention of m6A modification to affect gene expression for anti-tumor research (Huang et al., 2019).

Huang et al.also found that entacapone, which was previously approved by the FDA for marketing, could act as a specific inhibitor of FTO based on a virtual screening of the structure and a series of *in vitro* and *in vivo* bioactivity assays. It directly binds and inhibits FTO activity *in vitro*. In a diet-induced obesity mouse model, feeding entacapone with FOXO1 mRNA as a direct-acting substrate for FTO induced intrahepatic glycogen xenobiogenesis and adipose tissue thermogenesis, resulting in reduced body mass and fasting glucose concentrations in mice (Peng et al., 2019). In addition, FTO is not only closely related to obesity and tumors, but its common variants rs9939609 may be associated with central nervous system diseases such as brain volume loss and alcohol dependence (Qiao et al., 2016; Liu et al., 2018). Therefore, the potential use of FTO inhibitors, in addition to anti-tumor and weight loss, may also be developed as drugs for neurological diseases.

Other m6A-related protein inhibitors and chemical interventions

Both ALKBH5 and FTO belong to the ALKB subfamily of the Fe(II)/2-oxoglutarate (2OG) dioxygenase superfamily. 2OG dioxygenase superfamily members act on a variety of substrates and are involved in the regulation of protein biosynthesis. IOX3 can also bind covalently to ALKBH5. Under ALKBH5 crystallization conditions, citrate competes with 2OGs and Mn (ii) at the active site of alkanes and can be directed towards the development and modification of ALKHB5 as an inhibitor (Xu et al., 2014; Han et al., 2019). Among methylesterases, METTL3 is the main active catalytic site, while METTL14 plays a key role in the substrate recognition process (Wang et al., 2016). Due to the limited knowledge of the mechanism of methylesterase recognition and catalytic RNA methylation, the development of its inhibitors has been relatively slow. So far, only 3-deazaadenosine (DAA) has been found to inhibit METTL3, but the effect of DAA is broad-spectrum, inhibiting the activity of all RNA methylesterases, without specificity for m6A methylesterases.

In addition, chemical demethylation of m6A is another important chemical intervention strategy. Riboflavin was used as the exogenous photosensitive molecule riboflavin to selectively and efficiently oxidize m6A of mRNA catalyzed by

LED blue light, thus reducing the m6A modification of mRNA (Xie et al., 2017). They further developed flavin mononucleotide (FMN), which can perform m6A modification removal of mRNA on living cells, can chemically demethylate m6A in cells catalyzed by LED blue light, thus realizing mRNA m6A-specific demethylation in living cells using the compound.

m6A modification regulates progression of cancer mediated by microbiota

As a universally epigenetic modification, m6A modification is closely related to the occurrence and progression of cancer. Furthermore, it has been identified that intestinal bacteria are involved in supplying the methyl donor substances and modulating m6A RNA methylation, indicating that intestinal bacteria may play an essential role in occurrence and progression of cancers through regulating m6A RNA methylation. Luo et al. demonstrated that bioactive nonstarch polysaccharides dramatically ameliorate cancer by altering host m6A RNA methylation, which influences methyl donors mediated by intestinal microbiota (Luo et al., 2021). They provided us a scientific view that m6A RNA methylation may participate in the occurrence and progression of various types of cancer, including NSCLC, through intestinal microbiota. Additionally, Chen et al. found that Fusobacterium nucleatum significantly reduces m6A modifications in colorectal cancer cells and patient-derived xenograft (PDX) tissues through downregulation of METTL3, leading to enhancement of colorectal cancer aggressiveness (Chen et al., 2022). On the one hand, it was worth noting that lipopolysaccharide (LPS) simulation or reactive oxygen species (ROS) release promotes inflammation-related hepatocellular carcinoma progression in early stage of tumor and is related to unfavorable prognosis through increasing m6A methylation of GNAS mRNA (Ding et al., 2020). Therefore, the pathogen bacteria produce ROS or release LPS to induce oxidative stress to change m6A modification in host, causing poor response to chemotherapy and immunotherapy. On the other hand, Treg cell-mediated immunosuppression in patients at advanced stage of tumor can lead to secondary infection by some opportunistic pathogens like Staphylococcus aureus, Escherichia coli and Mycobacterium tuberculosis in a m6A-dependent manner (Figure 3). What's more, the secondary infection may activate TLR and TLR-mediated tumor-promoting responses, which promote the occurrence and development of lung cancer. Consequently, the above results indicated us that m6A modification may participate in the progression of virous types of cancer by microbiota and similar pattern of regulation probably existed in NSCLC. Nevertheless, the interactions between the commensal microbiota and m6A methylation remain incompletely understood and need further explored.

Conclusions and prospects

In recent years, the enzymes related to m6A methylation modification, the role and biological significance of m6A in mRNA modification, and the regulatory mechanism of m6A in malignant tumors have been revealed. m6A is precisely regulated

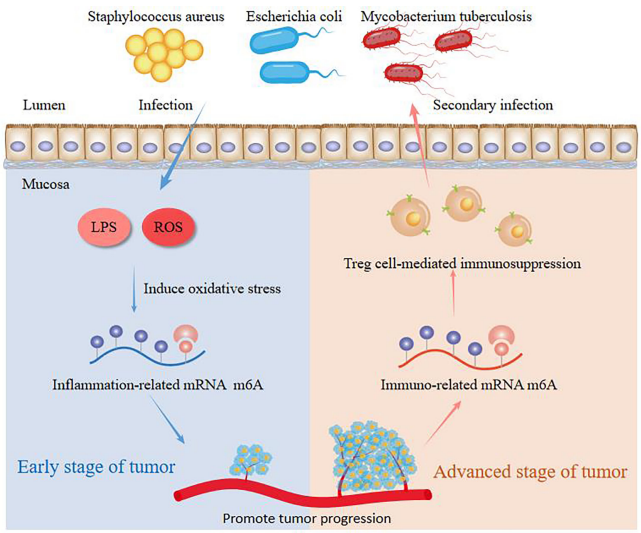

FIGURE 3
The molecular mechanisms of m6A in the interaction of host and microbes in cancer.

by methylesterases, demethylases and recognition proteins, and is involved in almost every step of mRNA biology from production to degradation, and is involved in regulating the production and biological functions of miRNAs and lncRNAs. m6A plays an important role in the metabolism, drug resistance and metastasis of many malignant tumors, suggesting that m6A modification can be a target for the prevention and treatment of human tumors.

The discovery of inhibitors of m6A-related factors (such as FTO, ALKBH5 and HIF) has helped to achieve oncogene-targeted therapy. So far, several FTO inhibitors have been reported (rhodopsin, meclofenamic acid, IOX3, etc.), most of which are not specific. Meclofenamic acid (MA), one of the selective FTO inhibitors, is a non-steroidal anti-inflammatory drug that competes with the FTO binding site. The FTO inhibitor MA2 (an ethyl ester derivative of MA) prolongs the lifespan of GSC-transplanted mice, implying that m6A modification may be a target for inhibiting tumor progression and reversing resistance to radiotherapy and chemotherapy in glioblastoma. In addition, the metabolite R-2HG caused by isocitrate dehydrogenase (IDH) mutation exerts anti-leukemic effects by inhibiting FTO/m6A/MYC/CEBPA signaling, providing a new target for clinical dosing in the treatment of leukemia. Although many inhibitors targeting m6A demethylases have been identified, there is limited *in vivo* evidence for their effects, and there is still room for extensive exploration of the development of m6A-related protein inhibitors and clinical indication options. Firstly, RNA modifications are spatially and temporally dynamic and tissue-specific, and their regulatory mechanisms require single-base precision high-resolution sequencing and measurement technologies, while the current mainstream antibody capture technologies cannot meet the single- base precision targeting of m6A. Secondly, there is no technology to edit the m6A site of the transcript single gene for specific genes. In addition, although some demethylase inhibitors have been identified and provide new targets for oncology drugs, their actions and specific mechanisms are not yet fully understood and lack specificity. Therefore, researchers expect to obtain more inhibitors targeting m6A-associated proteins, especially more specific ones, to provide a new strategy for epigenetic-based tumor targeting therapy.

Several new drugs targeting DNA methylesterases or histone modifying enzymes have been approved for the treatment of tumors with promising therapeutic effects and significant economic benefits. Epigenetic-based chemical interventions have become an active area of research for new drug targets in the international arena. Due to the late start, RNA epigenetic research with m6A modification as the core is still in the early stage. Since targeting the RNA epigenome, including its editing, degradation, translocation and translation, has good advantages in terms of safety and timeliness, the discovery of high-quality chemical probes and specific inhibitors, the development of single-gene specific editing technology, and the targeted intervention of m6A modifications can not only promote basic research in related fields, but also show great prospects for application in tumor therapy and other disease-related fields, and be used in both life science and new drug discovery. What's more, it has been identified that m6A methylation plays a crucial role in microbiota homeostasis for maintaining physiological balance and stabilization of body, which probably server as a messenger to participate in the crosstalk of host and microbiomes. For one thing, the change of microbiota induced by the m6A may participate in reshaping the microenvironment. For another, microbiota play an essential role in regulating host m6A RNA modification profiles through microbial metabolites, inducing ROS or releasing LPS. More importantly, m6A-related therapeutic strategies provide a promising direction for the targeted therapy in various diseases, especially in tumors.

Therefore, the development of single gene specific editing technologies to target m6A modifications will not only promote basic research in related fields, but also show great promise for applications in disease related fields such as tumor therapy, and show scientific importance in both life science and new drug discovery. These findings not only provide novel mechanistic insight into the biology of lung cancer but also shed light on new therapeutic targets and strategies for lung cancer prevention and treatment.

Author contributions

F-SQ and Y-SZ searched the articles and drafted the manuscript. M-YG checked the contents. J-QH and C-HY revised the manuscript and provided the funds. C-HY was responsible for the project administration. All authors contributed to the article and approved the submitted version.

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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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References

Aik, W., Demetriades, M., Hamdan, M. K., Bagg, E. A., Yeoh, K. K., Lejeune, C., et al. (2013). Structural basis for inhibition of the fat mass and obesity associated protein (FTO). *J. Med. Chem.* 56 (9), 3680–3688. doi: 10.1021/jm400193d

Chen, B., Ye, F., Yu, L., Jia, G., Huang, X., Zhang, X., et al. (2021). Development of cell-active N6-methyladenosine RNA demethylase FTO inhibitor. *J. Am. Chem. Soc* 134 (43), 17963–17971. doi: 10.1021/ja3064149

Chen, S., Zhang, L., Li, M., Zhang, Y., Sun, M., Wang, L., et al. (2022). Fusobacterium nucleatum reduces METTL3-mediated m6A modification and contributes to colorectal cancer metastasis. *Nat. Commun.* 13 (1), 1248. doi: 10.1038/s41467-022-28913-5

Chen, D. H., Zhang, J. G., Wu, C. X., and Li, Q. (2021). Non-coding RNA m6A modification in cancer: Mechanisms and therapeutic targets. *Front. Cell Dev. Biol.* 9, 778582. doi: 10.3389/fcell.2021.778582

Choe, J., Lin, S., Zhang, W., Liu, Q., Wang, L., Ramirez-Moya, J., et al. (2018). mRNA circularization by METTL3-eIF3h enhances translation and promotes oncogenesis. *Nature* 561 (7724), 556–560. doi: 10.1038/s41586-018-0538-8

Deng, L. J., Deng, W. Q., Fan, S. R., Chen, M. F., Qi, M., Lyu, W. Y., et al. (2022). m6A modification: recent advances, anticancer targeted drug discovery and beyond. *Mol. Cancer* 21 (1), 52. doi: 10.1186/s12943-022-01510-2

Desnoyers, G., Frost, L. D., Courteau, L., Wall, M. L., and Lewis, S. M. (2015). Decreased eIF3e expression can mediate epithelial-to-Mesenchymal transition through activation of the TGFβ signaling pathway. *Mol. Cancer Res.* 13 (10), 1421–1430. doi: 10.1158/1541-7786.MCR-14-0645

Desrosiers, R., Friderici, K., and Rottman, F. (1974). Identification of methylated nucleosides in messenger RNA from novikoff hepatoma cells. *Proc. Natl. Acad. Sci. U.S.A.* 71 (10), 3971–3975. doi: 10.1073/pnas.71.10.3971

Ding, H., Zhang, X., Su, Y., Jia, C., and Dai., C. (2020). GNAS promotes inflammation-related hepatocellular carcinoma progression by promoting STAT3 activation. *Cell Mol. Biol. Lett.* 25, 8. doi: 10.1186/s11658-020-00204-1

Du, Y., Hou, G., Zhang, H., Dou, J., He, J., Guo, Y., et al. (2018). SUMOylation of the m6A-RNA methyltransferase METTL3 modulates its function. *Nucleic Acids Res.* 46 (10), 5195–5208. doi: 10.1093/nar/gky156

Esteves, P., Dard, L., Brillac, A., Hubert, C., Sarlak, S., Rousseau, B., et al. (2020). Nuclear control of lung cancer cells migration, invasion and bioenergetics by eukaryotic translation initiation factor 3F. *Oncogene.* 39 (3), 617–636. doi: 10.1038/s41388-019-1009-x

Fang, C., Chen, Y. X., Wu, N. Y., Yin, J. Y., Li, X. P., Huang, H. S., et al. (2017). MiR-488 inhibits proliferation and cisplatin sensibility in non-small-cell lung cancer (NSCLC) cells by activating the eIF3a-mediated NER signaling pathway. *Sci. Rep.* 7, 40384. doi: 10.1038/srep40384

Gao, R., Ye, M., Liu, B., Wei, M., Ma, D., and Dong, K. (2021). m6A modification: A double-edged sword in tumor development. *Front. Oncol.* 11, 679367. doi: 10.3389/fonc.2021.679367

Han, X., Wang, N., Li, J., Wang, Y., Wang, R., and Chang., J. (2019). Identification of nafamostat mesilate as an inhibitor of the fat mass and obesity-associated protein (FTO) demethylase activity. *Chem. Biol. Interact.* 297, 80–84. doi: 10.1016/j.cbi.2018.10.023

He, J.-Q., Chen, Q., Wu, S.-J., Wang, D.-Q., Zhang, S.-Y., Zhang, S.-Z., et al. (2022). Potential implications of the lung microbiota in patients with chronic obstruction pulmonary disease and non-small cell lung cancer. *Front. Cell. Infect Microbiol.* 12. doi: 10.3389/fcimb.2022.937864

He, W., Zhou, B., Liu, W., Zhang, M., Shen, Z., Han, Z., et al. (2015). Identification of a novel small-molecule binding site of the fat mass and obesity associated protein (FTO). *J. Med. Chem.* 58 (18), 7341–7348. doi: 10.1021/acs.jmedchem.5b00702

Huang, Y., Su, R., Sheng, Y., Dong, L., Dong, Z., Xu, H., et al. (2019). Small-molecule targeting of oncogenic FTO demethylase in acute myeloid leukemia. *Cancer Cell* 35 (4), 677–691.e10. doi: 10.1016/j.ccell.2019.03.006

Huang, H., Wang, Y., Kandpal, M., Zhao, G., Cardenas, H., Ji, Y., et al. (2020). FTO-dependent n 6-methyladenosine modifications inhibit ovarian cancer stem cell self-renewal by blocking cAMP signaling. *Cancer Res.* 80 (16), 3200–3214. doi: 10.1158/0008-5472.CAN-19-4044

Huang, H., Weng, H., Sun, W., Qin, X., Shi, H., Wu, H., et al. (2018). Recognition of RNA N6-methyladenosine by IGF2BP proteins enhances mRNA stability and translation. *Nat. Cell Biol.* 20 (3), 285–295. doi: 10.1038/s41556-018-0045-z

Huang, Y., Yan, J., Li, Q., Li, J., Gong, S., Zhou, H., et al. (2015). Meclofenamic acid selectively inhibits FTO demethylation of m6A over ALKBH5. *Nucleic Acids Res.* 43 (1), 373–384. doi: 10.1093/nar/gku1276

Hu, J., Qiu, D., Yu, A., Hu, J., Deng, H., Li, H., et al. (2021). YTHDF1 is a potential pan-cancer biomarker for prognosis and immunotherapy. *Front. Oncol.* 11, 607224. doi: 10.3389/fonc.2021.607224

Jin, L., Chen, C., Yao, J., Yu, Z., and Bu, L. (2022). The RNA N^6-methyladenosine modulator HNRNPA2B1 is involved in the development of non-small cell lung cancer. *Clin. Exp. Pharmacol. Physiol.* 49 (3), 329–340. doi: 10.1111/1440-1681.13608

Jin, D., Guo, J., Wu, Y., Du, J., Yang, L., Wang, X., et al. (2019). m6A mRNA methylation initiated by METTL3 directly promotes YAP translation and increases YAP activity by regulating the MALAT1-miR-1914-3p-YAP axis to induce NSCLC drug resistance and metastasis. *J. Hematol. Oncol.* 12 (1), 135. doi: 10.1186/s13045-019-0830-6

Jin, D., Guo, J., Wu, Y., Du, J., Yang, L., Wang, X., et al. (2021). m6A mRNA methylation initiated by METTL3 directly promotes YAP translation and increases YAP activity by regulating the MALAT1-miR-1914-3p-YAP axis to induce NSCLC drug resistance and metastasis. *J. Hematol. Oncol.* 14 (1), 32. doi: 10.1186/s13045-021-01048-8

Jin, D., Guo, J., Wu, Y., Yang, L., Wang, X., Du, J., et al. (2020). m6A demethylase ALKBH5 inhibits tumor growth and metastasis by reducing YTHDFs-mediated YAP expression and inhibiting miR-107/LATS2-mediated YAP activity in NSCLC. *Mol. Cancer* 19 (1), 40. doi: 10.1186/s12943-020-01161-1

Kim, M. K., Choi, M. J., Lee, H. M., Choi, H. S., Park, Y. K., and Ryu, C. J. (2021). Heterogeneous nuclear ribonucleoprotein A2/B1 regulates the ERK and p53/HDM2 signaling pathways to promote the survival, proliferation and migration of non−small cell lung cancer cells. *Oncol. Rep.* 46 (2), 153. doi: 10.3892/or.2021.8104

Kumari, R., Ranjan, P., Suleiman, Z. G., Goswami, S. K., Li, J., Prasad, R., et al. (2021). mRNA modifications in cardiovascular biology and disease: with a focus on m6A modification. *Cardiovasc. Res.* 118 (7), 1680–1692. doi: 10.1093/cvr/cvab160

Li, J., Han, Y., Zhang, H., Qian, Z., Jia, W., Gao, Y., et al. (2019). The m6A demethylase FTO promotes the growth of lung cancer cells by regulating the m6A level of USP7 mRNA. *Biochem. Biophys. Res. Commun.* 512 (3), 479–485. doi: 10.1016/j.bbrc.2019.03.093

Lin, S., Choe, J., Du, P., Triboulet, R., and Gregory, R. I. (2016). The m(6)A methyltransferase METTL3 promotes translation in human cancer cells. *Mol. Cell.* 62 (3), 335–345. doi: 10.1016/j.molcel.2016.03.021

Lin, Z., Xiong, L., and Lin, Q. (2015). Knockdown of eIF3d inhibits cell proliferation through G2/M phase arrest in non-small cell lung cancer. *Med. Oncol.* 32 (7), 183. doi: 10.1007/s12032-015-0625-8

Liu, J., Ren, D., Du, Z., Wang, H., Zhang, H., and Jin, Y. (2018). m6A demethylase FTO facilitates tumor progression in lung squamous cell carcinoma by regulating MZF1 expression. *Biochem. Biophys. Res. Commun.* 502 (4), 456–464. doi: 10.1016/j.bbrc.2018.05.175

Li, J., Wang, Y., Han, X., Wang, N., Yu, W., Wang, R., et al. (2019). The role of chlorine atom on the binding between 2-phenyl-1H-benzimidazole analogues and fat mass and obesity-associated protein. *J. Mol. Recognit.* 32 (6), e2774. doi: 10.1002/jmr.2774

Luo, J., Yu, J., and Peng, X. (2021). Could partial nonstarch polysaccharides ameliorate cancer by altering m6A RNA methylation in hosts through intestinal microbiota? *Crit. Rev. Food Sci. Nutr.* 26, 1–16. doi: 10.1080/10408398.2021.1927975

Pei, Y., Lou, X., Li, K., Xu, X., Guo, Y., Xu, D., et al. (2020). Peripheral blood leukocyte N6-methyladenosine is a noninvasive biomarker for non-small-cell lung carcinoma. *OncoTargets Ther.* 13, 11913–11921. doi: 10.2147/ott.S267344

Peng, S., Xiao, W., Ju, D., Sun, B., Hou, N., Liu, Q., et al. (2019). Identification of entacapone as a chemical inhibitor of FTO mediating metabolic regulation through FOXO1. *Sci. Transl. Med.* 11 (488), eaau7116. doi: 10.1126/scitranslmed.aau7116

Prakash, M., Itoh, Y., Fujiwara, Y., Takahashi, Y., Takada, Y., Mellini, P., et al. (2021). Identification of potent and selective inhibitors of fat mass obesity-associated protein using a fragment-merging approach. *J. Med. Chem.* 64 (21), 15810–15824. doi: 10.1021/acs.jmedchem.1c01107

Qiao, Y., Zhou, B., Zhang, M., Liu, W., Han, Z., Song, C., et al. (2016). A novel inhibitor of the obesity-related protein FTO. *Biochemistry* 55 (10), 1516–1522. doi: 10.1021/acs.biochem.6b00023

Shen, J., Yin, J. Y., Li, X. P., Liu, Z. Q., Wang, Y., Chen, J., et al. (2014). The prognostic value of altered eIF3a and its association with p27 in non-small cell lung cancers. *PloS One* 9 (4), e96008. doi: 10.1371/journal.pone.0096008

Shi, Y., Fan, S., Wu, M., Zuo, Z., Li, X., Jiang, L., et al. (2019). YTHDF1 links hypoxia adaptation and non-small cell lung cancer progression. *Nat. Commun.* 10 (1), 4892. doi: 10.1038/s41467-019-12801-6

Shi, L., Gong, Y., Zhuo, L., Wang, S., Chen, S., and Ke, B. (2022). Methyltransferase-like 3 upregulation is involved in the chemoresistance of non-small cell lung cancer. *Ann. Trans. Med.* 10 (3), 139. doi: 10.21037/atm-21-6608

Shi, C. Y., Yu, C. H., Yu, W. Y., and Ying., H. Z. (2021). Gut-lung microbiota in chronic pulmonary diseases: Evolution, pathogenesis, and therapeutics. *Can. J. Infect. Dis. Med. Microbiol.* 2021, 9278441. doi: 10.1155/2021/9278441

Tang, Y., Chen, K., Song, B., Ma, J., Wu, X., Xu, Q., et al. (2021). m6A-atlas: a comprehensive knowledgebase for unraveling the N6-methyladenosine (m6A) epitranscriptome. *Nucleic Acids Res.* 49 (D1), D134–D143. doi: 10.1093/nar/gkaa692

Wang, X., Feng, J., Xue, Y., Guan, Z., Zhang, D., Liu, Z., et al. (2016). Structural basis of N(6)-adenosine methylation by the METTL3-METTL14 complex. *Nature* 534 (7608), 575–578. doi: 10.1038/nature18298

Wang, R., Han, Z., Liu, B., Zhou, B., Wang, N., Jiang, Q., et al. (2018). Identification of natural compound radicicol as a potent FTO inhibitor. *Mol. Pharm.* 15 (9), 4092–4098. doi: 10.1021/acs.molpharmaceut.8b00522

Wang, Y., Li, J., Han, X., Wang, N., Song, C., Wang, R., et al. (2019). Identification of clausine e as an inhibitor of fat mass and obesity-associated protein (FTO) demethylase activity. *J. Mol. Recognit.* 32 (10), e2800. doi: 10.1002/jmr.2800

Wang, Y., Li, M., Zhang, L., Chen, Y., and Zhang, S. (2021). m6A demethylase FTO induces NELL2 expression by inhibiting E2F1 m6A modification leading to metastasis of non-small cell lung cancer. *Mol. Ther. Oncolytics* 21, 367–376. doi: 10.1016/j.omto.2021.04.011

Wang, J., Wang, J., Gu, Q., Ma, Y., Yang, Y., Zhu, J., et al. (2020). The biological function of m6A demethylase ALKBH5 and its role in human disease. *Cancer Cell Int.* 20, 347. doi: 10.1186/s12935-020-01450-1

Xie, L. J., Wang, R. L., Wang, D., Liu, L., and Cheng, L. (2017). Visible-light-mediated oxidative demethylation of N6-methyl adenines. *Chem. Commun. (Camb).* 53 (77), 10734–10737. doi: 10.1039/c7cc05544g

Xu, X., Huang, J., Ocansey, D. K. W., Xia, Y., Zhao, Z., Xu, Z., et al. (2021). The emerging clinical application of m6A RNA modification in inflammatory bowel disease and its associated colorectal cancer. *J. Inflammation Res.* 14, 3289–3306. doi: 10.2147/JIR.S320449

Xu, C., Liu, K., Tempel, W., Demetriades, M., Aik, W., Schofield, C. J., et al. (2014). Structures of human ALKBH5 demethylase reveal a unique binding mode for specific single-stranded N6-methyladenosine RNA demethylation. *J. Biol. Chem.* 289 (25), 17299–17311. doi: 10.1074/jbc.M114.550350

Yang, F., Yuan, W. Q., Li, J., and Luo., Y. Q. (2021). Knockdown of METTL14 suppresses the malignant progression of non-small cell lung cancer by reducing twist expression. *Oncol. Lett.* 22 (6), 847. doi: 10.3892/ol.2021.13108

Yan, M., Sun, L., Li, J., Yu, H., Lin, H., Yu, T., et al. (2019). RNA-Binding protein KHSRP promotes tumor growth and metastasis in non-small cell lung cancer. *J. Exp. Clin. Cancer Res.* 38 (1), 478. doi: 10.1186/s13046-019-1479-2

Yin, Y., Long, J., Sun, Y., Li, H., Jiang, E., Zeng, C., et al. (2018). The function and clinical significance of eIF3 in cancer. *Gene.* 673, 130–133. doi: 10.1016/j.gene.2018.06.034

Zaccara, S., Ries, R. J., and Jaffrey, S. R. (2019). Writing and erasing mRNA methylation. *Nat. Rev. Mol. Cell Biol.* 20 (10), 608–624. doi: 10.1038/s41580-019-0168-5

Zeng, C., Huang, W., Li, Y., and Weng, H. (2020). Roles of METTL3 in cancer: mechanisms and therapeutic targeting. *J. Hematol. Oncol.* 13 (1), 117. doi: 10.1186/s13045-020-00951-w

Zhang, Y., Liu, S., Zhao, T., and Dang, C. (2021). METTL3–mediated m6A modification of Bcl–2 mRNA promotes non–small cell lung cancer progression. *Oncol. Rep.* 46 (2), 163. doi: 10.3892/or.2021.8114

Zhang, H., Shi, X., Huang, T., Zhao, X., Chen, W., Gu, N., et al. (2020). Dynamic landscape and evolution of m6A methylation in human. *Nucleic Acids Res.* 48 (11), 6251–6264. doi: 10.1093/nar/gkaa347

Zheng, G., Dahl, J. A., Niu, Y., Fedorcsak, P., Huang, C. M., Li, C. J., et al. (2013). ALKBH5 is a mammalian RNA demethylase that impacts RNA metabolism and mouse fertility. *Mol. Cell* 49 (1), 18–29. doi: 10.1016/j.molcel.2012.10.015

Zhou, J., Xiao, D., Qiu, T., Li, J., and Liu, Z. (2020). Loading MicroRNA-376c in extracellular vesicles inhibits properties of non-small cell lung cancer cells by targeting YTHDF1. *Technol. Cancer Res. Treat* 19, 1533033820977525. doi: 10.1177/153303382097752

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Geography, niches, and transportation influence bovine respiratory microbiome and health

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Bovine respiratory disease (BRD), one of the most common and infectious diseases in the beef industry, is associated with the respiratory microbiome and stressors of transportation. The impacts of the bovine respiratory microbiota on health and disease across different geographic locations and sampling niches are poorly understood, resulting in difficult identification of BRD causes. In this study, we explored the effects of geography and niches on the bovine respiratory microbiome and its function by re-analyzing published metagenomic datasets and estimated the main opportunistic pathogens that changed after transportation. The results showed that diversity, composition, structure, and function of the bovine nasopharyngeal microbiota were different across three worldwide geographic locations. The lung microbiota also showed distinct microbial composition and function compared with nasopharyngeal communities from different locations. Although different signature microbiota for each geographic location were identified, a module with co-occurrence of *Mycoplasma* species was observed in all bovine respiratory communities regardless of geography. Moreover, transportation, especially long-distance shipping, could increase the relative abundance of BRD-associated pathogens. Lung microbiota from BRD calves shaped clusters dominated with different pathogens. In summary, geography, sampling niches, and transportation are important factors impacting the bovine respiratory microbiome and disease, and clusters of lung microbiota by different bacterial species may explain BRD pathogenesis, suggesting the importance of a deeper understanding of bovine respiratory microbiota in health.

