THE ROLE OF MICROBIOTA IN THE ONSET AND DEVELOPMENT OF INTESTINE AND LIVER DISEASES AND CANCER: MOLECULAR AND CELL MECHANISMS

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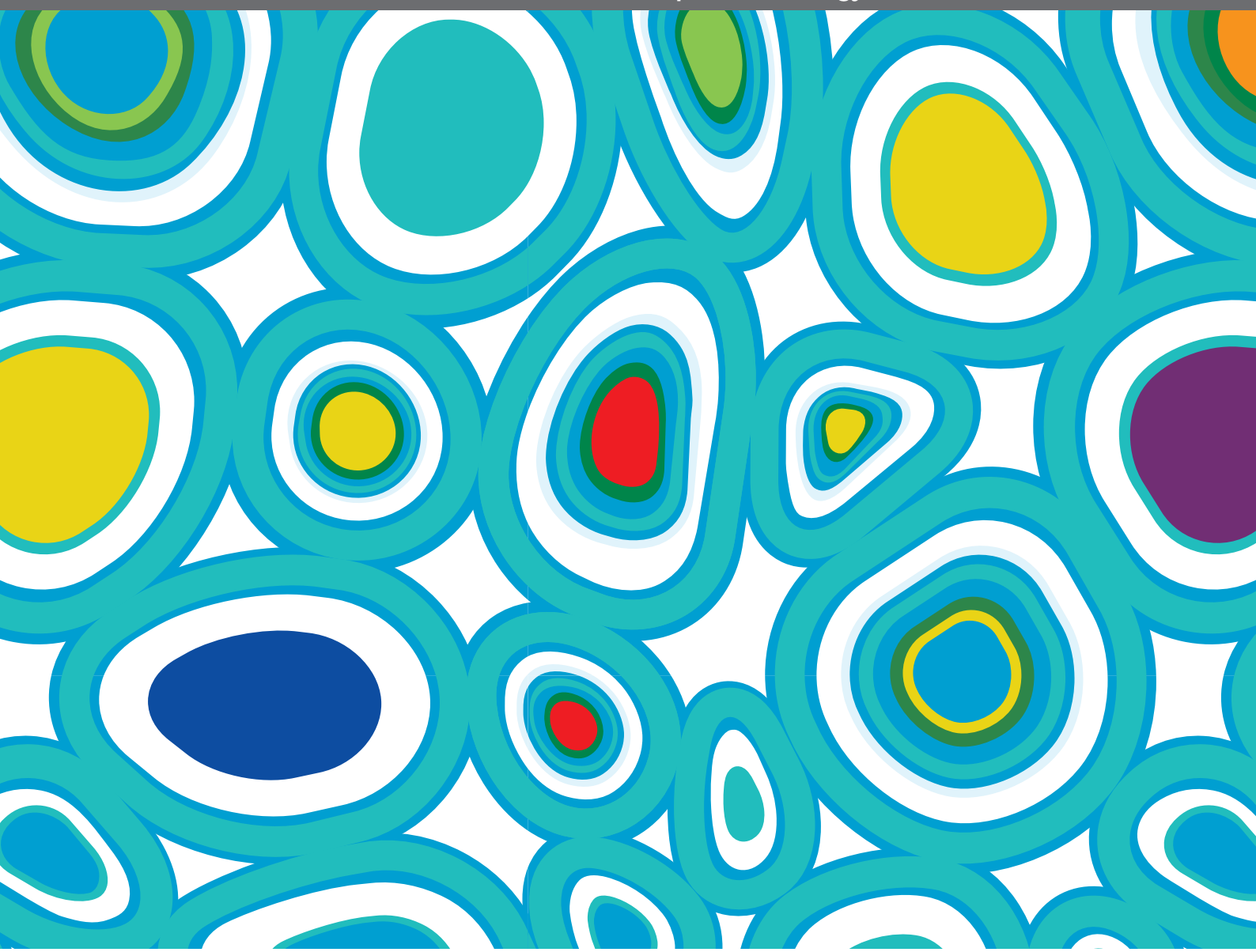

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THE ROLE OF MICROBIOTA IN THE ONSET AND DEVELOPMENT OF INTESTINE AND LIVER DISEASES AND CANCER: MOLECULAR AND CELL MECHANISMS

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EDITORIAL
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Editorial: The Role of Microbiota in the Onset and Development of Intestine and Liver Diseases and Cancer: Molecular and Cell Mechanisms

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Keywords: microbiota, cirrhosis, inflammatory bowel disease, nonalcoholic fatty liver disease, liver cancer, antibiotics, exercise, diet

Editorial on the Research Topic

The Role of Microbiota in the Onset and Development of Intestine and Liver Diseases and Cancer: Molecular and Cell Mechanisms

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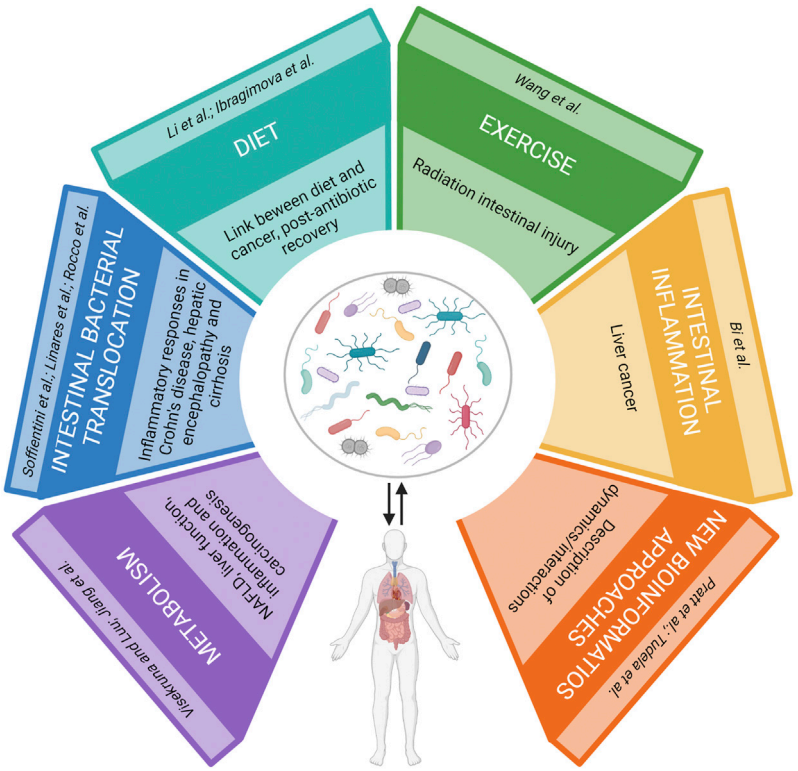
In the last decades, numerous studies highlighted the huge taxonomic and functional complexity that characterizes the human microbiota, demonstrating its role in several physiological processes necessary for host survival. This evidence supports the hypothesis that the microbiota constitutes an "essential organ" instead of a "simple" conglomerate of microbial symbionts. Furthermore, findings point out a mutual host-microbiota interaction, whose imbalance can trigger dysbiosis and, in turn, the onset of illnesses or vice versa. In the latter case, dysbiosis can magnify sicknesses. Several factors can alter microbiota homeostasis and the present Research Topic collects seven reviews and four original contributions focused on the cellular and molecular mechanisms involved in the interaction between host and microbiota which can help to unravel the possible cause of disease and find new therapeutic approaches.

Soffientini et al. and Bi et al. describe new cellular and molecular mechanisms that strengthen the role of the gut microbiota in pathogenesis and progression of liver diseases.

Using a mouse model, Soffientini et al. reported that the deficiency of caspase-11, a protease involved in the intracellular LPS sensing and triggering cell death pathways, gives protection against multi-organ injury induced by low-dose injection of LPS in CCl4-induced hepatic fibrosis. Furthermore, they found that high levels of the human orthologue, caspase-4, in the liver of patients with acute decompensation of cirrhosis is correlated with the degree of injury and clinical outcome. Overall, these data showed for the first time a causal relationship between translocation of gut-derived bacterial products and multi-organ injury in cirrhosis.

Bi et al. provided theoretical support for future clinical practice, discussing the more recent findings regarding the immuno-molecular mechanisms of the gut microbiota and their metabolites in the occurrence and development of liver cancer. They pointed out that a balanced composition in the gut microbiota is able to improve chemotherapy treatment in liver cancer and to reduce adverse reactions.



GRAPHICAL ABSTRACT |

Furthermore, in this Research Topic recent findings are reported on the identification of either new molecules derived from both bacteria (Visekruna and Luu) and the diet (Ibragimova et al.; Li et al.) or host-microorganism cellular interaction (Jiang et al.; Linares et al.; Rocco et al.) that act as modulators on the host-microbiota interplay and are involved in the development of cancer, intestine and liver diseases. Also, Wang et al. provides new clues regarding the recovery of microbiota after antibiotic therapy and the role of low-intensity exercise in its balance.

Visekruna and Luu discussed the molecular and cellular mechanisms by which short-chain fatty acids and bile acids as dominant classes of bacterial metabolites impact intestinal and liver function, inflammation, and carcinogenesis. The authors examined numerous mechanisms, including epigenetics and the more classical ligand-receptor ones, highlighting current gaps in the field and providing input on possible therapeutic interventions.

Li et al. questioned whether diet can affect the post-antibiotic recovery of the gut microbiome and host metabolism. Indeed, excessive antibiotics exposure leads to various detrimental impacts on host metabolism resulting from an imbalanced gut microbiome. In a mouse model, an integrated metagenomic and transcriptomic approach was used to demonstrate that the effects of an antibiotic intervention on host metabolism are long-lasting, antibiotic-specific, and diet-dependent. Furthermore, it was found that a high-fat diet could worsen the host metabolism recovery from short-term antibiotic perturbation in an antibiotic specific fashion, thus emphasizing the crucial role played by nutrition during post-antibiotic recovery.

Ibragimova et al. reviewed the emerging concepts regarding the relationship between diet, the microbiome, and cancer. They discuss the growing evidence indicating that a primary link between diet and cancer is mediated through dietary constituents influencing the composition and function of the gut microbiome. Furthermore, they underscore that future cancer prevention and treatment should possibly focus on optimizing favourable gut microbiomes and metabolomes.

Jiang et al. investigated the role of prolyl endopeptidase (PREP), an enzyme involved in the gut metabolic homeostasis, finding new clues regarding the crosstalk between gut microbiota and pathogenesis and progression of non-alcoholic fatty liver disease (NAFLD). In a mouse model of NAFLD induced by a high-fat diet, they described that the PREP-gene disruption can promote the abundance of several beneficial butyrate-producing bacteria and reduce hepatic inflammation, ameliorate liver lipogenesis and AMPK/SIRT1 signalling (involved in hepatic steatosis). They proposed PREP as a possible target for NAFLD.

Linares et al. reviewed the current literature regarding the interaction between intestinal bacterial translocation and the development of inflammatory responses in Crohn's disease. They outlined several factors that contribute to an uncontrolled bacterial translocation in patients with Crohn's disease, such as dysbiosis, increased permeability of the intestinal barrier, altered immune response, and a genetic

predisposition. They also focused in their discussion on the loose response to anti-TNF-alpha biologic therapy observed in patients with Crohn's disease and the role of bacterial translocation as a contributing factor.

Rocco et al. focused on the role of the gut milieu in the pathogenesis of hepatic encephalopathy. They critically reviewed the latest research findings in the field highlighting novelty and limitations and discussed the therapeutic options and novel treatment strategies.

Wang et al. studied the possible protective role of low-intensity exercise against radiation enteritis. They showed that in a mouse model walking and other comfortable forms of exercise can mitigate the radiation-induced gastrointestinal tract toxicity by restructuring the gut microbiota configuration. More specifically, they found that walking elevates the frequency of *Akkermansia muciniphila* in the digestive tract after radiation exposure and, furthermore, oral gavage of *Akkermansia muciniphila* protects against intestinal radiation toxicity. Their results suggest that *A. muciniphila* can be a useful agent to mitigate the radiation intestinal injury of patients who are clinically unfit to exercise.

In addition to wet lab experiments, bioinformatic based approaches have much to offer to microbiota research. Here, it is also highlighted the need to develop new bioinformatics approaches that describe the dynamics/interactions between the microbial population rather than the mere composition of the microbiota (Pratt et al.; Tudela et al.).

Pratt et al. illustrated the current "-omics" technologies for the study of the gut microbiome in order to identify metabolic biomarkers and patterns. Moreover, based on the results derived by other "-omics" studies, they discussed the significance of biological markers in the homeostasis and immune signalling pathways that affect inflammation or tumour development in the gut microenvironment. In particular, they focused on short-chain fatty acid and bile acid metabolism, inflammasome activation, and cytokine regulation in the context of inflammatory bowel disease and colorectal cancer.

Tudela et al. proposed that there is a need to identify specific keystone members of the gut microbiota ecosystem that carry essential functions that support a healthy symbiotic interaction with the host. Indeed, they underscored the weakness of current bioinformatic tools that focus on "presence or absence" information and do not provide a view of species interactions, thus missing the microbiota dynamics in disease status.

In this Research Topic, we discuss the latest findings and our current understanding of molecular and cellular mechanisms involved in the host-microbiota interaction and disease onset, improving the state of the art and emphasizing the need for further study.

AUTHOR CONTRIBUTIONS

FA, AQ, FS, and MF equally contributed to the writing of the manuscript. CM critically read the manuscript and conceptualized and made the graphical abstract. All authors approved the submitted version.

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ORIGINAL RESEARCH
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Prolyl Endopeptidase Gene Disruption Improves Gut Dysbiosis and Non-alcoholic Fatty Liver Disease in Mice Induced by a High-Fat Diet

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The gut-liver axis is increasingly recognized as being involved in the pathogenesis and progression of non-alcoholic fatty liver disease (NAFLD). Prolyl endopeptidase (PREP) plays a role in gut metabolic homeostasis and neurodegenerative diseases. We investigated the role of PREP disruption in the crosstalk between gut flora and hepatic steatosis or inflammation in mice with NAFLD. Wild-type mice (WT) and PREP gene knocked mice (PREP^{gt}) were fed a low-fat diet (LFD) or high-fat diet (HFD) for 16 or 24 weeks. Murine gut microbiota profiles were generated at 16 or 24 weeks. Liver lipogenesis-associated molecules and their upstream mediators, AMP-activated protein kinase (AMPK) and sirtuin1 (SIRT1), were detected using RT-PCR or western blot in all mice. Inflammatory triggers and mediators from the gut or infiltrated inflammatory cells and signal mediators, such as p-ERK and p-p65, were determined. We found that PREP disruption modulated microbiota composition and altered the abundance of several beneficial bacteria such as the butyrate-producing bacteria in mice fed a HFD for 16 or 24 weeks. The level of butyrate in HFD-PREP^{gt} mice significantly increased compared with that of the HFD-WT mice at 16 weeks. Interestingly, PREP disruption inhibited p-ERK and p-p65 and reduced the levels of proinflammatory cytokines in response to endotoxin and proline-glycine-proline, which guided macrophage/neutrophil infiltration in mice fed a HFD for 24 weeks. However, at 16 weeks, PREP disruption, other than regulating hepatic inflammation, displayed improved liver lipogenesis and AMPK/SIRT1 signaling. PREP disruption may target multiple hepatic mechanisms related to the liver, gut, and microbiota, displaying a dynamic role in hepatic steatosis and inflammation during NAFLD. PREP might serve as a therapeutic target for NAFLD.

Keywords: liver, gut microbiota, non-alcoholic fatty liver disease (NAFLD), gene knockout, prolyl endopeptidase

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a growing global health concern that affects around one-fourth of the general population worldwide (Younossi et al., 2016). The spectrum of NAFLD consists of non-alcoholic fatty liver (NAFL), the more advanced stage non-alcoholic steatohepatitis (NASH), NASH-related cirrhosis, and hepatocellular carcinoma (Saltzman et al., 2018; Aron-Wisnewsky et al., 2020). NAFLD pathogenesis is highly complex and involves numerous pathways, including insulin resistance, inflammation, lipotoxicity, increased *de novo* lipogenesis, oxidative stress, and gut dysbiosis (Tilg and Moschen, 2010; Suzuki and Diehl, 2017; Lonardo et al., 2018). Several factors, likely acting in parallel, contribute to NAFLD development and progression. These factors need to be better understood since no effective drug regimen that completely reverses the disease is currently available (Tilg and Moschen, 2010; Younossi et al., 2018).

A new model called "multiple organs-multiple hits" was proposed to explain NASH progression mechanisms (Schuster et al., 2018; Yan et al., 2020). A growing body of experimental and clinical evidence suggests that gut microbiota may be implicated in NAFLD pathogenesis (Abu-Shanab and Quigley, 2010; Safari and Gerard, 2019). Recently, studies found that certain plant extracts with prolyl endopeptidase (PREP) inhibitory function exert both intestinal flora and anti-NAFLD/NASH effects (Chen et al., 2014; Babkova et al., 2017; Wang et al., 2017). Consumption of chlorogenic acid (often through coffee) benefits intestinal functions and regulates the abundance of certain bacteria in the cecum (Chen et al., 2019). Berberine, commonly used for treating diarrhea in China (Kong et al., 2004; Yan et al., 2020), could induce gut microbiota-derived bioactive metabolite production, including butyrate, ultimately improving energy metabolism (Wang et al., 2017). It is worth mentioning that these extracts are naturally occurring PREP inhibitors (Adolpho et al., 2013; Babkova et al., 2017). As mechanisms may vary via different pathways in NAFLD development, various PREP roles in different organs need to be identified for further therapeutic applications.

Plant extracts with prolyl endopeptidase belongs to a unique family of serine proteases that specifically hydrolyze prolyl-containing bioactive peptides at the C-termini of proline residues (Shan et al., 2005). PREP is mainly found in the brain (Myohanen et al., 2007); however, significant PREP activities and protein levels have been measured in peripheral tissues, such as the liver and colorectal tumors (Larrinaga et al., 2014). One study has reported a beneficial effect of PREP in the intestine. PREP induction translated gluten into gluten immunogenic peptides in the intestine, thus improving metabolic homeostasis in mice fed a high-fat diet (HFD) (Olivares et al., 2019). However, another study showed PREP detrimental effect when collagen was cleaved by matrix metalloproteinases and PREP into proline-glycine-proline (PGP), which guided neutrophilic infiltration in the intestine and induced a vicious cycle in neutrophilic inflammation in the context of inflammatory bowel disease (Koelink et al., 2014). Our previous work found that N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP), generated from thymosin β4 (*T*β4) through hydrolysis of meprin-α and PREP, exerts a therapeutic effect on inflammatory bowel disease (Shi et al., 2020). Our studies also indicated that PREP inhibition improves hepatocyte steatosis *in vitro* and *in vivo* (Zhou et al., 2016; Jiang et al., 2020). However, the interactions between PREP and the gut environment in HFD-induced NAFLD and their potential multi-organ mechanisms remain unknown.