KEYWORDS

respiratory microbiota, bovine, metagenomics, geography, bovine respiratory disease, transportation, nasopharynx, lung

Introduction

Bovine respiratory disease (BRD) is one of the most common infectious causes of pneumonia in cattle worldwide and causes morbidity and mortality in newly transported and recently weaned feedlot cattle, resulting in a slower growth rate and economic cost for prevention and treatment (Chai et al., 2022). With developments of sequencing technology, the roles of the respiratory microbiome in maintaining homeostasis of the airway ecosystem and its association with disease are more deeply understood (Dickson et al., 2016; Man et al., 2017; Zeineldin et al., 2019). The most implicated bacterial pathogens in BRD, including *Mycoplasma bovis*, *Mannheimia haemolytica*, *Histophilus somni*, and *Pasteurella multocida*, have been identified (Nicola et al., 2017; Cirone et al., 2019). Until now, 16S rRNA sequencing has been the most popular technology to investigate the respiratory microbiome of both humans and cattle (Human Microbiome Project, 2012; Zeineldin et al., 2020). Metagenomics that capture sequences from nearly all the organisms inhabiting the respiratory system is more effective and accurate to investigate the composition and functions of the respiratory microbiome (Holman et al., 2017c; Cui et al., 2021). However, fewer studies regarding BRD using metagenomics have been performed.

Geography serves as one of the major factors influencing the respiratory microbiota (Gupta et al., 2017). As cattle are a main source of human protein and nutrition and BRD appears worldwide in cattle, research of the bovine respiratory microbiome at the world level is important and necessary. However, due to feeding strategy, diet, environment, climate, *etc*., the compositional differences of the bovine airway microbiota are affected by the geographic locations of farms. Using 16S sequencing technology, a study found that, in Canada, the most prominently identified genera in the nasopharynx of calves were *Mycoplasma*, *Lactococcus*, *Moraxella*, *Histophilus*, and *Pasteurella* (Mcmullen et al., 2018), while in the United States *Mannheimia*, *Mycoplasma*, *Moraxella*, *Psychrobacter*, and *Pseudomonas* were the top five genera in the nasopharynx of calves (Lima et al., 2016). However, to our knowledge, there are fewer studies to specifically investigate how bovine respiratory microbiota and its functions vary geographically.

Other factors, including sampling niche in the respiratory tract, weaning and transportation, time since arrival to the feedlot, and health status, can influence the microbial structure of the bovine respiratory ecosystem and are associated with an increased risk of BRD in recently weaned beef calves (Snowder et al., 2006; Taylor et al., 2010; Zeineldin et al., 2017a; Zeineldin et al., 2020). The biochemical and physiological environments of niches within the respiratory tracts are different, resulting in a different microbial composition (Zeineldin et al., 2019; Chai et al., 2022). A recent study reported that the dominant bacteria were *Moraxella* and *Mycoplasma* in the nasopharynx, as well as *Mycoplasma* in the lungs of healthy calves, and concluded that the nasopharynx could serve as a primary source for lung microbiota (Mcmullen et al., 2020). Although a recent theory that suggests microbiota colonize in the upper respiratory tracts firstly and then disperse to the lungs was reported in humans (Venkataraman et al., 2015), it has not been confirmed in cattle. Furthermore, the nasopharynx has been the most popular niche to determine the relationship between the respiratory microbiota and BRD (Holman et al., 2015a; Timsit et al., 2016a; Holman et al., 2017a; Zeineldin et al., 2017a; Holman et al., 2017d)—for instance, some studies mainly focused on the pathogens in the nasopharynx (Timsit et al., 2016b; Gaeta et al., 2017; Zeineldin et al., 2017a; Holman et al., 2017d; Holman et al., 2019; Mcmullen et al., 2019). In addition, transportation, as an important stressor, is associated with the bovine respiratory microbiota and BRD (Holman et al., 2017d; Mcmullen et al., 2018; Cui et al., 2021). There have been no studies, however, investigating whether consistent microbial changes by stressors (such as transportation) were found in calves from different geographic locations.

Three studies performed metagenomics to determine how transportation or BRD affects the nasopharyngeal microbiome of calves from Canada (Malmuthuge et al., 2021) and China (Cui et al., 2021) and the lung microbiota of calves from Canada (Klima et al., 2019). Their studies provide experimental data that can be analyzed to determine the effects of geography and transportation stress on bovine microbial structure and functional potential. This paper presents the results of our re-analysis of the shotgun metagenomic dataset from these three studies to determine the geographic and transportation effects on the bovine respiratory microbiome.

Materials and methods

Data collection

Our study was based on three public metagenomic datasets published by Malmuthuge et al. (2021); Cui et al. (2021), and Klima et al. (2019). Malmuthuge *et al*. investigated the effects of weaning and transportation on the temporal dynamics of the nasopharyngeal microbiota in Hereford-crossed calves in Canada, Cui and colleagues measured the longitudinal changes of the nasopharyngeal microbiome before and after long-distance transportation in Simmental calves in China, and Klima *et al*. collected and characterized the lung microbiota from feedlot calves that died from BRD (Supplementary Table S1). A total of 145 bovine respiratory samples collected from the feedlot calves 5 to 6 months of age were included in this study. In brief, the samples were from three geographic locations [Saskatoon in Canada, two cities (Qiqihaer and Guangan) in China, and Alberta in Canada] and two niches (nasopharynx

and lung). Moreover, transportation effects were estimated in the studies of Malmuthuge *et al.* and Cui *et al.* Details of the study design and sample collections were described in the original study. Metagenomic sequencing was performed using the Illumina HiSeq platform. Sequences were downloaded from the NCBI SRA database under accession codes PRJNA687519, PRJNA724913, and PRJNA395911.

Metagenomic sequence processing

KneadData (v 0.7.2) was used to remove host contamination and filter reads for the downloaded metagenomic data. Low-quality reads with a Phred score smaller than 15 within a 5-bp sliding window on reads were trimmed using Trimmomatic (v0.39). Clean reads of each sample were acquired, and then all reads were aligned to the *Bos taurus* reference genome (UMD v3.0) using bmtagger (v.3.102.4) for host contamination read removal. Next, clean reads of each sample were then uploaded into the MG-RAST metagenomic analysis server (v4.0) (Keegan et al., 2016) and against the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology database for function analysis and the RefSeq database for microbial taxa classification based on the recommended manual. After obtaining the count table of microbial composition and functional prediction from the MG-RAST server, R software was used to do downstream analyses.

Statistics and bioinformatics

Alpha diversity, including the Shannon index and richness, was calculated to evaluate the corresponding diversities by R (v4.1.2). A two-tailed Wilcoxon signed-rank test was used to test the significance of alpha diversity. Principal coordinate analysis based on Bray–Curtis distance was performed to visualize beta diversity, and dissimilarity among groups was determined using the "adonis2" function on the R "vegan" package. Signature microbiota for geographic locations were identified using the linear discriminant analysis (LDA) effect size (LEfSe) with default settings (*e.g.*, LDA score >2 as a criterion for judging the significant effect size), and the abundances of microbial signatures were visualized using a heat map. The Sankey plot was drawn in R to show the taxonomy of bovine respiratory microbiome in three geographic locations. Other boxplots were made using the "ggplot2" package in R.

To determine the correlation of the bovine respiratory microbiome in the network interface, correlation matrixes by calculating pairwise Spearman's rank correlations among the top 200 bacteria were constructed. A correlation between two bacteria was considered statistically robust if Spearman's correlation coefficient (R) was greater than 0.5, and the P-value was less than 0.01. To avoid false positives, all P-values were adjusted with a multiple testing correction using the Benjamini–Hochberg method. The pairwise correlations of the bacteria formed their co-occurrence networks. Network analyses were performed using "VEGAN", "igraph", and "Hmisc" packages in R, and the interactive platform of Gephi was employed for network visualization. The size of each node is proportional to the number of connections.

Hierarchical clustering based on the relative abundance of the bronchoalveolar lavage (BAL) microbiome was performed using the Bray–Curtis distance and the "hclust" function in R. Taxa of the top 30 lung microbiota were included in hierarchical clustering. All BAL samples were subjected to unsupervised hierarchical clustering.

Results

Sample characteristics and sequencing analysis

Metagenomic samples (n = 145) during the feedlot period were obtained from three worldwide studies with publicly available datasets (Klima et al., 2019; Cui et al., 2021; Malmuthuge et al., 2021). The characteristics of the samples are summarized in Supplementary Table S1. Three geographic locations, including Saskatoon in Canada, two cities (Qiqihaer and Guangan) in China, and Alberta in Canada, were selected. The respiratory microbial samples were collected from the nasopharynx and lung using nasopharyngeal swabs (NPS; n = 130) and bronchoalveolar lavage (BAL; n = 15). Notably, the effects of short- and long-distance transportation, respectively, were included in the NPS, which were analyzed to estimate the shipping stress on the nasopharyngeal microbiota. After quality control, an average of 3,374,490 clean reads in the respiratory metagenomics were used for downstream analysis, including microbial classification using the RefSeq database and functional prediction by the KEGG Orthology database.

Bovine respiratory microbiome influenced by geographic locations and sampling niches

To analyze the effects of geographic location and sampling niches on the bovine respiratory microbiome, microbial diversities were estimated at the community level. The Shannon index of NPS of Qiqihaer and Guangan was higher compared with the NPS of Saskatoon and the BAL of Alberta ($P < 0.05$) (Figure 1A). Interestingly, the BAL of Alberta had greater alpha diversity than the NPS of Saskatoon ($P < 0.05$). Consistently, the NPS of Qiqihaer and Guangan showed distinct clustering compared with the NPS of Saskatoon (analysis of similarity, ANOSIM: R = 0.99, R = 1.0, p = 0.001) and the BAL of Alberta (ANOSIM: R = 0.78, R = 0.49, p = 0.001) based on the Bray–Curtis distance (Figure 1B). At the same

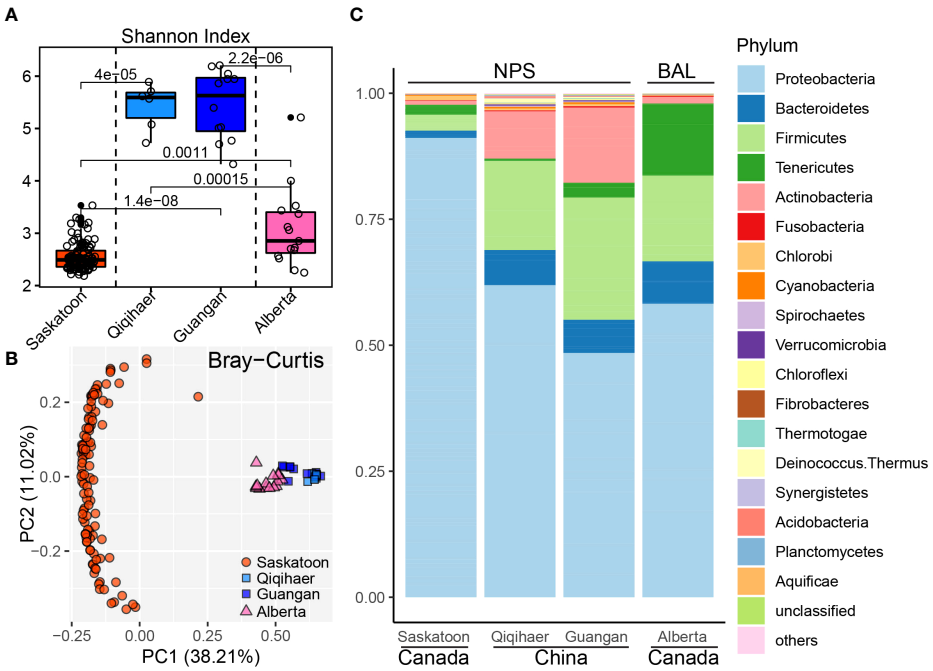

FIGURE 1
Bovine respiratory microbiome influenced by geographic locations and sampling niches. **(A)** Shannon index of bovine respiratory microbiota among NPS of Saskatoon (Canada), NPS of Qiqihaer and Guangan (China), and BAL of Alberta (Canada). Alpha diversity was tested using the Kruskal–Wallis test. The line inside the box denotes the median, and the boxes denote the interquartile range between the first and third quartiles (25th and 75th percentiles, respectively). **(B)** Principal coordinate analysis of the Bray–Curtis distances between microbiota. **(C)** Bacterial abundances at the phylum level. NPS, nasopharyngeal swab; BAL, bronchoalveolar lavage.

time, significant differences between Saskatoon and Alberta (ANOSIM: $R = 0.99$, $p = 0.001$) were also observed.

Using the Refseq database, 96.72% high-quality reads were classified as the bacterial kingdom, indicating the bacteria as the main microbiome in the bovine respiratory tracts (Supplementary Figure S1). At the phylum level, a total of 27 bacterial phyla were identified across 145 bovine respiratory samples. The predominant phylum in the NPS of Saskatoon was Proteobacteria (91.19%). In the NPS niche of Qiqihaer and Guangan (China), the most dominant phyla were Proteobacteria (48.49 and 61.94%), Firmicutes (24.26 and 17.68%), Actinobacteria (14.90 and 9.40%), and Bacteroidetes (6.59 and 6.98%) (Figure 1C). The BAL of Alberta was dominated by Proteobacteria (58.27%), Firmicutes (17.03%), Tenericutes (14.27%), and Bacteroidetes (8.41%). At the genus level, *Burkholderia* (81.71%) was the dominant genus in the NPS of Saskatoon (Supplementary Figure S2). The top genera in the NPS of Qiqihaer and Guangan were *Psychrobacter* (8.07 and 11.63%), *Moraxella* (4.94 and 7.13%), and *Corynebacterium* (4.63 and 2.08%). In the BAL microbiome, the top genera included *Mannheimia* (16.55%), *Mycoplasma* (14.11%), *Actinobacillus* (10.51%), *Clostridium* (5.73%), *Psychrobacter* (5.66%), and *Haemophilus* (5.60%).

The signature microbiota associated with geographic locations and sampling niches

LEfSe was performed to identify the microbial species differentiating both cities and sampling niches. *Burkholderia* species, including *Burkholderia cenocepacia*, *Burkholderia* sp. *383*, *Burkholderia ambifaria*, *Burkholderia multivorans*, *Burkholderia vietnamiensis*, *Burkholderia xenovorans*, *Burkholderia dolosa*, *Burkholderia ubonensis*, and *Burkholderia phymatum*, were enriched in the NPS of Saskatoon (Figure 2), while the NPS of Qiqihaer and Guangan had greater abundances of bacteria such as *Moraxella catarrhalis*, *Psychrobacter* sp. *PRwf-1*, *Enhydrobacter aerosaccus*, *Psychrobacter arcticus*, *Corynebacterium efficiens*, *Corynebacterium glutamicum*, and *Mycoplasma conjunctivae*. In the BAL of Alberta (Canada), the abundant bacteria identified by LefSe were *Mannheimia haemolytica*, *Mannheimia succiniciproducens*, *Mycoplasma* species (*Mycoplasma agalactiae*, *Mycoplasma arthritidis*, *Mycoplasma bovis*, *Mycoplasma hominis*, and *Mycoplasma mycoides*), *Histophilus somni*, *Haemophilus* species (*Haemophilus ducreyi*, *Haemophilus influenzae*, and *Haemophilus parasuis*), *Actinobacillus* species (*Actinobacillus*

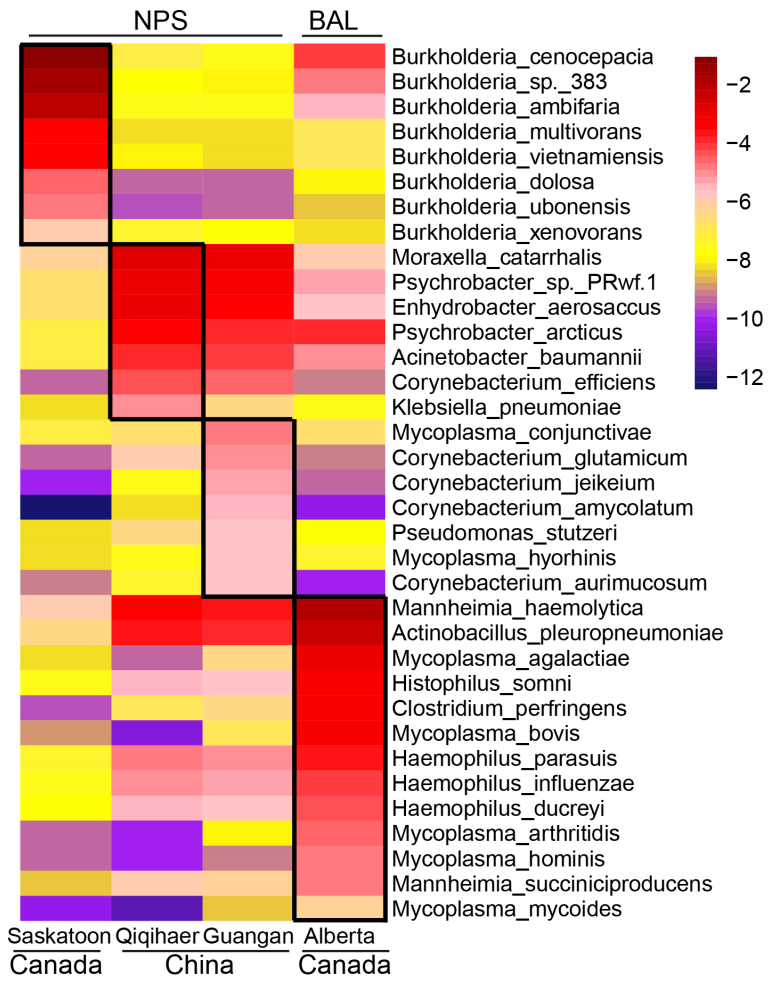

FIGURE 2
Geographic and niche-associated bacterial signatures that were identified by LEfSe analysis. The heat map shows the average relative abundance of ages on a log scale. The log-scaled relative abundance heat map of city-related bacterial species screened by LEfSe (linear discriminant analysis >2) in the bovine respiratory microbiome. The color of cells from purple to red corresponds to the relative abundance of bacteria from low to high. NPS, nasopharyngeal swab; BAL, bronchoalveolar lavage.

pleuropneumoniae and *Actinobacillus succinogenes*), *Clostridium perfringens*, and *Prevotella* species (*Prevotella melaninogenica*, *Prevotella copri*, *Prevotella ruminicola*, *Prevotella buccae*, *Prevotella oris*, and *Prevotella bryantii*).

To better visualize the microbial composition of the bovine respiratory microbiome among geographic locations, a Sankey diagram of bacterial taxonomy was drawn (Figure 3). The most abundant bacteria in the NPS of Saskatoon were *Burkholderia* species, including *B. cenocepacia*, *B.* sp. *383*, and *B. ambifaria* (*Burkholderiaceae* family, Proteobacteria phylum) (Figure 3A). The top bacteria in the NPS of Qiqihaer and Guangan were species under the *Moraxellaceae* family (*Moraxella catarrhalis*, *Psychrobacter* sp. *PRwf-1*, and *Enhydrobacter aerosaccus*) and the *Pasteurellaceae* family (*Mannheimia haemolytica* and *Actinobacillus pleuropneumoniae)* belonging to the Proteobacteria phylum, followed by the *Streptococcaceae* family (*Streptococcus salivarius*) and the *Eubacteriaceae* family (*Eubacterium hallii*) belonging to the Firmicutes phylum (Figure 3B). In the BAL of Alberta (Canada), species members of the *Pasteurellaceae* family (*e.g.*, *Mannheimia haemolytica*, *Actinobacillus pleuropneumoniae*, and *Histophilus somni*), the *Pseudomonadaceae* family (*e.g.*, *Pseudomonas fluorescens*), and the *Moraxellaceae* family (*e.g.*, *Psychrobacter cryohalolentis*) belonging to the Proteobacteria phylum, the *Clostridiaceae* family (*e.g.*, *Clostridium perfringens*) belonging to the Firmicutes phylum, the *Bacteroidaceae* family (*e.g.*, *Bacteroides* sp. *1_1_6* and *Bacteroides thetaiotaomicron*) belonging to the Bacteroidetes phylum, and the *Mycoplasmataceae* family (*e.g.*, *Mycoplasma bovis* and *Mycoplasma agalactiae*) belonging to the Tenericutes phylum formed the major bacterial composition (Figure 3C).

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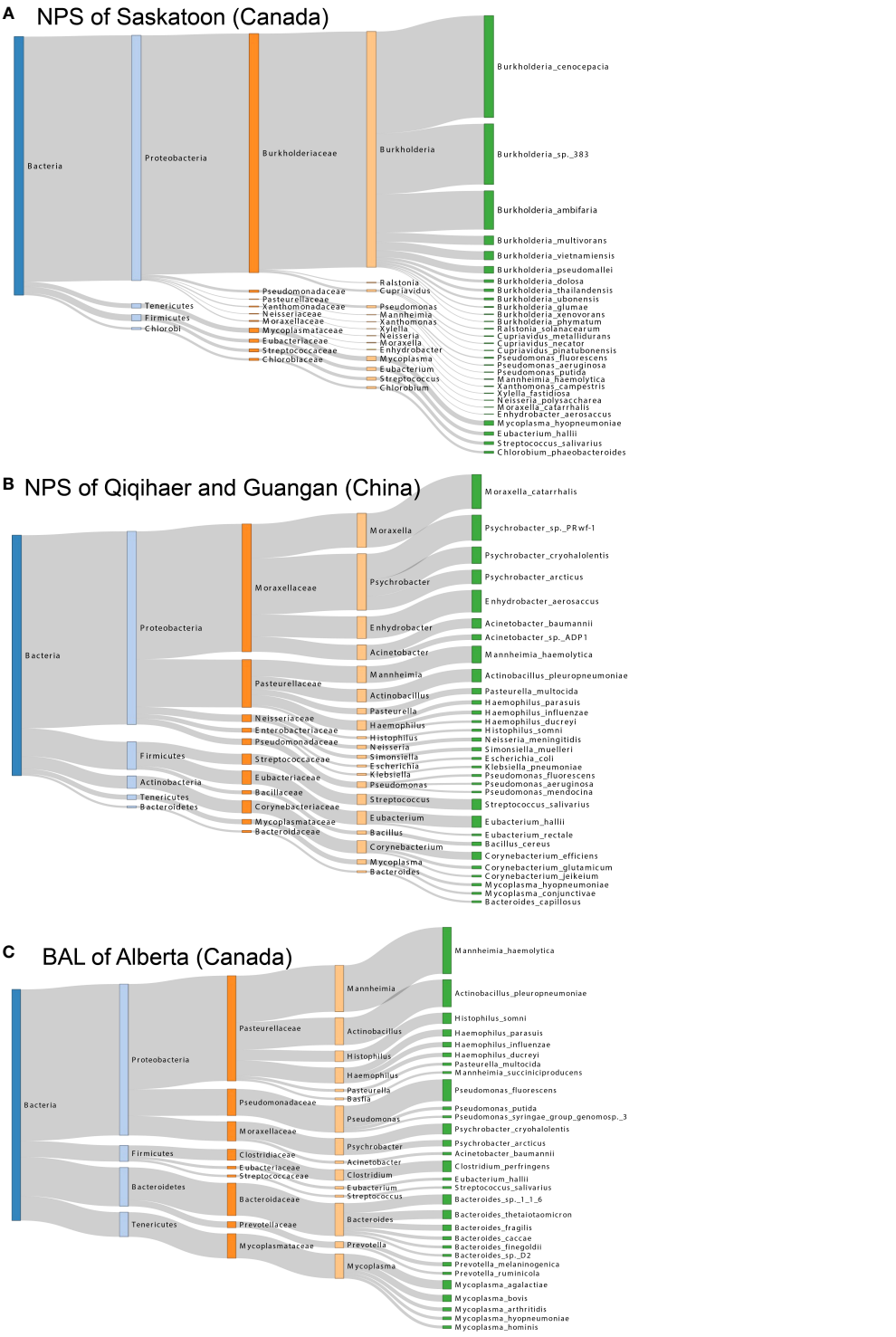

FIGURE 3
Sankey diagram for the major taxonomy structure of bovine respiratory microbiome. **(A**, **B**, and **C)** Sankey diagrams of bovine airway microbiota among NPS of Saskatoon (Canada), NPS of Qiqihaer and Guangan (China), and BAL of Alberta (Canada), which depict the bacterial flow of taxonomy from the kingdom to species level. NPS, nasopharyngeal swab; BAL, bronchoalveolar lavage.

Co-occurrence analysis of bacterial interconnections within geographic locations and niches

Based on the modularity class, the entire network in the NPS of Saskatoon can be parsed into six major modules (Figure 4A). *Burkholderia* species, including *B. cenocepacia*, *B.* sp. *383*, *B. ambifaria*, *B. multivorans* and *B. pseudomallei*, formed a module, while *Mycoplasma* species (*M. agalactiae*, *Mycoplasma synoviae*, *Mycoplasma alligatoris*, *Mycoplasma crocodyli*, *Mycoplasma hyopneumoniae*, and *Mycoplasma hyorhinis*) co-occurred in another module. Regarding the NPS of Qiqihaer and Guangan (China), six modules with more complex bacterial co-occurrences were found. Interestingly, *Mycoplasma* species (*M. conjunctivae*, *M. hyopneumoniae*, *M. hyorhinis*, and *M. agalactiae*) also co-occurred (Figure 4B). In the BAL of Alberta community, *Mycoplasma* species (*M. agalactiae*, *M. bovis*, *M. arthritidis*, *M. hyopneumoniae*, and *M. hominis*), which were inter-connected with each other, were also observed (Figure 4C). Moreover, *Mannheimia haemolytica*, *Histophilus somni*, *Pasteurella multocida*, and *Actinobacillus pleuropneumoniae* were correlated with *Haemophilus* species (*H. ducreyi*, *H. influenzae*, and *H. parasuis*).

Geographic locations and sampling niches affect the functions of the bovine respiratory microbiome

Consistent with the bovine microbial structure, the KEGG functional configuration showed significant differences among cities and niches (Figures 5A, B). The Shannon index in the NPS of Qiqihaer and Guangan was higher compared with the NPS of Saskatoon and the BAL of Alberta ($P < 0.05$) (Figure 5A) and had distinct clustering compared with the NPS of Saskatoon (ANOSIM: $R = 0.99$, $R = 0.98$, $p = 0.001$) and the BAL of Alberta (ANOSIM: $R = 0.30$, $R = 0.66$, $p = 0.001$) based on the Bray–Curtis distance (Figure 5B).

The NPS of Saskatoon (Canada), the NPS of Qiqihaer and Guangan, and the BAL of Alberta had relative abundances of "cellular processes" (12.00, 8.92, 8.30, and 5.02%), "environmental information processing" (21.24, 14.99, 14.15, and 17.11%), and "genetic information processing" (11.79, 22.49, 22.16, and 23.28%) at level 1 (Supplementary Figure S3). At level 2, the NPS of Saskatoon had higher relative abundance of "membrane transport" (11.92%), "signal transduction" (6.60%), "transcription" (2.41%), "cell growth and death" (6.45%), "cell motility" (2.62%), "amino acid metabolism" (18.57%), "carbohydrate metabolism" (16.53%), "metabolism of cofactors and vitamins" (4.98%), "nucleotide metabolism" (3.06%), and "energy metabolism" (3.22%) (Figure 5C). The NPS of Qiqihaer and Guangan was greater in "folding, sorting, and degradation" (3.98%) and "signal transduction" (5.95%). The BAL of Alberta had higher relative abundances of "translation" (22.34%), "carbohydrate metabolism" (13.53%), "membrane transport" (10.34%), "replication and repair" (6.84%), and "cell communication" (5.51%).

Long-distance transportation changes bovine nasopharyngeal microbiota

Three groups including non-transportation (Control) and short-distance (Short) and long-distance transportation (Long) were selected. Three days was chosen as the long-distance transportation from a study of Cui et al. (2021), and metagenomic data in the Long group at the time before loading to truck (Bfload), unloading (Unload), and 7 days after placement and adaptive feeding (Adfeed) were screened. Correspondingly, we selected data at the same time points from two other groups in the study of Malmuthuge et al. (2021). Interestingly, richness in the Long group at Unload and Adfeed time points significantly decreased ($P < 0.05$) compared with that at Bfload, while there were no temporal changes of alpha diversity in the Control and Short groups. Beta diversity based on the Bray–Curtis distance had consistent results (Figures 6A, B).

Next, LefSe was used to identify the longitudinal changes of microbiota following transportation (Supplementary Figure S4). Due to the variation from different studies and across multiple cities, the signature microbiota after transportation among the three groups were different. Then, we focused on the bacteria among studies to test if they have patterns across transportation distance and time. Although the microbial data were from different studies, shared bacteria, such as *Pseudomonas aeruginosa*, were not influenced by either short or long transportation, while *Mycoplasma agalactiae* was influenced by both short- and long-distance transportations such as higher abundances at Adfeed time in the Short and Long groups (Figures 6C, D). Specifically, some bacteria associated with BRD pathogens were influenced by long-distance transportation, such as the greater abundances of *Mycoplasma bovis*, *Mycoplasma conjunctivae*, *Mycoplasma pulmonis*, and *Mycoplasma hyorhinis* at the ADfeed of the Long group compared with at the BFload and Unload time points (Figures 6E–H).

Colonization of the opportunistic pathogens at the nasopharynx changes with time following weaning and transportation

Measurement of the temporal dynamics of the bovine respiratory microbiome affected by stressors including weaning

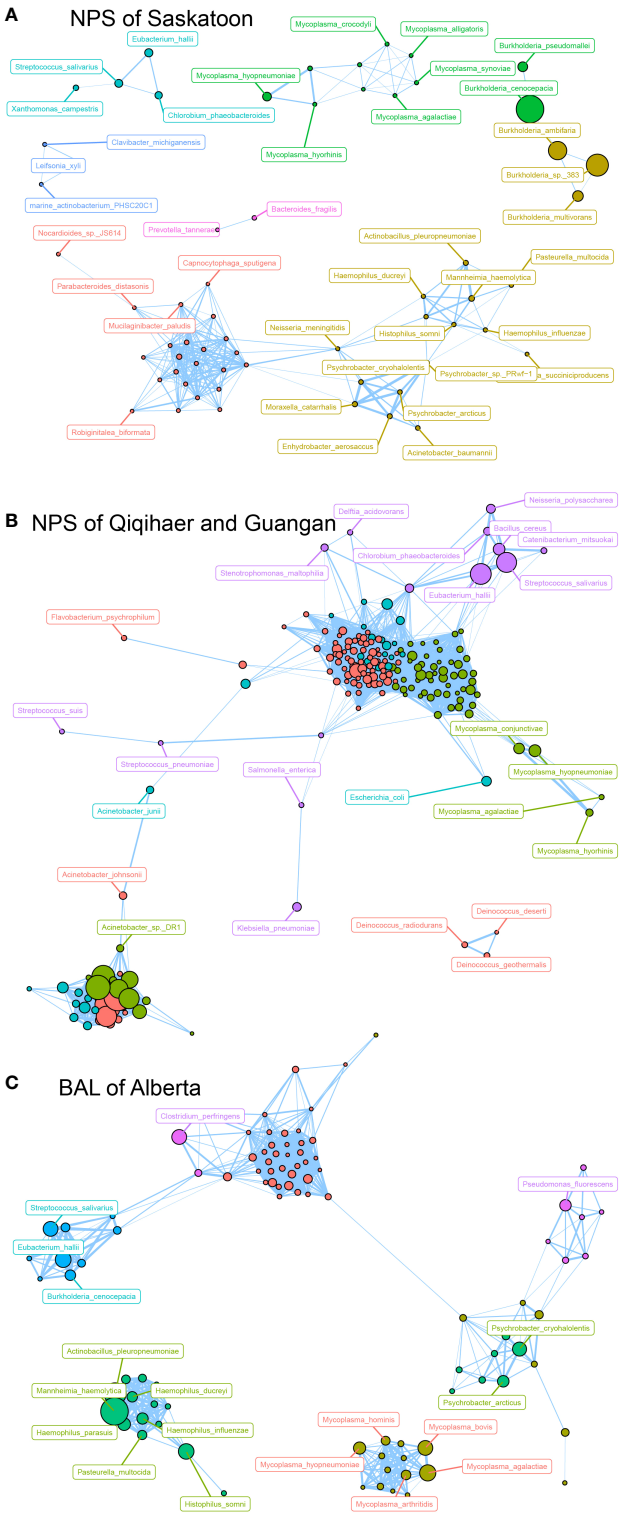

FIGURE 4
Network analysis revealing the co-occurrence patterns among airway microbial species. The nodes were colored according to modularity class. **(A–C)** Network analysis of bovine airway microbial species in samples of NPS of Saskatoon (Canada), NPS of Qiqihaer and Guangan (China), and BAL of Alberta (Canada). A connection represents a strong (Spearman's correlation coefficient $R > 0.8$) and significant ($P < 0.01$) correlation. The size of each node is proportional to the number of connections, that is, the degree. NPS, nasopharyngeal swab; BAL, bronchoalveolar lavage.

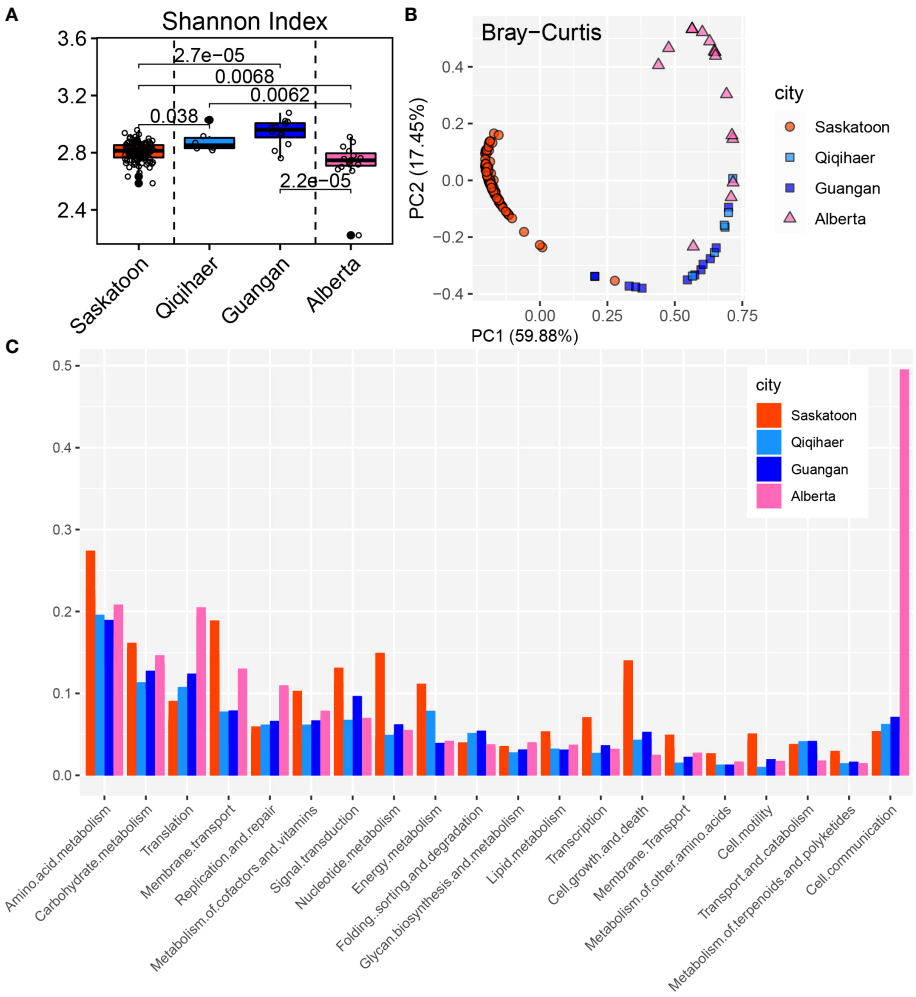

FIGURE 5
Functional analysis of bovine respiratory microbiome. **(A)** Shannon index of Kyoto Encyclopedia of Genes and Genomes (KEGG) function. **(B)** Principal coordinate analysis of the Bray–Curtis distance of the KEGG pathways. NPS, nasopharyngeal swab; BAL, bronchoalveolar lavage. **(C)** KEGG annotation at level 2.

and short-distance transportation allows us to better understand the changes of the bovine respiratory microbiome at the feedlot, leading to a better understanding of BRD pathogenesis (Chai et al., 2022). Metagenomics of the bovine respiratory microbiome in treatment groups on days 0 (prior to weaning and short-distance transportation, WT), 2, 4, 8, 14, and 28 was used to compare with the control group that stayed with their dams (suckling). Slight differences between suckling and WT were found based on alpha and beta diversities (Supplementary Figure S5), while the temporal dynamics of the bovine respiratory microbiome changed significantly as more statistically significant differences were found among time points (Supplementary Figure S6).

The major bacterial genera associated with BRD were influenced by time and WT (Figure 7). *Moraxella* was more abundant in the suckling group on day 14 (Figure 7A). Another genus, *Pseudomonas*, was not influenced by WT since no differences were observed between the two groups during the trial (Figure 7B). *Mannheimia* was greater in WT at day 14, and *Mycoplasma* had higher medians than suckling, although there were no statistically significant differences (Figures 7C, D).

Individual variances and clusters of the bovine lung microbiome

In the BAL microbial samples collected from BRD calves, through phylum and genus bar plots (Supplementary Figure S7), we did observe a distinct individual variation of the bovine lung microbiome. Regarding similar microbial composition found in

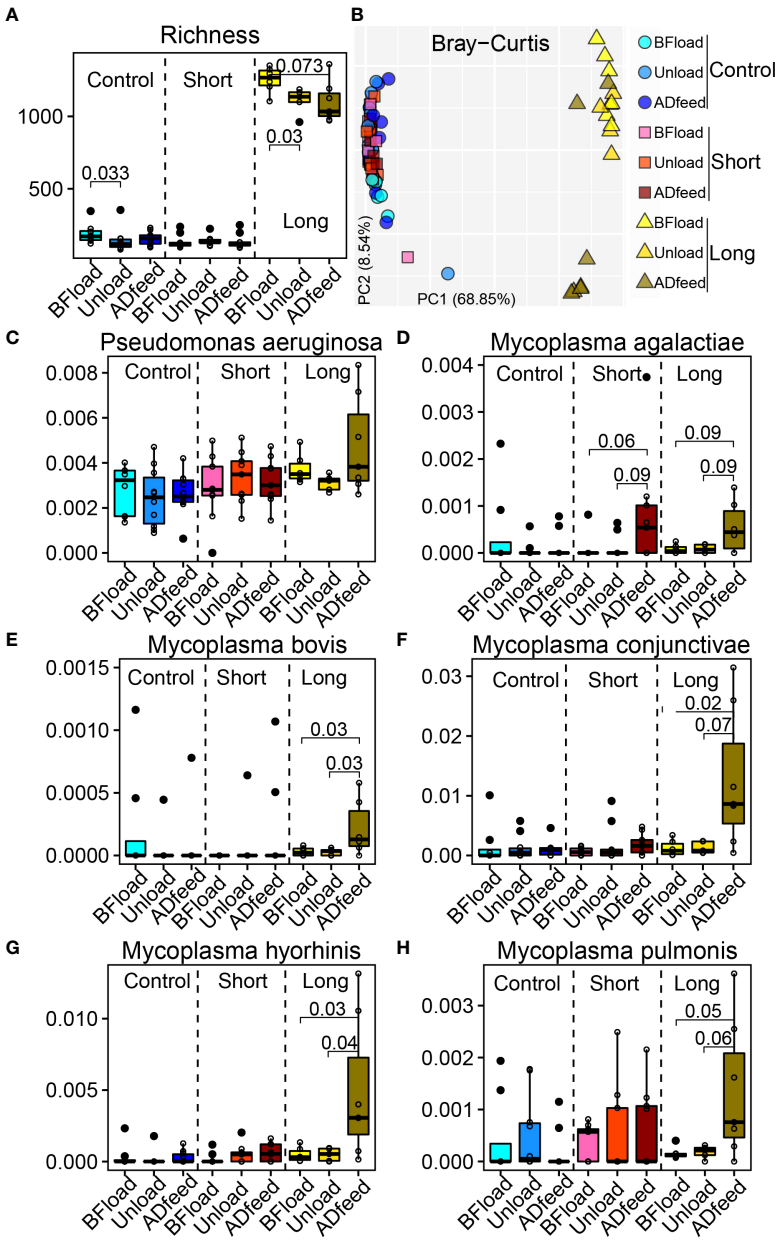

FIGURE 6
Long-distance transportation changes the bovine nasopharyngeal microbiota. **(A)** Richness. **(B)** Principal coordinate analysis plot based on the Bray–Curtis distance. **(C–H)** Main species associated with transportation. Control, non-transportation; Short, short-distance transportation; Long, long-distance transportation; BFload, time before loading to truck; Unload, unloading; ADfeed, 7 days after placement and adaptive feeding.

part of the subjects, clustering analysis was performed at the species level (Figure 8A). Four clusters were observed among 15 BAL samples. Cluster 1, including animal IDs Yellow6373, 713_157, and W7839, mainly consisted of *Mycoplasma bovis*, *Histophilus somni*, and *Mycoplasma agalactiae* (Figure 8B). Cluster 2 (animal IDs B2_406LL, Peach2295, Org79, F1_109, 302_62, and 405_202) was abundant with *Mannheimia haemolytica* and *Actinobacillus pleuropneumoniae*. Cluster 3 (animal IDs 8577, 302_45, and 511_157WL) was dominated by *Mannheimia haemolytica* and *Psychrobacter cryohalolentis*. However, cluster 4 (animal IDs B6_216, G2516, and G3758) did not have a good pattern. The B6_216 had greater abundance of *Eubacterium hallii* and *Streptococcus salivarius*, while G3758 was dominated *by Clostridium perfringens*.

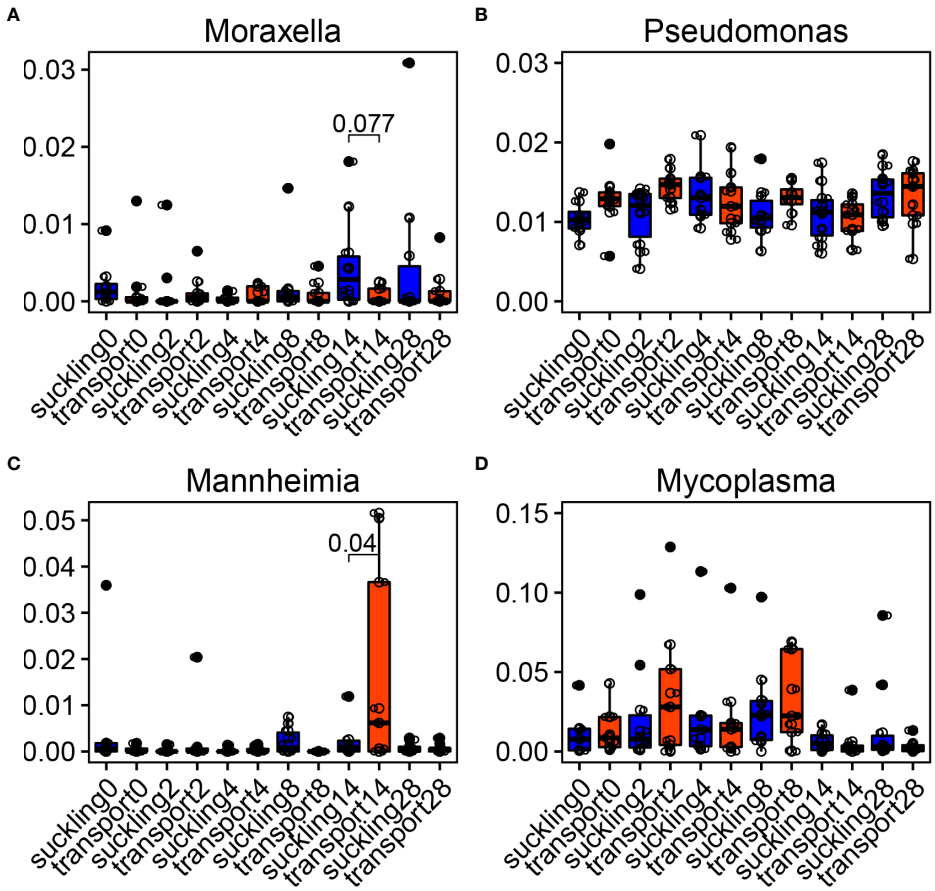

FIGURE 7
Temporal dynamics of bovine respiratory microbiota after weaning + transportation (WT). **(A–D)** Main bacterial genera changed after WT compared with the control group that stayed with their dams (suckling). Significances were labeled between suckling and WT at the time when they were statistically different.

Discussion

The investigation of bovine respiratory microbiome and its function benefits us in order to understand microbial functions in health and disease. Although 16S rRNA sequencing technology has been broadly used, less metagenomics of bovine respiratory microbiome is reported. This study characterized microbial communities using metagenomics in the nasopharynx and lungs of calves from worldwide geographic locations, which provides evidence that geography and niches are important factors to impact the bovine respiratory microbiome and its function. Beyond this, long-distance transportation had greater impacts on the nasopharyngeal microbiota, especially BRD pathogens, than short-distance transportation. Consistent changes in microbiota influenced by transportation were found across samples from different countries. Metagenomics results from both healthy and diseased calves suggested the associations between bovine respiratory microbiome and BRD.

Calves in different geographic locations experienced huge variations in living environment, such as breed, gender, feeding strategy, diet, altitude, climate, *etc*. These factors together result in the variation of the microbial composition in the nasopharynx across different cities or countries. In this study, although the calves were of similar age and maintained healthy throughout the trial, calves in Canada were Hereford-crossed females that had access to water and brome-alfalfa hay, while calves in China were Simmental males that were restricted from eating and drinking. It is not surprising that the diversity, structure, and composition of the nasopharyngeal microbiota in calves from different geographic locations showed distinct variations. A previous report found that weaned calves that consumed selenium-biofortified alfalfa hay for 9 weeks resulted in favorably reformed microbial communities in the nostrils

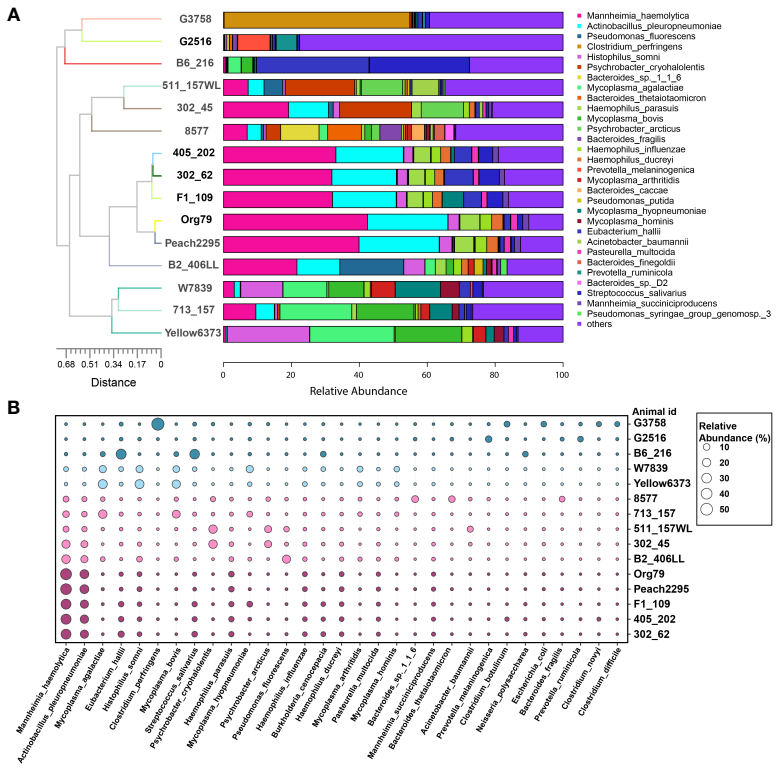

FIGURE 8
Individual variations and clusters of bovine lung microbiome. **(A)** Top 30 bacterial species in bronchoalveolar lavage were used for clustering analysis. **(B)** The major bacterial species distribution in the lungs. Circle size proportionally represents the relative abundance of bacterial species at a certain animal.

(Hall et al., 2017). Differences of nasopharyngeal microbiota among Charolais and Angus–Hereford calves were also reported (Zeineldin et al., 2017a; Holman et al., 2017d; Zeineldin et al., 2017c). Thus, geography and breed effects resulting in differences of bovine respiratory microbiota increase the difficulties to understand BRD pathogenesis when using calves from different countries. In addition, signature microbiota in the nasopharynx for each geographic location were identified. *Burkholderia cenocepacia* (*Burkholderiaceae* family, Proteobacteria phylum) was the dominant species in the NPS of Saskatoon (Canada), while the NPS of Qiqihaer and Guangan had greater abundances of *Moraxellaceae* family (Proteobacteria phylum). *B. cenocepacia* is an important opportunistic respiratory pathogen of cystic fibrosis patients (Berlutti et al., 2008). *Moraxellaceae* is often found to be one of the most abundant genera in the upper respiratory tract of cattle (Mcmullen et al., 2019; Mcmullen et al., 2020), and one previous study found an association between *Moraxella* and the development of pneumonia and/or otitis in the early life of dairy calves (Lima et al., 2016). Moreover, co-occurrence between the species-associated BRD pathogens was observed in all nasopharyngeal communities. Therefore, although the calves for nasopharyngeal sampling that were from different geographic locations were clinically healthy during the trial, the nasopharynx could be a reservoir of the opportunistic pathogens for BRD. The effect of geographic locations was also a key factor to affect the functions of the bovine respiratory microbiome. As we have observed, the structure and the abundance of the function were different across geographic locations. Regarding the main functions at KEGG level 2, “membrane transport”, “signal transduction”, “transcription”, “cell growth and death”, and “cell motility” were found in the nasopharynx of Saskatoon, indicating the active, healthy, and balanced respiratory ecosystem. The calves in Saskatoon were kept healthy during the trial, which implied that the microbial community had more interactions with the host. It is not surprising that more cell growth and death and nutrient metabolism were found in the calves of Saskatoon when compared with the calves in China that experienced long-distance transportation. Therefore, homeostasis of the respiratory microbiome plays critical roles in bovine airway health, and disease or environmental factors can lead to disequilibrium in the respiratory ecosystem.

The lung microbiome is a critical point for BRD (pneumonia). In this study, the lung microbiome and its

functions showed a significantly different composition and structure compared with nasopharyngeal communities regardless of their geographic locations. The BAL samples were collected at necropsy from 15 feedlot cattle that were confirmed to have died of BRD. Higher abundances of *Mannheimia haemolytica*, *Mycoplasma bovis*, and *Histophilus somni* were observed, which is in agreement with previous studies that these bacteria are BRD-associated pathogens (Rice et al., 2007; Singh et al., 2011; Nicola et al., 2017). Notably, *Mycoplasma* species (*M. agalactiae*, *M. bovis*, *M. arthritidis*, *M. hyopneumoniae*, and *M. hominis*) inter-connected with each other were observed in BRD lungs, which reflects the cooperation of pathogens to cause BRD. Based on the hypothesis that the nasopharynx is a reservoir of the opportunistic pathogens for BRD, we assumed that *Mycoplasma* species may come from the nasopharynx. A study reported that the best recovery condition for *Mycoplasma bovis* is at 36.91°C (± 0.07) and pH = 7.13 (± 0.05) under an aerobic environment (Parker et al., 2016). Bovine nasopharynx is the niche with pH of about 7 and temperature of about 37°C, which is good for the colonization and growth of *Mycoplasma* species (Chai et al., 2022). Therefore, the opportunistic pathogens for BRD may colonize the nasopharynx and disperse in and infect the lungs, which provides a new way to explore BRD pathogenesis and prevent BRD. In addition, the interactions of *Mycoplasma* species might be another reason causing BRD, which need to be investigated in future research.

BRD, also known as "shipping fever", is caused by various stressors (*i.e.*, weaning, transportation) that also influence the bovine respiratory microbiota (Chai et al., 2022). Understanding the association between transportation and microbial pathogen proliferation may explain BRD pathogenesis. A previous study demonstrated that transportation to a feedlot caused an abrupt shift in the nasopharyngeal microbiota of cattle using 16S rRNA sequencing, and major differences were driven mostly by *Mycoplasma* (Holman et al., 2017d). A metagenomic study confirmed that stress from long-distance shipping influences the nasopharyngeal microbiota (Cui et al., 2021). Similarly, in our study, long-distance transportation had bigger impacts on the bovine respiratory microbiota, and *Mycoplasma* species, including *M. bovis*, *M. conjunctivae*, *M. pulmonis*, and *M. hyorhinis*, increased the abundances after feedlot arrival. Thus, stressors (*i.e.*, long-distance transportation) and the feedlot environment may provide conditions that allow for the proliferation of *Mycoplasma* in the nasopharynx. In addition, abundance of *Mycoplasma agalactiae* by both short- and long-distance transportation was observed, which gives more evidence that transportation can cause changes in *Mycoplasma* species (Stroebel et al., 2018). It was reported that stress can exacerbate respiratory hyperreactivity and inflammation in an animal model of allergic bronchial asthma (Joachim et al., 2003). Newly weaned calves experience huge stress during transportation or shipping, especially for a long distance (Zulkifli et al., 2019; Malmuthuge et al., 2021), which may cause unclear changes in the airway for *Mycoplasma* proliferation. Keeping microbial balances in bovine airway during transportation might be an alternative strategy to decrease BRD morbidity.

BRD is usually diagnosed in cattle within 4 weeks after having been transported to a feedlot (Currin and Whittier, 2005). Understanding of the temporal dynamics of the bovine respiratory microbiome from the time since arrival to the feedlot can allow us to recognize the association between microbiota and BRD. Here we found that genera associated with BRD, such as *Mycoplasma* and *Mannheimia*, were increased at a specific time after weaning and transportation. Since all of the calves were healthy and had never been diagnosed with BRD in this study, these increased pathogens might be due to the time after feedlot arrival or changes of the respiratory health status. Moreover, *Moraxella* was higher in calves that stayed with their dams. *Moraxella* is often found to be one of the most abundant genera in the upper respiratory tract of healthy cattle (Zeineldin et al., 2017a; Mcmullen et al., 2019). Therefore, *Mycoplasma* and *Mannheimia* may reach a specific level to cause clinical BRD signs, and a greater abundance of *Moraxella* in the nasopharynx might resist BRD.

The inter-individual variability of the respiratory microbiota was found in cattle and humans (Dickson et al., 2015; Holman et al., 2015b; Zeineldin et al., 2017a), which is not surprising due to individual variation. The bacterial clusters and their association with clinical signs were investigated in human respiratory microbiome studies (Zhou et al., 2019; Widder et al., 2022). However, in cattle, there were no studies to characterize the community type of the respiratory microbiota. In this study, microbial clusters of BRD lungs were found, although there was significant inter-individual variability—for instance, bacterial pathogens, including *Mycoplasma bovis*, *Histophilus somni*, and *Mycoplasma agalactiae*, were enriched in cluster 1, while *Mannheimia haemolytica* plus different pathogens formed a different cluster in the BRD lung microbiome. Previous studies found that *Mycoplasma bovis* co-infection with other respiratory bacteria (*M. haemolytica* and *H. somni*) can lead to severe pneumonic lesions, and *Mannheimia haemolytica* might be associated with *Actinobacillus pleuropneumoniae* in pigs (Gagea et al., 2006; Radaelli et al., 2008). Although the cooperation of different pathogens causing BRD is still unclear, this study provides insights that pathogenic symbiosis is one of the important causes of respiratory diseases. In future BRD studies, large-scale sampling is needed for characterizing the community type of the lung microbiome, which may reveal the interactions of bacterial pathogens and provide an alternative therapeutic strategy for BRD.

A limitation of this study may be the small sample size of lung samples. However, the dominant bacterial pathogens in each cluster could explain the subtypes of BRD pathogenesis since these samples were collected from the lungs of calves that died from BRD. The strengths of this study are the analysis of bovine nasopharyngeal microbiome from different geographic locations using metagenomics and the finding that the general pathogens were influenced by transportation regardless of geography. Future studies to assess more geographic locations of bovine respiratory microbiome and use omics to explore the interactions of the airway microbiome and the host are urgently needed.

Conclusions

Metagenomic sequencing offers the chance to illustrate the species level of community structure and functional profile of the bovine respiratory microbiome. The geographic locations affect the composition and the functions of the bovine respiratory microbiome, and the microbiota from different sampling niches were also distinct. Additionally, transportation is an important factor driving the bovine respiratory microbiome and diseases, and long-distance transportation had a bigger chance to cause increased abundances of pathogens in the nasopharynx. Furthermore, we found that the cluster of lung microbiota from different BRD calves might provide an opportunity to come up with a new guidebook for the category of BRD pathogenesis.

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 in the article/Supplementary Material.

Ethics statement

All data related to animals were downloaded from the published NCBI database.