Herein, we conducted *in vivo* experiments at different times and in different organs to explore the role of PREP disruption on HFD-induced steatohepatitis, focusing on its controversial role in gut flora and its relationship with HFD-induced hepatic steatosis and inflammatory responses, and to elucidate its possible mechanism of action.

MATERIALS AND METHODS

Animal Model and Diets

Wild-type (WT) C57BL/6J and PREP-disrupted ($PREP^{gt}$) mice were obtained from the Shanghai Model Organisms Center, Inc. The details of PREP knockout mice are provided in the Methods section of our previous study (Jiang et al., 2020). Mice were fed a standard chow diet or a HFD (fat 30 kcal%, carbohydrates 52 kcal%, protein 18 kcal%, and cholesterol 2%) for 16 or 24 weeks. All mice were housed under a 12:12 h light/dark cycle at 25 ± 2°C and were allowed free access to food and water. All animal experiments followed the National Research Council's Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of SHRM (SHRM-IACUC-022).

Gut Microbiota Analysis

Cecal content samples were snap-frozen and stored at −80°C. Bacterial DNA was isolated from the cecal contents using a DNeasy PowerSoil kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. The quality and quantity of DNA were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, United States) and agarose gel electrophoresis, respectively. The V3–V4 regions of the bacterial 16S ribosomal RNA gene were amplified in a 25-μl reaction using PCR. The amplicons were purified using Agencourt AMPure XP beads (Beckman Coulter Co., United States). Purified amplicons were then applied to the Illumina MiSeq platform (Illumina Inc., San Diego, United States). After paired-end reads were preprocessed using Trimmomatic software (Bolger et al., 2014) to detect and cut off ambiguous bases, FLASH software was used to assemble paired-end reads (Reyon et al., 2012). All results were based on sequenced reads and operational taxonomic units (OTUs).

Hematoxylin and Eosin and Immunohistochemistry Staining

Liver tissue and ileum were fixed in 4% paraformaldehyde at 4°C overnight, then embedded in paraffin wax or snap-frozen in liquid nitrogen and stored at −80°C. Paraffin sections were stained with hematoxylin-eosin (H&E) for

pathological analysis. NALFD activity score (NAS) is calculated from the semi-quantitative evaluation of hepatic steatosis, lobular inflammation, and hepatocyte ballooning, as the previous review concluded (Aron-Wisnewsky et al., 2020). For immunohistochemistry, liver sections were incubated with antibodies against F4/80 (gb11027, Servicebio, China) and myeloperoxidase (MPO) (gb11224, Servicebio, China). The number of positive cells in the liver sections was normalized to the tissue area. Ileum sections were incubated with antibodies against zonula occludens 1 (ZO-1) (ab96587, Abcam, United Kingdom) and occludin (ab216327, Abcam, United Kingdom). Images were captured using an optical microscope (Olympus BX51, China).

PREP Activity Fluorometric Assay

The PREP activity assay was performed as described previously (Zhou et al., 2016). Briefly, 60 mg liver tissues were homogenized in 500 μl assay buffer (10 mmol/L Tris–HCl buffer, pH 7.4) and then centrifuged for 20 min at 4°C. Thereafter, 465 μl Tris–HCl (pH 7.4) was added to 10 μl supernatant for 30 min at 37°C. Next, 25 μl of the substrate (4 mmol/L Suc-Gly-Pro-AMC, Bachem) was added. The reagents were mixed, and the reaction was incubated for 60 min at 37°C. After adding the stop solution (500 μl, 1 mol/L sodium acetate buffer, pH 4.2), the fluorescence intensity was read at Ex/Em = 360/460 nm. The final concentrations were normalized to protein content and reaction time.

MMP9 Fluorometric Assays

Liver tissue samples were homogenized in assay buffer and centrifuged for 15 min at 10,000 × *g* 4°C, followed by their activation with APMA (1 mM; AnaSpec, United States) for 2 h at 37°C. The active MMP-9 was detected using SensoLyte 520 MMP-9 Assay Kit (fluorometric) using a 5-FAM/QXL^{TM}520 fluorescence resonance energy transfer peptide (AS-71155, AnaSpec, United States), according to the manufacturer's instructions. The reagents were mixed, and the fluorescence intensity was read at Ex/Em = 490/520 nm after adding the stop solution. The final concentrations were normalized to protein content.

Immunoblots

Liver or ileum tissue was homogenized and lysed in ice-cold RIPA lysis buffer (Beyotime, Shanghai, China). Total protein concentrations were measured using the BCA Protein Assay Kit (Beyotime, Shanghai, China). For immunoblotting, the protein extracts were loaded onto SDS-polyacrylamide gels (SDS-PAGE) and separated. Then, the proteins were transferred onto polyvinylidene difluoride membranes and blocked with 5% skimmed milk. Next, the membranes were incubated with primary antibodies, followed by incubation with secondary antibodies and enhanced chemiluminescence. Antibodies against sirtuin1 (SIRT1, 9475T), phosphorylated-adenosine 5′-monophosphate-activated protein kinase (PAMPK, 2535T), AMPK (4150P), fatty acid synthase (FAS, 3180S), phosphorylated-p65 (3033P), p65 (8242P), phosphorylated-ERK1/2 (4370T), and ERK1/2 (4695T) were obtained from Cell Signaling Technology (Beverly, MA, United States). Antibodies against sterol regulatory element-binding transcription factor 1 (SREBP1 and GB11524) and GAPDH (GB11002) were obtained from Servicebio (Wuhan, China). Antibodies against MMP9 (ab38898) were obtained from Abcam. The bands were quantified using Image Lab Version 2.0.1 (Bio-Rad, Hercules, CA, United States). The western blots used for analysis are included in the **Supplementary Files** (**Supplementary Figures 3, 4**).

Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

The tissue samples were homogenized using TRIzol reagent (Takara, Dalian, China) to extract total RNAs, which were reverse transcribed to cDNA using reverse transcriptase (Takara, Dalian, China). Thereafter, cDNA was used to perform real-time PCR using SYBR Premix Ex Taq (Tli RNase H Plus) (Takara, Dalian, China) using a ViiA7 real-time PCR system (Applied Biosystems, United States). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as an internal control. Relative mRNA expression levels were determined using the $2^{-\Delta\Delta Ct}$ method. The gene-specific primers in this experiment are listed in **Supplementary Table 1**.

Statistical Analysis

All data are expressed as the means ± SEM. Comparisons were performed using a one-way analysis of variance (ANOVA) in GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, United States). Tukey's post-hoc comparisons were applied for comparisons between multiple experimental groups. Differences were considered significant at P-values < 0.05.

RESULTS

Hepatic Steatosis and Liver Injury Were Ameliorated by PREP Disruption in the Liver of HFD-Fed Mice at Different Time Points

After ingesting an HFD for 16 weeks (w), HFD-WT mice developed enlarged and yellow greasy livers compared to LFD-WT mice, while the gross picture was more evident after 24 weeks feeding (**Figure 1A**). The general view of the liver improved after PREP disruption (**Figure 1B**). The HFD-WT mice gained more body weight than LFD-WT mice, while the weights of HFD-PREP^{gt} mice decreased to varying degrees after 16 and 24 weeks feeding (5.24 and 10.26%, respectively) compared to HFD-WT mice (**Figure 1B**). Additionally, ALT and AST serum levels were greatly elevated in HFD-WT mice and significantly decreased in HFD-PREP^{gt} mice after 24 weeks feeding (**Figure 1C**). However, after 16 weeks feeding, only the ALT serum results displayed statistical significance. H&E staining demonstrated substantially increased fat accumulation in the livers of the HFD-WT mice (16 and 24 weeks) compared with that in the LFD-WT, respectively, while lobular inflammation is more evident in the 24 weeks HFD-WT mice (**Figure 2A**). Specifically, histological changes

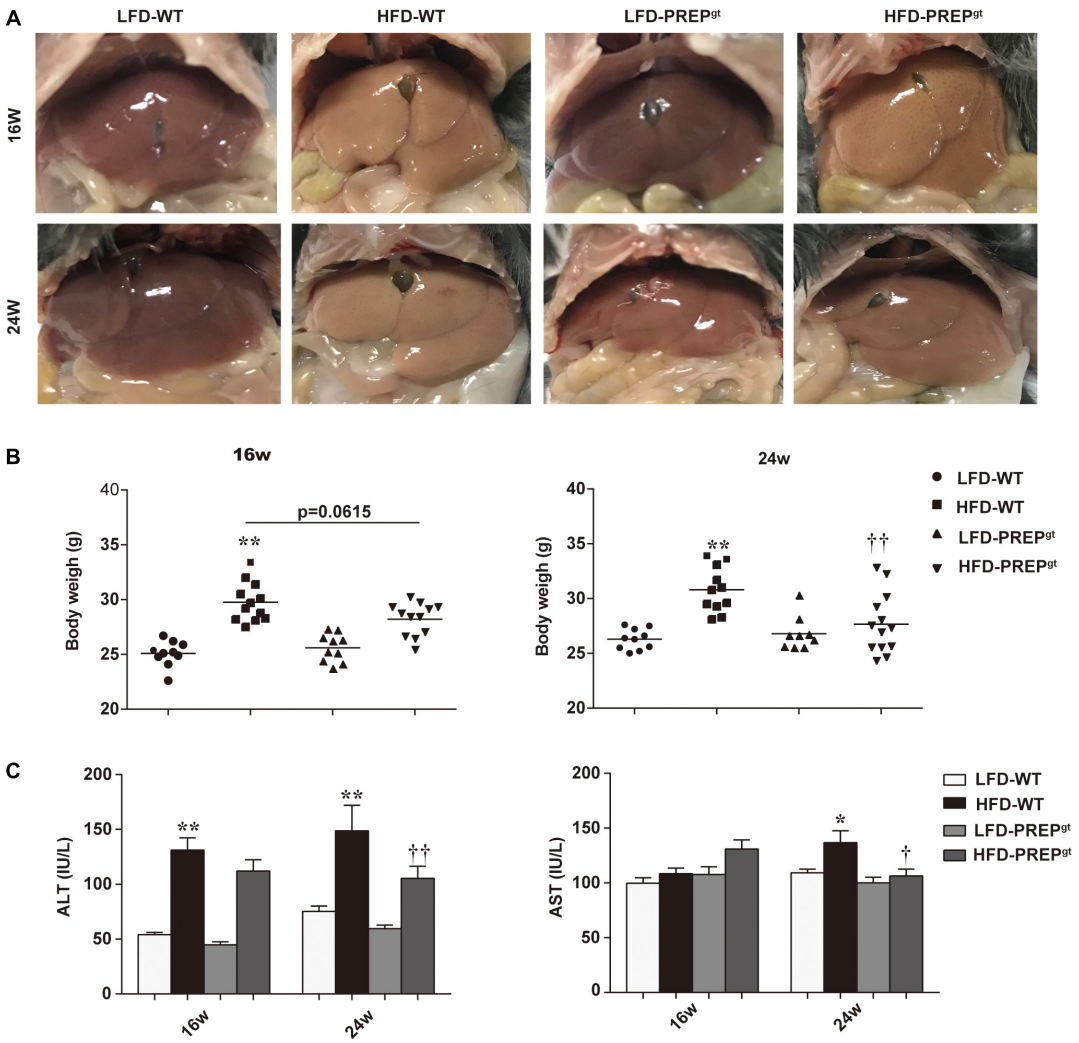

FIGURE 1 | PREP disruption reduced mice weight and protected against liver injury in HFD-fed mice as NAFLD progressed. **(A)** Representative gross pictures of the livers in different groups; **(B)** Body weight was measured at 16 and 24 w, respectively; **(C)** Plasma levels of ALT and AST were measured. All data are presented as the means or mean ± SEM (n = 8–13). $^{*}P < 0.05$ and $^{**}P < 0.01$, LFD-WT vs. HFD-WT; $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$, HFD-WT vs. HFD-PREP^{gt}.

improved in HFD-PREP^{gt} mice at both 16 and 24 weeks, NAS decreased by 29.51 and 27.82% compared with HFD-WT mice, respectively (**Figures 2A,B**). Hepatic triglyceride content increased significantly at both time points with HFD feeding, while the indexes of 16 and 24 weeks HFD-PREP^{gt} mice decreased by 35.75 and 43.41% compared with HFD-WT mice, respectively (**Figure 2C**). Hepatic cholesterol results from both time points were similar to hepatic triglycerides to a certain extent (**Figure 2D**).

PREP Disruption Dynamically Activates the AMPK/SIRT1 Pathway to Regulate Hepatic Lipid Synthesis in HFD-Induced NAFLD Mice at Different Time Points

To further understand the mechanisms that exacerbate NAFLD progression, we measured the hepatic AMPK/SIRT1 pathway's protein levels in mice. We observed downregulation of PAMPK and SIRT1 protein expression in 16 weeks HFD-WT mice compared with LFD-WT mice at the corresponding time (25.79 and 31.63%, respectively), while significant upregulation was observed in 16 weeks HFD-PREP^{gt} mice compared with the HFD-WT mice (150.47 and 54.54%, respectively; **Figures 3A,B**). The differences in P62 levels and the LC3B-II/LC3B-I ratios (autophagy-related proteins) between HFD-WT mice and HFD-PREP^{gt} mice at 16 weeks display no significance (**Supplementary Figure 1a**). We also determined the levels of downstream factors, such as sterol regulatory element-binding protein 1c (SREBP1c) and fatty acid synthase (FASN), to evaluate PREP disruption effects on lipid metabolism. Upregulation of SREBP1c and FASN were observed in 16 weeks HFD-WT mice, while significant downregulation was observed in HFD-PREP^{gt} mice at the corresponding time compared with the HFD-WT mice (43.17 and 37.1%, respectively; **Figures 3A,B**). In addition, liver

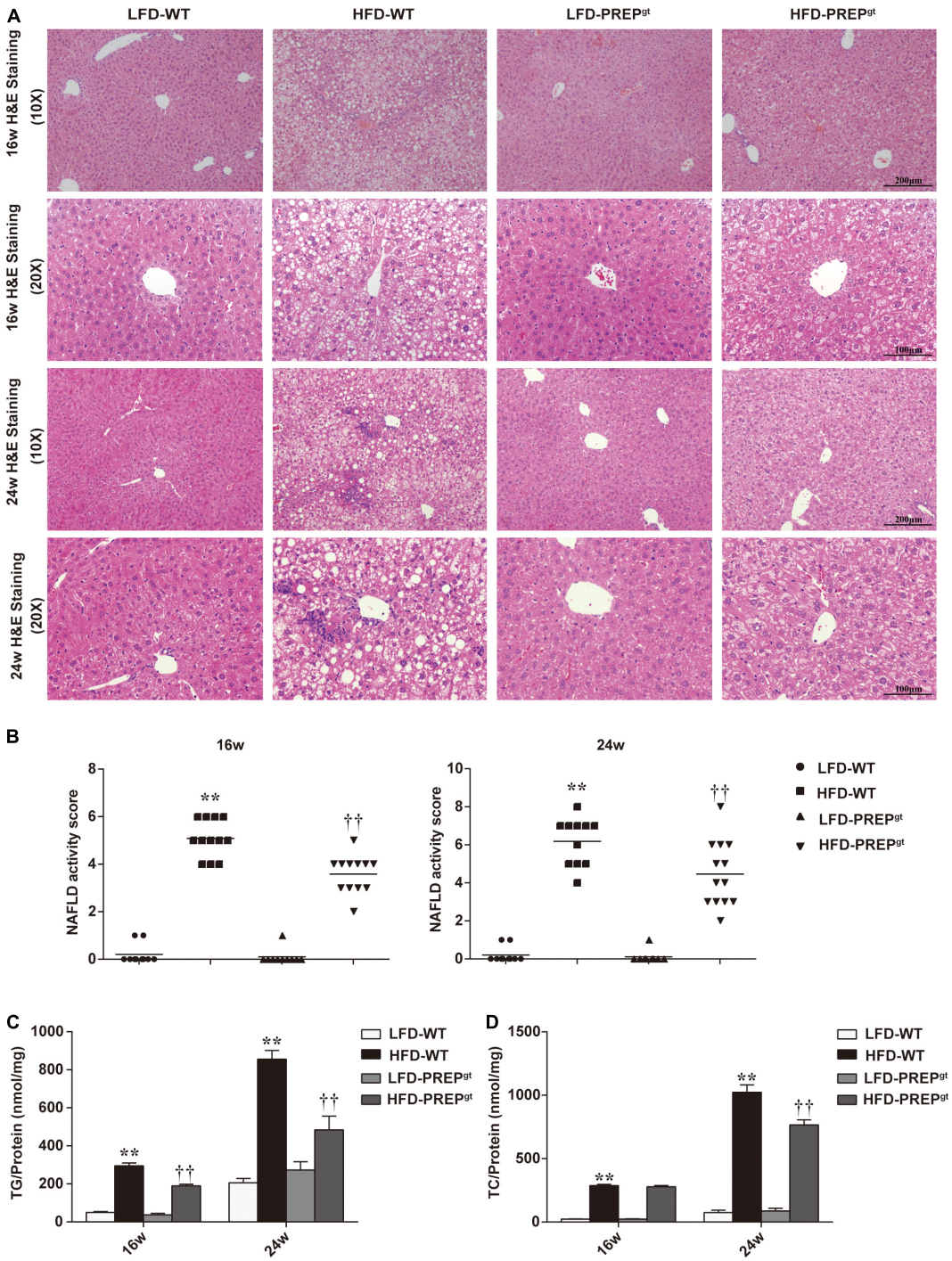

FIGURE 2 | Lipid accumulation and hepatic steatosis ameliorated by PREP disruption in the liver of HFD-fed mice as NAFLD progresses. **(A)** Liver sections were harvested and stained with hematoxylin-eosin (200× magnification) at 16 and 24 w; **(B)** NAS is calculated from the semi-quantitative evaluation of hepatic steatosis, lobular inflammation, and hepatocyte ballooning in different groups; hepatic levels of triglycerides **(C)** and cholesterol **(D)** were detected in mice. All data are presented as the mean or mean ± SEM (n = 8–13). $^{**}P < 0.01$, LFD-WT vs. HFD-WT; $^{\dagger\dagger}P < 0.01$, HFD-WT vs. HFD-PREP^{gt}.

mRNA levels of AMPK/SIRT1-mediated lipogenesis enzymes, such as acetyl-coenzyme A carboxylase (ACC), FASN, stearoyl-CoA desaturase1 (SCD1), SREBP1c, and CD36, were lower in the 16 weeks LFD-WT mice (40.17∼84.47%) and HFD-PREP^{gt} mice (44.58∼51.23%) compared with the corresponding levels in the HFD-WT mice (**Figure 3C**). However, PAMPK and SIRT1 protein levels were not significantly upregulated in 24 weeks HFD-PREP^{gt} mice compared to HFD-WT mice (31.47 and