Author contributions

JC contributed to data collection, analysis and interpretation, and figure organization and drafted the manuscript. XL and HU contributed to draft proofing. FD contributed to data collection. JZ and YL contributed to conception, manuscript proofing, and project supervision. All authors contributed to the article and approved the submitted version.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.961644/full#supplementary-material

References

Berlutti, F., Superti, F., Nicoletti, M., Morea, C., Frioni, A., Ammendolia, M. G., et al. (2008). Bovine lactoferrin inhibits the efficiency of invasion of respiratory A549 cells of different iron-regulated morphological forms of pseudomonas aeruginosa and burkholderia cenocepacia. *Int. J. Immunopathol. Pharmacol.* 21, 51–59. doi: 10.1177/039463200802100107

Chai, J., Capik, S. F., Kegley, B., Richeson, J. T., Powell, J. G., and Zhao, J. (2022). Bovine respiratory microbiota of feedlot cattle and its association with disease. *Vet. Res.* 53, 4. doi: 10.1186/s13567-021-01020-x

Cirone, F., Padalino, B., Tullio, D., Capozza, P., Lo Surdo, M., Lanave, G., et al. (2019). Prevalence of pathogens related to bovine respiratory disease before and

after transportation in beef steers: Preliminary results. *Anim. an Open Access J. MDPI* 9, 1093. doi: 10.3390/ani9121093

Cui, Y., Qi, J., Cai, D., Fang, J., Xie, Y., Guo, H., et al. (2021). Metagenomics reveals that proper placement after long-distance transportation significantly affects calf nasopharyngeal microbiota and is critical for the prevention of respiratory diseases. *Front Microbiol.* 12, 700704. doi: 10.3389/fmicb.2021.700704.

Currin, J. F., and Whittier, W. D. (2005). Recognition and treatment of bovine respiratory disease complex. *Vet Med* 8, 1–3.

Dickson, R. P., Erb-Downward, J. R., Freeman, C. M., Mccloskey, L., Beck, J. M., Huffnagle, G. B., et al. (2015). Spatial variation in the healthy human lung microbiome and the adapted island model of lung biogeography. *Ann. Am. Thorac. Soc.* 12, 821–830. doi: 10.1513/AnnalsATS.201501-029OC

Dickson, R. P., Erb-Downward, J. R., Martinez, F. J., and Huffnagle, G. B. (2016). The microbiome and the respiratory tract. *Annu. Rev. Physiol.* 78, 481–504. doi: 10.1146/annurev-physiol-021115-105238

Gaeta, N. C., Lima, S. F., Teixeira, A. G., Ganda, E. K., Oikonomou, G., Gregory, L., et al. (2017). Deciphering upper respiratory tract microbiota complexity in healthy calves and calves that develop respiratory disease using shotgun metagenomics. *J. Dairy Sci.* 100, 1445–1458. doi: 10.3168/jds.2016-11522

Gagea, M. I., Bateman, K. G., Shanahan, R. A., Van Dreumel, T., Mcewen, B. J., Carman, S., et al. (2006). Naturally occurring mycoplasma bovis-associated pneumonia and polyarthritis in feedlot beef calves. *J. Veterinary Diagn. Invest.* 18, 29–40. doi: 10.1177/104063870601800105

Gupta, V. K., Paul, S., and Dutta, C. (2017). Geography, ethnicity or subsistence-specific variations in human microbiome composition and diversity. *Front. Microbiol.* 8, 1162. doi: 10.3389/fmicb.2017.01162

Hall, J. A., Isaiah, A., Estill, C. T., Pirelli, G. J., and Suchodolski, J. S. (2017). Weaned beef calves fed selenium-biofortified alfalfa hay have an enriched nasal microbiota compared with healthy controls. *PloS One* 12 (6), e0179215. doi: 10.1371/journal.pone.0179215

Holman, D., Hallewell, J., and Alexander, T. W. (2017a). The nasopharyngeal microbiota of beef cattle before and after transport to a feedlot. *J. Anim. Sci.* 95, 29–29. doi: 10.1186/s12866-017-0978-6

Holman, D. B., Klima, C. L., Ralston, B. J., Niu, Y. D., Stanford, K., Alexander, T. W., et al. (2017c). Metagenomic sequencing of bronchoalveolar lavage samples from feedlot cattle mortalities associated with bovine respiratory disease. *Genome Announc* 5 (40), e01045-17. doi: 10.1128/genomeA.01045-17

Holman, D., Mcallister, T. A., Topp, E., Wright, A.-D. G., and Alexander, T. W. (2015a). The nasopharyngeal microbiota of feedlot cattle that develop bovine respiratory disease. *Veterinary Microbiol.* 180, 90–95. doi: 10.1016/j.vetmic.2015.07.031

Holman, D., Timsit, E., and Alexander, T. (2015b). The nasopharyngeal microbiota of feedlot cattle. *Sci. Rep.* 5, 15557. doi: 10.1038/srep15557

Holman, D. B., Timsit, E., Amat, S., Abbott, D. W., Buret, A. G., and Alexander, T. W. (2017d). The nasopharyngeal microbiota of beef cattle before and after transport to a feedlot. *BMC Microbiol.* 17, 70. doi: 10.1186/s12866-017-0978-6

Holman, D. B., Yang, W., and Alexander, T. W. (2019). Antibiotic treatment in feedlot cattle: a longitudinal study of the effect of oxytetracycline and tulathromycin on the fecal and nasopharyngeal microbiota. *Microbiome* 7, 86. doi: 10.1186/s40168-019-0696-4

Human Microbiome Project, C. (2012). Structure, function and diversity of the healthy human microbiome. *Nature* 486, 207–214. doi: 10.1038/nature11234

Joachim, R. A., Quarcoo, D., Arck, P. C., Herz, U., Renz, H., and Klapp, B. F. (2003). Stress enhances airway reactivity and airway inflammation in an animal model of allergic bronchial asthma. *Psychosom Med.* 65, 811–815. doi: 10.1097/01.PSY.0000088582.50468.A3

Keegan, K. P., Glass, E. M., and Meyer, F. (2016). MG-RAST, a metagenomics service for analysis of microbial community structure and function. *Methods Mol. Biol.* 1399, 207–233. doi: 10.1007/978-1-4939-3369-3_13

Klima, C. L., Holman, D. B., Ralston, B. J., Stanford, K., Zaheer, R., Alexander, T. W., et al. (2019). Lower respiratory tract microbiome and resistome of bovine respiratory disease mortalities. *Microb. Ecol.* 78 (2), 446–456. doi: 10.1007/s00248-019-01361-3

Lima, S. F., Teixeira, A. G., Higgins, C. H., Lima, F. S., and Bicalho, R. C. (2016). The upper respiratory tract microbiome and its potential role in bovine respiratory disease and otitis media. *Sci. Rep.* 6, 29050. doi: 10.1038/srep29050

Malmuthuge, N., Howell, A., Arsic, N., Prysliak, T., Perez-Casal, J., and Griebel, P. (2021). Effect of maternal separation and transportation stress on the bovine upper respiratory tract microbiome and the immune response to resident opportunistic pathogens. *Anim. Microbiome* 3, 62. doi: 10.1186/s42523-021-00123-2

Man, W. H., De Steenhuijsen Piters, W. A., and Bogaert, D. (2017). The microbiota of the respiratory tract: gatekeeper to respiratory health. *Nat. Rev. Microbiol.* 15, 259–270. doi: 10.1038/nrmicro.2017.14

Mcmullen, C., Alexander, T. W., Leguillette, R., Workentine, M., and Timsit, E. (2020). Topography of the respiratory tract bacterial microbiota in cattle. *Microbiome* 8, 91. doi: 10.1186/s40168-020-00869-y

Mcmullen, C., Orsel, K., Alexander, T. W., van der Meer, F., Plastow, G., and Timsit, E. (2018). Evolution of the nasopharyngeal bacterial microbiota of beef calves from spring processing to 40 days after feedlot arrival. *Veterinary Microbiol.* 225, 139–148. doi: 10.1016/j.vetmic.2018.09.019

Mcmullen, C., Orsel, K., Alexander, T. W., van der Meer, F., Plastow, G., and Timsit, E. (2019). Comparison of the nasopharyngeal bacterial microbiota of beef calves raised without the use of antimicrobials between healthy calves and those diagnosed with bovine respiratory disease. *Veterinary Microbiol.* 231, 56–62. doi: 10.1016/j.vetmic.2019.02.030

Nicola, I., Cerutti, F., Grego, E., Bertone, I., Gianella, P., D'angelo, A., et al. (2017). Characterization of the upper and lower respiratory tract microbiota in piedmontese calves. *Microbiome* 5 (1), 152. doi: 10.1186/s40168-017-0372-5

Parker, A. M., House, J. K., Hazelton, M. S., Bosward, K. L., Mohler, V. L., Maunsell, F. P., et al. (2016). Milk acidification to control the growth of mycoplasma bovis and salmonella Dublin in contaminated milk. *J. Dairy Sci.* 99, 9875–9884. doi: 10.3168/jds.2016-11537

Radaelli, E., Luini, M., Loria, G. R., Nicholas, R. A., and Scanziani, E. (2008). Bacteriological, serological, pathological and immunohistochemical studies of mycoplasma bovis respiratory infection in veal calves and adult cattle at slaughter. *Res. Vet. Sci.* 85, 282–290. doi: 10.1016/j.rvsc.2007.11.012

Rice, J. A., Carrasco-Medina, L., Hodgins, D. C., and Shewen, P. E. (2007). Mannheimia haemolytica and bovine respiratory disease. *Anim. Health Res. Rev.* 8, 117–128. doi: 10.1017/S1466252307001375

Singh, K., Ritchey, J. W., and Confer, A. W. (2011). Mannheimia haemolytica: bacterial-host interactions in bovine pneumonia. *Vet. Pathol.* 48, 338–348. doi: 10.1177/0300985810377182

Snowder, G. D., Van Vleck, L. D., Cundiff, L. V., and Bennett, G. L. (2006). Bovine respiratory disease in feedlot cattle: environmental, genetic, and economic factors. *J. Anim. Sci.* 84, 1999–2008. doi: 10.2527/jas.2006-046

Stroebel, C., Alexander, T., Workentine, M. L., and Timsit, E. (2018). Effects of transportation to and co-mingling at an auction market on nasopharyngeal and tracheal bacterial communities of recently weaned beef cattle. *Veterinary Microbiol.* 223, 126–133. doi: 10.1016/j.vetmic.2018.08.007

Taylor, J. D., Fulton, R. W., Lehenbauer, T. W., Step, D. L., and Confer, A. W. (2010). The epidemiology of bovine respiratory disease: What is the evidence for predisposing factors? *Can. Vet. J.* 51, 1095–1102.

Timsit, E., Holman, D. B., Hallewell, J., and Alexander, T. W. (2016a). The nasopharyngeal microbiota in feedlot cattle and its role in respiratory health. *Anim. Front.* 6, 44–50. doi: 10.2527/af.2016-0022

Timsit, E., Workentine, M., Schryvers, A. B., Holman, D. B., van der Meer, F., and Alexander, T. W. (2016b). Evolution of the nasopharyngeal microbiota of beef cattle from weaning to 40 days after arrival at a feedlot. *Veterinary Microbiol.* 187, 75–81. doi: 10.1016/j.vetmic.2016.03.020

Venkataraman, A., Bassis, C. M., Beck, J. M., Young, V. B., Curtis, J. L., Huffnagle, G. B., et al. (2015). Application of a neutral community model to assess structuring of the human lung microbiome. *MBio* 6 (1), e02284-14. doi: 10.1128/mBio.02284-14

Widder, S., Zhao, J., Carmody, L. A., Zhang, Q., Kalikin, L. M., Schloss, P. D., et al. (2022). Association of bacterial community types, functional microbial processes and lung disease in cystic fibrosis airways. *ISME J.* 16, 905–914. doi: 10.1038/s41396-021-01129-z

Zeineldin, M., Elolimy, A. A., and Barakat, R. (2020). Meta-analysis of bovine respiratory microbiota: link between respiratory microbiota and bovine respiratory health. *FEMS Microbiol. Ecol.* 96(8), fiaa127. doi: 10.1093/femsec/fiaa127

Zeineldin, M., Lowe, J., and Aldridge, B. (2019). Contribution of the mucosal microbiota to bovine respiratory health. *Trends Microbiol.* 27 (9), 753–770. doi: 10.1016/j.tim.2019.04.005

Zeineldin, M., Lowe, J., De Godoy, M., Maradiaga, N., Ramirez, C., Ghanem, M., et al. (2017a). Disparity in the nasopharyngeal microbiota between healthy cattle on feed, at entry processing and with respiratory disease. *Veterinary Microbiol.* 208, 30–37. doi: 10.1016/j.vetmic.2017.07.006

Zeineldin, M., Lowe, J., Grimmer, E., De Godoy, M., Ghanem, M., Abd El-Raof, Y., et al. (2017c). Relationship between nasopharyngeal and bronchoalveolar microbial communities in clinically healthy feedlot cattle. *BMC Microbiol.* 17 (1), 138. doi: 10.1186/s12866-017-1042-2

Zhou, Y., Jackson, D., Bacharier, L. B., Mauger, D., Boushey, H., Castro, M., et al. (2019). The upper-airway microbiota and loss of asthma control among asthmatic children. *Nat. Commun.* 10, 5714. doi: 10.1038/s41467-019-13698-x

Zulkifli, I., Abubakar, A. A., Sazili, A. Q., Goh, Y. M., Imlan, J. C., Kaka, U., et al. (2019). The effects of Sea and road transport on physiological and electroencephalographic responses in Brahman crossbred heifers. *Anim. an Open Access J. MDPI* 9, 199. doi: 10.3390/ani9050199

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Gut dysbiosis in nonalcoholic fatty liver disease: pathogenesis, diagnosis, and therapeutic implications

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The incidence of nonalcoholic fatty liver disease (NAFLD) is increasing recently and has become one of the most common clinical liver diseases. Since the pathogenesis of NAFLD has not been completely elucidated, few effective therapeutic drugs are available. As the "second genome" of human body, gut microbiota plays an important role in the digestion, absorption and metabolism of food and drugs. Gut microbiota can act as an important driver to advance the occurrence and development of NAFLD, and to accelerate its progression to cirrhosis and hepatocellular carcinoma. Growing evidence has demonstrated that gut microbiota and its metabolites directly affect intestinal morphology and immune response, resulting in the abnormal activation of inflammation and intestinal endotoxemia; gut dysbiosis also causes dysfunction of gut-liver axis *via* alteration of bile acid metabolism pathway. Because of its composition diversity and disease-specific expression characteristics, gut microbiota holds strong promise as novel biomarkers and therapeutic targets for NAFLD. Intervening intestinal microbiota, such as antibiotic/probiotic treatment and fecal transplantation, has been a novel strategy for preventing and treating NAFLD. In this article, we have reviewed the emerging functions and association of gut bacterial components in different stages of NAFLD progression and discussed its potential implications in NAFLD diagnosis and therapy.

KEYWORDS

gut dysbiosis, nonalcoholic fatty liver disease, bile acid metabolism, probiotics, novel treatment strategies

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver injury disease worldwide, with a broad spectrum ranging from simple steatosis, nonalcoholic steatohepatitis (NASH) to cirrhosis and even hepatocellular carcinoma (HCC). Epidemiological investigation shows that NAFLD has become one of the most common liver diseases, with a global prevalence of 25.2%, 27.4% in Asia (Lonardo et al., 2016; Wiest et al., 2017), but more than 33% in China (Zhou et al., 2020; Wong and Chan, 2021). As NAFLD is a "silent" disease, the actual number of people with this disease is likely to be higher. Recently, growing evidence displays that NAFLD is multisystem metabolic disease associated with high insulin resistance and genetic susceptibility, in which the accumulation of triglycerides in hepatocytes is the main pathological change (Brunt et al., 2015). Besides liver-related complications, NAFLD also increase the risk of type 2 diabetes mellitus (T2DM), cardiovascular and chronic kidney disease. Conversely, obesity, T2DM, lifestyle changes and druggable genetic alterations also rise the morbidity and mortality of NAFLD (Mohammadi et al., 2020). Despite advance in the pathogenesis of NAFLD, few therapeutic drugs are available. Although nonspecific antioxidative, anti-inflammatory, hypolipemic and hypoglycemic drugs have been widely used in clinic to improve the symptoms, knowledge on NAFLD pathogenesis remains incomplete and hence there are few FDA-approved medications that directly treat NAFLD. In addition, interventions for NAFLD have focused on diet and lifestyle changes, but outcomes are unsatisfactory due to poor patient compliance (Notarnicola et al., 2021). Due to the hidden lesions, 20% patients have an irreversible advanced fibrosis and even HCC before the initial diagnosis (Leung et al., 2022). Therefore, the early diagnosis is important for ensuring appropriate treatment to delay or control the NASH progression. Current clinical diagnosis of NAFLD involves various procedures such as blood tests, B-ultrasound, transient elastography and MRI, but misdiagnosis of nonspecific symptoms (HBV-associated hepatitis, alcoholic hepatitis, and cirrhosis) is difficult to be distinguished in clinical practice, indicating that effective and reproducible biomarkers are still needed in clinical application (Cusi et al., 2022).

The popular theory for the pathological progression of NAFLD is "multi-hit" theory (Ore and Akinloye, 2021). According to this theory, the vicious cycle of "lipid accumulation/steatosis → hepatic lipotoxicity → metabolic disorders → inflammatory response → insulin resistance → aggravation of metabolic disorders" forms in the liver through interactions among various factors. The dysbiosis of the gut microbiota can be involved in multiple attacks on the liver and plays a key role in the pathogenesis of NAFLD. The intestinal bacteria and their metabolites can enter into the liver through the portal vein and subsequently affect the pathophysiological processes of liver. The gut microbiota and its metabolites can act as the molecular vehicles between intestine and liver (Wiest et al., 2017; Nawrot et al., 2021). Amazingly, some specific gut microbiota and metabolites, including short-chain fatty acids (SCFAs), bile acids (BAs), lipopolysaccharide (LPS), choline and trimethylamine (TMA), would be changed associated with disease severity and fibrosis stage, indicating the potential of diagnostic markers for NAFLD (Del Chierico et al., 2017; Loomba et al., 2019; Lee et al., 2020). Several clinical trials involving gut microbiota intervention, including probiotic and prebiotic supplementation, fecal microbial transplantation (FMT), and microbiome-targeted therapies (MTT), are underway for nearly every type of liver disease (Behrouz et al., 2020; Liu et al., 2021). As reported, the overall survival rates of patients with severe liver diseases who received FMT from healthy donors could be increased by 54.2% compared with those without FMT, indicating that the gut microbiota directly affects NAFLD progression (Philips et al., 2017). Therefore, the regulation, modification, and reconstruction of the gut microbiota may become a personalized strategy for the prevention and treatment of NAFLD.

Roles of the gut microbiota dysbiosis on NAFLD occurrence and development

The gut microbiota is a complex community that exist in the human gastrointestinal tract in a symbiotic manner and maintain 3 important physiological functions of the human digestive system, namely digestion, metabolism, and protection (Stražar et al., 2021; Zheng et al., 2021). Growing studies have demonstrated several mechanistic pathways of gut microbiota involved in the progression of NAFLD (Figure 1).

Gut microbiota affects host energy metabolism by altering intestinal metabolites

The gut microbiota breaks down undigested polysaccharides into monosaccharides and dietary fibers into SCFAs by utilizing a variety of enzymes, both of which provides energy support for the host cells. Most notably, SCFAs exert multiple benefits in the regulation of metabolism and immune, and could be emerged as a potential therapeutic agent against various diseases. Beside the main source of energy for host colonocytes, SCFAs have distinctive pharmacological and physiological functions, such as promoting colonic motility, protecting intestinal mucosal barrier, altering carbohydrate and lipid metabolism, participating in immune regulation, and improving the

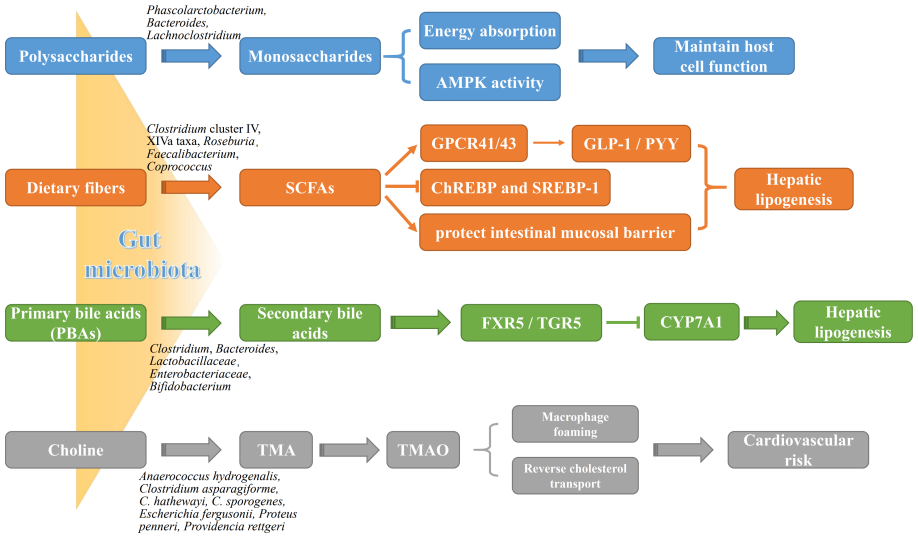

FIGURE 1
The roles of gut microbiota metabolites in NAFLD pathogenesis. Gut microbial metabolites, such as monosaccharides, SCFAs, BAs and TMA, not only are potently involved in energy metabolism of liver and intestinal epithelial cells, but affect directly liver lipogenesis and systemic inflammation. AMPK, adenosine monophosphate-dependent protein kinase; ChREBP, carbohydrate-responsive element-binding protein; CYP7A1, cytochrome P450 7A1; FXR, farnesoid X receptor; GLP-1, glucagon-like peptide-1; GPCR41/43, G protein-coupled receptor 41/43; PYY, peptide YY; SREBP-1, sterol regulatory element-binding protein 1; TGR5, takeda G protein receptor 5; TMAO, trimethylamine oxide.

absorption of electrolytes and nutrients as well as anti-inflammatory and antitumor activities (Rauf et al., 2022). Depending on the amount of dietary fiber intake, approximately 500-600 mmol of SCFAs are produced in the colon daily. Among all the metabolic products of carbohydrate fermentation by the microbiota, the acetic acid (C2), propionic acid (C3) and butyric acid (C4) are the most abundant SCFAs in the intestinal tract. Acetic acid is an important source of host energy, providing approximately 10% of total energy daily for the body. Butyric acid as the main source of energy for supplement epithelial cells plays a crucial role in the function of the intestinal barrier; and is the main metabolic substrate for the gastrointestinal microbiota, providing at least 60-70% of its energy requirements for their proliferation and differentiation (Zhou et al., 2018). It has been demonstrated that the intestinal epithelial cells both in germ-free mice and in antibiotic-induced microbiome depletion mice present shorter turnover and lower proliferative activity than the normal mice, and decreased availability of SCFAs shifts colonocyte energy metabolism toward glucose utilization (Slezak et al., 2014; Park et al., 2016). Moreover, butyric acid inhibited the activation of ChREBP and SREBP-1, resulting in the repressing of lipogenesis (Park et al., 2016). Propionic acid is mainly catabolized in the liver, where it involves in the transformation from pyruvate to glucose, and it also has been found to reduce lipid accumulation in overweight and adipose sufferers (Canfora et al., 2017). Unlike butyric acid and acetic acid acting as sources of energy for host cells, propionic acid is the precursor for adipogenesis and gluconeogenesis, which plays more important roles in the pathogenesis of NAFLD (Liu et al., 2021). Interestingly, no matter what type of dietary fiber the patients have taken, it will present obvious benefits as soon as it enters the intestinal tract, especially, to those who are already low in dietary fiber intake (Letourneau et al., 2022). Mechanically, SCFAs activate G protein-coupled receptors, such as GPR41 and GPR43, in intestinal and adipose tissues (Lu et al., 2016). The activation of GPR41 increases glucagon-like peptide 1 and peptide YY (PYY) secretion by enteroendocrine cells, resulting in the decreased intestinal motility but enhanced nutrient absorption. GPR43 activation inhibits adipocyte differentiation, and increases hepatic lipogenesis, thereby promoting the development of NAFLD (Kimura et al., 2020).

BAs are the main components of bile and can be divided into primary and secondary bile acids. Primary bile acids (PBAs), including cholic acid (CA) and chenodeoxycholic acid (CDCA), are produced from cholesterol in hepatocytes and excreted to the bile duct. Secondary bile acids (SBAs), including lithocholic acid (LCA), deoxycholic acid (DCA), ursodeoxycholic acid (UDCA) and their respective isoforms (such as isolithocholic acid), are then transformed from the primary bile acids by bacteria in the small intestine. PBAs not only maintain intestinal microbiota homeostasis through directly inhibiting overgrowth of pathogenic bacteria, but

also act as the naturally occurring agonists of intestinal FXR (FXR activation: CDCA>DCA>LCA>CA; FXR inhibition: UDCA) and subsequently activate its downstream defense genes in ileal mucosa (Fiorucci and Distrutti, 2019) to protect intestinal epithelial cells against bacterium and microorganism's corrosion. In addition, FXR down-regulates the expression of LXR and SREBP-1c to reduce fatty acid and triglyceride synthesis in the liver, thereby reducing steatogenesis and gluconeogenesis. FXR also up-regulates hepatic glycogen synthesis *via* the activation of fibroblast growth factor (FGF) 15/19, PPARγ, GLUT-4 and GLP-1 to improve insulin sensitivity (Yang et al., 2010; Han et al., 2019). TGR5 is another classical BA receptor, mainly activated by SBAs (TGR5 activation: LCA>DCA> CDCA>CA). Too few SBAs lead to reduced FXR activity but increased inflammation in the body, while too many SBAs can cause cellular DNA damage through the production of reactive oxygen species (ROS), leading to the development of HCC. It had been found that activation of TGR5 by SBAs induces transcription of the type 2 iodothyronine deiodinase (Dio2) gene, which in turn converts thyroid hormone (T4) to the more active triiodothyronine (T3), thereby increasing basal metabolism and promoting energy metabolism in brown adiposes and muscle tissues of the high-fat diet (HFD)-fed mice (Zietak and Kozak, 2016). Activation of TGR5 in intestinal secretion cells by SBAs promotes the expression of GLP1, which increases insulin synthesis and release, resulting in the protection against islet β-cell apoptosis and improvement of blood glucose (Maczewsky et al., 2019). However, alteration of gut microbiota and hepatic function directly affects the ultimate compositions and the contents of BAs, resulting in the differential expression profiles of BA receptors (e.g., FXR vs. TGR5) among different stages of NAFLD process. Anyway, these two BA receptors, FXR and TGR5, have emerged as putative therapeutic targets for obesity and NAFLD.

Choline and its derivatives, such as TMA and TMAO, are the major metabolites of intestinal microorganisms, and their elevated levels not only contribute to atherosclerosis but also are closely related to cholesterol and triglyceride metabolism (Li et al., 2021). Clinical trials have found that reduced choline can lead to lipid deposition in the liver by reducing the synthesis and secretion of very low-density lipoproteins (VLDL) in hepatocytes, leading to the steatohepatitis. It is the consequence which could be commonly found in methionine/choline-deficient (MCD) diet-fed rodents (Cao et al., 2022; Ding et al., 2022; Zhang et al., 2022). It has been known that the food containing dietary methylamine, choline, phosphatidylcholine and carnitine will break down into various metabolites including TMA through the catabolism of trimethylamine lytic enzymes in Proteobacteria and Firmicutes (Guerrerio et al., 2012). TMA is transferred to the liver *via* the portal vein and converted by flavin-containing monooxygenases into TMAO, which promotes the accumulation of activated leukocytes into human endothelial cells, leading to endothelial dysfunction and significantly increases the risk of atherosclerosis and cardiovascular diseases (Tan et al., 2019; Shi et al., 2022).

Gut microbiota affects intestinal and hepatic immune function

Growing investigations held the intestinal barrier dysfunction blame for inflaming the NAFLD progression (Liu et al., 2021; Gupta et al., 2022; He et al., 2022; Leng et al., 2022; Kaushal et al., 2022; Yu et al., 2022). During the initiation and progression of NAFLD, large amounts of gut bacterial metabolites, bacterial components, and other hazards enter the liver through the portal vein due to the intestinal mucosal barrier disruption induced by various stimuli, with the increased intestinal permeability. Those attacks can further accelerate liver injury and fibrosis *via* elevating inflammation, oxidative stress, and lipid accumulation (Lechner et al., 2020) as shown in Figure 2. The investigation by *in situ* hybridization revealed the presence of gut bacterial metabolites and DNA fragments in the livers of HFD-fed mice, but presence of bacteria in the liver of NASH patients remains unkown (Mouries et al., 2019). Compared with normal individuals, the number of enteric bacteria, especially Gram-negative bacteria, was significantly increased in obesity or NAFLD patients, and presented obvious endotoxemia (Li et al., 2022). The excessive amounts of LPS activates adenylate cyclase in the intestinal mucosa and damages the mitochondria and lysosomes of epithelial cells, leading to necrosis of the apical cells of the intestinal villi and autolysis of the epithelial cells. Moreover, the gut-derived lipopolysaccharides (LPS) are the key factor in conducting the liver inflammation and chronic damage through activating LPS-dependent pattern recognition receptor signaling (An et al., 2022). LPS activates TLR4 in endothelial cells and TLR9 in dendritic cells, resulting in the release of a large number of pro-inflammatory cytokines (such as TNF-α, IL-1β, and IL-6) and chemokines (CCL2, CXCL2, CXCL10 and CXCL16) that mediates inflammation and pathological damage to the liver (Arelaki et al., 2022; Han et al., 2022; Nagata et al., 2022; Tiegs and Horst, 2022). Therefore, LPS causes inflammation and serious metabolic changes in the body, such as increasing fat consumption, and elevating circulating free fatty acid (FFA) and triglyceride (TG). The deposition of FFA in the liver may also induce inflammation, and insulin resistance (IR), further contributing to the development of NAFLD (Rennert et al., 2020; Zheng et al., 2022).

On the other hand, the gut microbiota can alter the balance of anti-inflammatory and proinflammatory cytokines secreted by M1 and M2 macrophages by affecting the metabolism of SBAs, thereby affecting the immune function of the liver (Yang et al., 2021). Even small amounts of SBAs produced by the gut microbiota can decrease FXR activity and increase inflammation in the body; however, high amounts of SBAs can produce large amounts of ROS, cause cellular DNA damage, and lead to the development of HCC (Sun et al., 2020). In addition, PBAs can upregulate CXCL16 in hepatic vascular endothelial cells, which in turn leads to the recruitment of NKT cells that can kill tumor cells in a CD1d-dependent manner (Ma et al., 2018).

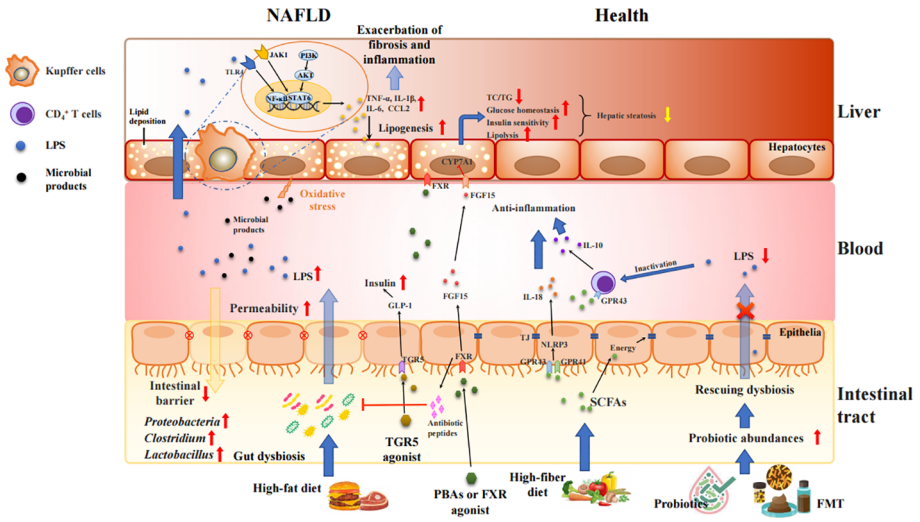

FIGURE 2
Intestinal barrier disrupted by gut dysbiosis facilitates bacterial endotoxins to liver and exacerbates inflammatory process and fat accumulation in the pathogenesis of NAFLD. Unhealth lifestyle (e.g., high-fat, low-fiber diet) alters the microbiota colonization in gut, increases gut permeability, and produces various proinflammatory molecules, such as LPS, TMAO, SBAs, and bacterial 16sDNA. These proinflammatory molecules worsen the liver inflammation and fibrosis and potentially accelerate NAFLD progression. Treatment with FXR/TGR5 agonist, probiotics and FMT strengthens intestinal tight junction, and mediates the glucose and lipid metabolism *via* activation of FXR and TGR5 signaling as well as inhibition of TLR4//NF-κB and JAK1/STAT6 pathways.

Gut microbiota as a screening marker and therapeutic target for NAFLD

Several studies have shown that Patients with NAFLD have the remarkable gut dysbiosis, in which the relative abundances of *Proteus* and *Enterobacter* bacteria were increased while and *Ruminococcus* and *Lactobacillus* were decreased (Loomba et al., 2019; Oh et al., 2020). As simple steatosis progresses to advanced liver fibrosis, the number of Gram-negative bacteria was increases, especially *Proteus* bacteria (Caussy et al., 2018; Pettinelli et al., 2022). Fecal BAs may reflect alterations in two bile acid metabolic pathways: "glycine, serine, and threonine metabolism" and "taurine and hypotaurine metabolism." *Escherichia*, *Bilophila* and *Rhodobacter* involved in BAs-mediating taurine and glycine metabolism were significantly increased in the faeces of NASH patients (Jiao et al., 2018). Moreover, 60% of NAFLD patients were associated with infection with high alcohol *Klebsiella pneumoniae* (HiAlc-Kpn), and more interestingly, transplantation of this HiAlc-Kpn isolated from the feces of NASH patients could induce NAFLD in the mice (Yuan et al., 2019). However, the profiles of gut microbiota found in different studies often vary greatly due to differences in study subjects, dietary habits, medication history, and detection methods. Although close interactions between gut microbiota and NAFLD pathogenesis have been demonstrated over the past decades, the reliable microbiome markers for early NAFLD still remain to be further verified. Recently, with the aid of machine learning model integrating baseline microbial signatures to distinguish liver fat accumulation status of NAFLD participants has displayed good perspective in clinical practice, with 80% of overall accuracy (vs 60% by blood biochemical detection) (Leung et al., 2022). Given the obvious individual differences and dynamic changes of gut microbiota, it is difficult to classify disease status by stool-based biomarker detection using 16s rDNA amplification alone rather than multi-omics analysis, such as metagenomics and other culture-independent technologies. To increase the robustness and generalizability of predictors, the set of predictive metabolites identified by multiple methods could be more potential for future trials to validate prediction biomarkers in larger and independent cohorts.

Novel strategy for NAFLD treatment based on gut microbiota

Up to now, the basic steps in the prevention of NAFLD are still diet, exercise and management of complications (such as type 2 diabetes and dyslipidemia) (Yaskolka et al., 2021; Cui et al., 2022; Cheng et al., 2022). There are few effective drugs for treatment and prevention of NAFLD/NASH. Pioglitazone may be effective in advanced NASH patients or associated with type 2 diabetes, but reliable clinical data are lacking (Shaaban et al., 2022); vitamin E and D have some efficacy, but the safety of long-term use is unclear and the course of treatment is inconclusive (Perumpail et al., 2018; Ravaioli et al., 2022); statins can reduce serum LDL levels and prevent cardiovascular complications, but do not address the progression of liver disease (Torres-Peña et al., 2021). Some

promising drugs, including BA derivatives, BA metabolic-mediated agents, and probiotics, are still being evaluated in clinical trials (Table 1).

Microbiome-targeted therapies

MTT is a novel strategy for the treatment of NAFLD by correcting or reshaping gut microbiota by using antibiotics, probiotics, prebiotics, synbiotics and FMT to maintain gut homeostasis (Sharpton et al., 2019). Commercialized *Streptococcus*, *Lactobacillus*, and *Bifidobacterium* improve the gut inflammatory microenvironment, promote the growth and survival of intestinal epithelial cells, and inhibit pathogenic bacteria by modulating the immune system and host defense (Xie et al., 2018). As safe edible probiotics, *Lactobacillus* and *Bifidobacterium* can lower blood cholesterol levels (Wu and Chiou, 2021). FMT is a comprehensive approach for regulating the gut microbiota that has been shown to be effective in restoring microbiota composition and can be used to treat *C. difficile*-associated diarrhea and *Pseudomembranous colitis*, as well as other chronic intestinal inflammatory diseases, such as ulcerative colitis and Crohn's disease (Costello et al., 2019; Sokol et al., 2020). After FMT treatment, the gut microbiota in both HFD-fed mice and NAFLD patients would be reshaped, small intestinal permeability was elevated, and the symptom of NASH was partly improved (Zhou et al., 2017; Craven et al., 2020; Xue et al., 2022). However, unlike the

TABLE 1 Current clinical status of targeting gut microbiota-related agents for NAFLD treatment.

Agent	Clinical status	Target	Target diseases	Effects	References
BAs sequestrant	Approved	Increase BAs excretion	Hyperlipidemia, NAFLD	Increase hepatic LDLR and insulin sensitivity; decrease plasma LDL	(Le et al., 2012)
CDCA	Approved	FXR agonist	NAFLD	Improve liver function and reduce endogenous BAs synthesis	(Panzitt et al., 2022)
Obeticholic acid (INT-747, 6-ECDCA, OCA)	Approved	FXR agonist	NAFLD, Primary Biliary Cholangitis	Activate FXR but indirectly inhibit CYP7A1	(Younossi et al., 2019)
PX-104	Phase II	FXR agonist	Non-diabetic NAFLD	Improve insulin sensitivity and liver enzymes	(Traussnigg et al., 2021)
Nidufexor (LMB763)	Phase II	FXR agonist	NASH and diabetic nephropathy	Reduce NAFLD activity scores, triglyceride levels, and liver fibrosis	(Chianelli et al., 2020)
Cilofexor	Phase III	FXR agonist, insulin sensitizer	NASH and fibrosis	Decrease serum γ-glutamyltransferase, C4, and primary bile acids	(Patel et al., 2020)
EDP-305	Phase II	FXR agonist	NASH, cholangiopathies, renal fibrosis	Reduce ALT levels and liver fat content	(Ratziu et al., 2022)
INT-767	Phase I	TGR5/FXR dual agonist	NASH, alcoholic liver disease	Decrease hepatic steatosis, associated with reduced liver fatty acid synthase protein expression	(Iracheta-Vellve et al., 2018)
Allogenic FMT	Clinical investigation	Gut microbiota	Lean NAFLD	Decrease liver fat accumulation and improve gut dysbiosis	(Xue et al., 2022)
Allogenic FMT	Phase I	Gut microbiota	NAFLD	Not improve insulin resistance and hepatic PDFF, but reduce small intestinal permeability	(Craven et al., 2020)
Clostridium butyricum combined with Rosuvastatin	Clinical investigation	Gut microbiota and HMG-CoA reductase inhibitor	NAFLD	Increase contents of *Bacteroides thetaiotaomicron* and *Bifidobacteria*; reduce serum lipid, hepatic fibrotic indexes and pro-inflammatory cytokines	(Zhu et al., 2022)
VSL#3 (8 probiotic mixture)	Phase I	GLP-1	NAFLD	No effects on triglycerides, HOMA and ALT; decrease BMI and increase GLP-1 and activated GLP1	(Alisi et al., 2014)
Six probiotic mixtures	Phase I	Gut microbiota	Obese NAFLD	Reduce intrahepatic fat and body weight	(Ahn et al., 2019)
Synbiotics	Phase I	Gut microbiota	NAFLD	Increase proportions of Bifidobacterium and Faecalibacterium, and decrease Oscillibacter and Alistipes; not reduce liver fat content or markers of liver fibrosis.	(Scorletti et al., 2020)
Probiotics	Clinical investigation	Gut barrier	NAFLD	No significant clinical improvement	(Mohamad et al., 2021)
Probiotics	Clinical investigation	Insulin resistance	NAFLD	Decrease blood glucose, insulin, insulin resistance, TNF-α, and IL-6	(Sepideh et al., 2016)

necessity for ulcerative colitis and Crohn's disease, FMT is not often the first choice for NAFLD patients due to the distaste for the gut contents. Compared with FMT, prebiotic supplementation has a higher safety and operability. The oral administration of prebiotics, such as lactulose, oligofructose, and inulin, can stimulate the release of gastrointestinal peptides, which have the effect of regulating appetite and energy metabolism of the body (Cerdó et al., 2019). However, the excessive intake of inulin may cause liver damage, and thus prebiotics should be supplemented appropriately under the guidance of a doctor to prevent harm to the body (Singh et al., 2018). Although probiotics, prebiotics and synbiotics have been proposed for treating the patients with obesity-related NAFLD and indeed alleviate intestinal permeability, they did not show any significant clinical improvement in NAFLD patients (Behrouz et al., 2020; Scorletti et al., 2020; Chong et al., 2021; Mohamad et al., 2021). Therefore, their therapeutic effects should be further investigated by high-quality clinical trials.

Selective intervention of specific flora (phage therapy)

The use of a specific bacteria or bacterial products to interfere with NASH may be a novel therapeutic strategy for anti-NAFLD drug discovery. The colonization of *Akkermansia muciniphila* in gut could improve liver function, reduce oxidative stress, inhibit inflammation, normalize gut microbiota, and reverse metabolic disturbances caused by HFD (Jiao et al., 2018). Some SBA-producing bacteria, such as *Lactobacillaceae* and *Lachnospiraceae*, exhibit cholesterol-lowering potential and may improve metabolic abnormalities under HFD (Zeng et al., 2020).

Recently, phage therapy is being given great attention as a new antimicrobial agent. Understanding endogenous phage-gut microbiota interactions in health and in NAFLD may enable phage utilization in precise gut microbiome editing, towards treating NAFLD and other obesity-associated metabolic diseases. As previously reported, the targeted eradication of HiAlc-Kpn with phages effectively alleviates bacterial auto-wine syndrome and NASH in NAFLD model mice (Yuan et al., 2019). Treatment with bacteriophages can specifically target cytolytic *Enterococcus faecalis* and alleviate liver injury in humanized mice colonized with faecal bacteria of patients with alcoholic hepatitis (Duan et al., 2019). Unlike the major therapeutic hurdles of antibiotics and other chemotherapies caused by high degrees of bacteria resistance in clinic, phage therapy provides a new therapeutic approach for precisely editing the intestinal microbiota, completely resolving bacteria resistance and treating liver diseases. Although great advances have presented in various bacterial infection (Mitropoulou et al., 2022; Van Nieuwenhuyse et al., 2022), due to the diversity and complexity of gut microbiome, more clinical trials with larger cohorts are required to validate the relevance of the findings in NAFLD patients.

The interaction between gut microbiota and immunotherapy in NAFLD

The gut microbiota also has been demonstrated to enhance the therapeutic response to immune checkpoint inhibitors though targeting immunomodulatory molecules or their ligands on the surfaces of T cells (McCulloch et al., 2022; Wu et al., 2022; Zhang et al., 2022; Zhao et al., 2022). The combination has been approved for treating various malignant tumors, including NAFLD-associated HCC (Baruch et al., 2021; Davar et al., 2021; Shiraishi et al., 2022). Compared with those untreated with antibiotics, progression-free survival and overall survival of patients treated with antibiotics were significantly lower during the treatment of anti-PD-1/PD-L1 antibodies (He et al., 2021). This finding was consistent with the results of the mouse experiment, in which the transplantation of fecal flora from tumor patients who responded to PD-1 inhibitor into germ-free mice enhanced the antitumor effects of PD-1 inhibitor, while Fecal flora from non-responding patients did not exhibit this improvement (Gao et al., 2021). These results suggest that the regulation of gut microbiota may be a new target for NAFLD-related HCC and cirrhosis therapy. Given that patients with HCC and cirrhosis often have severe intestinal ecological dysregulation, which may lead to immunotherapy failure in some patients, the modulation of the gut microbiota may be more relevant for the therapeutic response in HCC and cirrhosis than other solid tumors.

Notably, recent data indicate that the IL-17/IL-17R originating from TH17 cells drives intestinal neutrophil migration, limits gut dysbiosis, maintains intestinal barrier integrity and attenuates LPS translocation to visceral adipose tissue, resulting in protection to metabolic syndrome and comorbidities such as NAFLD and obesity (Pérez et al., 2019; He et al., 2022). Paradoxically, there is also some evidence that IL-17 accelerated hepatic steatosis through activation of the JNK-PPARα pathway in the HFD mice and oleic acid-preloaded hepatocytes (Shen et al., 2017; Sugimura et al., 2019; Pan et al., 2019; Wang et al., 2020; Wang et al., 2021; Takamura et al., 2022). Circulating levels of IL-17, released by the visceral adipose tissue, induces eotaxin secretion *via* the smooth muscle cells, both of which are associated with early atherosclerosis that is the main factor drastically reducing the survival of NAFLD patients (Tarantino et al., 2014). These results indicated the versatile of IL-17 in the development of NAFLD and the significance of target delivery in different tissues.

FXR and TGR5 agonists

Besides the central roles in bile acid metabolism, FXR and TGR5 are closely linked to the metabolism of lipids, glucose and lipoproteins, and hence have emerged as the new targets for the treatment of NAFLD (Yang et al., 2010; Han et al., 2019; Maczewsky et al., 2019). FXR agonists include natural ligands (such as CDCA), semi-synthetic BAs (such as obeticholic acid (OCA)) or synthetic non-steroidal molecules (such as GW4064, WAY-362450). These FXR agonists prevent hepatic steatosis in obese and insulin-resistant rodents. Synthetic FXR agonists are associated with increased body mass and reduced glucose tolerance with prolonged use, whereas the BA class of natural FXR agonists are not. There are two published clinical trials on the FXR agonist OCA. Short-term oral administration of OCA improved insulin resistance and reduced markers of liver fibrosis in patients with NASH combined with type 2 diabetes (Mudaliar et al., 2013; Pockros et al., 2019). However, in the FLINT (clinical trials gov identifier NCT01265498) trial, long-term oral administration of OCA at 25 mg/d for 72 weeks in patients with NASH did not improve insulin sensitivity and increased homeostasis model assessment (HOMA) indices (Neuschwander-Tetri et al., 2015). The difference between these two trials may lie in the length of time the medication is taken. Indeed, OCA treatment significantly improved liver histological outcomes, with a reduction in NASH activity scores compared with placebo-treated patients. However, the improvement rate was only 22% and hardly seen in patients with severe fibrosis (Younossi et al., 2019). Also, the adverse reactions caused by FXR agonist are the key problems that have to be faced. Therefore, further clinical trials are needed to assess the efficacy and safety of OCA for the clinical treatment of NASH.

Up to now, most synthetic FXR agonists are ongoing at preclinical or phase I status (Panzitt et al., 2022). Recent study has shown that hepatic FXR subtypes (α1 and α2) differ in their mechanisms of limiting lipid accumulation in hepatocytes, in which FXRα2 has a stronger inhibition on hepatic TG levels (Ramos et al., 2020). This has implications for the further optimization of FXR agonists and the improvement of therapeutic efficacy.

TGR5 agonists are theoretically able to maintain glucose homeostasis, increase energy consumption to maintain healthy body mass, and reduce liver inflammation in NASH. The CA derivative INT-777 is a selective TGR5 agonist that increases energy metabolism, reduces liver fat deposition and prevents body mass gain in HFD-fed mice (de Oliveira et al., 2016; Gillard et al., 2022). The TGR5/FXR dual agonist INT-767 showed good affinity to both receptors, and reduced hepatic steatosis in obese diabetic mice *via* repressing the production of pro-inflammatory cytokines and inducing M2 macrophage polarization (McMahan et al., 2013; Iracheta-Vellve et al., 2018). Despite good results in animal studies, clinical trials with TGR5 agonists have been less effective.

UDCA is a classical drug with immunomodulatory, anti-oxidant and anti-apoptotic effects. However, its effect on NAFLD/NASH has not been confirmed in clinical trials (Marchianò et al., 2022). However, some derivatives of UDCA have shown good therapeutic effects in mouse NASH models, such as norUDCA, which is currently in phase II clinical trials.

The BA sequestrants colesevelam interrupts the hepatic-intestinal circulation of BA and has been shown to affect cholesterol, triglyceride and glucose homeostasis, reducing hepatic fat deposition in NASH patients (Kessoku et al., 2020). Several additional ASBT antagonists have been developed for the treatment of hyperlipidemia and hypercholesterolemia, but have not been tested in NAFLD (Ge et al., 2019; Karpen et al., 2020).

Future challenges and prospects

Abnormal gut microbiota can promote the occurrence, progression, and even deterioration and carcinogenesis of NAFLD. How to use gut microbiota to alter or block the progression of NASH and find bacteria and bacterial products, as potential targets, has become the critical for NAFLD intervention and treatment. First, although new strategies, such as probiotics, prebiotics, FMT, phage therapy and FXR/TGR5 agonists, have shown promise in the treatment of NAFLD, challenges in developing safe and effective therapeutic drugs, including the selectivity, tissue specificity and drug resistance for long-term use, remain further assessed.

Second, whether specific bacterial strain or microbiota are causally related to the occurrence of NASH and could be served as effective therapeutics has not yet been established mechanistically. Moreover, most studies have remained at the animal stage, and how differences in gut bacterial species between animals and humans affect microbial metabolism remains unclear.

Third, other microbial factors, such as metabolites associated with gut virus, may be also important to the progression of NAFLD. To date, the microbial origins of many detected metabolites are unclear, and few microbial sources have been reported for several metabolites. The metabolomic profiles of patients with NAFLD are inconsistent and sometimes contradictory probably because of the relatively small number of patients and the lack of uniform methods and standards. Therefore, in-depth studies are required to explore these metabolites and their microbial sources and to refine the characteristics of floras and their metabolites in different NAFLD subtypes. It is believed that the developments in technology and research may facilitate the use of the gut microbiota in NAFLD intervention or treatment, and the in-

depth exploration of the composition and function of gut microbiota will bring new diagnostic tools and personalized treatments for NAFLD.

Author contributions

JF, and C-HY: Wrote the manuscript; X-JL, J-MY, S-HY, and Z-YF: Searched literatures; C-HY, and JF: Illustrated figures and tables; Z-YF, and W-YY: Edited the manuscript; C-HY and W-YY: Designed and supervised the review. All authors contributed to the article and approved the submitted version.

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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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References

Ahn, S. B., Jun, D. W., Kang, B. K., Lim, J. H., Lim, S., and Chung, M. J. (2019). Randomized, double-blind, placebo-controlled study of a multispecies probiotic mixture in nonalcoholic fatty liver disease. *Sci. Rep.* 9 (1), 5688. doi: 10.1038/s41598-019-42059-3

Alisi, A., Bedogni, G., Baviera, G., Giorgio, V., Porro, E., Paris, C., et al. (2014). Randomised clinical trial: The beneficial effects of VSL#3 in obese children with non-alcoholic steatohepatitis. *Aliment. Pharmacol. Ther.* 39 (11), 1276–1285. doi: 10.1111/apt.12758

An, L., Wirth, U., Koch, D., Schirren, M., Drefs, M., Koliogiannis, D., et al. (2022). The role of gut-derived lipopolysaccharides and the intestinal barrier in fatty liver diseases. *J. Gastrointest Surg.* 26 (3), 671–683. doi: 10.1007/S11605-021-05188-7

Arelaki, S., Koletsa, T., Sinakos, E., Papadopoulos, V., Arvanitakis, K., Skendros, P., et al. (2022). Neutrophil extracellular traps enriched with IL-1β and IL-17A participate in the hepatic inflammatory process of patients with non-alcoholic steatohepatitis. *Virchows Arch.* 481 (3), 455–465. doi: 10.1007/S00428-022-03330-7

Baruch, E. N., Youngster, I., Ben-Betzalel, G., Ortenberg, R., Lahat, A., Katz, L., et al. (2021). Fecal microbiota transplant promotes response in immunotherapy-refractory melanoma patients. *Science* 371 (6529), 602–609. doi: 10.1126/SCIENCE.ABB5920

Behrouz, V., Aryaeian, N., Zahedi, M. J., and Jazayeri, S. (2020). Effects of probiotic and prebiotic supplementation on metabolic parameters, liver aminotransferases, and systemic inflammation in nonalcoholic fatty liver disease: A randomized clinical trial. *Food Sci.* 85 (10), 3611–3617. doi: 10.1111/1750-3841.15367

Brunt, E. M., Wong, V. W., Nobili, V., Day, C. P., Sookoian, S., Maher, J. J., et al. (2015). Nonalcoholic fatty liver disease. *Nat. Rev. Dis. Primers* 1, 15080. doi: 10.1038/nrdp.2015.80

Canfora, E. E., van der Beek, C. M., Jocken, J. W. E., Goossens, G. H., Holst, J. J., Olde Damink, S. W. M., et al. (2017). Colonic infusions of short-chain fatty acid mixtures promote energy metabolism in overweight/obese men: a randomized crossover trial. *Sci. Rep.* 7 (1), 2360. doi: 10.1038/s41598-017-02546-x

Cao, Y. N., Yue, S. S., Wang, A. Y., Xu, L., Hu, Y. T., Qiao, X., et al. (2022). Antrodia cinnamomea and its compound dehydroeburicoic acid attenuate nonalcoholic fatty liver disease by upregulating ALDH2 activity. *Ethnopharmacol* 292, 115146. doi: 10.1016/J.JEP.2022.115146

Caussy, C., Hsu, C., Lo, M. T., Liu, A., Bettencourt, R., Ajmera, V. H., et al. (2018). Genetics of NAFLD in twins consortium. link between gut-microbiome derived metabolite and shared gene-effects with hepatic steatosis and fibrosis in NAFLD. *Hepatology* 68 (3), 918–932. doi: 10.1002/hep.29892

Cerdó, T., García-Santos, J. A., G. Bermúdez, M., and Campoy, C. (2019). The role of probiotics and prebiotics in the prevention and treatment of obesity. *Nutrients* 11 (3), 635. doi: 10.3390/nu11030635

Cheng, R., Wang, L., Le, S., Yang, Y., Zhao, C., Zhang, X., et al. (2022). A randomized controlled trial for response of microbiome network to exercise and diet intervention in patients with nonalcoholic fatty liver disease. *Nat. Commun.* 13 (1), 2555. doi: 10.1038/S41467-022-29968-0

Chianelli, D., Rucker, P. V., Roland, J., Tully, D. C., Nelson, J., Liu, X., et al. (2020). Nidufexor (LMB763), a novel FXR modulator for the treatment of nonalcoholic steatohepatitis. *J. Med. Chem.* 63 (8), 3868–3880. doi: 10.1021/acs.jmedchem.9b01621

Chong, P. L., Laight, D., Aspinall, R. J., Higginson, A., and Cummings, M. H. (2021). A randomised placebo controlled trial of VSL#3® probiotic on biomarkers of cardiovascular risk and liver injury in non-alcoholic fatty liver disease. *BMC Gastroenterol.* 21 (1), 144. doi: 10.1186/S12876-021-01660-5

Costello, S. P., Hughes, P. A., Waters, O., Bryant, R. V., Vincent, A. D., Blatchford, P., et al. (2019). Effect of fecal microbiota transplantation on 8-week remission in patients with ulcerative colitis: A randomized clinical trial. *Jama* 321 (2), 156–164. doi: 10.1001/jama.2018.20046

Craven, L., Rahman, A., Nair Parvathy, S., Beaton, M., Silverman, J., Qumosani, K., et al. (2020). Allogenic fecal microbiota transplantation in patients with nonalcoholic fatty liver disease improves abnormal small intestinal permeability: A randomized control trial. *Am. J. Gastroenterol.* 115 (7), 1055–1065. doi: 10.14309/ajg.0000000000000661

Cui, N., Li, H., Dun, Y., Ripley-Gonzalez, J. W., You, B., Li, D., et al. (2022). Exercise inhibits JNK pathway activation and lipotoxicity *via* macrophage migration inhibitory factor in nonalcoholic fatty liver disease. *Front. Endocrinol. (Lausanne)* 13. doi: 10.3389/FENDO.2022.961231

Cusi, K., Isaacs, S., Barb, D., Basu, R., Caprio, S., Garvey, W. T., et al. (2022). American Association of clinical endocrinology clinical practice guideline for the diagnosis and management of nonalcoholic fatty liver disease in primary care and endocrinology clinical settings: Co-sponsored by the American association for the study of liver diseases (AASLD). *Endocr. Pract.* 28 (5), 528–562. doi: 10.1016/J.EPRAC.2022.03.010

Davar, D., Dzutsev, A. K., McCulloch, J. A., Rodrigues, R. R., Chauvin, J. M., Morrison, R. M., et al. (2021). Fecal microbiota transplant overcomes resistance to anti-PD-1 therapy in melanoma patients. *Science* 371 (6529), 595–602. doi: 10.1126/SCIENCE.ABF3363