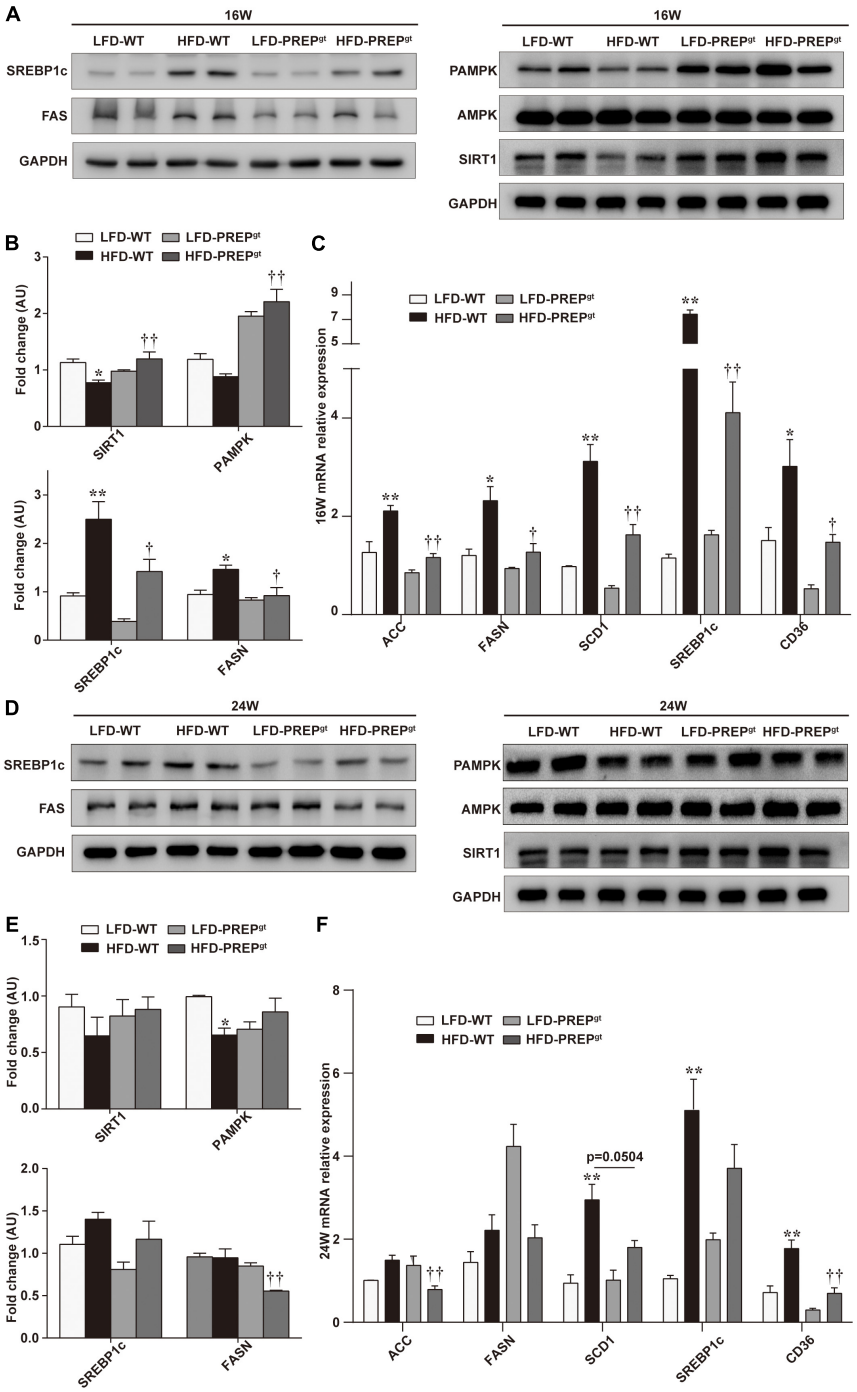

FIGURE 3 | PREP disruption dynamically activates the AMPK/SIRT1 pathway to regulate hepatic lipid synthesis in HFD-fed mice as lipid accumulation progresses. **(A)** Liver expression of total and phosphorylated AMPK (PAMPK) and SIRT1 proteins and their downstream molecules (SREBP1c and FASN) were detected at 16 w in mice using western blot analysis; **(B)** relative bar graphs display blot quantification analysis for 16 w mice; **(C)** hepatic mRNA levels of lipid synthesis-associated genes of 16 w mice were examined using RT-PCR; **(D)** protein levels of PAMPK/SIRT1, SREBP1c, and FASN in livers were detected in 24 w mice; **(E)** relative bar graphs display blot quantification analysis for 16 weeks mice; **(F)** hepatic mRNA levels of lipid synthesis-associated genes of 24 w mice were examined using RT-PCR. All data are presented as the mean ± SEM ($n = 4$). $^{*}P < 0.05$ and $^{**}P < 0.01$, LFD-WT vs. HFD-WT; $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$, HFD-WT vs. HFD-PREP^{gt}.

36.34%), along with the protein levels of SREBP1c and FASN, which were downregulated (16.78 and 41.46%; **Figures 3D,E**). Reduced P62 levels were observed in 24 weeks HFD-PREP^{gt} mice compared with HFD-WT mice, but the LC3B-II/LC3B-I ratios did not display a significant difference (**Supplementary Figure 1b**). However, the reduced LC3B-II/LC3B-I ratio in

24 weeks HFD-WT mice was more evident than that observed in 24 weeks HFD-PREP^{gt} mice than LFD mice, respectively (**Supplementary Figure 1b**). After 24 weeks, liver mRNA levels associated with the *de novo* lipogenesis pathway (ACC, FASN, SREBP1c, SCD1, and CD36) were lower in LFD-WT mice (32.38∼79.36%) and HFD-PREP^{gt} mice (7.95∼47.15%) relative to HFD-WT mice (**Figure 3F**). However, some differences did not display significance.

Hepatic Inflammation and Related Signal Molecules Were Attenuated by PREP Disruption in HFD-Fed Mice

Upon examination of hepatic inflammatory status, we observed that liver sections from HFD-PREP^{gt} mice contained fewer MPO-positive cells (neutrophils) and F4/80-positive cells (macrophages and Kupffer cells) compared with HFD-WT mice at 16 weeks (22.76 ± 3.31 vs. 39.76 ± 4.46, 22.62 ± 2.23 vs. 33.46 ± 13.32, respectively, **Figure 4A**). Moreover, the phosphorylation states of ERK (p-ERK) and nuclear factor κB (NFκB) p65 (p-p65) and related proinflammatory cytokines in livers were detected in different groups. However, differences in protein expressions of p-p65 and MMP9, including total MMP9 and its active form, were not statistically significant between HFD-WT mice and HFD-WT mice at 16 weeks (**Figure 4B,C**). p-ERK protein expression was significantly higher in HFD-WT mice than in LFD-WT mice, while this appeared downregulated in HFD-PREP^{gt} mice (P = 0.073; **Figure 4B**). Besides, hepatic mRNA levels of CCL2, tumor necrosis factor α (TNFα), interleukin 1β (IL1β), and IL6 were increased in the livers of HFD-WT mice and HFD-PREP^{gt} mice at 16 weeks; however, these differences were not statistically significant (**Figure 4D**). Interestingly, we detected the same indexes in mice's liver at 24 weeks, while results differed in terms of hepatic inflammation progression. Liver sections from 24 weeks HFD-PREP^{gt} mice contained fewer MPO-positive cells and F4/80-positive cells compared with HFD-WT mice at 24 weeks (13.71 ± 2.28 vs. 19.88 ± 3.91, 26.05 ± 2.53 vs. 57.82 ± 4.21, respectively; **Figure 5A**). HFD-fed PREP^{gt} mice also showed significant decreases in the phosphorylation states of p-ERK and p-p65, and mRNA levels of CCL2 and TNFα, compared with HFD-fed WT mice at 24 weeks (**Figures 5B,D**). Moreover, the active form of MMP9 was downregulated in HFD-PREP^{gt} mice compared with HFD-WT mice (32.6%), consistently with its protein level (**Figures 5B,C**). Furthermore, PGP production of HFD-WT mice markedly increased as hepatic inflammation progressed, and it was further downregulated in the liver of 24 weeks HFD-PREP^{gt} mice compared with 24 weeks HFD-WT mice (47.56%; **Figure 5E**).

PREP Gene Disruption Alleviated Gut Microbiota Dysbiosis in Mice Fed a HFD

The PREP^{gt} mice used in this study carry a partial exon three deletion in the PREP gene, which caused complete PREP protein loss. The liver PREP activity of HFD-WT mice was higher than that of LFD-WT mice at both time points. **Figures 6A,B** shows representative PREP western blot images (**Figure 6A**) and activity measurements (**Figure 6B**) of the liver in mice, respectively. In PREP^{gt} mice, specific PREP activity was significantly downregulated compared with WT mice (**Figure 6B**).

Fecal samples were collected at 16 and 24 weeks, and the microbiota composition was analyzed using 16S rRNA gene amplicon sequencing. First, the gut microbial profile at the phylum level was assessed. At the phylum level, we observed increased *Firmicutes* abundance and decreased *Bacteroidetes* abundance in the HFD-WT mice and the HFD-PREP^{gt} mice at 16 weeks (**Figures 6C,D**). However, the difference in the *Firmicutes* to *Bacteroidetes* ratio between the LFD-WT, and HFD-WT mice, was not statistically significant at 24 weeks (**Figures 6C,E**). Non-metric multidimensional scaling analysis and principal coordinate analysis showed that the overall composition of the gut flora expectedly changed in the 24 weeks HFD-WT mice, and the microbial profile slightly shifted in 24 weeks HFD-PREP^{gt} mice (**Figures 6F,G**). The overall composition of the gut flora in 16 weeks HFD-WT and 16 weeks HFD-PREP^{gt} mice was similar (**Figures 6F,G**). However, pathways related to energy and nutrient (amino acid, lipid, and glucose) metabolism were upregulated and downregulated in HFD-WT in HFD-PREP^{gt} mice, respectively, at both time points, suggesting that PREP may affect metabolic processes by regulating the gut flora (**Figure 6H**).

After analyzing the microbial profile in detail, levels of *Ruminiclostridium 9*, *Blautia, Corprocccus 2, Lachnospiraceae NK4A139, Oscillibacter*, and *Odoribacter* increased in the 16 weeks mice (**Figure 7A**). The levels of *Ruminiclostridium 9*, *Blautia*, *Lachnospiraceae NK4A139, Odoribacter*, *Intestinimonas*, and *Faecalibaculum* decreased in 24 weeks HFD-WT mice and increased after PREP gene knockout (**Figure 7B**). *Desulfovibrio*, *Romboutsia*, and *Bilophila* increased in HFD-WT mice and decreased in HFD-PREP^{gt} mice at 16 and 24 weeks (**Figures 7A,B**). The expression of SCFAs receptors (GPR41 and GPR43) decreased in HFD-WT mice and slightly improved after PREP disruption at 16 and 24 weeks (**Supplementary Figures 2a–d**). The level of butyrate in 16 weeks HFD-PREP^{gt} mice significantly increased compared with HFD-WT mice (**Supplementary Figure 2e**).

PREP Gene Disruption Diminished Damage to the Intestinal Epithelial Barrier in Mice Fed a HFD

We explored whether PREP loss exerted beneficial effects on the integrity of the intestinal barrier in mice under HFD stimulation. As shown in **Figure 8A**, we observed abnormal morphological alterations of intestinal mucosa in HFD-WT mice compared with LFD-WT mice and HFD-PREP^{gt} mice. A loss of normal villus structure in the terminal ileac epithelium was observed in HFD-WT mice (**Figure 8A**). The villus height and crypt depth were significantly decreased in the terminal ileum of HFD-WT mice compared with LFD-WT mice and HFD-PREP^{gt} mice; however, changes in the villus to crypt ratio were not evident (**Figures 8A,B**). Besides, we detected protein and mRNA expression levels of zonula occludens 1 (ZO1) and occludin. Protein levels of ZO1 and

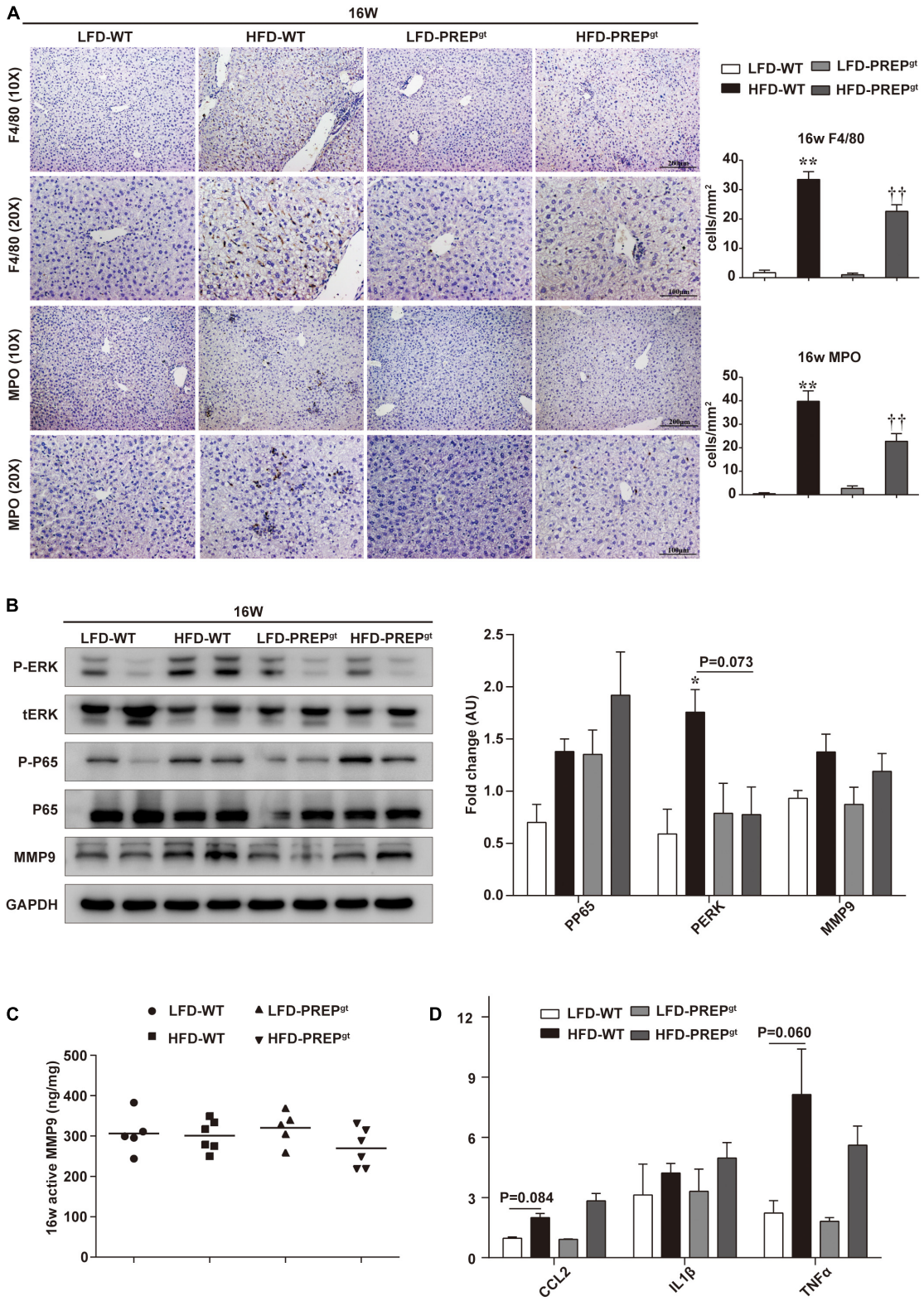

FIGURE 4 | Hepatic inflammation and expression of related signaling molecules were slightly attenuated by PREP disruption in HFD-fed mice at 16 w. **(A)** Liver sections harvested from mice at 16 w were stained with anti-F4/80 and anti-MPO; **(B)** protein levels of p-ERK, p-p65, and MMP9 were detected using western blot analysis; **(C)** hepatic active MMP9 levels were measured using fluorometric assays; **(D)** mRNA expression of proinflammatory cytokines. All data are presented as the mean or mean ± SEM (n = 4–6). *P < 0.05 and **P < 0.01, LFD-WT vs. HFD-WT. ††P < 0.01, HFD-WT vs. HFD-PREP^{gt}.