Del Chierico, F., Nobili, V., Vernocchi, P., Russo, A., De Stefanis, C., Gnani, D., et al. (2017). Gut microbiota profiling of pediatric nonalcoholic fatty liver disease

and obese patients unveiled by an integrated meta-omics-based approach. *Hepatology* 65 (2), 451–464. doi: 10.1002/hep.28572

de Oliveira, M. C., Gilglioni, E. H., de Boer, B. A., Runge, J. H., de Waart, D. R., Salgueiro, C. L., et al. (2016). Bile acid receptor agonists INT747 and INT777 decrease oestrogen deficiency-related postmenopausal obesity and hepatic steatosis in mice. *Biochim. Biophys. Acta* 1862 (11), 2054–2062. doi: 10.1016/j.bbadis.2016.07.012

Ding, N., Wang, K., Jiang, H., Yang, M., Zhang, L., Fan, X., et al. (2022). AGK regulates the progression to NASH by affecting mitochondria complex I function. *Theranostics* 12 (7), 3237–3250. doi: 10.7150/THNO.69826

Duan, Y., Llorente, C., Lang, S., Brandl, K., Chu, H., Jiang, L., et al. (2019). Bacteriophage targeting of gut bacterium attenuates alcoholic liver disease. *Nature* 575 (7783), 505–511. doi: 10.1038/s41586-019-1742-x

Fiorucci, S., and Distrutti, E. (2019). The pharmacology of bile acids and their receptors. *Handb. Exp. Pharmacol.* 256, 3–18. doi: 10.1007/164_2019_238

Gao, G., Ma, T., Zhang, T., Jin, H., Li, Y., Kwok, L. Y., et al. (2021). Adjunctive probiotic lactobacillus rhamnosus probio-M9 administration enhances the effect of anti-PD-1 antitumor therapy *via* restoring antibiotic-disrupted gut microbiota. *Front. Immunol.* 12. doi: 10.3389/fimmu.2021.772532

Ge, M. X., Niu, W. X., Ren, J. F., Cai, S. Y., Yu, D. K., Liu, H. T., et al. (2019). A novel ASBT inhibitor, IMB17-15, repressed nonalcoholic fatty liver disease development in high-fat diet-fed Syrian golden hamsters. *Acta Pharmacol. Sin.* 40 (7), 895–907. doi: 10.1038/s41401-018-0195-3

Gillard, J., Picalausa, C., Ullmer, C., Adorini, L., Staels, B., Tailleux, A., et al. (2022). Enterohepatic takeda G-protein coupled receptor 5 agonism in metabolic dysfunction-associated fatty liver disease and related glucose dysmetabolism. *Nutrients* 14 (13), 2707. doi: 10.3390/NU14132707

Guerrerio, A. L., Colvin, R. M., Schwartz, A. K., Molleston, J. P., and Murray, K. F. (2012). Choline intake in a large cohort of patients with nonalcoholic fatty liver disease. *Am. J. Clin. Nutr.* 95 (4), 892–900. doi: 10.3945/ajcn.111.020156

Gupta, B., Rai, R., Oertel, M., and Raeman, R. (2022). Intestinal barrier dysfunction in fatty liver disease: Roles of microbiota, mucosal immune system, and bile acids. *Semin. Liver Dis.* 42 (2), 122–137. doi: 10.1055/S-0042-1748037

Han, Y. H., Choi, H., Kim, H. J., and Lee, M. O. (2022). Chemotactic cytokines secreted from kupffer cells contribute to the sex-dependent susceptibility to non-alcoholic fatty liver diseases in mice. *Life Sci.* 306, 120846. doi: 10.1016/J.LFS.2022.120846

Han, X., Cui, Z. Y., Song, J., Piao, H. Q., Lian, L. H., Hou, L. S., et al. (2019). Acanthoic acid modulates lipogenesis in nonalcoholic fatty liver disease *via* FXR/LXRs-dependent manner. *Chem. Biol. Interact.* 311, 108794. doi: 10.1016/j.cbi.2019.108794

He, S., Cui, S., Song, W., Jiang, Y., Chen, H., Liao, D., et al. (2022). Interleukin-17 weakens the NAFLD/NASH process by facilitating intestinal barrier restoration depending on the gut microbiota. *mBio* 13 (2), e0368821. doi: 10.1128/MBIO.03688-21

He, D., Li, X., An, R., Wang, L., Wang, Y., Zheng, S., et al. (2021). Response to PD-1-Based immunotherapy for non-small cell lung cancer altered by gut microbiota. *Oncol. Ther.* 9 (2), 647–657. doi: 10.1007/s40487-021-00171-3

Iracheta-Vellve, A., Calenda, C. D., Petrasek, J., Ambade, A., Kodys, K., Adorini, L., et al. (2018). FXR and TGR5 agonists ameliorate liver injury, steatosis, and inflammation after binge or prolonged alcohol feeding in mice. *Hepatol. Commun.* 2 (11), 1379–1391. doi: 10.1002/hep4.1256

Jiao, N., Baker, S. S., Chapa-Rodriguez, A., Liu, W., Nugent, C. A., Tsompana, M., et al. (2018). Suppressed hepatic bile acid signalling despite elevated production of primary and secondary bile acids in NAFLD. *Gut* 67 (10), 1881–1891. doi: 10.1136/gutjnl-2017-314307

Karpen, S. J., Kelly, D., Mack, C., and Stein, P. (2020). Ileal bile acid transporter inhibition as an anticholestatic therapeutic target in biliary atresia and other cholestatic disorders. *Hepatol. Int.* 14 (5), 677–689. doi: 10.1007/s12072-020-10070-w

Kaushal, K., Agarwal, S., Sharma, S., Goswami, P., Singh, N., Sachdev, V., et al. (2022). Demonstration of gut-barrier dysfunction in early stages of non-alcoholic fatty liver disease: A proof-Of-Concept study. *Clin. Exp. Hepatol.* 12 (4), 1102–1113. doi: 10.1016/J.JCEH.2022.01.006

Kessoku, T., Kobayashi, T., Ozaki, A., Iwaki, M., Honda, Y., Ogawa, Y., et al. (2020). Rationale and design of a randomised, double-blind, placebo-controlled, parallel-group, investigator-initiated phase 2a study to investigate the efficacy and safety of elobixibat in combination with cholestyramine for non-alcoholic fatty liver disease. *BMJ Open* 10 (9), e037961. doi: 10.1136/bmjopen-2020-037961

Kimura, T., Pydi, S. P., Pham, J., and Tanaka, N. (2020). Metabolic functions of G protein-coupled receptors in hepatocytes-potential applications for diabetes and NAFLD. *Biomolecules* 10 (10), 1445. doi: 10.3390/biom10101445

Le, T. A., Chen, J., Changchien, C., Peterson, M. R., Kono, Y., Patton, H., et al. (2012). Effect of colesevelam on liver fat quantified by magnetic resonance in nonalcoholic steatohepatitis: a randomized controlled trial. *Hepatology* 56 (3), 922–932. doi: 10.1002/hep.25731

Lechner, S., Yee, M., Limketkai, B. N., and Pham, E. A. (2020). Fecal microbiota transplantation for chronic liver diseases: Current understanding and future direction. *Dig. Dis. Sci.* 65 (3), 897–905. doi: 10.1007/s10620-020-06100-0

Lee, G., You, H. J., Bajaj, J. S., Joo, S. K., Yu, J., Park, S., et al. (2020). Distinct signatures of gut microbiome and metabolites associated with significant fibrosis in non-obese NAFLD. *Nat. Commun.* 11 (1), 4982. doi: 10.1038/s41467-020-18754-5

Leng, J., Tian, H. J., Fang, Y., Hu, Y. Y., and Peng, J. H. (2022). Amelioration of non-alcoholic steatohepatitis by atractylodes macrocephala polysaccharide, chlorogenic acid, and geniposide combination is associated with reducing endotoxin gut leakage. *Front. Cell Infect. Microbiol.* 12. doi: 10.3389/FCIMB.2022.827516

Letourneau, J., Holmes, Z. C., Dallow, E. P., Durand, H. K., Jiang, S., Carrion, V. M., et al. (2022). Ecological memory of prior nutrient exposure in the human gut microbiome. *ISME J.* 10, 114. doi: 10.1038/S41396-022-01292-X

Leung, H., Long, X., Ni, Y., Qian, L., Nychas, E., Siliceo, S. L., et al. (2022). Risk assessment with gut microbiome and metabolite markers in NAFLD development. *Sci. Transl. Med.* 14 (648), eabk0855. doi: 10.1126/scitranslmed.abk0855

Li, Q., Rempel, J. D., Yang, J., and Minuk, G. Y. (2022). The effects of pathogen-associated molecular patterns on peripheral blood monocytes in patients with non-alcoholic fatty liver disease. *Clin. Exp. Hepatol.* 12 (3), 808–817. doi: 10.1016/j.jceh.2021.11.011

Li, X., Su, C., Jiang, Z., Yang, Y., Zhang, Y., Yang, M., et al. (2021). Berberine attenuates choline-induced atherosclerosis by inhibiting trimethylamine and trimethylamine-n-oxide production *via* manipulating the gut microbiome. *NPJ Biofilms Microbiomes* 7 (1), 36. doi: 10.1038/s41522-021-00205-8

Liu, W., Luo, X., Tang, J., Mo, Q., Zhong, H., Zhang, H., et al. (2021). A bridge for short-chain fatty acids to affect inflammatory bowel disease, type 1 diabetes, and non-alcoholic fatty liver disease positively: by changing gut barrier. *Eur. J. Nutr.* 60 (5), 2317–2330. doi: 10.1007/s00394-020-02431-w

Liu, C., Wang, Y. L., Yang, Y. Y., Zhang, N. P., Niu, C., Shen, X. Z., et al. (2021). "Novel approaches to intervene gut microbiota in the treatment of chronic liver diseases.". *FASEB J.* 35 (10), e21871. doi: 10.1096/fj.202100939R

Lonardo, A., Byrne, C. D., Caldwell, S. H., Cortez-Pinto, H., and Targher, G. (2016). Global epidemiology of nonalcoholic fatty liver disease: Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology* 64 (4), 1388–1389. doi: 10.1002/hep.28431

Loomba, R., Seguritan, V., Li, W., Long, T., Klitgord, N., Bhatt, A., et al. (2019). Gut microbiome-based metagenomic signature for non-invasive detection of advanced fibrosis in human nonalcoholic fatty liver disease. *Cell Metab.* 30 (3), 607. doi: 10.1016/j.cmet.2019.08.002

Lu, Y., Fan, C., Li, P., Lu, Y., Chang, X., and Qi, K. (2016). Short chain fatty acids prevent high-fat-diet-induced obesity in mice by regulating G protein-coupled receptors and gut microbiota. *Sci. Rep.* 6, 37589. doi: 10.1038/srep37589

Maczewsky, J., Kaiser, J., Gresch, A., Gerst, F., Düfer, M., Krippeit-Drews, P., et al. (2019). TGR5 activation promotes stimulus-secretion coupling of pancreatic β-cells *via* a PKA-dependent pathway. *Diabetes* 68 (2), 324–336. doi: 10.2337/db18-0315

Ma, C., Han, M., Heinrich, B., Fu, Q., Zhang, Q., Sandhu, M., et al. (2018). Gut microbiome-mediated bile acid metabolism regulates liver cancer *via* NKT cells. *Science* 360 (6391), eaan5931. doi: 10.1126/science.aan5931

Marchianò, S., Biagioli, M., Roselli, R., Zampella, A., Di Giorgio, C., Bordoni, M., et al. (2022). Beneficial effects of UDCA and norUDCA in a rodent model of steatosis are linked to modulation of GPBAR1/FXR signaling. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* 1867 (11), 159218. doi: 10.1016/J.BBALIP.2022.159218

McCulloch, J. A., Davar, D., Rodrigues, R. R., Badger, J. H., Fang, J. R., Cole, A. M., et al. (2022). Intestinal microbiota signatures of clinical response and immune-related adverse events in melanoma patients treated with anti-PD-1. *Nat. Med.* 3), 545–556. doi: 10.1038/S41591-022-01698-2

McMahan, R. H., Wang, X. X., Cheng, L. L., Krisko, T., Smith, M., El Kasmi, K., et al. (2013). Bile acid receptor activation modulates hepatic monocyte activity and improves nonalcoholic fatty liver disease. *Biol. Chem.* 288 (17), 11761–11770. doi: 10.1074/jbc.M112.446575

Mitropoulou, G., Koutsokera, A., Csajka, C., Blanchon, S., Sauty, A., Brunet, J. F., et al. (2022). Phage therapy for pulmonary infections: lessons from clinical experiences and key considerations. *Eur. Respir. Rev.* 31 (166), 220121. doi: 10.1183/16000617.0121-2022

Mohamad Nor, M. H., Ayob, N., Mokhtar, N. M., Raja Ali, R. A., Tan, G. C., Wong, Z., et al. (2021). The effect of probiotics (MCP® BCMC® strains) on hepatic steatosis, small intestinal mucosal immune function, and intestinal barrier in patients with non-alcoholic fatty liver disease. *Nutrients* 13 (9), 3192. doi: 10.3390/nu13093192

Mohammadi, Z., Poustchi, H., Motamed-Gorji, N., Eghtesad, S., Hekmatdoost, A., Saniee, P., et al. (2020). Fecal microbiota in non-alcoholic fatty liver disease and non-alcoholic steatohepatitis: A systematic review. *Arch. Iran Med.* . 23 (1), 44–52.

Mouries, J., Brescia, P., Silvestri, A., Spadoni, I., Sorribas, M., Wiest, R., et al. (2019). Microbiota-driven gut vascular barrier disruption is a prerequisite for non-alcoholic steatohepatitis development. *Hepatol* 71 (6), 1216–1228. doi: 10.1016/j.jhep.2019.08.005

Mudaliar, S., Henry, R. R., Sanyal, A. J., Morrow, L., Marschall, H. U., Kipnes, M., et al. (2013). Efficacy and safety of the farnesoid X receptor agonist obeticholic acid in patients with type 2 diabetes and nonalcoholic fatty liver disease. *Gastroenterology* 145 (3), 574–82.e1. doi: 10.1053/j.gastro.2013.05.042

Nagata, N., Chen, G., Xu, L., and Ando, H. (2022). An update on the chemokine system in the development of NAFLD. *Medicina (Kaunas)* 58 (6), 761. doi: 10.3390/MEDICINA58060761

Nawrot, M., Peschard, S., Lestavel, S., and Staels, B. (2021). Intestine-liver crosstalk in type 2 diabetes and non-alcoholic fatty liver disease. *Metabolism* 123, 154844. doi: 10.1016/j.metabol.2021.154844

Neuschwander-Tetri, B. A., Loomba, R., Sanyal, A. J., Lavine, J. E., Van Natta, M. L., Abdelmalek, M. F., et al. (2015). NASH clinical research network Farnesoid X nuclear receptor ligand obeticholic acid for non-cirrhotic, non-alcoholic steatohepatitis (FLINT): a multicentre, randomised, placebo-controlled trial. *Lancet* 385 (9972), 956–965. doi: 10.1016/S0140-6736(14)61933-4

Notarnicola, M., Osella, A. R., Pesole, P. L., Lippolis, A., Tutino, V., Caruso, M. G., et al. (2021). Nonalcoholic fatty liver disease: Focus on new biomarkers and lifestyle interventions. *Int. J. Mol. Sci.* 22 (8), 3899. doi: 10.3390/ijms22083899

Oh, T. G., Kim, S. M., Caussy, C., Fu, T., Guo, J., Bassirian, S., et al. (2020). A universal gut-Microbiome-Derived signature predicts cirrhosis. *Cell Metab.* 32 (5), 878–888.e6. doi: 10.1016/j.cmet.2020.06.005

Ore, A., and Akinloye, O. A. (2021). Phytotherapy as multi-hit therapy to confront the multiple pathophysiology in non-alcoholic fatty liver disease: A systematic review of experimental interventions. *Medicina (Kaunas)* 57 (8), 822. doi: 10.3390/medicina57080822

Pan, Y. J., Ren, X., Zhang, Y. Y., Lv., J., Zeng, Q. L., Zhang, H. Y., et al. (2019). IL-17A-mediated ERK1/2/p65 signaling pathway is associated with cell apoptosis after non-alcoholic steatohepatitis. *IUBMB Life.* 71 (3), 302–309. doi: 10.1002/iub.1960

Panzitt, K., Zollner, G., Marschall, H. U., and Wagner, M. (2022). Recent advances on FXR-targeting therapeutics. *Mol. Cell Endocrinol.* 552, 111678. doi: 10.1016/j.mce.2022.111678

Park, J. H., Kotani, T., Konno, T., Setiawan, J., Kitamura, Y., Imada, S., et al. (2016). Promotion of intestinal epithelial cell turnover by commensal bacteria: Role of short-chain fatty acids. *PloS One* 11 (5), e0156334. doi: 10.1371/journal.pone.0156334

Patel, K., Harrison, S. A., Elkhashab, M., Trotter, J. F., Herring, R., Rojter, S. E., et al. (2020). Cilofexor, a nonsteroidal FXR agonist, in patients with noncirrhotic NASH: A phase 2 randomized controlled trial. *Hepatology* 72 (1), 58–71. doi: 10.1002/hep.31205

Pérez, M. M., Martins, L. M. S., Dias, M. S., Pereira, C. A., Leite, J. A., Gonçalves, E. C. S., et al. (2019). Interleukin-17/interleukin-17 receptor axis elicits intestinal neutrophil migration, restrains gut dysbiosis and lipopolysaccharide translocation in high-fat diet-induced metabolic syndrome model. *Immunology* 156 (4), 339–355. doi: 10.1111/imm.13028

Perumpail, B. J., Li, A. A., John, N., Sallam, S., Shah, N. D., Kwong, W., et al. (2018). The role of vitamin e in the treatment of NAFLD. *Diseases* 6 (4), 86. doi: 10.3390/diseases6040086

Pettinelli, P., Arendt, B. M., Schwenger, K. J. P., Sivaraj, S., Bhat, M., Comelli, E. M., et al. (2022). Relationship between hepatic gene expression, intestinal microbiota, and inferred functional metagenomic analysis in NAFLD. *Clin. Transl. Gastroenterol.* 13 (7), e00466. doi: 10.14309/CTG.0000000000000466

Philips, C. A., Pande, A., Shasthry, S. M., Jamwal, K. D., Khillan, V., Chandel, S. S., et al. (2017). Healthy donor fecal microbiota transplantation in steroid-ineligible severe alcoholic hepatitis: A pilot study. *Clin. Gastroenterol. Hepatol.* 15 (4), 600–602. doi: 10.1016/j.cgh.2016.10.029

Pockros, P. J., Fuchs, M., Freilich, B., Schiff, E., Kohli, A., Lawitz, E. J., et al. (2019). CONTROL: A randomized phase 2 study of obeticholic acid and atorvastatin on lipoproteins in nonalcoholic steatohepatitis patients. *Liver Int.* 39 (11), 2082–2093. doi: 10.1111/liv.14209

Ramos Pittol, J. M., Milona, A., Morris, I., Willemsen, E. C. L., van der Veen, S. W., Kalkhoven, E., et al. (2020). FXR isoforms control different metabolic functions in liver cells *via* binding to specific DNA motifs. *Gastroenterology* 159 (5), 1853–1865.e10. doi: 10.1053/j.gastro.2020.07.036

Ratziu, V., Rinella, M. E., Neuschwander-Tetri, B. A., Lawitz, E., Denham, D., Kayali, Z., et al. (2022). EDP-305 in patients with NASH: A phase II double-blind placebo-controlled dose-ranging study. *J. Hepatol.* 76 (3), 506–517. doi: 10.1016/j.jhep.2021.10.018

Rauf, A., Khalil, A. A., Rahman, U. U., Khalid, A., Naz, S., Shariati, M. A., et al. (2022). Recent advances in the therapeutic application of short-chain fatty acids (SCFAs): An updated review. *Crit. Rev. Food Sci. Nutr.* 62 (22), 6034–6054. doi: 10.1080/10408398.2021.1895064

Ravaioli, F., Pivetti, A., Di Marco, L., Chrysanthi, C., Frassanito, G., Pambianco, M., et al. (2022). Role of vitamin d in liver disease and complications of advanced chronic liver disease. *Int. J. Mol. Sci.* 23 (16), 9016. doi: 10.3390/IJMS23169016

Rennert, C., Heil, T., Schicht, G., Stilkerich, A., Seidemann, L., Kegel-Hübner, V., et al. (2020). Prolonged lipid accumulation in cultured primary human hepatocytes rather leads to ER stress than oxidative stress. *Int. J. Mol. Sci.* 21 (19), 7097. doi: 10.3390/ijms21197097

Scorletti, E., Afolabi, P. R., Miles, E. A., Smith, D. E., Almehmadi, A., Alshathry, A., et al. (2020). Synbiotics alter fecal microbiomes, but not liver fat or fibrosis, in a randomized trial of patients with nonalcoholic fatty liver disease. *Gastroenterology* 158 (6), 1597-1610.e7. doi: 10.1053/j.gastro.2020.01.031

Sepideh, A., Karim, P., Hossein, A., Leila, R., Hamdollah, M., Mohammad, E. G., et al. (2016). Effects of multistrain probiotic supplementation on glycemic and inflammatory indices in patients with nonalcoholic fatty liver disease: A double-blind randomized clinical trial. *J. Am. Coll. Nutr.* 35 (6), 500–505. doi: 10.1080/07315724.2015.1031355

Shaaban, H. H., Alzaim, I., El-Mallah, A., Aly, R. G., El-Yazbi, A. F., and Wahid, A. (2022). Metformin, pioglitazone, dapagliflozin and their combinations ameliorate manifestations associated with NAFLD in rats *via* anti-inflammatory, anti-fibrotic, anti-oxidant and anti-apoptotic mechanisms. *Life Sci.* 308, 120956. doi: 10.1016/J.LFS.2022.120956

Sharpton, S. R., Maraj, B., Harding-Theobald, E., Vittinghoff, E., and Terrault, N. A. (2019). Gut microbiome-targeted therapies in nonalcoholic fatty liver disease: a systematic review, meta-analysis, and meta-regression. *Am. J. Clin. Nutr.* 110 (1), 139–149. doi: 10.1093/ajcn/nqz042

Shen, T., Chen, X., Li, Y., Tang, X., Jiang, X., Yu, C., et al. (2017). Interleukin-17A exacerbates high-fat diet-induced hepatic steatosis by inhibiting fatty acid β-oxidation. *Biochim. Biophys. Acta Mol. Basis Dis.* 1863 (6), 1510–1518. doi: 10.1016/j.bbadis.2017.01.027

Shi, C., Pei, M., Wang, Y., Chen, Q., Cao, P., Zhang, L., et al. (2022). Changes of flavin-containing monooxygenases and trimethylamine-n-oxide may be involved in the promotion of non-alcoholic fatty liver disease by intestinal microbiota metabolite trimethylamine. *Biochem. Biophys. Res. Commun.* 594, 1–7. doi: 10.1016/J.BBRC.2022.01.060

Shiraishi, Y., Hakozaki, T., Nomura, S., Kataoka, T., Tanaka, K., Miura, S., et al. (2022). A multicenter, randomized phase III study comparing platinum combination chemotherapy plus pembrolizumab with platinum combination chemotherapy plus nivolumab and ipilimumab for treatment-naive advanced non-small cell lung cancer without driver gene alterations: JCOG2007 (NIPPON study). *Clin. Lung Cancer* 23 (4), e285–e288. doi: 10.1016/J.CLLC.2021.10.012

Singh, V., Yeoh, B. S., Chassaing, B., Xiao, X., Saha, P., Aguilera Olvera, R., et al. (2018). Dysregulated microbial fermentation of soluble fiber induces cholestatic liver cancer. *Cell* 175 (3), 679–694.e622. doi: 10.1016/j.cell.2018.09.004

Slezak, K., Krupova, Z., Rabot, S., Loh, G., Levenez, F., Descamps, A., et al. (2014). Association of germ-free mice with a simplified human intestinal microbiota results in a shortened intestine. *Gut Microbes* 5 (2), 176–182. doi: 10.4161/gmic.28203

Sokol, H., Landman, C., Seksik, P., Berard, L., Montil, M., Nion-Larmurier, I., et al. (2020). Fecal microbiota transplantation to maintain remission in crohn's disease: a pilot randomized controlled study. *Microbiome* 8 (1), 12. doi: 10.1186/s40168-020-0792-5

Stražar, M., Temba, G. S., Vlamakis, H., Kullaya, V. I., Lyamuya, F., Mmbaga, B. T., et al. (2021). Gut microbiome-mediated metabolism effects on immunity in rural and urban African populations. *Nat. Commun.* 12 (1), 4845. doi: 10.1038/s41467-021-25213-2

Sugimura, N., Otani, K., Watanabe, T., Nakatsu, G., Shimada, S., Fujimoto, K., et al. (2019). High-fat diet-mediated dysbiosis exacerbates NSAID-induced small intestinal damage through the induction of interleukin-17A. *Sci. Rep.* 9 (1), 16796. doi: 10.1038/s41598-019-52980-2

Sun, Y., Zhu, M., Zhao, H., Ni, X., Chang, R., Su, J., et al. (2020). Serum fibroblast growth factor 19 and total bile acid concentrations are potential biomarkers of hepatocellular carcinoma in patients with type 2 diabetes mellitus. *BioMed. Res. Int.* 2020, 1751989. doi: 10.1155/2020/1751989

Takamura, S., Teraki, Y., Katayama, E., Kawaguchi, T., Kawaguchi, M., Nakano, D., et al. (2022). Effects of interleukin-17 inhibitors on hepatic fibrosis index in patients with psoriasis and metabolic dysfunction-associated fatty liver disease: Directed acyclic graphs. *Clin. Mol. Hepatol.* 28 (2), 269–272. doi: 10.3350/cmh.2022.0040

Tan, X., Liu, Y., Long, J., Chen, S., Liao, G., Wu, S., et al. (2019). Trimethylamine n-oxide aggravates liver steatosis through modulation of bile acid metabolism and inhibition of farnesoid X receptor signaling in nonalcoholic fatty liver disease. *Mol. Nutr. Food Res.* 63 (17), e1900257. doi: 10.1002/mnfr.201900257

Tarantino, G., Costantini, S., Finelli, C., Capone, F., Guerriero, E., La Sala, N., et al. (2014). Is serum interleukin-17 associated with early atherosclerosis in obese patients? *J. Transl. Med.* 12, 214. doi: 10.1186/s12967-014-0214-1

Tiegs, G., and Horst, A. K. (2022). TNF in the liver: targeting a central player in inflammation. *Semin. Immunopathol.* 44 (4), 445–459. doi: 10.1007/S00281-022-00910-2

Torres-Peña, J. D., Martín-Piedra, L., and Fuentes-Jiménez, F. (2021). Statins in non-alcoholic steatohepatitis. *Front. Cardiovasc. Med.* 8. doi: 10.3389/FCVM.2021.777131

Traussnigg, S., Halilbasic, E., Hofer, H., Munda, P., Stojakovic, T., Fauler, G., et al. (2021). Open-label phase II study evaluating safety and efficacy of the non-steroidal farnesoid X receptor agonist PX-104 in non-alcoholic fatty liver disease. *Wien. Klin. Wochenschr.* 133 (9-10), 441–451. doi: 10.1007/s00508-020-01735

Van Nieuwenhuyse, B., van der Linden, D., Chatzis, O., Lood, C., Wagemans, J., Lavigne, R., et al. (2022). Bacteriophage-antibiotic combination therapy against extensively drug-resistant pseudomonas aeruginosa infection to allow liver transplantation in a toddler. *Nat. Commun.* 13 (1), 5725. doi: 10.1038/s41467-022-33294-w

Wang, X., Ji, D., Zhu, B., Jiang, S., Han, L., Wang, Y., et al. (2020). Contribution of endotoxin to Th17 bias in patients with non-alcoholic steatohepatitis. *Microb. Pathog.* 142, 104009. doi: 10.1016/j.micpath.2020.104009

Wang, Y., Li, W., Zhao, T., Zou, Y., Deng, T., Yang, Z., et al. (2021). Interleukin-17-Producing CD4+ T cells promote inflammatory response and foster disease progression in hyperlipidemic patients and atherosclerotic mice. *Front. Cardiovasc. Med.* 8. doi: 10.3389/fcvm.2021.667768

Wiest, R., Albillos, A., Trauner, M., Bajaj, J. S., and Jalan, R. (2017). Targeting the gut-liver axis in liver disease. *Hepatol* 67 (5), 1084–1103. doi: 10.1016/j.jhep.2017.05.007

Wong, W. K., and Chan, W. K. (2021). Nonalcoholic fatty liver disease: A global perspective. *Clin. Ther.* 43 (3), 473–499. doi: 10.1016/j.clinthera.2021.01.007

Wu, H., and Chiou, J. (2021). Potential benefits of probiotics and prebiotics for coronary heart disease and stroke. *Nutrients* 13 (8), 2878. doi: 10.3390/nu13082878

Wu, H., Zheng, X., Pan, T., Yang, X., Chen, X., Zhang, B., et al. (2022). Dynamic microbiome and metabolome analyses reveal the interaction between gut microbiota and anti-PD-1 based immunotherapy in hepatocellular carcinoma. *Int. J. Cancer* 151 (8), 1321–1334. doi: 10.1002/IJC.34118

Xie, H., Lu, Q., Wang, H., Zhu, X., and Guan, Z. (2018). Effects of probiotics combined with enteral nutrition on immune function and inflammatory response in postoperative patients with gastric cancer. *buon* 23 (3), 678–683.

Xue, L., Deng, Z., Luo, W., He, X., and Chen, Y. (2022). Effect of fecal microbiota transplantation on non-alcoholic fatty liver disease: A randomized clinical trial. *Front. Cell Infect. Microbiol.* 12. doi: 10.3389/FCIMB.2022.759306

Yang, Z. H., Liu, F., Zhu, X. R., Suo, F. Y., Jia, Z. J., and Yao, S. K. (2021). Altered profiles of fecal bile acids correlate with gut microbiota and inflammatory responses in patients with ulcerative colitis. *World J. Gastroenterol.* 27 (24), 3609–3629. doi: 10.3748/wjg.v27.i24.3609

Yang, Z. X., Shen, W., and Sun, H. (2010). Effects of nuclear receptor FXR on the regulation of liver lipid metabolism in patients with non-alcoholic fatty liver disease. *Hepatol. Int.* 4 (4), 741–748. doi: 10.1007/s12072-010-9202-6

Yaskolka Meir, A., Keller, M., Müller, L., Bernhart, S. H., Tsaban, G., Zelicha, H., et al. (2021). Effects of lifestyle interventions on epigenetic signatures of liver fat: Central randomized controlled trial. *Liver Int.* 41 (9), 2101–2111. doi: 10.1111/liv.14916

Younossi, Z. M., Ratziu, V., Loomba, R., Rinella, M., Anstee, Q. M., Goodman, Z., et al. (2019). Obeticholic acid for the treatment of non-alcoholic steatohepatitis: interim analysis from a multicentre, randomised, placebo-controlled phase 3 trial. *Lancet* 394 (10215), 2184–2196. doi: 10.1016/S0140-6736(19)33041-7

Yuan, J., Chen, C., Cui, J., Lu, J., Yan, C., Wei, X., et al. (2019). Fatty liver disease caused by high-Alcohol-Producing klebsiella pneumoniae. *Cell Metab.* 30 (4), 675–688.e677. doi: 10.1016/j.cmet.2019.08.018

Yu, S., Jiang, J., Li, Q., Liu, X., Wang, Z., Yang, L., et al. (2022). Schisantherin a alleviates non-alcoholic fatty liver disease by restoring intestinal barrier function. *Front. Cell Infect. Microbiol.* 12. doi: 10.3389/FCIMB.2022.855008

Zeng, H., Larson, K. J., Cheng, W. H., Bukowski, M. R., Safratowich, B. D., Liu, Z., et al. (2020). Advanced liver steatosis accompanies an increase in hepatic inflammation, colonic, secondary bile acids and Lactobacillaceae/Lachnospiraceae bacteria in C57BL/6 mice fed a high-fat diet. *Nutr. Biochem.* 78, 108336. doi: 10.1016/j.jnutbio.2019.108336

Zhang, S. L., Han, B., Mao, Y. Q., Zhang, Z. Y., Li, Z. M., Kong, C. Y., et al. (2022). Lacticaseibacillus paracasei sh2020 induced antitumor immunity and synergized with anti-programmed cell death 1 to reduce tumor burden in mice. *Gut Microbes* 14 (1), 2046246. doi: 10.1080/19490976.2022.2046246

Zhang, J., Zhao, Y., Wang, S., Li, G., and Xu, K. (2022). CREBH alleviates mitochondrial oxidative stress through SIRT3 mediating deacetylation of MnSOD and suppression of Nlrp3 inflammasome in NASH. *Free Radic. Biol. Med.* 190, 28–41. doi: 10.1016/J.FREERADBIOMED.2022.07.018

Zhao, J., Wang, Y., Wang, J., Lv, M., Zhou, C., Jia, L., et al. (2022). Lactobacillus kefiranofaciens ZW18 from kefir enhances the anti-tumor effect of anti-programmed cell death 1 (PD-1) immunotherapy by modulating the gut microbiota. *Food Funct.* 13 (19), 10023–10033. doi: 10.1039/D2FO01747D

Zheng, Y., Shi, H., Zhou, Y., Wang, A., Kang, D., and Kang, L. (2022). Effects of endoplasmic reticulum stress, liver function, insulin resistance and vascular endothelial function in patients with nonalcoholic fatty liver disease. *Cell Mol. Biol. (Noisy-le-grand)* 67 (5), 210–217. doi: 10.14715/cmb/2021.67.5.29

Zheng, P., Wu, J., Zhang, H., Perry, S. W., Yin, B., Tan, X., et al. (2021). The gut microbiome modulates gut-brain axis glycerophospholipid metabolism in a region-specific manner in a nonhuman primate model of depression. *Mol. Psychiatry* 26 (6), 2380–2392. doi: 10.1038/s41380-020-0744-2

Zhou, D., Chen, Y. W., Zhao, Z. H., Yang, R. X., Xin, F. Z., Liu, X. L., et al. (2018). Sodium butyrate reduces high-fat diet-induced non-alcoholic steatohepatitis through upregulation of hepatic GLP-1R expression. *Exp. Mol. Med.* 50 (12) 1–12. doi: 10.1038/s12276-018-0183-1

Zhou, D., Pan, Q., Shen, F., Cao, H. X., Ding, W. J., and Chen, Y. W. (2017). Total fecal microbiota transplantation alleviates high-fat diet-induced steatohepatitis in mice *via* beneficial regulation of gut microbiota. *Sci. Rep.* 7 (1), 1529. doi: 10.1038/s41598-017-01751-y

Zhou, J., Chen, Y. W., Wang, W., Zhang, X. J., Ji, Y. X., Zhang, P., et al. (2020). Epidemiological features of NAFLD from 1999 to 2018 in China. *Hepatology* 71 (5), 1851–1864. doi: 10.1002/hep.31150

Zhu, W., Yan, M., Cao, H., Zhou, J., and Xu, Z. (2022). Effects of clostridium butyricum capsules combined with rosuvastatin on intestinal flora, lipid metabolism, liver function and inflammation in NAFLD patients. *Cell Mol. Biol. (Noisy-le-grand)* 68 (2), 64–69. doi: 10.14715/cmb/2022.68.2.10

Zietak, M., and Kozak, L. P. (2016). Bile acids induce uncoupling protein 1-dependent thermogenesis and stimulate energy expenditure at thermoneutrality in mice. *Am. J. Physiol. Endocrinol. Metab.* 310 (5), E346–E354. doi: 10.1152/ajpendo.00485.2015

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Gut microbiota signatures in tissues of the colorectal polyp and normal colorectal mucosa, and faeces

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Background: Colorectal polyps are the most common precursors of colorectal cancer (CRC). The close relationship has been observed between colorectal polyps and gut microbiota. However, gut microbiota signatures among sampling sites in patients with colorectal polyps and healthy adults remain elusive.

Aims: To learn about gut microbiota signatures in tissues of the colorectal polyp and normal colorectal mucosa, and faeces.

Methods: We performed 16S rRNA gene sequencing and bioinformatic analysis for the microbiota in the normal colorectal mucosa, the colorectal polyps and faeces of adults with colorectal polyps (n = 24) and in faeces and normal mucosa of healthy adults (n = 16) in this preliminary trial.

Results: The Ace and Chao indexes were higher in the normal colorectal mucosa and polyp tissues compared to faecal samples ($P < 0.05$). The composition of microbiota based on PCoA and ANOSIM analysis showed the significant differences only between faeces and tissues of the normal mucosa and polyp ($P < 0.05$). Based on the LEfSe analysis, the abundances of *Bacteroides*, *Prevotella-2* and *Agathobacter* were higher, whereas the abundances of *Haemophilus, Escherichia_Shigella*, *Fusobacterium* and *Streptococcus* were lower in faeces both in patients with colorectal polyp and healthy individuals, compared with those in the normal mucosa in two groups or polyp tissues. In healthy individuals, the abundance of *Fusobacterium* was significantly higher in the normal colorectal mucosa than in faeces. Moreover, there was no significant difference in the abundance of *Fusobacterium* between the normal colorectal mucosa and polyps in patients with colorectal polyps, but it was significantly higher in the mucosa and polyps than in faeces. Remarkably, the abundance of *Fusobacterium* in the normal colorectal mucosa was significantly higher in healthy individuals than in the polyp group.

Conclusions: The microbial structure in faeces differs from that in tissues of polyp and normal mucusa. Additionally, *Fusobacterium* may be a normal colonizer in colonic mucosa, and an abnormal increase of *Fusobacterium* detected in faeces may be related with the injury of the colorectal mucosa. The difference of the faecal microbiota and mucosal microbiota should be carefully considered in studies on gut microbiota in patients with colorectal lesions.

KEYWORDS

colorectal polyps, gut microbiota, 16S rRNA, mucosa, *Fusobacterium*

1 Introduction

Colorectal polyps are protrusions on the surface of the colorectum, which are the most common precursors of colorectal cancer (CRC) (Sung et al., 2021). Colorectal cancer is mostly related to colorectal polyps because polyps are prone to transforming to a malignant carcinoma (Barberis et al., 2021). Modern medicine proves that the risk factors of colorectal polyps include ageing, male sex, high protein consumption (especially red meat), high-fat and low-fibre diet, smoking and excessive drinking (Zeller et al., 2014; Kordahi et al., 2021).

Studies have shown that there is a direct or indirect interaction between gut microbiota and intestinal diseases, such as inflammatory bowel disease, irritable bowel syndrome and CRC (Biondi et al., 2021; Cai et al., 2022). Our previous study found significant differences between mucosal and faecal microbiota in CRC patients, and the relative abundance of *Fusobacterium* was significantly higher in mucosa than in faeces (Li et al., 2022). Besides, a study confirmed that *Bacteroides fragilis* in faeces from patients with colorectal polyps can serve as a risk predictor for CRC (Kordahi et al., 2021).

Some studies have investigated the microbiota changes in the colorectal polyp microenvironment. For example, a study showed that *Fusobacterium mortiferum* increased in patients with intestinal adenomatous polyps (Liang S et al., 2020). In addition, compared with normal people, another study detected a higher number of *Fusobacterium nucleatum* in faecal samples from patients with adenomatous polyps (Rezasoltani et al., 2018). Besides, a study showed the regression of cap polyposis six months after oral administration of antibiotics (Okamoto et al., 2018). On the contrary, another study observed that the use of antibiotics increase the risk of colorectal polyps (Song et al., 2021). In general, the microbiota signatures among sampling sites in patients with colorectal polyps and healthy adults remain elusive.

This study analyzed the characteristics of faecal and mucosal microbiota in patients with colorectal polyps and healthy individuals by using 16S rRNA gene sequencing. In addition, the microbial signature of the colorectal polyp tissue and normal intestinal mucosal tissue was also compared. Overall, this study attempted to provide a reference for subsequent studies regarding gut microbial changes in the whole process of development from polyps to adenocarcinoma.

2 Materials and methods

2.1 Study population

Ethical approval was granted by the Ethics Committee of the Affifiliated Hospital of Jiangnan University, Wuxi, China (LS2022022), and this preliminary trial was registered at the Chinese Clinical Trial Registry (ChiCTR2200063806). All participants were 18–80 years old and were voluntarily enrolled prior to presenting for colonoscopy. Twenty-four patients with colorectal polyps were classified as the polyps group, while 16 healthy individuals were classified as the control group. The inclusion criteria including patients: 1. were diagnosed with proliferative polyps, inflammatory polyps and adenomatous polyps by colonoscopy and pathological results; Those patients: 1. with history of colonic cancer, colonic polyps or diabetes; 2. with use of antibiotics or probiotics in the past three months; 3. with symptoms of infection within 1 week; 4. with other intestinal diseases were excluded.

2.2 Sample collection

Faecal samples were self-collected by participants after enrollment and before bowel preparation. Colorectal polyps and normal mucosal biopsies were collected by the sterile biopsy forceps with colonoscopy following a bowel preparation. These tissues together with the mucus were collected, but the mucus was washed away with saline before

DNA extraction. All specimens were labeled and immediately archived at -80°C (within 1 hour) until further processing. These specimens were labeled as the normal colorectal mucosal tissues (NC_), colorectal polyp tissues (CP_) and faecal sample (FS_) in the colorectal polyps group (_P) and the healthy control group (_C).

2.3 DNA extraction

DNA extraction was performed according to E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, Norcross, GA, U.S.) instructions from manufacturer. 1% agarose gel electrophoresis was used to check the DNA integrity. The NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientifific, Waltham, MA) was used to measure the DNA concentration and purity.

2.4 16S rRNA amplicon sequencing

The variable region V3-V4 was amplified by an ABI Gene Amp® 9,700 polymerase chain reaction thermocycler (ABI, CA, United States) using the following primer pair: 338F, 5'-ACTCCtacGGGagGCAGcagCAG-3' and 806R, 5 '-GGACTACHVGGGTWTCTAAT - 3 '. Purified amplicons were pooled in equimolar and paired-end sequenced on the Illumina MiSeq PE300 platform (Illumina, San Diego, United States) according to the standard protocols by HonSunBio Technology Co. Ltd (Shanghai, China). The 16S rRNA sequencing was performed using a MiSeq Reagent Kit v3 (2 x 300, 600 cycles, Illumina Inc., San Diego, CA, USA). The OTU clustering of sequences based on 97% similarity was performed using UPARSE software (version 8.1) (Edgar, 2013), and individual sequences and chimeras were removed during clustering. The species classification of each sequence was annotated using the RDP Classifier and compared with the Silva database (SSU138), and the matching threshold was set to 70%.

2.5 Microbial analysis of sequences

After sequencing, the raw 16S rRNA gene sequencing reads were quality-fifiltered by fastp (version 0.21.0) (Chen et al., 2018) and merged by FLASH (version 1.2.12) (Magoc and Salzberg, 2011). The OTUs were clustered with a 97% similarity cutoff using UPARSE platform (version 8.1) (Edgar, 2013). Alpha-diversity focuses on the abundance and diversity of microbial communities, and commonly used diversity indexes include Ace, Chao, Shannon and Simpson indexes. Principal co-ordinates analysis (PCoA) and Anoism analysis were drawn based on Bray-Curtis distance matrix. The closer the distance, the closer the composition of the samples. Linear discriminant analysis effect size (LEfSe) was used for screening the key strains between groups (Segata et al., 2011). Besides, for revealing the potential differences in metabolism, phylogenetic investigation of communities by reconstruction of unobserved state analysis (PICRUSt2) (version 2-2.0.3-b) was employed for predicting the functional contents based on 16S rRNA gene data (Langille et al., 2013). The functional potential of the microbiota was predicted according to the guidelines with the rarefied OTU abundance table as the input. Relative predicted abundance of MetaCyc pathways was calculated by dividing the abundance of each pathway by the sum of all pathway abundances per sample. Relative contribution of each OTU to predicted pathways was calculated by dividing the contribution of each OTU by the sum of all contributions per sample.

2.6 Bioinformatic analysis together with statistical methods

Both the R software (version 4.1.2) (https://www.R-project.org) and QIIME (version 1.9.1) (Caporaso et al., 2010) were employed to analyze the sequencing data. The α-diversity indexes at OTU level, including richness estimator (Ace and Chao) and diversity index (Shannon and Simpson), were determined based on OTU table using QIIME. Spearman correlation analysis was performed between the relevant indicators and the corresponding gut microbiota, and the correlation coefficient was set as $R > 0.4$ or $R < -0.4$, and $P < 0.05$ were considered to be associated. The results of continuous variables were expressed as Mean ± standard error (Mean ± SEM), and categorical variables were expressed as frequency and percentage. All statistical analyses were performed by one-way ANOVA or t-test using GraphPad Prism software (version 9.0.0), and $P < 0.05$ represented statistically significant difference. Besides, "*" was marked in the corresponding plot for $P < 0.05$, and "**" was marked in the corresponding plot for $P < 0.01$.

3 Results

3.1 α-diversity among the microbiota in the normal colorectal mucosa, polyps and faeces

As shown in Figure 1, the Ace and Chao indexes were higher in the normal colorectal mucosa and polyp tissues compared to fecal samples in patients with colorecctal polyps ($P < 0.01$). However, there was no significant difference in the Ace and Chao indexes between their normal colorectal mucosa and polyp tissues. In healthy individuals, the Ace and Chao indexes in the normal colorectal mucosa were higher than that in faeces

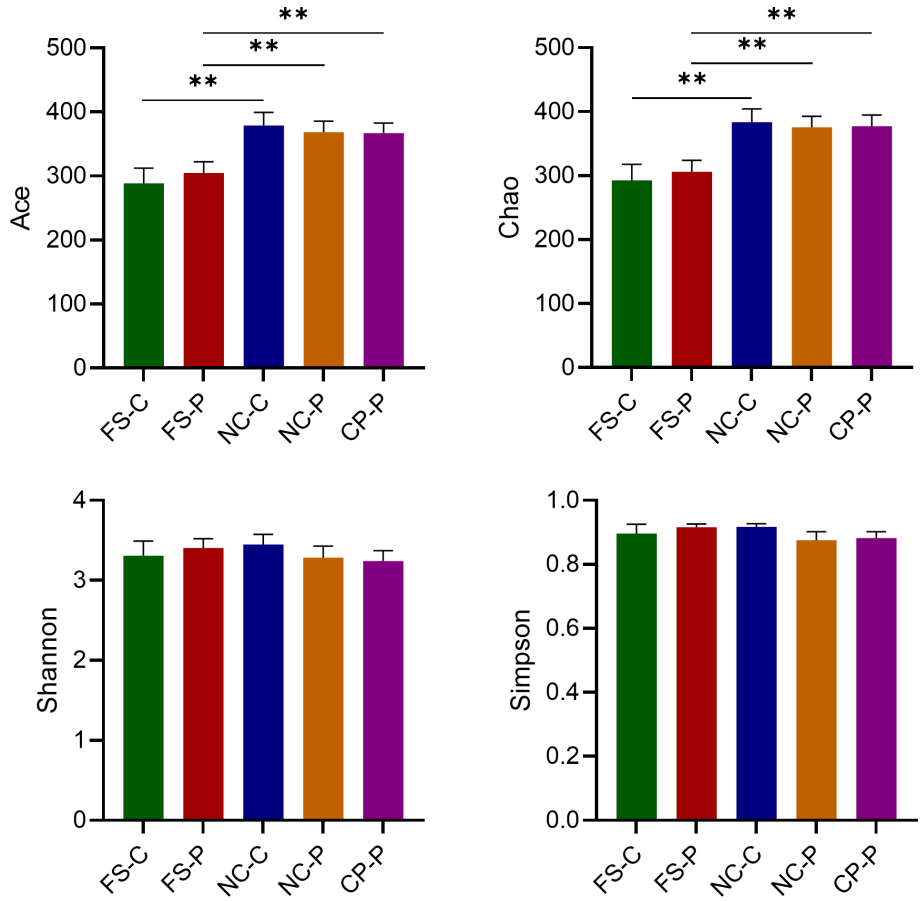

FIGURE 1
Analysis of α-diversity of microbiota in the normal colorectal mucosal tissues (NC_), colorectal polyp tissues (CP_) and faecal samples (FS_) in the colorectal polyps group (_P) and the healthy control group (_C). $^{**}P < 0.01$.

($P < 0.05$). Besides, the Shannon and Simpson index suggested that there was no difference in the community diversity among sampling sites both in patients with colorectal polyps and healthy individuals ($P > 0.05$).

3.2 Microbial communities in the normal colorectal mucosa, polyps and faeces

The overall composition of faecal microbial communities appeared similar at the levels of phylum and genus in patients with colorectal polyps and healthy individuals, and so did the normal mucosal microbial communities. In patients with colorectal polyps, the composition of the microbial communities in colorectal polyps tissue appeared similar with that in the normal mucosa at the levels of phylum and genus. Nevertheless, microbial communities in faeces appeared different from that in tissues of the normal colorectal mucosa and polyp (Figure 2).

3.3 β-diversity among the microbiota in the normal colorectal mucosa, polyps and faeces

As shown in Figures 3A-C, the structure of the faecal microbiota was significantly different from that of the microbiota in tissues of normal mucosa and colorectal polyp using PCoA based on Bray-Curtis distance (Figures 3A-C). Between healthy participants and patients with colorectal polyps, the structure of microbiota showed no difference in faeces, nor did in normal mucosa (Figures 3D, E). In addition, there is no difference in the composition of the microbiota in tissues of colorectal polyps and normal mucosa in patients with colorectal polyps (Figure 3F). Besides, the intra- and inter-individual Bray-Curtis distances were also evaluated at the OTU level using PCoA analysis. Actually, the inter-individual Bray-Curtis distance was significantly higher than the intra-individual distance among sampling sites in patients with colorectal polyps as well as in healthy individuals (Figure 3G).

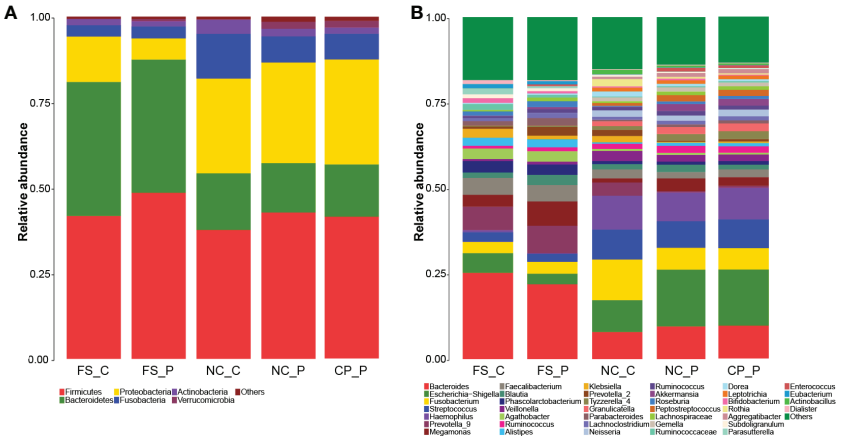

FIGURE 2
Microbial communities among sampling sites. **(A)** microbial communities at the levels of phylum; **(B)** microbial communities at the levels of genus.

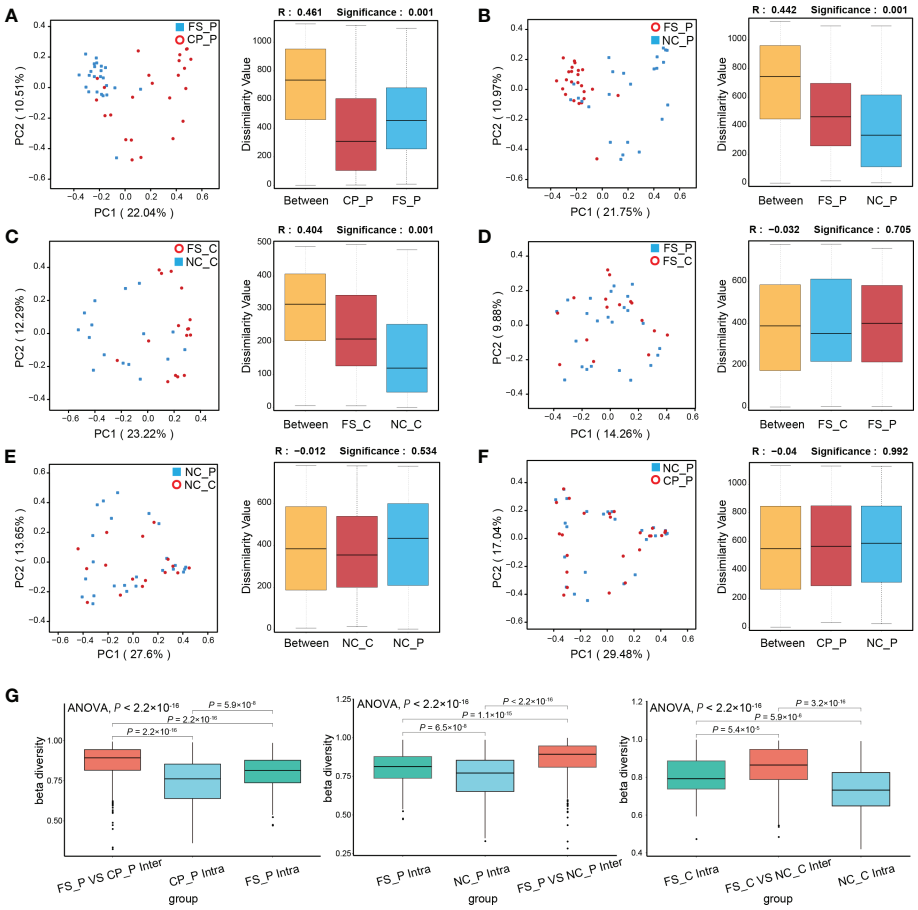

FIGURE 3
Analysis of β-diversity of microbiota among sampling sites. **(A-F)** PCoA and ANOSIM between groups; **(G)** the intra- and inter-individual Bray-Curtis distances using PCoA analysis.

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3.4 Diffirential microbiota among sampling sites

Histograms of LDA scores (> 4.0) for the differential bacterial taxa in normal colorectal mucosa, polyp tissue and faecal samples were screened out using the LEfSe analysis. Compared with the faecal sample, in the polyp tissues, *Escherichia_Shigella*, *Haemophilus*, *Streptococcus*, *Fusobacterium* and *Granulicatella* were the dominant genera, while the abundance of *Bacteroides*, *Agathobacter*, *Phascolarctobacterium* and *Prevotella-2* were lower (Figure 4A). The differential genera in the normal colorectal mucosa and the faecal sample of patients with colorectal polyps were consistent with those between the faecal sample and the polyp tissue (Figure 4B). In the healthy participants, the abundance of *Haemophilus*, *Fusobacterium*, *Streptococcus*, *Escherichia_Shigella* and *Veillonella* were higher, while the abundance of *Bacteroides* and *Agathobacter* were lower in the normal colorectal mucosa compared with the faeecal sample (Figure 4C).

We further compared genera in different sampling sites using Mann-Whitney statistic analysis. Consistent with the results of the LEfSe analysis, *Bacteroides*, *Prevotella-2* and *Agathobacter* were enriched in faeces, whereas *Haemophilus*, *Escherichia_Shigella*, *Fusobacterium* and *Streptococcus* were enriched in normal mucosa and polyps (Figure 5A). Remarkably, *Fusobacterium* was detected in the tissues of both colorectal polyps and the normal mucosa, and the relative abundance of *Fusobacterium* in the normal colorectal mucosa decreased in patients with colorectal polyps when compared to

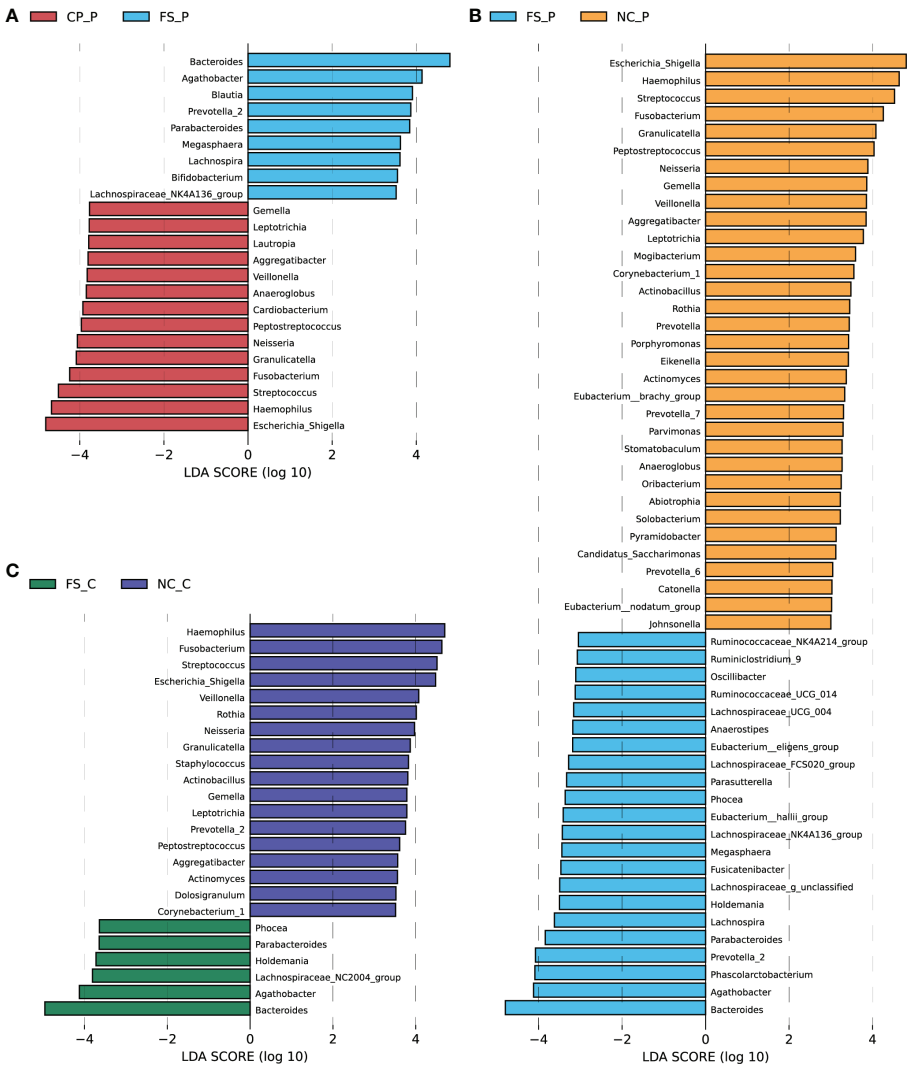

FIGURE 4
Differential abundant genera were screened out using LEfSe analysis **(A)** between colorectal polyps and faeces in the polyp group, **(B)** between normal mucosa and faeces in the polyp group, and **(C)** between normal mucosa and faeces in the control group.