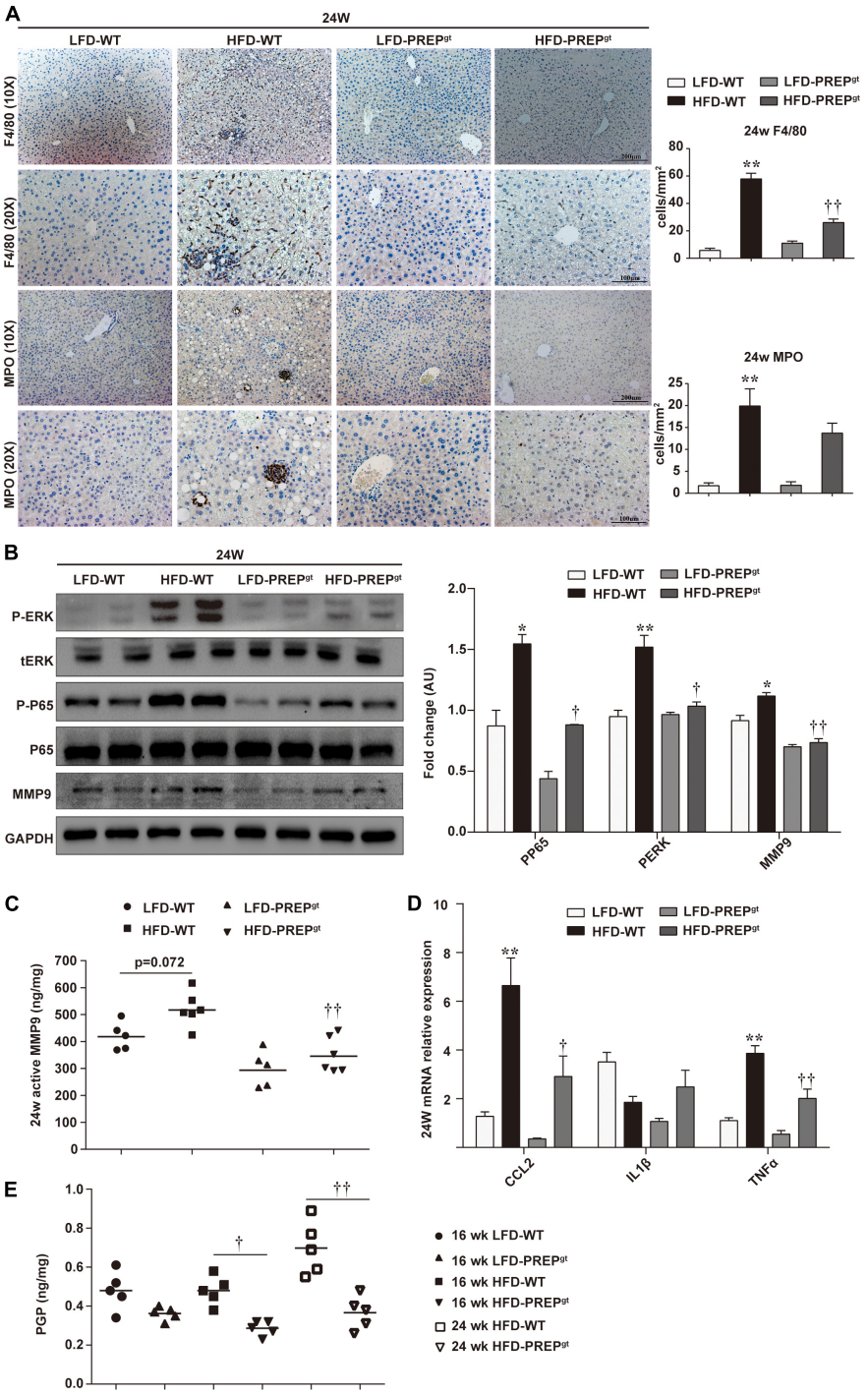

FIGURE 5 | Hepatic inflammation increased as NASH progressed, while PREP disruption suppressed the detrimental microenvironment at later stages of HFD-induced NASH. **(A)** Liver sections harvested from mice after 24 w were stained with anti-F4/80 and anti-MPO; **(B)** protein levels of p-ERK, p-p65, and MMP9 were detected using western blot analysis; **(C)** hepatic active MMP9 levels were measured using fluorometric assays; **(D)** mRNA expression of proinflammatory cytokines; **(E)** Production of PGP in the liver. All data are presented as the mean or mean ± SEM (n = 4–6). *P < 0.05 and **P < 0.01, LFD-WT vs. HFD-WT; $^{\dagger}P$ < 0.05 and $^{\dagger\dagger}P$ < 0.01, HFD-WT vs. HFD-PREP^{gt}.

occludin were reduced in the intestine of HFD-WT mice compared with LFD-WT mice, and the two were increased in HFD-PREP^{gt}, as shown by immunostaining and immunoblots (**Figures 8A,C**). mRNA expression of ZO1 and occludin were consistent with protein expression, respectively (**Figure 8D**). As shown in **Figure 8E**, elevated liver endotoxin levels were

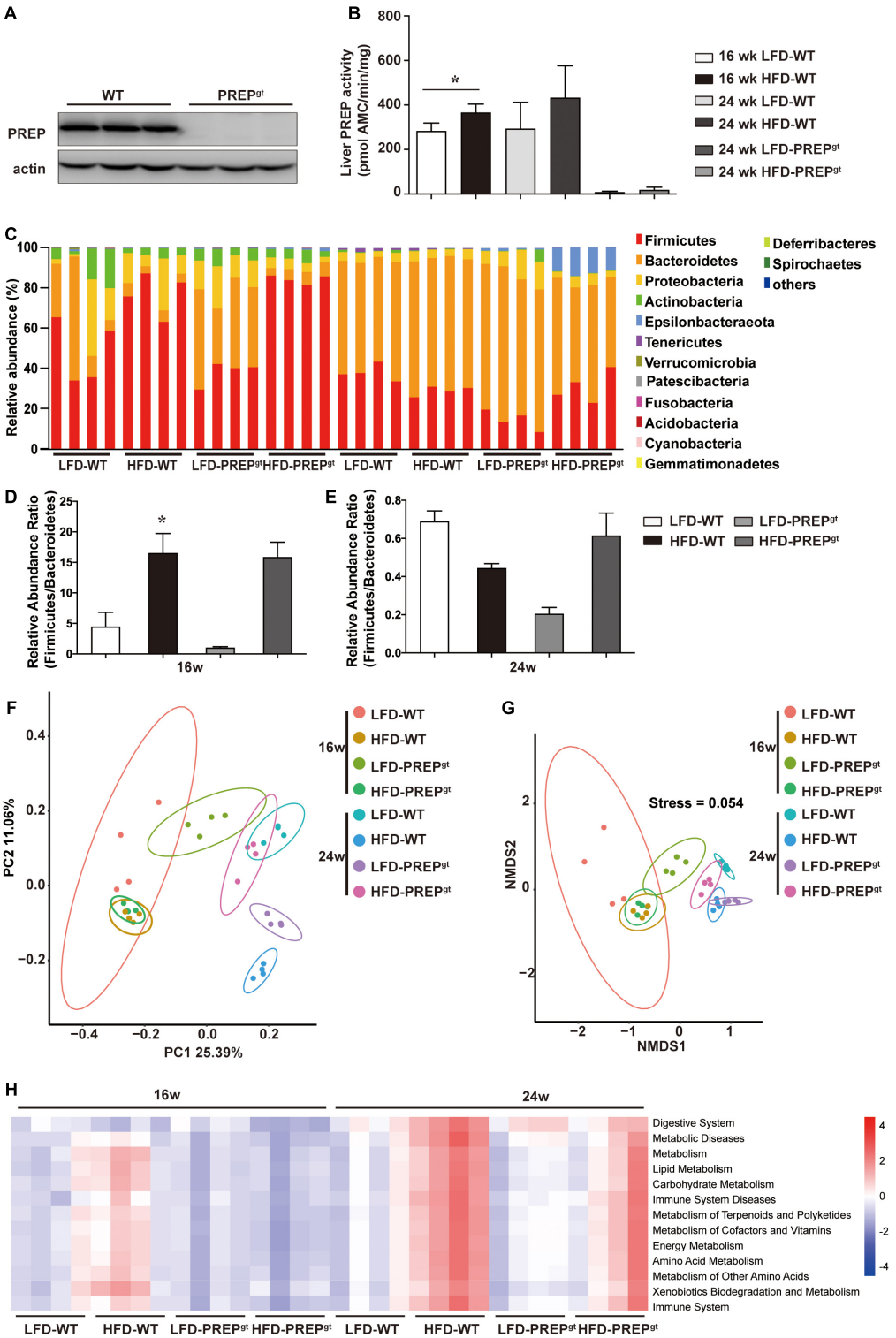

FIGURE 6 | PREP gene disruption improved the gut flora structure and altered the metabolic pathways involved with the microbiota of mice fed a HFD. **(A)** Representative anti-PREP western blot images of the liver of WT and PREP^{gt} mice; **(B)** PREP enzymatic activity measured (± SEM) in liver samples from WT and PREP^{gt} mice, $^*P < 0.05$; **(C)** relative abundance of taxa at the phylum level as 16S rRNA-based gut microbial profiling was performed; **(D)** the *Firmicutes* to *Bacteroidetes* ratio at 16 weeks mice, $^*P < 0.05$, LFD-WT vs. HFD-WT; **(E)** the *Firmicutes* to *Bacteroidetes* ratio at 24 weeks mice, $^*P < 0.05$, LFD-WT vs. HFD-WT; **(F)** principal coordinate analysis of an unweighted unifrac distance matrix; **(G)** non-metric multidimensional scaling analysis of an unweighted unifrac distance matrix. **(H)** prediction of the functional genes in the bacterial community in the gut, performed using PICRUSt ($n = 4$).

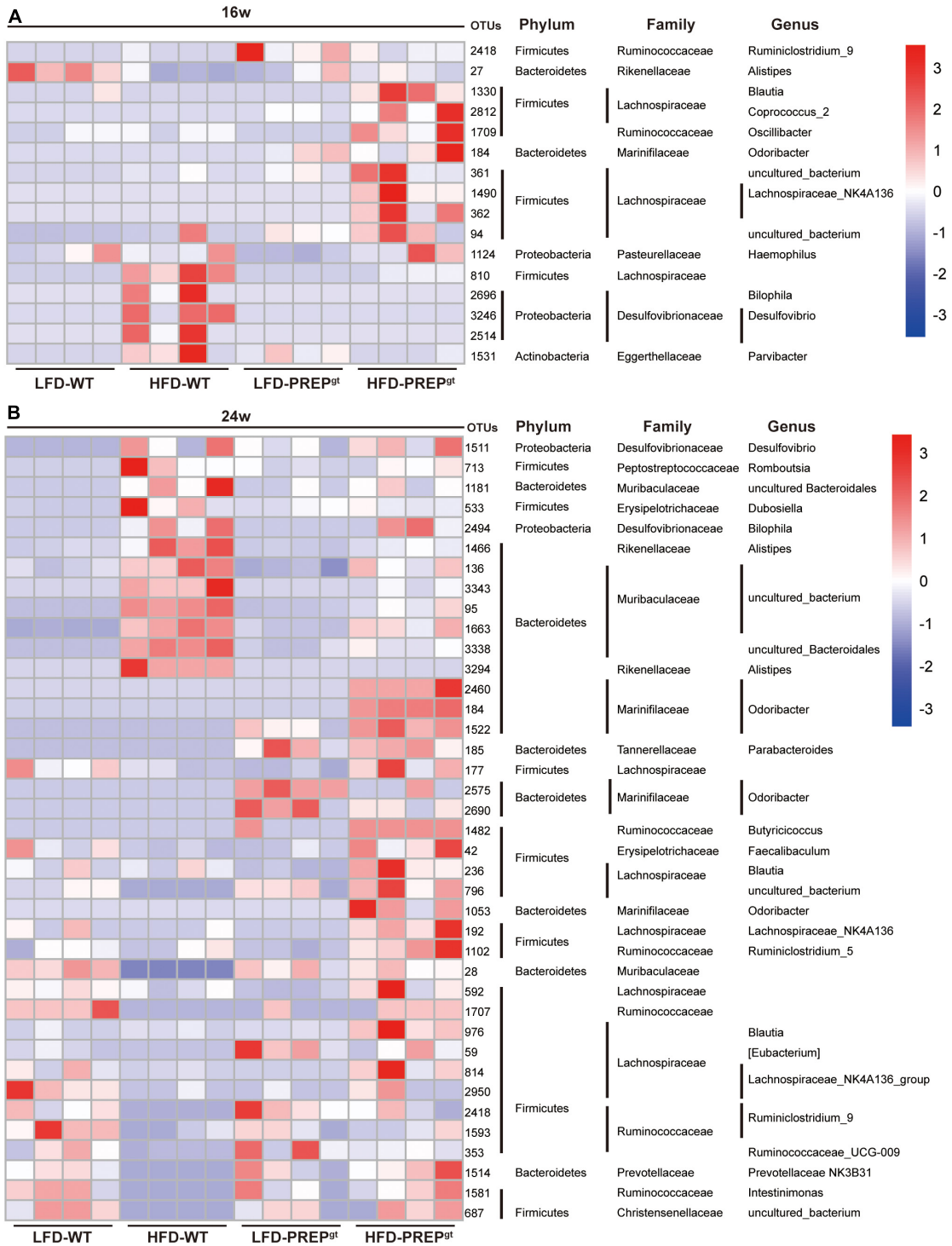

FIGURE 7 | (A) The abundance of OTUs and representative bacterial taxa information (phylum, family, and genus) of 16 w mice (n = 4); **(B)** the abundance of OTUs and representative bacterial taxa information (phylum, family, and genus) of 24 w mice (n = 4).

significantly increased in HFD-WT mice compared with LFD-WT mice. Although the indexes were slightly decreased in HFD-PREP^{gt} mice, this difference was not statistically significant (**Figure 8E**).

DISCUSSION

The involvement of the gut-liver axis in the pathogenesis and progression of NAFLD is increasingly being recognized. PREP

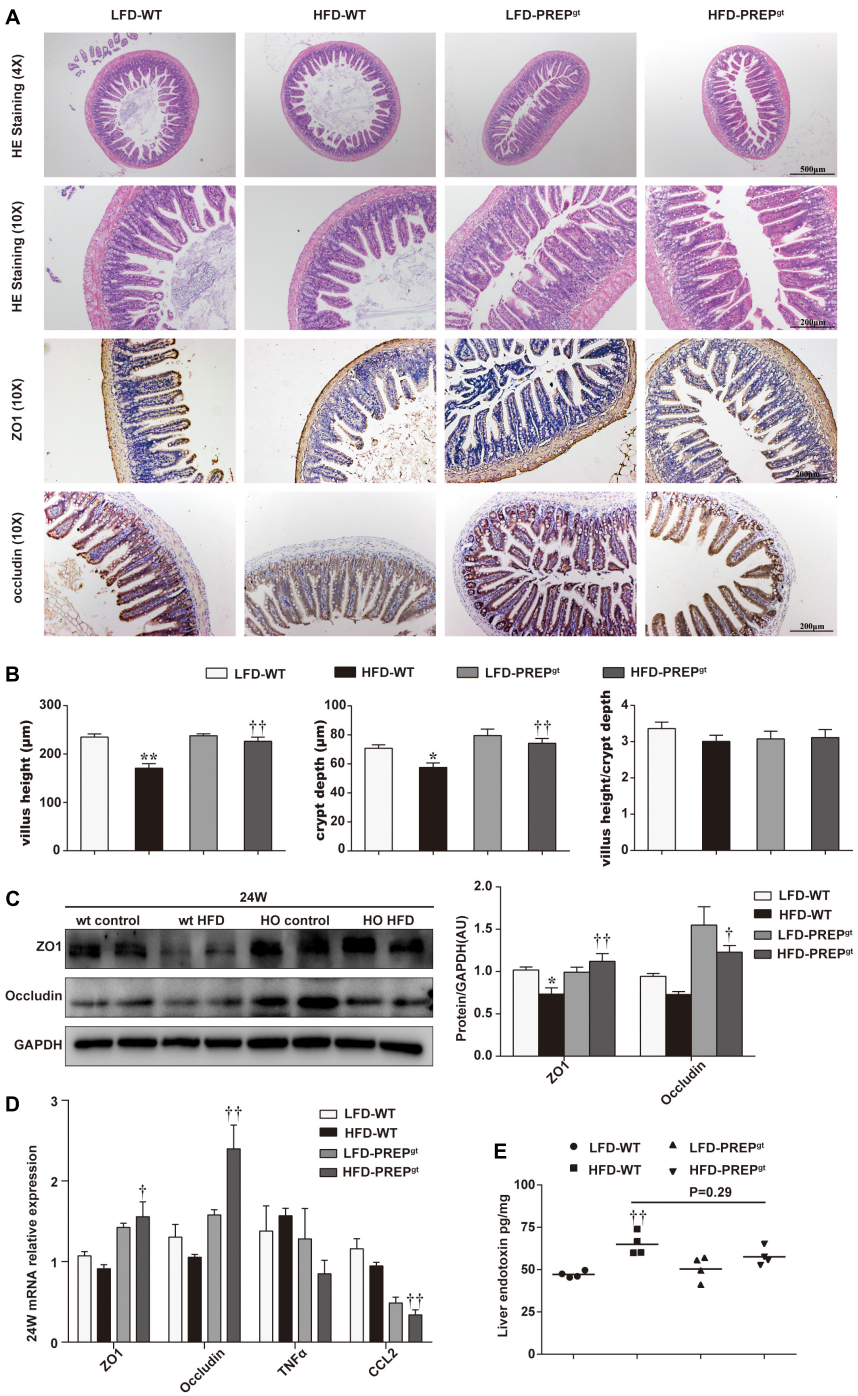

FIGURE 8 | Damage to the intestinal epithelial barrier in mice fed a HFD was reversed by PREP gene knockout. **(A)** Representative H&E staining and immunostaining of ZO-1 and occluding of the terminal ileum sections are shown; **(B)** as the villus height and the depth of the crypts were measured, the ratio of villus height to crypt depth was calculated; **(C)** protein levels of ZO-1 and occludin in the intestine were measured using western blot. Bar graphs display the quantification of western blots; **(D)** intestinal mRNA levels of tight junction protein-associated genes and inflammatory factors were examined using RT-PCR; **(E)** the hepatic level of endotoxin was measured using a mouse lipopolysaccharide ELISA kit. All data are presented as the mean or mean ± SEM (n = 4–6). *$P < 0.05$ and **$P < 0.01$, LFD-WT vs. HFD-WT; †$P < 0.05$ and ††$P < 0.01$, HFD-WT vs. HFD-PREP^{gt}.

action is quite complex, as it can induce metabolic benefits or proinflammatory damage to the intestinal environment (Koelink et al., 2014; Olivares et al., 2019). Our previous study found that PREP disruption plays a beneficial role in NAFLD progression, mainly through decreases in the number of chemokines (such as PGP) and inflammatory cell accumulation

(Jiang et al., 2020). Notably, different hepatic pathogenesis mechanisms have previously been described, and complex PREP actions have been reported; therefore, we carried out this study to imitate the different NAFLD stages to uncover PREP's influence on the mice gut. We found that (Younossi et al., 2016) hepatic lipid metabolism improved dynamically by activating the SIRT1/AMPK pathway via PREP disruption under HFD feeding conditions; (Saltzman et al., 2018) PREP disruption markedly improved hepatic proinflammatory status progression by inhibiting phosphorylated ERK and p65 as NASH progresses to more severe stages; (Aron-Wisnewsky et al., 2020) PREP disruption improves intestinal dysbiosis and protects against intestinal epithelial barrier damage induced by a HFD. Therefore, we provided compelling evidence to demonstrate that PREP inhibition effects may play various roles in NAFLD progression.

Numerous studies revealed that regulating the gut dysbiosis contributed to restraining NAFLD development (Si et al., 2018; Wang X. et al., 2019; Aron-Wisnewsky et al., 2020). When the gut microbiota is in dysbiosis, the host's health is compromised as the gut microbiota is unable to maintain control of local homeostasis, thereby increasing intestinal permeability (Saltzman et al., 2018). The modulation of the gut microbial profile by PREP disruption may prevent NAFLD by altering the relative abundance of several "beneficial indicators" in the cecum, thereby promoting homeostasis. *Odoribacter* and *Oscillibacter* are closely associated with intestinal epithelial homeostasis, while *Lachnospiraceae NK4A139* negatively correlated with serum lipid levels (Zhao et al., 2019; Mu et al., 2020). Besides, *Ruminiclostridium 9* belongs to the *Ruminococcaceae* family; reportedly, a high abundance of *Ruminococcaceae* in the cecum could effectively prevent malnutrition (Million et al., 2016). The short-chain fatty acid butyrate-producing bacteria, such as *Ruminococcaceae*, *Odoribacter*, *Intestinimonas*, and *Faecalibaculum* (Kang et al., 2021; Liu et al., 2021). Notably, the abovementioned bacteria were more abundant in HFD-PREP^{gt} than HFD-WT mice.