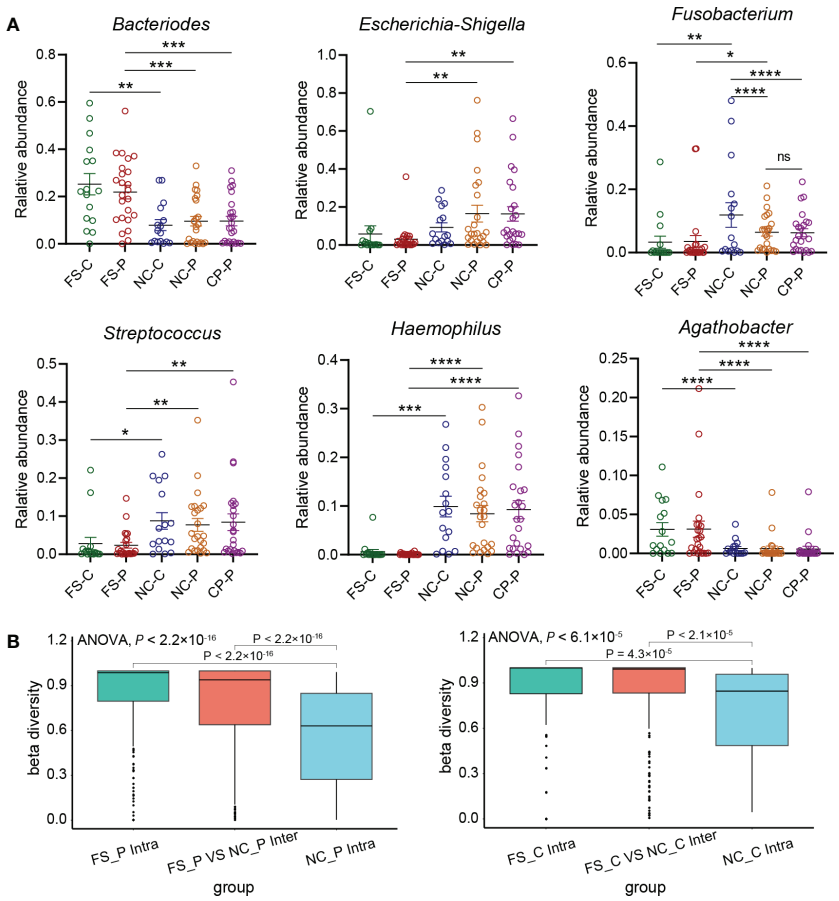

FIGURE 5
Key genera in five groups. **(A)** relative abundance of key genera in five groups selected by LEfSe including *Bacteroides, Escherichia_Shigella, Fusobacterium, Streptococcus, Haemophilus, Agathobacter*. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. **(B)** the difference of *Fusobacterium* in inter-groups and intra-groups based on the Bray-Curtis distances using PCoA analysis.

healthy subjects. Compared with faecal samples in healthy individuals, *Fusobacterium* was enriched in colorectal mucosa. Besides, when compared to faecal samples from patients with colorectal polyps, *Fusobacterium* was enriched in colorectal mucosa and polyps tissue. Moreover, the difference of *Fusobacterium* in inter-groups exceeds the difference in intra-groups based on the Bray-Curtis distances (Figure 5B). However, there was no significant difference in the abundance of *Fusobacterium* in their normal colorectal mucosa and polyp tissues. In addition, *Haemophilus* was rarely detected in faeces, while it was significantly enriched in tissues of colorectal mucosa and polyp.

Although there is no significance in the overall structure, some abundant genera were screened out using LEfSe analysis. As shown in Supplementary Figure 1, *Klebsiella* is more abundant in healthy individuals than in patients with colorectal polyps, both in faeces and in normal mucosa. The abundance of *Eubacterium_eligens* was higher, whereas the abundance of *Enterococcus* was lower in the normal mucosa of healthy individuals, compared with those of patients with colorectal polyps (Supplementary Figure 1A). Compared with healthy individuals, the abundance of *Butyricicoccus* in faeces was higher, whereas the abundances of *Lachnoclostridium* and *Erysipelatoclostridium* in faeces were lower in patients with colorectal polyps (Supplementary Figure 1B).

3.5 The functional differences among sampling sites

For revealing the potential taxonomic differences corresponded to functional changes, we performed a predictive functional analysis using PICRUSt2 based on the 16S rRNA gene data. In patients with colorectal polyps, the metabolic pathways of microbiota in the normal mucosa were significantly different from those in faeces and polyp tissues, whereas those in faeces showed no difference from those in polyp tissues (Figures 6A-C). In healthy individuals, the metabolic pathways of microbiota in the normal mucosa were not different from those in faeces (Figures 6D). Significantly, the metabolic pathways of microbiota in the normal mucosa showed differences,

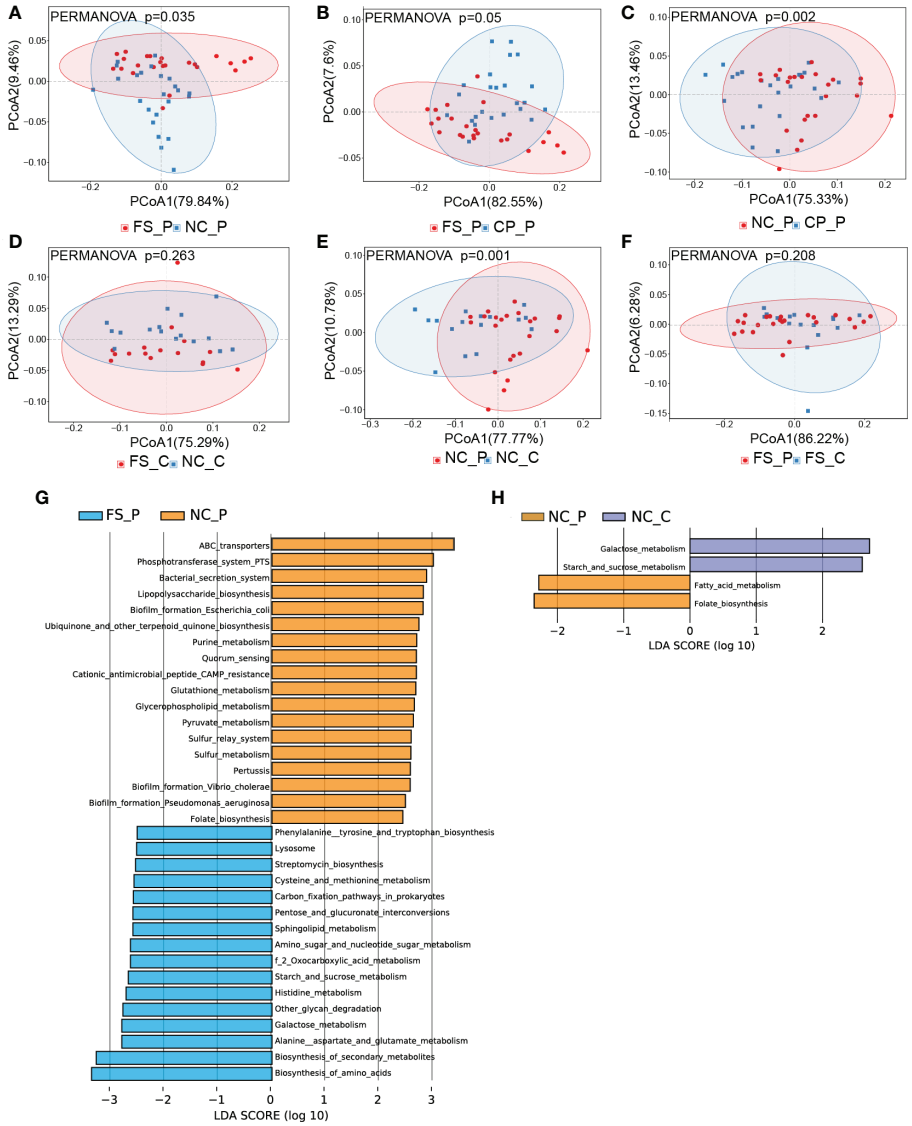

FIGURE 6
The overall functional differences in pathways among sampling sites. **(A-F)** the functional differences in pathways were displayed using PCoA analysis, and **(G, H)** the histograms of LDA scores (> 2.0) for the detailed differences in metabolic potential were displayed using LEfSe analysis.

while the metabolic pathways of microbiota in faeces showed no differences between healthy participants and patients with colorectal polyps (Figures 6E, F). In addition, compared with microbiota in faeces of patients with colorectal polyps, microbiota in the normal mucosa exhibits increased biofilm formation; and increased lipopolysaccharide, ubiquinone, and other terpenoid_quinone biosynthesis; and increased purine, glutathione, glycerophospholipid, and pyruvate metabolism (Figure 6G). By contrast, the microbiota in faeces is predicted to exhibit increased biosynthesis of amino_acids and increased alanine, aspartate, glutamate, glycan, histidine, starch, sucrose, cysteine, and methionine metabolism when compared with microbiota in normal mucosa of patients with colorectal polyps (Figure 6G). Besides, compared with microbiota in the normal mucosa of patients with colorectal polyps, the pathways of galactose, starch, and sucrose metabolism are predicted to increased, whereas the pathways of folate and fatty acid metabolism are predicted to decreased in those of healthy individual (Figure 6H).

4 Discussion

In this study, we found the differences in the microbial composition among different sampling sites from individuals

with and without colorectal polyps. The β-diversity of the microbiota in the normal colorectal mucosa and polyp tissue are significantly different from that in faecal samples. Meanwhile, the metabolic pathways of microbiota in the normal mucosa showed differences, while those of microbiota in faeces showed no difference between healthy participants and patients with colorectal polyps, indicating there is a potential risk when we define colorectal mucosal microbiota with faecal microbiota. Microbial communities in the normal colorectal mucosa showed no difference between patients with colorectal polyps and healthy individuals, nor did in their faecal sample. However, it has been reported that the inter-individual differences in the mucosa-associated gastrointestinal microbiota were remarkable in healthy individuals (Kashiwagi et al., 2020). Collectively, the dysbiosis of fecal and mucosal microbiota was not remarkable in patients with colorectal polyps.

In this study, compared with faecal samples of healthy individuals, *Fusobacterium* was enriched in their normal colorectal mucosa. The relative abundance of *Fusobacterium* in the normal mucosa was higher in healthy individuals than in patients with colorectal polyps. Remarkably, we previously proved that *Fusobacterium* was extremely abundant in the tumor tissue, and gradually decreased in the normal colorectal mucosa and faeces (Li et al., 2022). Consequently, *Fusobacterium* was detected both in the lesion tissue and in the normal colorectal mucosa, which is consistent with the report that *Fusobacterium* can be detected in the intestinal mucosa of healthy individuals (Kashiwagi et al., 2020). In this study, the abundance of *Fusobacterium* did not increased in faeces when compared with that in the normal mucosa, which may be associated with the undamaged polyp tissue of the patients with colorectal polyps. Hence, there was no difference in the abundance of faecal *Fusobacterium* between healthy individuals and patients with colorectal polyps. Therefore, *Fusobacterium* may be the commensal colonizing bacterium in the normal colorectal mucosa. The abnormal increase of *Fusobacterium* detected in faeces may be associated with the injury of the colorectal mucosa.

Besides, it has been reported that *Klebsiella* spp. are opportunistic pathogens which are normally found in gut microbiota of healthy individuals (Dong et al., 2022). Studies have proved that *Eubacterium_eligens* and *Butyricicoccus* strongly promote the production of the anti-inflammatory cytokine (Eeckhaut et al., 2013; Chung et al., 2017). The genus *Enterococcus* and *Erysipelatoclostridium* are of great relevance to human diseases for their role as major causative agents of severe infections (García-Solache and Rice, 2019; Zakham et al., 2019). A study revealed a novel faecal *Lachnoclostridium* marker for the non-invasive diagnosis of colorectal adenoma and cancer (Liang J et al., 2020). In this study, *Enterococcus, Lachnoclostridium* and *Erysipelatoclostridium* are potentially to be causes of aggravating the condition in patients with colorectal polyps. Although there is no significance in the overall structure, these abundant genera could be explored as potential target for the treatment of infection.

In this study, the richness of gut microbiota in the normal colorectal mucosa and polyps was significantly higher than in faeces, which was inconsistent with the results of some studies (Pop et al., 2020; Zhou et al., 2021). Accumulating studies have explored the gut microbiota in individuals and identified a wide range of different bacterial groups associated with carcinogenesis, including *Bacteroides, Fusobacterium*, *Escherichia and Streptococcus* (Mangifesta et al., 2018). An abnormal regulation of TLRs in relation to gut microbial quantity may contribute to carcinogenesis. TLR2 and TLR4 expression was directly associated with the *Fusobacterium, Enterococcus and Streptococcus* (Rezasoltani et al., 2020). One study showed that *Enterococcus* release enterotoxins and reactive oxygen contributing to DNA damage, inflammation, and injury to the epithelial barrier (Pop et al., 2020). In contrast, *Agathobacter*, mainly in faeces, positively correlates with the outcome of patients with CRC (Martini et al., 2022). Besides, *Sutterella* and *Escherichia_ Shigella* being the most representative genera, appeared to be associated with malignancy (Mori et al., 2018). Nevertheless, in this study, *Escherichia_Shigella* was not detected abundant in faeces but in the polyp and the mucosal sample. These discrepancies showed that the dysbiosis was more severe in colorectal mucosa than in faeces. Notably, the overall hospital mortality of 28.1% was observed among patients with bacteremia due to *Haemophilus* and *Aggregatibacter* species (Chien et al., 2021). However, *Haemophilus* is rarely detected in faeces in this study, while it is significantly enriched in normal colorectal mucosa and polyps. Therefore, it may indicate the damaged colorectal mucosa once the *Haemophilus* is detected with high abundance in faeces.

Overall, faeces were usually adopted as the sample for the convenience of sample collection in studies on gut microbiota. However, the colorectal faecal microbiota is a mixture of bacteria in the intestinal lumen and drops from mucosa. The differential taxa in faeces between healthy individuals and patients with colorectal polyps are not the same as those in colorectal mucosa (Eckburg et al., 2005). Besides, it has been reported that the biofilm plays a protective role in adherent microbiota (Baumgartner et al., 2021). In this study, the microbiota in faeces is predicted to exhibit increased nutrient metabolism when compared with microbiota in the normal mucosa of patients with colorectal polyps. On the contrary, the microbiota in the normal mucosa is predicted to exhibit increased biofilm formation, which helps to avoid environmental influences and to protect mucosal bacterium. It has been found that the mucosal microbiota is superior to faecal bacteria in distinguishing disease phenotypes (Čipčić Paljetak et al., 2022), which was also proved by this study. Since bacteria in close contact with the epithelium may have greater potential

to impact the progression of colorectal pathological changes (DeDecker et al., 2021). Therefore, this study provides a perspective on the unrepresentative role of faecal microbiota for mucosal microbiota.

Nevertheless, some limitations existed in this study. Although the process of library preparation and DNA sequencing are identical for faeces and tissues, a few differences in DNA extraction may lead to bias in the results. Another limitation was the number of cases enrolled. In addition, gut microbiota in patients with colorectal polyps is not significantly different from that in healthy individuals. The dramatically change of gut microbiota can be provoked in colorectal carcinogenesis rather than the formation of colorectal polyps.

5 Conclusions

This study suggests that the richness of the colorectal polyps and normal mucosal microbiota were significantly higher than that of faecal microbiota. Significantly, *Fusobacterium* may be the normal colonizing bacteria of colonic mucosa, and an abnormal increase of *Fusobacterium* detected in faeces may be related with the injury of the colorectal mucosa. Meanwhile, the signature of mucosal microbiota cannot be replaced by the faecal microbiota, and both should be carefully considered in studies of gut microbiota in patients with colorectal lesions. Nonetheless, during the development of colorectal polyps, the contribution of faecal and mucosal microbiota remains to be investigated.

Data availability statement

The data presented in the study are deposited in the NCBI repository, accession number PRJNA888242. The data has been released.

Ethics statement

This study was approved by the Ethics Committee of the Affifiliated Hospital of Jiangnan University, Wuxi, China (LS2022022). The trial has been registered at the Chinese Clinical Trial Registry (ChiCTR2200063806). Written and verbal informed consent was obtained from all subjects.

Author contributions

YW, JX and FZ designed the trial. YW, JX and XZ conducted the study. XZ and FZ analyzed and interpreted the data. XW, FZ and HC were responsible for project administration and supervision. XZ wrote the first draft of the manuscript and had primary responsibility for the manuscript's final content. All authors critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.1054808/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Differential abundant genera were screened out using LEfSe analysis between healthy individuals and patients with colorectal polyps. **(A)** the genera in the normal colorectal mucosa, **(B)** the genera in faeces.

References

Barberis, E., Joseph, S., Amede, E., Clavenna, M., La Vecchia, M., Sculco, M., et al. (2021). A new method for investigating microbiota-produced small molecules in adenomatous polyps. *Anal. chim. Acta* 1179, 338841. doi: 10.1016/j.aca.2021.338841

Baumgartner, M., Lang, M., Holley, H., Crepaz, D., Hausmann, B., Pjevac, P., et al. (2021). Mucosal biofilms are an endoscopic feature of irritable bowel syndrome and ulcerative colitis. *Gastroenterology* 161, 1245–1256. doi: 10.1053/j.gastro.2021.06.024

Biondi, A., Basile, F., and Vacante, M. (2021). Familial adenomatous polyposis and changes in the gut microbiota: New insights into colorectal cancer carcinogenesis. *World J. gastrointest. Oncol.* 13, 495–508. doi: 10.4251/wjgo.v13.i6.495

Cai, J., Sun, L., and Gonzalez, F. (2022). Gut microbiota-derived bile acids in intestinal immunity, inflammation, and tumorigenesis. *Cell Host Microbe* 30, 289–300. doi: 10.1016/j.chom.2022.02.004

Caporaso, J.G., Kuczynski, J., and Stombaugh, J. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Chen, S., Zhou, Y., Chen, Y., and Gu, J. (2018). Fastp: an ultra-fast all-In-One FASTQ preprocessor. *Bioinformatics* 34, i884–i890. doi: 10.1093/bioinformatics/bty560

Chien, Y., Huang, Y., Liao, C., Chien, J., and Hsueh, P. (2021). Clinical characteristics of bacteremia caused by haemophilus and aggregatibacter species and antimicrobial susceptibilities of the isolates. *J. microbiol. immunol. infect.* 54, 1130–1138. doi: 10.1016/j.jmii.2020.12.002

Chung, W., Meijerink, M., Zeuner, B., Holck, J., Louis, P., Meyer, A., et al. (2017). Prebiotic potential of pectin and pectic oligosaccharides to promote anti-inflammatory commensal bacteria in the human colon. *FEMS Microbiol. Ecol.* 93, 112–119. doi: 10.1093/femsec/fix127

DeDecker, L., Coppedge, B., Avelar-Barragan, J., Karnes, W., and Whiteson, K. (2021). Microbiome distinctions between the CRC carcinogenic pathways. *Gut Microbes* 13, 1854641. doi: 10.1080/19490976.2020.1854641

Dong, N., Yang, X., Chan, E., Zhang, R., and Chen, S. (2022). Klebsiella species: Taxonomy, hypervirulence and multidrug resistance. *EBioMedicine* 79, 103998. doi: 10.1016/j.ebiom.2022.103998

Eckburg, P., Bik, E., Bernstein, C., Purdom, E., Dethlefsen, L., Sargent, M., et al. (2005). Diversity of the human intestinal microbial flora. *Sci. (New York N.Y.)* 308, 1635–1638. doi: 10.1126/science.1110591

Edgar, R. C. (2013). UPARSE: Highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. doi: 10.1038/nmeth.2604

Eeckhaut, V., Machiels, K., Perrier, C., Romero, C., Maes, S., Flahou, B., et al. (2013). Butyricicoccus pullicaecorum in inflammatory bowel disease. *Gut* 62, 1745–1752. doi: 10.1136/gutjnl-2012-303611

García-Solache, M., and Rice, L. (2019). The enterococcus: a model of adaptability to its environment. *Clin. Microbiol. Rev.* 32. doi: 10.1128/CMR.00058-18

Kashiwagi, S., Naito, Y., Inoue, R., Takagi, T., Nakano, T., Inada, Y., et al. (2020). Mucosa-associated microbiota in the gastrointestinal tract of healthy Japanese subjects. *Digestion* 101, 107–120. doi: 10.1159/000496102

Kordahi, M., Stanaway, I., Avril, M., Chac, D., Blanc, M., Ross, B., et al. (2021). Genomic and functional characterization of a mucosal symbiont involved in early-stage colorectal cancer. *Cell Host Microbe* 29, 1589–1598.e6. doi: 10.1016/j.chom.2021.08.013

Langille, M., Zaneveld, J., Caporaso, J., Mcdonald, D., Knights, D., Reyes, J., et al. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat. Biotechnol.* 31, 814–821. doi: 10.1038/nbt.2676

Liang, J., Li, T., Nakatsu, G., Chen, Y., Yau, T., Chu, E., et al. (2020). LachnoclostridiumA novel faecal marker for the non-invasive diagnosis of colorectal adenoma and cancer. *Gut* 69, 1248–1257. doi: 10.1136/gutjnl-2019-318532

Liang, S., Mao, Y., Liao, M., Xu, Y., Chen, Y., Huang, X., et al. (2020). Gut microbiome associated with APC gene mutation in patients with intestinal adenomatous polyps. *Int. J. Biol. Sci.* 16, 135–146. doi: 10.7150/ijbs.37399

Li, Y., Cao, H., Fei, B., Gao, Q., Yi, W., Han, W., et al. (2022). Gut microbiota signatures in tumor, para-cancerous, normal mucosa, and feces in colorectal cancer patients. *Front. Cell Dev. Biol.* 10, 916961. doi: 10.3389/fcell.2022.916961

Magoc, T., and Salzberg, S. L. (2011). FLASH: Fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963. doi: 10.1093/bioinformatics/btr507

Mangifesta, M., Mancabelli, L., Milani, C., Gaiani, F., De'angelis, N., De'angelis, G., et al. (2018). Mucosal microbiota of intestinal polyps reveals putative biomarkers of colorectal cancer. *Sci. Rep.* 8, 13974. doi: 10.1038/s41598-018-32413-2

Martini, G., Ciardiello, D., Dallio, M., Famiglietti, V., Esposito, L., Corte, C., et al. (2022). Gut microbiota correlates with antitumor activity in patients with mCRC and NSCLC treated with cetuximab plus avelumab. *Int. J. Cancer* 151, 473–480. doi: 10.1002/ijc.34033

Mori, G., Rampelli, S., Orena, B., Rengucci, C., De Maio, G., Barbieri, G., et al. (2018). Shifts of faecal microbiota during sporadic colorectal carcinogenesis. *Sci. Rep.* 8, 10329. doi: 10.1038/s41598-018-28671-9

Okamoto, K., Watanabe, T., Komeda, Y., Okamoto, A., Minaga, K., Kamata, K., et al. (2018). Dysbiosis-associated polyposis of the colon-cap polyposis. *Front. Immunol.* 9, 918. doi: 10.3389/fimmu.2018.00918

Paljetak, H. Č, Barešić, A., Panek, M., Perić, M., Matijašić, M., Lojkić, I., et al. (2022). Gut microbiota in mucosa and feces of newly diagnosed, treatment-naïve adult inflammatory bowel disease and irritable bowel syndrome patients. *Gut Microbes* 14, 2083419. doi: 10.1080/19490976.2022.2083419

Pop, O., Vodnar, D., Diaconeasa, Z., Istrati, M., Binţinţan, A., Binţinţan, V., et al. (2020). An overview of gut microbiota and colon diseases with a focus on adenomatous colon polyps. *Int. J. Mol. Sci.* 21, 7359–7380. doi: 10.3390/ijms21197359

Rezasoltani, S., Aghdaei, H.A., Dabiri, H., Sepahi, A.A., Modarressi, M., and Mojarad, E.N. (2018). The association between fecal microbiota and different types of colorectal polyp as precursors of colorectal cancer. *Microb pathogen.* 124, 244–249. doi: 10.1016/j.micpath.2018.08.035

Rezasoltani, S., Ghanbari, R., Looha, M., Mojarad, E., Yadegar, A., Stewart, D., et al. (2020). Expression of main toll-like receptors in patients with different types of colorectal polyps and their relationship with gut microbiota. *Int. J. Mol. Sci.* 21, 8968–8978. doi: 10.3390/ijms21238968

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12, R60. doi: 10.1186/gb-2011-12-6-r60

Song, M., Nguyen, L., Emilsson, L., Chan, A., and Ludvigsson, J. (2021). Antibiotic use associated with risk of colorectal polyps in a nationwide study. *Clin. Gastroenterol. Hepatol.* 19, 1426–1435.e6. doi: 10.1016/j.cgh.2020.05.036

Sung, H., Ferlay, J., Siegel, R., Laversanne, M., Soerjomataram, I., Jemal, A., et al. (2021). Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: Cancer J. Clin.* 71, 209–249. doi: 10.3322/caac.21660

Zakham, F., Pillonel, T., Brunel, A., Zambelli, P., Greub, G., Croxatto, A., et al. (2019). Molecular diagnosis and enrichment culture identified a septic pseudoarthrosis due to an infection with erysipelatoclostridium ramosum. *Int. J. Infect. Dis.* 81, 167–169. doi: 10.1016/j.ijid.2019.02.001

Zeller, G., Tap, J., Voigt, A., Sunagawa, S., Kultima, J., Sobhani, I., et al. (2014). Potential of fecal microbiota for early-stage detection of colorectal cancer. *Mol. Syst. Biol.* 10, 766. doi: 10.15252/msb.20145645

Zhou, X., Zhang, S., Liu, D., Qian, H., Zhang, D., and Liu, Q. (2021). The differences between fecal microbiota and intestinal fluid microbiota in colon polyps: An observational study. *Medicine* 100, e28028. doi: 10.1097/MD.0000000000028028

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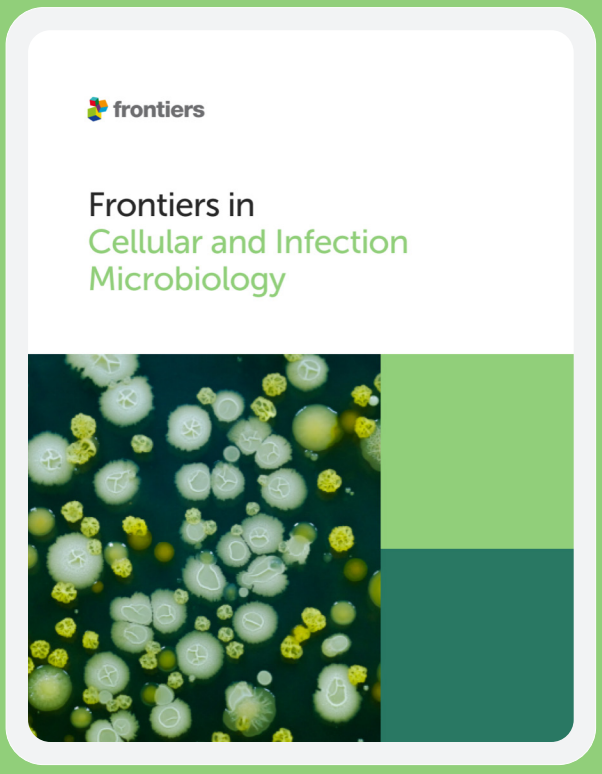

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