PREP inhibition may benefit flora homeostasis by suppressing protein fermentation, which reduces indole and phenol production, thus preventing the thinning of the intestinal mucous barrier. In addition, increased phosphorylation and activation of AMPK and its downstream lipid metabolism targets (Chiu et al., 2015) in the liver are associated with butyrate-producing bacteria (Leung et al., 2016). Butyrate bounds to endogenous GPR41- and GPR43-containing receptors in the liver, impacting lipid *de novo* synthesis (Lu et al., 2016). We only detected butyrate levels in the colon of 16 weeks mice; however, a previous study reported that sodium butyrate could delay the onset of early signs of NAFLD in mice (Jin et al., 2016). Our previous studies also indicated that PREP is closely related to energy metabolism and the downstream lipid metabolism targets of AMPK (Jiang et al., 2020). The functional consequences of this taxa shift and our previous work provide clues about how PREP inhibition may regulate flora homeostasis, trigger the AMPK signaling pathway, and improve liver lipid metabolism. Protective intestinal microbiota also associates other metabolites such as the specific bile acids, which promotes protection against NAFLD (Petrov et al., 2019). However, the potential relationship between the bile acids and PREP warrants further study.

Our study detected phosphorylated AMPK (PAMPK) in the liver after 24 weeks HFD feeding. The changes were not evident as the activation of PAMPK appeared slightly elevated with no statistical significance alongside its downstream molecules (SIRT1/SREBP1c/FASN (Teng et al., 2019)), although we observed a noticeable improvement in lipid accumulation in PREP knockout mice after 24 weeks HFD feeding. It is known that lipogenesis can be promoted by SIRT1-mediated inhibition of AMPK phosphorylation and activation, leading to hepatic steatosis (Srivastava et al., 2012; Teng et al., 2019). We should further consider that PREP expression levels, protein distribution, and activity correlate with aging and are reported in many neurodegenerative conditions (Svarcbahs et al., 2019). Besides, aging promotes the development of diet-induced murine steatohepatitis, but not steatosis (Fontana et al., 2013), and hepatic steatosis and inflammation may contribute to the development of NAFLD via different pathways, respectively (Mahli et al., 2018). We hypothesized that PREP might affect NAFLD progression at different time points. To verify our conjecture, we investigated the early stage of NASH – before the 24-week HFD feeding model. Interestingly, activation of PAMPK/SIRT1 and improvements in lipid metabolism were pronounced in the liver of 16 weeks HFD-PREP^{gt} mice. Autophagy could be activated through PREP inhibition via protein phosphatase 2A in the brain (Svarcbahs et al., 2020). Further, autophagy may be mediated directly by the AMPK/SIRT1 pathway in hepatic steatosis (Wang Y. et al., 2019). As NASH progressed, we observed apparent autophagy damage in 24 weeks HFD-WT mice compared with LFD-WT mice; however, this improved upon PREP gene knockout. However, it is unclear which pathway is responsible for the dynamic autophagy changes observed during NASH, and further research on the subject is warranted. Since PREP disruption improved intestinal flora disorders and results showed the liver in different NAFLD stages, we concluded that PREP inhibition might improve lipid metabolism via the PAMPK/SIRT1 pathway in early NAFLD stages when lipogenesis plays a major role compared to inflammation.

However, hepatic lipotoxicity and inflammation are not easily separated, as hepatic lipotoxicity-induced wound healing requires subsequent inflammation, remodeling of the hepatic vasculature and matrix, and outgrowth of liver progenitors (Suzuki and Diehl, 2017). Tissue outside the liver (such as adipose tissue or the gut) and processes within the organ (for instance, lipotoxicity) contribute to NASH development (Schuster et al., 2018). Besides tracking the lipid metabolic benefits from gut dysbiosis improvement, our results indicated that PREP gene disruption attenuates mucosal lesions caused by HFD feeding. Dysbiosis increases gut permeability to bacterial products and increases hepatic exposure to injurious substances that increase hepatic inflammation and fibrosis (Leung et al., 2016). Notably, *Bilophila* and *Desulfovibrio* are gram-negative endotoxin-producing bacteria known to increase intestinal permeability and circulate gut-derived antigens, primarily LPS (Moreno-Indias et al., 2016; Zhuang et al., 2020).

On the one hand, compared with the 24 weeks HFD-WT mice, the corresponding HFD-PREP^{gt} mice display a lower

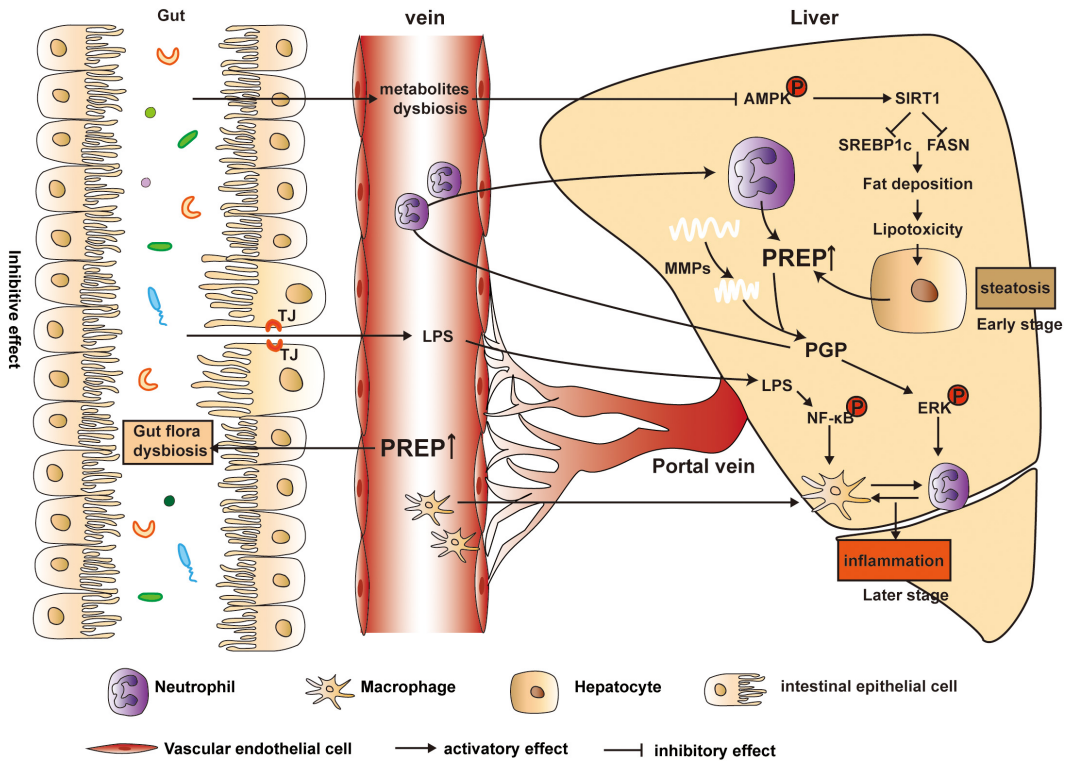

FIGURE 9 | Model of the proposed mechanism underlying PREP disruption-mediated dynamic amelioration of hepatic steatosis and inflammation via intestinal dysbiosis regulation, AMPK/SIRT1 pathway activation, and inflammatory signaling pathway inhibition.

abundance of *Desulfovibrio* and decreased hepatic LPS content, although this was not statistically significant. On the other hand, a former study indicated that PREP might play a role in microglial activation since PREP knockout mice lack a response to LPS (Hofling et al., 2016). Therefore, we hypothesize that the combined effects of PREP disruption on gut dysbiosis and its response to inflammatory triggers inhibit NASH progression. Interestingly, factors associated with proinflammation and its related signaling molecules showed dynamic changes in HFD-PREP^{gt} mice upon hepatic inflammation progression. Our previous works demonstrated that PREP could potentially affect the progression of hepatic inflammation, possibly by regulating chemotactic factors (such as PGP and MMPs) (Jiang et al., 2020), and in this study, we found this effect was more important and evident in the later and more severe stage of NAFLD. This observation may be partially explained by the fact that NASH is considered a potentially progressive disorder, as liver inflammation may prompt collagen matrix synthesis and deposition (Suzuki and Diehl, 2017), which PREP and MMP9 hydrolyze to produce PGP (chemotaxis of neutrophils) (Weathington et al., 2006; Gaggar et al., 2008). Nevertheless, endotoxin-mediated TLR4/NF-κB pathway activation in macrophages reportedly plays a pivotal role in NASH pathogenesis (Zhao et al., 2019). PREP increased PGP production, possibly activating ERK and facilitating crosstalk between neutrophils, which release MPO and lipocalin2, and macrophages to exacerbate their migration and activation (Ye et al., 2016; Jiang et al., 2020). Based on the above, our findings demonstrate that HFD-induced NAFLD status in mice was alleviated to varying degrees by PREP disruption, contributing to the remission of gut flora dysbiosis and hepatic inflammation (**Figure 9**).

Our study also has some limitations. First, the specific PREP deletion mechanisms (such as in the liver and gut) in mice need to be further explored. We believe that their complexity and heavy workload warrant further study. Second, we have reported the effect of PREP-specific inhibitors (S17092) on lipid synthesis *in vitro* (Zhou et al., 2016). A PREP inhibitor suitable for use in vivo experiments is still in progress. Third, we did not explore the complex relationship between PREP, autophagy, and the microbiota during NAFLD progression, which warrants future studies.

CONCLUSION

In summary, PREP disruption may target multiple detrimental hepatic mechanisms related to systems, including the liver, macrophages, neutrophils, the gut, and microbiota, which may show dynamic changes during NAFLD progression. Our study demonstrates that PREP disruption dynamically ameliorates hepatic steatosis and inflammation by regulating intestinal dysbiosis, activating the AMPK/SIRT1 pathway, and inhibiting the inflammatory signaling pathway. Therefore, targeting PREP

may be a viable therapeutic or preventive approach for the management of NAFLD.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by all animal experiments followed the National Research Council's Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of SHRM (SHRM-IACUC-022).

AUTHOR CONTRIBUTIONS

DJ and JZ performed most experiments. DJ and SL analyzed and interpreted the data. YC, YW, and JF designed and coordinated the research. DJ and YC drafted 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/fcell.2021.628143/full#supplementary-material

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Integrated Metagenomic and Transcriptomic Analyses Reveal the Dietary Dependent Recovery of Host Metabolism From Antibiotic Exposure

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The balance of gut microbiome is essential for maintaining host metabolism homeostasis. Despite widespread antibiotic use, the potential long-term detrimental consequences of antibiotics for host health are getting more and more attention. However, it remains unclear whether diet affects the post-antibiotic recovery of gut microbiome and host metabolism. In this study, through metagenomic sequencing and hepatic transcriptome analysis, we investigated the divergent impacts of short-term vancomycin (Vac), or combination of ciprofloxacin and metronidazole (CM) treatment on gut microbiome and host metabolism, as well as their recovery extent from antibiotic exposure on chow diet (CD) and high-fat diet (HFD). Our results showed that short-term Vac intervention affected insulin signaling, while CM induced more functional changes in the microbiome. However, Vac-induced long-term (45 days) changes of species were more apparent when recovered on CD than HFD. The effects of antibiotic intervention on host metabolism were long-lasting, antibiotic-specific, and diet-dependent. The number of differentially expressed gene was doubled by Vac than CM, but was comparable after recovery on CD as revealed by the hepatic transcriptomic analysis. In contrast, HFD intake during recovery could worsen the extent of post-antibiotic recovery by altering infection, immunity, and cancer-related pathways in short-term Vac-exposed rats and by shifting endocrine system-associated pathways in CM-exposed rats. Together, the presented data demonstrated the long-term recovery extent after different antibiotic exposure was diet-related, highlighting the importance of dietary management during post-antibiotic recovery.

Keywords: antibiotics, post-antibiotic recovery, diet, gut microbiota, metabolism

INTRODUCTION

The human intestine is colonized by trillions of microbes that participate in many physiological and pathological processes of host including energy metabolism, xenobiotic metabolism, and immune response (Lozupone et al., 2012; Festi et al., 2014; Valdes et al., 2018). A stable and healthy gut microbiota structure is essential for maintaining metabolic homeostasis (Rinninella et al., 2019; Fan and Pedersen, 2021), preventing colonization by pathogens (Pickard et al., 2017; Ducarmon et al., 2019), improving immunity (Pickard et al., 2017), and extending lifespan (Biagi et al., 2016; Vaiserman et al., 2017). Mounting evidence in humans and rodents has shown that disruption of gut microbiota homeostasis and loss of bacterial diversity could cause metabolic disorders, neurological and immunological diseases, and impaired immunotherapy response (Gilbert et al., 2016; Gopalakrishnan et al., 2018a,b; Routy et al., 2018; Aron-Wisnewsky et al., 2020).

Many factors affect the composition of gut microbiota, such as antibiotic administration (Quigley, 2017). Antibiotics have been widely used to prevent and treat bacterial infections in humans and animals since the 1940s (Lewis, 2013; McPherson et al., 2018). However, exposure to antibiotics reduces the diversity of gut microbiota and leads to dysbiosis sometimes (Ianiro et al., 2016). There is increasing concern about the potential long-term detrimental consequences of antibiotic use for host health (Blaser, 2014; Cox and Blaser, 2015; Neuman et al., 2018). *Clostridium difficile* infection-associated diarrhea is a common consequence of antibiotic use (Guh and Kutty, 2018). Early life exposure to low-dose penicillin led to long-term increased adiposity, amplified diet-induced obesity (Cox et al., 2014), increased brain cytokine expression, and altered behavior in animal models (Leclercq et al., 2017). In addition, antibiotic-driven changes in microbiota affected glucose homeostasis and murine immune response and increased susceptibility to allergic asthma (Russell et al., 2012; Russell et al., 2013; Fujisaka et al., 2016). Epidemiological studies have found that early exposure to antibiotics is associated with subsequent development of obesity, inflammatory bowel diseases, allergic diseases, and detrimental neurodevelopmental outcomes (Hviid et al., 2011; Trasande et al., 2013; Forrest et al., 2017; Han et al., 2017; Hirsch et al., 2017; Slykerman et al., 2017; Mitre et al., 2018; Slykerman et al., 2019). These findings suggest that antibiotic-induced microbiome disruption can have long-term substantial effects on host health. However, how to eliminate the detrimental consequences of antibiotic use for host metabolism should particularly be given more attention.

Post-antibiotic recovery of gut microbiota composition is essential for the long-term health of host. Many studies have demonstrated that gut microbiota recovery following antibiotic treatment can be incomplete and the differential recovery of gut microbiota to the same antibiotic treatment is associated with their initial gut microbiota structure (Dethlefsen and Relman, 2011; Raymond et al., 2016a,b; Chng et al., 2020). For example, higher initial microbial diversity is positively correlated with better recovery from antibiotic-induced dysbiosis (Dethlefsen and Relman, 2011; Raymond et al., 2016a,b). In addition, certain bacterial species and enriched carbohydrate-degradation and energy-production pathways exhibit a robust association with post-antibiotic recovery (Chng et al., 2020). Nevertheless, besides the initial gut microbiota composition, our understanding on the factors that affect post-antibiotic recovery process is very limited. Diet is another critical factor influencing the composition of the gut microbiota within days (Carmody et al., 2015; Gentile and Weir, 2018; Kolodziejczyk et al., 2019; Zmora et al., 2019). Unbalanced diet was found to affect host health, such as causing metabolic disorders, immunological diseases, and neurological diseases (Brandsma et al., 2015; Gentile and Weir, 2018; Riccio and Rossano, 2018; Zmora et al., 2019; Tong et al., 2020). However, it is not clear whether and how environmental factors, such as diet, accelerate or impede the process of post-antibiotic recovery from different antibiotic-induced dysbiosis, which, in turn, affects the host phenotype and metabolism.

In this study, we investigated the divergent impacts of a 5-day intervention with two antibiotic regimens (vancomycin, Vac, or combination of ciprofloxacin and metronidazole, CM) on gut microbiota composition and host metabolism in rats, as well as the recovery from antibiotic exposure on either chow diet (CD) or high-fat diet (HFD) for 45 days. Our results revealed that the impacts of antibiotic exposure on host metabolism were long-lasting, antibiotic-specific, and diet-dependent. Compared to the effect of CD on post-Vac recovery, HFD affected post-Vac recovery of host metabolism and significantly regulated infection, immunity, and cancer-related pathways. In sum, diet plays a critical role in the recovery of host metabolism after antibiotic intervention, which points to the fact that the dietary management should particularly be given more attention at the post-antibiotic recovery stage.

MATERIALS AND METHODS

Antibiotic Intervention Experiment

Male SD rats of 200-g body weight (BW) were provided by the Laboratory Animal Center of Shanghai University of Traditional Chinese Medicine (Shanghai, China). Rats were orally administrated with vehicle or vancomycin (100 mg/kg per dose) or combination of ciprofloxacin (50 mg/kg per dose) and metronidazole (50 mg/kg per dose) twice daily for 5 days. The dose was doubled at the first and last administration. For the insulin intervention experiment, 8 min after insulin injection (10 U/kg), rats were euthanized and tissues were collected for protein analysis (Stahel et al., 2017). For the recovery experiment, rats were fed with chow diet or high-fat diet for 45 days after antibiotic intervention. All rats were housed in a 12-h light (7 am to 7 pm) and 12-h dark (7 pm to 7 am) cycle, with free access to water and diet. The experiments were conducted under the Guidelines for Animal Experiment of Shanghai University of Traditional Chinese Medicine, and the protocol was approved by the institutional Animal Ethics Committee.

Glucose and GTTs

Glucose-tolerance tests were performed on fasted rats (15 h, paper bedding) by monitoring glucose levels after a glucose bolus

(1 g/kg of BW) by intraperitoneal injection (IP). Data acquisition was carried out at 0, 15, 30, 60, 90, and 120 min after injection. For the diet stimulation experiment, blood glucose was measured on fasted rats (15 h) and re-fed rats (2 h) (Dick et al., 2015).

Biochemical Analysis

Serum biochemical indices such as triglycerides (TG), cholesterol (TC), alanine aminotransferase (ALT), aspartate aminotransferase (AST), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) were determined according to the instructions of specific kits (Nanjing Jiancheng Bioengineering Institute, China). Serum insulin was measured according to the instructions of Elisa kit (Briggs et al., 2014). The liver tissues were added with lysate and ground with magnetic beads. After centrifugation, the supernatant was taken for the determination of TC and TG according to the instructions.

Quantification of Protein

Liver and muscle protein lysates (20 mg) were subjected to polyacrylamide gel electrophoresis under reducing conditions followed by transfer to polyvinylidene difluoride membranes. The membranes were incubated with 5% skimmed milk followed by antibodies specific for p-AKT (Cell Signaling Technology, Danvers, MA, United States), AKT (Cell Signaling Technology, Danvers, MA, United States), p-IR (Cell Signaling Technology, Danvers, MA, United States), IR (Cell Signaling Technology, Danvers, MA, United States), and β-ACTIN (Sigma-Aldrich, Shanghai, China). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies. The signals were detected using an enhanced chemiluminescence (ECL) system with Pierce SuperSignal West Pico chemiluminescent substrates (Biyuntian Biotechnology, Shanghai, China).

Histological Evaluation on the Degree of Hepatic Steatosis

Liver tissues were fixed with 10% neutral formalin for 24 h, embedded in paraffin, stained with hematoxylin–eosin staining (H&E), and sections were observed for the degree of hepatic steatosis under the light microscope. The degree of hepatic steatosis was evaluated according to a previous publication in a blinded way (Kleiner et al., 2005). The criteria for scoring include 0 (absent), 1 (rare), 2 (mild), 3 (moderate), and 4 (severe).

16S rRNA Sequencing

Fecal DNA was isolated using the Qiagen QIAamp DNA Stool Mini Kit (Qiagen, Dusseldorf, Germany). Illumina sequencing was done based on published methods (Ma et al., 2020). The V3–V4 region of the 16S ribosomal RNA gene was amplified and sequenced. Sequence reads were analyzed using QIIME software 1.9.1 (Caporaso et al., 2010). Functional profiles of microbial communities for 16S rRNA sequencing were predicted using PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States). Sequence data associated with this project have been deposited in the NCBI Short Read Archive (SRA) database (Accession Number: PRJNA702866).

Metagenomics

Total genomic DNA was extracted from fecal samples using the E.Z.N.A. tissue DNA Kit (Omega Bio-tek, Norcross, GA, United States) according to the manufacturer's instructions. The concentration and purity of extracted DNA were determined with TBS-380 and NanoDrop 2000, respectively. DNA extract quality was checked on 1% agarose gel. Read analysis was performed according to our previous publication (Hong et al., 2020). Adapter sequences were stripped from the 3′ and 5′ end of paired-end Illumina reads using SeqPrep[1]. Reads with length <50 bp or a quality value <20 or having N bases would be considered as low-quality and be removed by Sickle[2]. The hit would be removed if it was associated with the reads, which was aligned to the Rattus norvegicus genome by BWA,[3] or their mated reads. MEGAHIT, which utilized succinct de Bruijn graphs, was used to assemble data[4] (Li et al., 2015). Only contigs with the length being or over 300 bp were selected as the final assembling result and were used for further gene prediction and annotation. Open reading frames (ORFs) from each assembled contig were predicted using MetaGene[5] (Noguchi et al., 2006). The predicted ORFs with length being or over 100 bp were retrieved and translated into amino acid sequences using the NCBI translation table[6]. Then, CD-HIT would be used for clustering predicted genes with a 95% sequence identity (90% coverage[7]) (Fu et al., 2012); the longest sequences from each cluster were selected as representative sequences to construct a nonredundant gene catalog. Reads after quality control were mapped to the representative sequences with 95% identity using SOAPaligner[8] (Li et al., 2008), and gene abundance in each sample was evaluated. The Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation was conducted using BLASTP (Version 2.2.28+) against the KEGG database (Xie et al., 2011[9]) with an e-value cutoff of $1e^{-5}$. The higher-order functional information is stored in the KEGG PATHWAY database, which contains three different classification levels (Kanehisa and Goto, 2000). In this study, we focused on KEGG pathway level 1 and level 3. Sequence data have been deposited in the NCBI SRA database (Accession Number: PRJNA719272).

RNA Sequencing Analysis

Total RNA was extracted from the liver using TRIzol Reagent according to the manufacturer's instructions (Invitrogen), and genomic DNA was removed using DNase I (TaKara). Then, RNA quality was determined by 2100 Bioanalyzer (Agilent) and quantified using ND-2000 (NanoDrop Technologies). Only the high-quality RNA sample was used to construct the sequencing

[1]https://github.com/jstjohn/SeqPrep
[2]https://github.com/najoshi/sickle
[3]http://bio-bwa.sourceforge.net
[4]https://github.com/voutcn/megahit
[5]http://metagene.cb.k.u-tokyo.ac.jp/
[6]http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/index.cgi?chapter=tgencodes#SG1
[7]http://www.bioinformatics.org/cd-hit/
[8]http://soap.genomics.org.cn/
[9]http://www.genome.jp/keeg/

library. Illumina HiSeq X Ten/NovaSeq 6000 Sequencing and reading were finished based on the published method (Ma et al., 2020). Sequence data were deposited in the NCBI SRA database (Accession Number: PRJNA713932).

Bioinformatics and Statistical Analysis

Shannon index and Simpson index are the measures of biodiversity (α diversity). Shannon index is intended to quantify both richness and evenness of the species/individuals in the ecosystem or community (Shannon, 1997). Shannon index was calculated as follows: $H_{shannon} = -\sum_{i=1}^{S_{obs}} \frac{n_i}{N} ln \frac{n_i}{N}$. Based on the assumption on the probability of obtaining similar species samples randomly in an infinite community, Simpson index takes into account the number of species present, as well as the abundance of each species (Simpson, 1949). Simpson index is calculated as follows: $D_{simpson} = \frac{\sum_{i=1}^{S_{obs}} n_i(n_i-1)}{N(N-1)}$, where S_{obs} = the number of OTUs actually observed; n_i = the number of sequences contained in the ith OTU; and N = number of all sequences. Bray–Curtis as the distance algorithm is used to represent β diversity; its calculation is based on the independent taxa (such as OTU, genus, etc.), the weighted calculation method is adopted, and the existence and abundance of species are considered at the same time, but without considering the evolutionary relationship or association information among species (Beals, 1984). The Bray–Curtis dissimilarity index was calculated as follows: $D_{Bray-Curtis} = 1 - 2\frac{\sum \min(S_{A,i}, S_{B,i})}{\sum S_{A,i} + \sum S_{B,i}}$, where $S_{A,i}$ = the number of sequences contained in the ith taxon in sample A; $S_{B,i}$ = the number of sequences contained in the ith taxon in sample B. Data shown in this study are expressed as mean ± standard error of mean (SEM) unless otherwise noted. Differences between groups at microbiota phylum and genus levels were calculated by the Mann–Whitney U test. Other comparisons were calculated by two-tailed Student's *t*-test. $p < 0.05$ was considered statistically significant.

RESULTS

Short-Term Antibiotic Exposure Divergently Alters the Composition and Function of Gut Microbiota

SD rats were given vancomycin (Vac group), combination of ciprofloxacin and metronidazole (CM group), or water (Con group) twice daily for 5 days on CD (**Figure 1A**). The 16S rDNA amplicon sequencing of fecal bacteria showed reduced richness and evenness after antibiotic interventions (**Figures 1B,C**). The three groups clustered separately as shown by Bray–Curtis analysis and hierarchical cluster tree on OTU level, suggesting a significant alternation of the gut microbiota structure (**Figures 1D,E**). Compared with the Con group, the Vac group had increased relative abundance of Proteobacteria and Tenericutes and reduced Bacteroidetes, while the CM group showed increased relative abundance of Firmicutes and reduced Proteobacteria and Bacteroidetes, resulting in an increased Firmicutes-to-Bacteroidetes ratio in both Vac and CM groups (**Figure 1F**). At the genus level, the relative abundance of 36 genera were differentially altered by both Vac and CM, while Vac uniquely altered 18 genera (11 upregulated and 7 downregulated) and CM uniquely altered 5 genera (4 upregulated and 1 downregulated) (**Supplementary Figure 1**). Among them, the dominant bacteria were changed from *Norank_f_Bacteroidates_S24-7_group* (19.4%) and *Helicobacter* (10.3%) in the Con group to *Norank_f_clostridiates_vadinBB60_group* (23.8%) and *Anaeroplasma* (20.5%) in the Vac group (**Figure 1G**). Most strikingly, the proportion of *Lactobacillus* genus increased to 81% after CM intervention.

These compositional and diversity changes of gut microbiota induced by Vac or CM were accompanied by functional alternations. Based on the KEGG pathway at level 1, Vac intervention impacted metabolism and organismal systems, whereas CM intervention affected all pathways except cellular processes (**Figure 1H**). At KEGG level 3, CM induced more pathway changes ($p < 0.01$, fold change (FC) < 0.5 or > 2) compared with Vac-induced changes (130 vs. 48 pathways) (**Figure 1I**). Among them, 32 pathways were found to be in common between Vac- and CM-induced pathway changes, and half of them were metabolic pathways (**Figure 1J**). In addition, most of the 16 unique pathway changes caused by Vac intervention were related to metabolism and human diseases based on KEGG pathway analysis at level 1 (**Figure 1K**), while the top 15 of CM-induced pathways were metabolic pathways including amino acid biosynthesis and fatty acid metabolism (**Figure 1L**). In summary, short-term intervention with Vac or CM significantly decreased microbial diversity and altered potential functions of gut microbiota, in which CM had more impact on regulating gut microbial function than Vac in rats.

Short-Term Antibiotic Exposure Affects Insulin-Signaling Pathway

Since the influences of antibiotic intervention on glucose tolerance have been previously reported (Suez et al., 2014; Vrieze et al., 2014; Fujisaka et al., 2016), we expected to test whether the impacts on glucose homeostasis were different when different antibiotics regimens were applied. Results showed that the fasting serum insulin level was comparable among the three groups, whereas the fasting serum glucose levels were higher in both Vac and CM groups than in the Con group (**Figure 2A**). Interestingly, the serum glucose level of the Vac group remained higher than that of the Con group, but was normalized in CM group 2 h after feeding (**Figure 2A**). These findings suggested that short-term exposure of Vac and CM affected glucose homeostasis under fasting condition, while Vac caused worse glucose control compared to CM under fed conditions.

Impaired insulin signaling is the important cause for dysglycemia (Kubota et al., 2017). Thus, the expression of proteins involved in insulin signaling pathways was measured in rats that were challenged with insulin injection in the context of either Vac or CM pretreatment. The results showed that insulin increased the protein levels of both phosphate-insulin

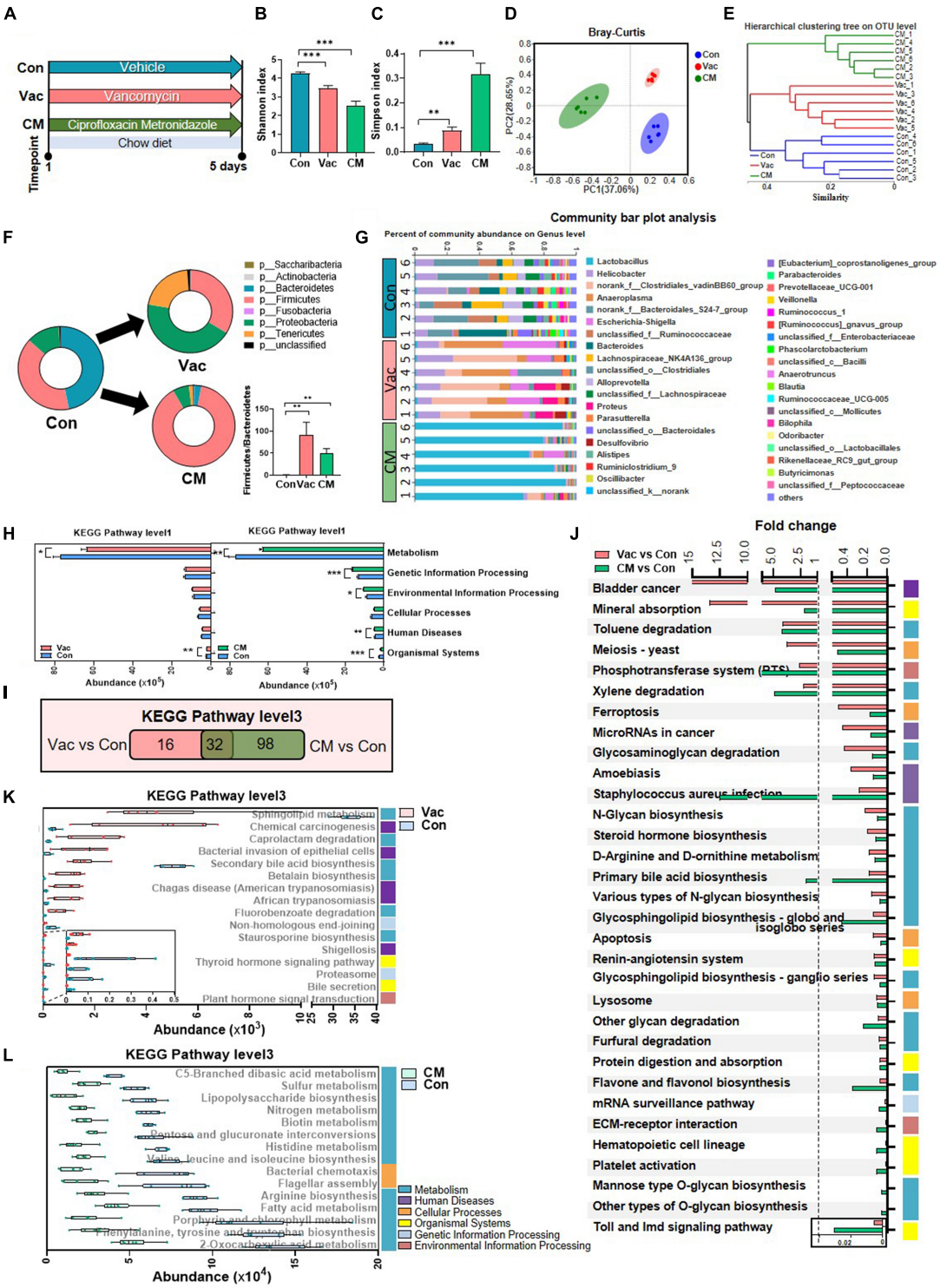

FIGURE 1 | The effects of Vac and CM intervention on regulating gut microbiota composition and function. Rats were given vancomycin (Vac) or combination of ciprofloxacin and metronidazole (CM) by oral gavage twice daily. Rats in the control group (Con) were orally administrated with water. **(A)** Intervention process chart. **(B)** Shannon index. **(C)** Simpson index. $^{**}p < 0.01$, $^{***}p < 0.001$ by Student's *t*-test. **(D)** Principal co-ordinate analysis based on the Bray–Curtis distance algorithm on OTU level. **(E)** Hierarchical tree of sample clustering. **(F)** Fecal microbiota at phylum level by 16S rRNA sequencing. **(G)** Fecal microbiota at the genus level. **(H)** The function differences between Vac vs. Con and CM vs. Con on KEGG pathway level 1 predicted by PICRUSt2. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ by the Mann–Whitney U test. **(I)** Venn diagram of the overlap of differential enriched pathways between Vac vs. Con and CM vs. Con by PICRUSt2 analysis on KEGG pathway level 3 ($p < 0.01$ under the Mann–Whitney U test, fold change (FC) <0.5 or >2). **(J)** The 32 common pathways between Vac vs. Con and CM vs. Con. **(K)** The 16 unique pathways between Vac and Con groups. **(L)** Top 15 unique pathways between CM and Con groups. $n = 6$ per group.

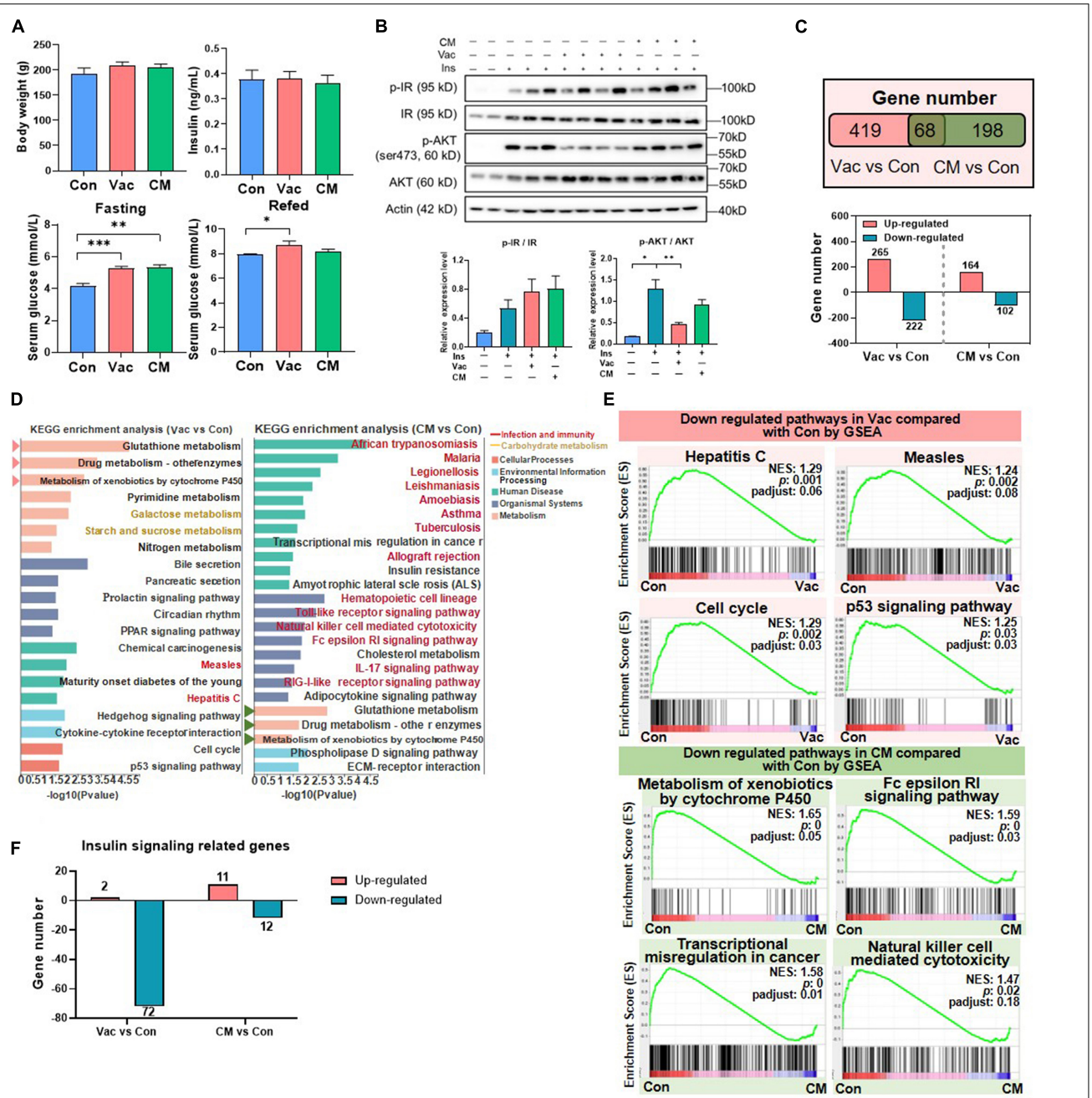

FIGURE 2 | The impact of antibiotic intervention on rat phenotype and liver transcriptome. **(A)** Body weight, fasting serum insulin, fasting serum glucose, and fed serum glucose after antibiotic intervention. **(B)** Western blot analysis of hepatic protein level. Rats were injected with insulin 8 min before tissues were collected. For index determination, n = 5–6 per group; for protein analysis, n = 2–4 per group; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ by Student's t-test. **(C)** Venn diagram of the overlap of differential expressed genes between Vac vs. Con and CM vs. Con ($p < 0.05$ based on edgeR software, and FC $\leq$0.5 or $\geq$2). **(D)** KEGG pathway enrichment analysis of 487 and 266 differential genes. Triangles on the left showed the same pathways between Vac vs. Con and CM vs. Con. **(E)** The same pathways between Gene Set Enrichment analysis (GSEA) and KEGG pathway enrichment analysis. **(F)** Different regulatory effects of Vac and CM on insulin related genes. n = 4 per group for transcriptome.

receptor (p-IR) and IR, resulting in an increased trend of the p-IR/IR ratio in the liver, and this trend was not changed by either Vac or CM. Meanwhile, insulin challenge also significantly elevated the expression of both p-AKT and AKT, as well as the p-AKT/AKT ratio. Vac, not CM, pretreatment markedly reduced p-AKT expression leading to a decreased p-AKT/AKT ratio, suggesting impaired hepatic insulin signaling in Vac-pretreated rats (**Figure 2B**). In contrast, Vac or CM pretreatment did not affect the insulin signaling pathway in the gastrocnemius (**Supplementary Figure 2B**). Together,

although the fasting insulin levels were comparable among the three groups, Vac and CM elevated the fasting blood glucose level, demonstrating that impaired insulin signaling occurred in Vac- and CM-treated rats. Further re-fed experiment and reduced hepatic p-AKT level and p-AKT/AKT ratio suggested impaired insulin signaling under fed conditions, especially in the Vac group.

Short-Term Antibiotic Exposure Alters Hepatic Transcriptome

Given the critical roles of gut microbiota in host metabolism (Fan and Pedersen, 2021), we further compared the impacts of short-term Vac or CM pretreatment on host metabolism by using the hepatic transcriptome approach. Compared with the Con group, Vac pretreatment induced 487 differentially expressed genes, while there were only 266 genes that were regulated in the CM group (**Figure 2C**). The KEGG enrichment analysis of these differentially expressed genes revealed that 20 and 24 pathways were significantly altered by Vac and CM, respectively (**Figure 2D**). The top three altered metabolic pathways in the Vac group were also found to be changed in CM. Additionally, Vac regulated carbohydrate metabolism, such as galactose metabolism and starch and sucrose metabolism. CM mainly affected infection and immunity-related pathways, as well as insulin resistance and cholesterol metabolism.

To further explore Vac- and CM-induced changes of biological function, Gene Set Enrichment Analysis (GSEA) was employed to identify significantly enriched biological pathways on the basis of normalized enrichment score (NES) ranking. Compared with the Con group, Vac and CM regulated 67 and 44 gene sets (NES $>$ 1 or $<$-1, $p < 0.05$, p-adjust < 0.25), respectively (**Supplementary Figures 2C–E**). Some pathways that were shifted by both KEGG enrichment analysis and GSEA are shown in **Figure 2E**, suggesting the importance and significance of these pathways that respond to different antibiotic treatments. We also noticed that Vac intervention has a more extensive disturbance effect on insulin signaling-related genes than CM intervention, and most genes were downregulated (**Figure 2F** and **Supplementary Figure 2F**). Because orally administered vancomycin is poorly absorbed in the gut, we speculate that Vac-induced changes of gut microbiota might be correlated with hepatic insulin dysregulation. In sum, Vac and CM had divergent effects on regulating hepatic gene expression where Vac pretreatment affected more gene expression than CM. The metabolic pathways were mainly altered by Vac, whereas pathways of infection and immunity were predominantly changed by CM.

Gut Microbiota Composition and Function Are Partly Restored After 45 Days Recovery on Chow Diet

Gut dysbiosis caused by antibiotics can persist for extended periods. To study the recovery extent of gut microbial profile and function after a short-term Vac or CM pretreatment, metagenomic analysis was performed when rats recovered on CD for 45 days (**Figure 3A**). Although long-term (45 days) CD intake recovered the general structure of gut microbiota in the Vac_CD (post-Vac recovery under CD) and CM_CD (post-CM recovery under CD) groups, revealed by alpha and beta diversity analysis (**Figures 3B-D**), many bacterial species and potential microbial functions were still significantly different among groups. At the species level, the relative abundance of 63 species and 15 species was different between CD and Vac_CD and between CD and CM_CD ($p < 0.05$), respectively. In particular, four species of top 10 species that changed in the Vac_CD group belonged to *Lactobacillus*, indicating that the difference of *Lactobacillus* might account for the difference of the gut microbiota profile between the Vac_CD and CD groups after recovery (**Figure 3E**). Additionally, among the significantly changed species in the CM_CD group, four belonged to *Firmicutes bacterium* in CM_CD and two species belonged to *Faecalibacterium*. Moreover, the biggest reduction at species level after recovery was *Firmicutes bacterium* CAG:110, which was consistently reduced in the Vac_CD and CM_CD groups (**Figure 3F**).

We next predicated the potential function of gut microbiota to study whether bacterial function was restored after recovery from antibiotic exposure. At KEGG level 1, environmental information processing, organismal systems, and cellular processes were increased in the CM_CD group, while no pathway was altered in the Vac_CD group (**Figure 3G**). At KEGG level 3, 11 and 49 pathways were significantly different between Vac_CD and CD groups and between CM_CD and CD groups ($p < 0.05$), respectively (**Figures 3H–J**). Pathways of starch and sucrose metabolism and cyanoamino acid metabolism, which were not different after antibiotic perturbation, were found to be increased in both the Vac_CD and CM_CD groups. In addition, the number of differential pathways narrowed after recovery under CD when compared with the number after antibiotic intervention, suggesting partly recovery of intestinal bacterial structure and function in rats.

Physiological Status After 45 Days Recovery From Antibiotic Exposure on Chow Diet

Pretreatment with CM reduced energy intake during the prolonged CD feeding, resulting in slightly lower BW compared to the CD group (**Figures 4A,B**). In addition, the levels of serum TG and HDL tended to be decreased in the Vac_CD and CM_CD groups, while serum LDL tended to be increased (**Figures 4C–E**). Moreover, CM_CD has reduced the hepatic TC level, while the levels of serum TC, LPS, hepatic TG, and liver histology were comparable between groups (**Figure 4F** and **Supplementary Figures 3A,B**). The fasting glucose level was a little bit lower in the Vac_CD group, and the fasting insulin levels were significantly lower in both Vac_CD and CM_CD groups (**Figures 4G,H**). However, the area under the curve (AUC) of the glucose tolerance test revealed a similar glucose clearance capacity in the three groups (**Figure 4I**). These findings suggested the enhanced insulin capacity of regulating glucose homeostasis in the Vac_CD and CM_CD groups. Post-antibiotic

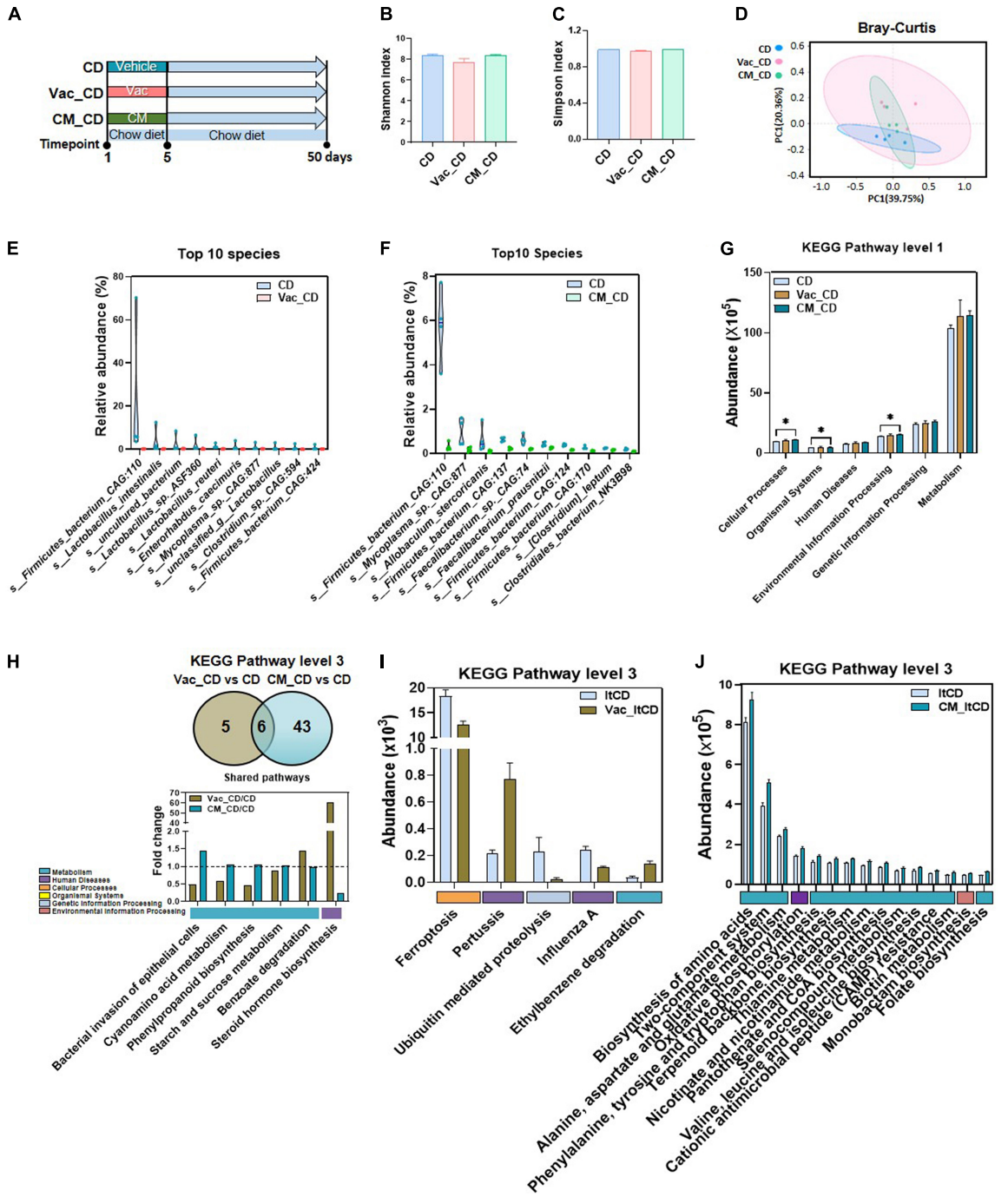

FIGURE 3 | The long-lasting impact of antibiotic treatment on bacterial composition and function when rats were fed with chow diet. **(A)** Intervention process chart. Rats were treated with vehicle, Vac, or CM for 5 days and then fed with chow diet for another 45 days. **(B)** Shannon index. **(C)** Simpson index. **(D)** Principal co-ordinate analysis based on the Bray–Curtis distance algorithm on species level. **(E,F)** Top 10 differential species between Vac_CD and CD and between CM_CD and CD ($p < 0.05$ by the Mann–Whitney U test). **(G)** The function differences among groups on KEGG pathway level 1 predicted by PICRUSt2. *$p < 0.05$ by Student's *t*-test. **(H)** Venn diagram of the overlap of differential enriched pathways between Vac_CD vs. CD and CM_CD vs. CD by PICRUSt2 analysis on KEGG pathway level 3 ($p < 0.05$ under the Mann–Whitney U test). The overlapped pathways between Vac_CD vs. CD and CM_CD vs. CD. **(I)** The unique shifted pathways between Vac_CD and CD. **(J)** Top 15 unique shifted pathways between CM_CD and CD.

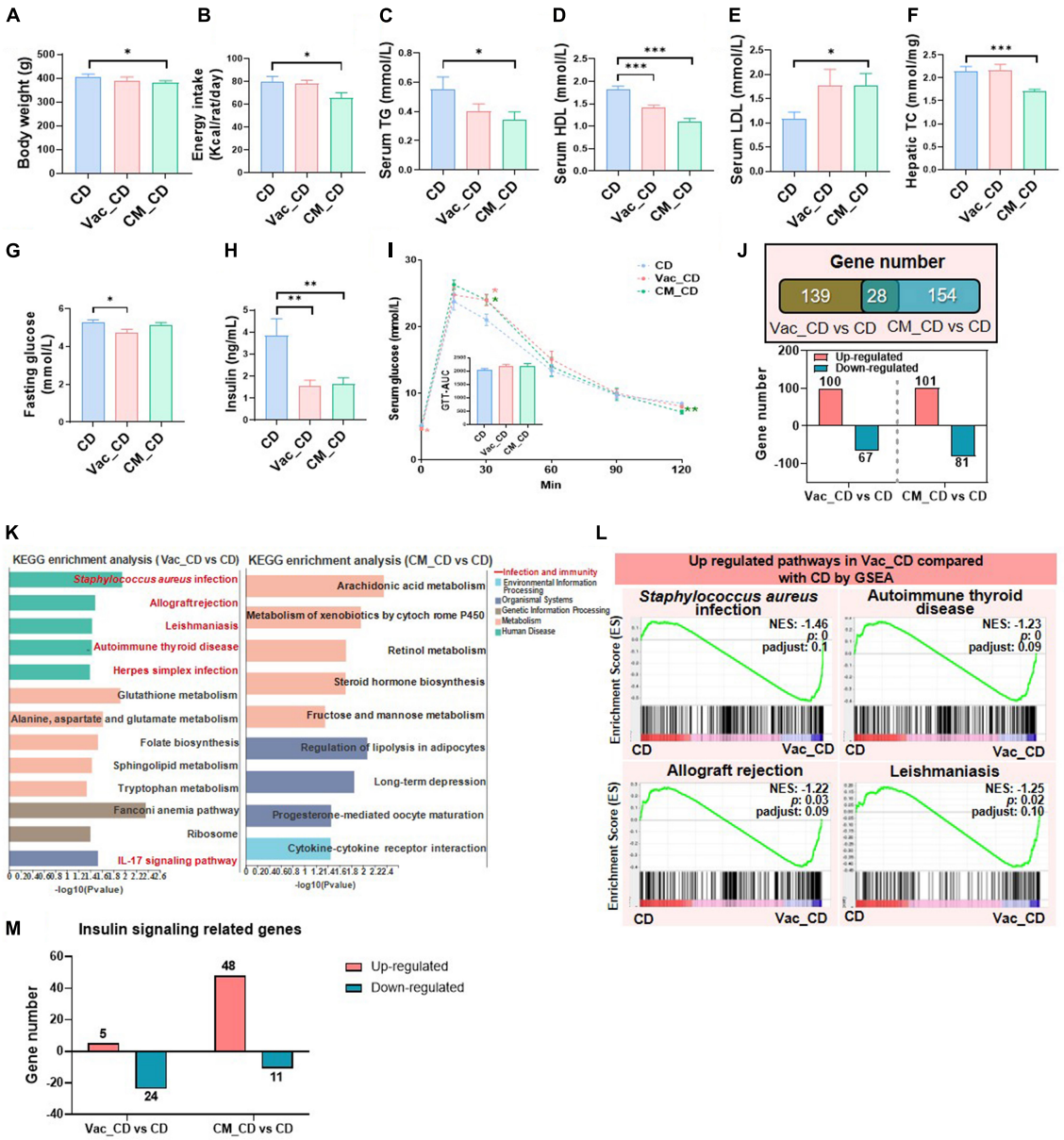

FIGURE 4 | The long-lasting impact of antibiotic treatment on rat phenotype and liver transcriptome when rats were fed with chow diet. The body weight **(A)**, energy intake **(B)**, serum triglyceride level **(C)**, serum high-density lipoprotein level **(D)**, serum low-density lipoprotein level **(E)**, and liver total cholesterol level **(F)** were shown at the end of the 45-day recovery. **(G–I)** Fasting blood glucose level, fasting blood insulin level, and glucose tolerance test result. **(J)** Venn diagram of the overlap of differential expressed genes between Vac_CD vs. CD and CM_CD vs. CD ($p < 0.05$ based on edgeR software, and FC ≤0.5 or ≥2). **(K)** KEGG pathway enrichment analysis of 167 and 182 differential genes. **(L)** The same pathways between Gene Set Enrichment analysis (GSEA) and KEGG pathway enrichment analysis. **(M)** Different regulatory effects of Vac and CM on insulin-related genes. n = 9–10 per group for biochemical analysis, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ by Student's t-test; n = 3 per group for transcriptome, Mann–Whitney U test.

recovery of gut microbiota reversed impaired insulin signaling in rats fed with CD.

Liver Gene Expression Is Partly Restored After 45 Days Recovery on Chow Diet

Since gut microbiota composition was partly restored after 45 days CD feeding, we next assessed the status of host metabolism by analyzing the hepatic transcriptome. First of all, the number of differentially expressed genes was comparable between Vac_CD and CM_CD groups, both of which were greatly reduced after 45 days recovery on CD (**Figures 2C**, **4J**). The KEGG enrichment analysis revealed that 13 pathways were altered in the Vac_CD group ($p < 0.05$) (**Figure 4K**). However, nine pathways were enriched in the CM_CD group ($p < 0.05$), four of which were related to sugar and lipid metabolism, such as arachidonic

acid metabolism, steroid hormone biosynthesis, fructose and mannose metabolism, and regulation of lipolysis in adipocytes (**Figure 4K**). Meanwhile, four pathways were commonly found by KEGG enrichment analysis and GSEA in the Vac_CD group, including *Staphylococcus aureus* infection, allograft rejection, leishmaniasis, and autoimmune thyroid disease (**Figure 4L** and **Supplementary Figure 3C**). Interestingly, post-Vac recovery on CD increased the number of differentially expressed genes of the insulin-related pathway when compared with short-term Vac intervention, while post-CM recovery on CD had the opposite effect (**Figures 2F**, **4M**). In addition, the number and specific expression panel of altered genes in this pathway were largely different in the Vac_CD or CM_CD group, where more genes were kept upregulated in CM_CD but downregulated in the Vac_CD group compared with the CD group (**Figure 4M** and **Supplementary Figure 3D**). Altogether, these results suggested that the host metabolic responses to short-term antibiotic exposure were long-lasting and antibiotic-specific.

The Recovery of Gut Dysbiosis After the 45-Day High-Fat Diet Feeding

High-fat diet is an important factor for influencing gut microbiota composition resulting in the changes of host metabolism (Gentile and Weir, 2018; Zmora et al., 2019). To further evaluate the dietary impact on post-antibiotic recovery, rats were switched to HFD for 45 days after a short-term antibiotic intervention (**Figure 5A**). In general, similar with the results after recovery on CD, the Shannon and Simpson indices as well as PCoA results showed no significant difference among HFD, Vac_HFD (post-Vac recovery under HFD), and CM_HFD (post-CM recovery under HFD) groups (**Figures 5B–D**). At species level, the number of significant differential species between pre-Vac-treated and untreated groups was dramatically reduced from 63 when rats were recovered on CD to 14 when recovered on HFD, while the differential number between pre-CM-treated and untreated groups was increased from 15 when recovered on CD to 21 when recovered on HFD. Among the top 10 significant differential species between Vac_HFD and HFD groups, four species belonged to *Desulfovibrio*, indicating the importance of *Desulfovibrio* for the difference of gut microbiota structure during recovery on HFD (**Figure 5E**). In addition, the CM_HFD group had markedly increased *s_Lactobacillus_johnsonii* (the fold change was 2.85) and decreased three species of *Lachnospiraceae_bacterium* and three species of *Desulfovibrio* compared to the HFD group (**Figure 5F**).

Further prediction on the potential function of gut microbiota demonstrated that the cellular process pathway was reduced in the CM_HFD group compared to the HFD group at KEGG pathway level 1 (**Figure 5G**). At level 3, the number of altered pathways was greatly reduced after recovery on HFD than recovery on CD. Vac_HFD and CM_HFD only altered 14 and 22 pathways, respectively, in which two pathways were commonly changed by both (**Figure 5H**). The most significant changes after recovery on HFD was the induction of sugar metabolism and insulin signaling-related pathways in Vac_HFD, including fructose and mannose metabolism, glucagon signaling pathway, insulin resistance, and insulin signaling pathway, while the glucagon signaling pathway was increased in both Vac_HFD and CM_HFD (**Figures 5I,J**). Different with the findings that most KEGG pathways were elevated in Vac_HFD, most pathways were decreased in CM_HFD compared to the HFD group. Together, the results suggested that the gut dysbiosis which resulted from short-term Vac or CM intervention was long-lasting and antibiotic-related after the 45-day recovery on HFD feeding.

The Recovery of Glucose Homeostasis After the 45-Day High-Fat Diet Feeding

Since recovery on HFD shifted sugar metabolism and insulin signaling-related pathways, we further studied the phenotype changes after long-term HFD feeding post-antibiotic intervention. There was no difference among the three groups regarding BW and energy intake post-antibiotic recovery on HFD (**Figures 6A,B**). Different from the obvious changes of lipid profiles in serum and liver observed during post-antibiotic recovery on CD, HFD feeding after antibiotic exposure had little effects on regulating these phenotypes (**Figures 6C–F** and **Supplementary Figures 4A,B**). Although short-term Vac intervention reduced fasting blood glucose when rats were recovered on CD, it had little effect on improving glucose homeostasis when recovered on HFD. By contrast, short-term CM intervention followed by HFD feeding lowered the fasting blood glucose level and improved glucose tolerance as revealed by the decreased AUC in CM_HFD (**Figures 6G,I**). In addition, fasting insulin levels were slightly higher in Vac_HFD and CM_HFD groups without significant difference (**Figure 6H**). These findings suggested that different early antibiotic exposures divergently affected glycemia at the recovery stage on HFD.

The Recovery of Hepatic Gene Expression After Antibiotic Exposure Is Affected by High-Fat Diet

The number of differential expressed genes between groups was markedly increased when rats were recovered on HFD than on CD. Specifically, the expressions of 283 and 215 hepatic genes were changed by Vac_HFD and CM_HFD, respectively, in which more genes were downregulated (**Figure 6J**). KEGG enrichment analysis revealed that 78 pathways were shifted between Vac_HFD and HFD ($p < 0.05$), while there were only 13 differential pathways when comparing Vac_CD with CD, suggesting that HFD feeding enlarged short-term Vac treatment-induced changes of liver function at the recovery condition. Among them, 30 pathways were related to infection and immunity and 10 pathways were associated with cancer (**Figure 6K**). However, only 15 pathways were shifted between CM_HFD and HFD ($p < 0.05$) and 7 of them were associated with the endocrine system (**Figure 6K**). GSEA results further showed that 37 gene sets were changed by Vac_HFD, many of which were related to infectious disease or the immune system (**Figure 6L** and **Supplementary Figures 4C–E**). Regarding insulin signaling-related genes, when compared

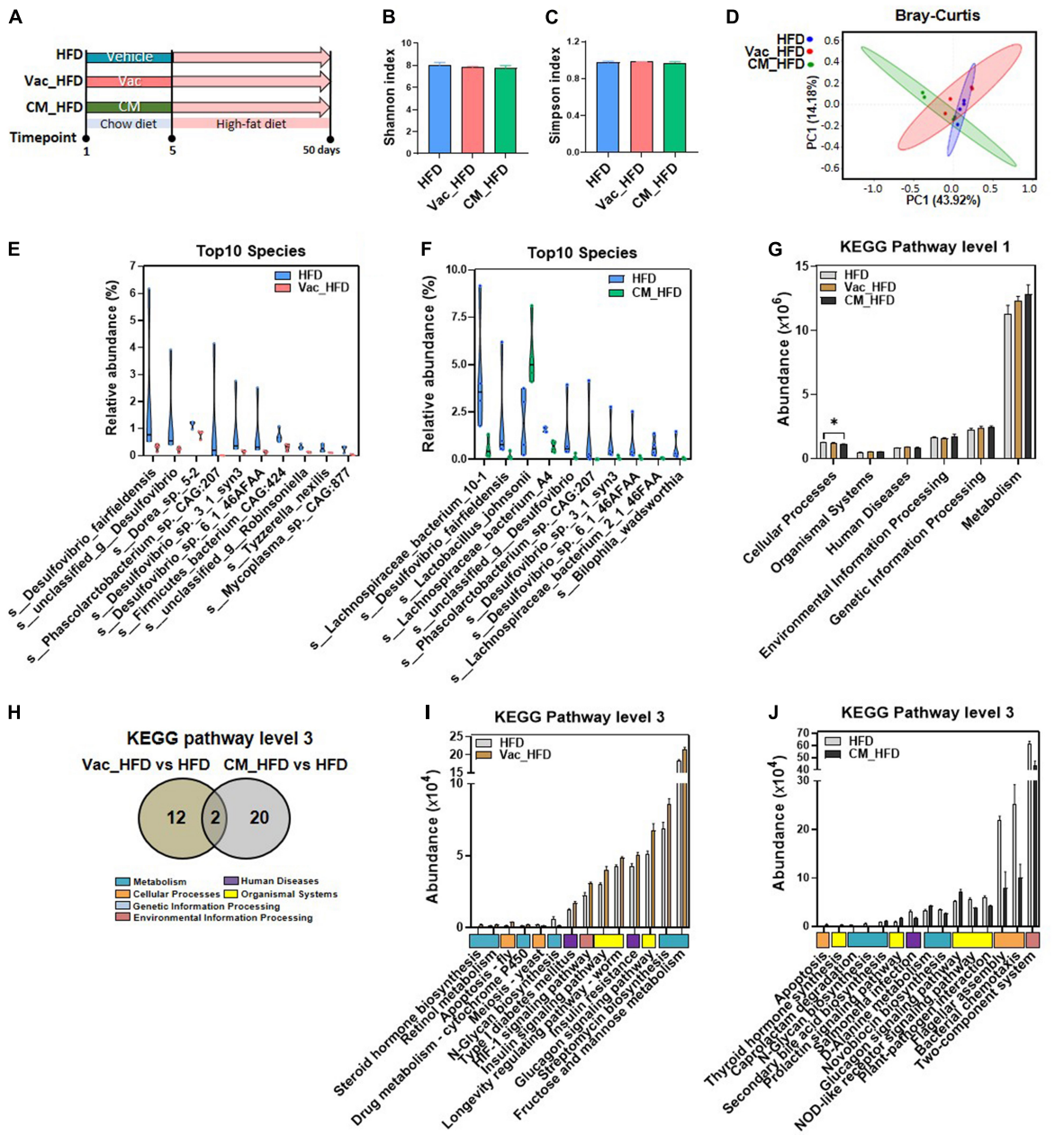

FIGURE 5 | HFD affects post-antibiotic recovery of gut microbiota composition and function. **(A)** Intervention process chart. Rats were treated with vehicle, Vac, or CM for 5 days and then fed with a high-fat diet for another 45 days. **(B)** Shannon index. **(C)** Simpson index. **(D)** Principal co-ordinate analysis based on the Bray–Curtis distance algorithm at species level. **(E,F)** Top 10 differential species between Vac_HFD and HFD and between CM_HFD and HFD ($p < 0.05$ by the Mann–Whitney U test). **(G)** The function differences among groups on KEGG pathway level 1 predicted by PICRUSt2. $^*p < 0.05$ by Student's *t*-test. **(H)** Venn diagram of the overlap of differential enriched pathways between Vac_HFD vs. HFD and CM_HFD vs. HFD by PICRUSt2 analysis on KEGG pathway level 3 ($p < 0.05$ under the Mann–Whitney U test). **(I)** The shifted pathways between Vac_HFD and HFD. **(J)** Top 15 shifted pathways between CM_HFD and HFD.

with the number of differential genes between CM_CD and CD (most genes were upregulated in the CM_CD group), HFD intake reduced the number of differential genes between CM_HFD and HFD, with most genes downregulated in both cases (**Figure 6M** and **Supplementary Figure 4F**). These results might correlate with the improved glucose tolerance in the CM_HFD group. These results might correlate with the improved glucose tolerance in the CM_HFD group. Together, short-term Vac and CM interventions have unique long-term effects on modulating liver function. The long-lasting effects of early Vac and CM perturbation after recovery on HFD were associated with the changes of infection, immunity, cancer-related pathways, and endocrine system-related pathways, respectively.

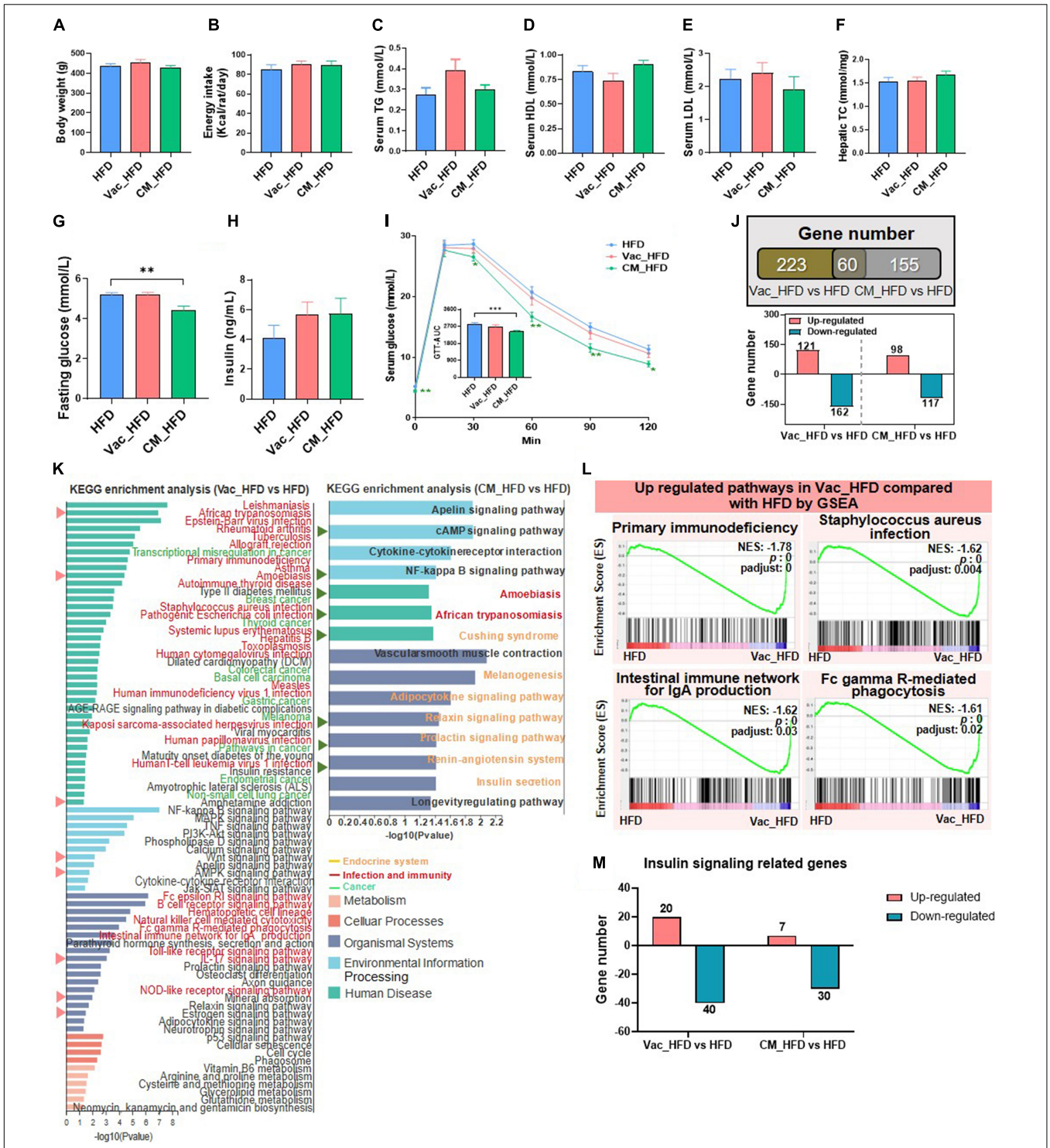

FIGURE 6 | High-fat diet affects post-antibiotic recovery of rat phenotype and hepatic transcriptome. The body weight **(A)**, energy intake **(B)**, serum triglyceride level **(C)**, serum high-density lipoprotein level **(D)**, serum low-density lipoprotein level **(E)**, and liver total cholesterol level **(F)** were shown at the end of the 45-day recovery. **(G–I)** Fasting blood glucose level, fasting blood insulin level, and glucose tolerance test result. **(J)** Venn diagram of the overlap of differential expressed genes between Vac_HFD vs. HFD and CM_HFD vs. HFD ($p < 0.05$ based on edgeR software, and FC ≤ 0.5 or ≥ 2). **(K)** KEGG pathway enrichment analysis of 283 and 215 differential genes. Triangles on the left showed the same pathways between Vac_HFD vs. HFD and CM_HFD vs. HFD. **(L)** The same pathways between Gene Set Enrichment analysis (GSEA) and KEGG pathway enrichment analysis. **(M)** Different regulatory effects of Vac and CM on insulin-related genes. $n = 9$–10 per group for biochemical analysis, $^{**}p < 0.01$ and $^{***}p < 0.001$ by Student's t-test; $n = 3$ per group for transcriptome, Mann–Whitney U test.

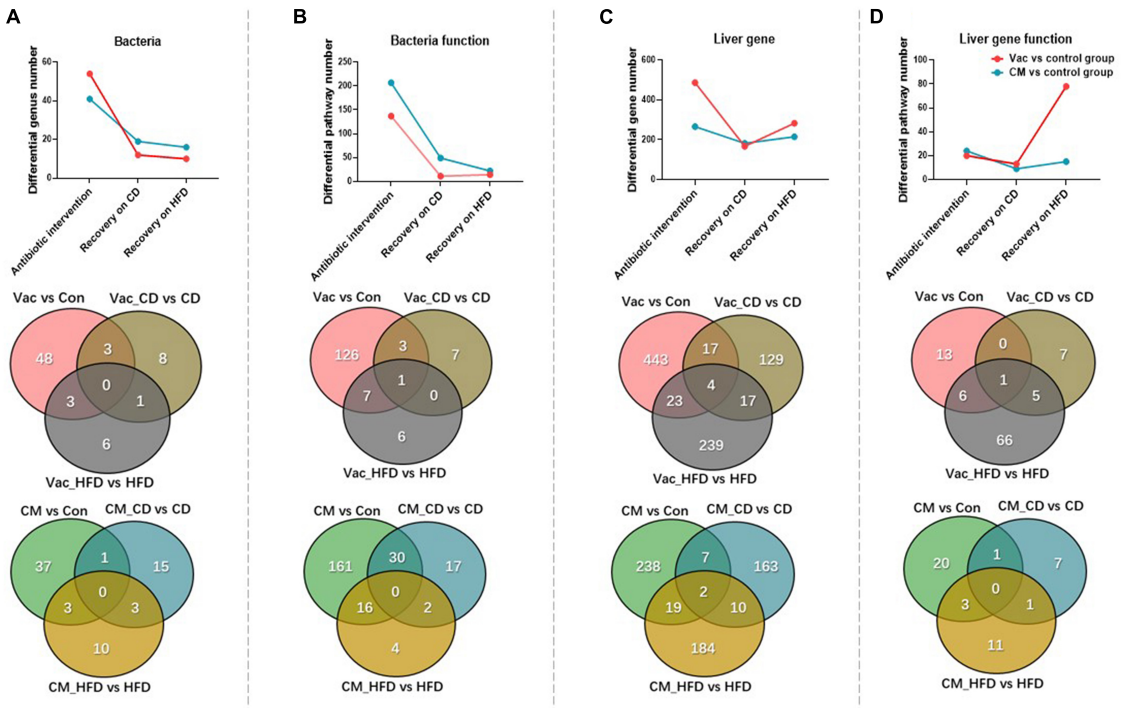

FIGURE 7 | The changes of gut microbiota and liver gene after antibiotic intervention and post-antibiotic recovery. **(A–D)** Venn diagram and line chart show the number changes of differential genus, functional prediction of bacteria at KEGG pathway level 3, differential expression of liver genes, and functional prediction of genes among the three conditions under the same screening conditions.

Post-antibiotic Recovery on HFD Enlarges Antibiotic-Induced Metabolic Differences

To investigate how diet affects host recovery after different antibiotic perturbations, we compared the composition and function of gut microbiota as well as the gene expression and biological function of the liver after short-term Vac and CM exposure and at post-antibiotic recovery state when rats were fed with CD or HFD. After short-term antibiotic intervention, compared with the control group, the number of differential genera was higher in Vac than in the CM group. However, the differential genus number was dramatically reduced after recovery on both CD and HFD with a slightly higher number in CM than in Vac (**Figure 7A**). The Venn diagram showed that only a few genera were commonly altered by any two conditions with no genus commonly regulated by three conditions. Although short-term Vac induced more changes at the genus level, CM affected more pathway changes of the bacterial function pathway, and the differential pathway number was reduced post-antibiotic recovery, suggesting partly recovery of gut bacterial function (**Figure 7B** and **Supplementary Figure 5**).

Liver gene expression was affected more significantly by Vac than by CM based on the number of regulated genes (**Figure 7C**). Post-antibiotic recovery with CD reduced the number of differential expression genes, but recovery with HFD increased this number especially in the Vac_HFD group (**Figure 7C**). In addition, the shifted pathway number between Vac- and CM-induced changes was comparable in short-term antibiotic intervention and recovery on CD condition. However, post-Vac recovery on HFD markedly elevated the differential pathway number to 78, in which most pathways were infection-, immunity-, and cancer-related, whereas only 15 pathways were changed with post-CM recovery on HFD, and most of them were endocrine system-related (**Figure 7D**). Glutathione metabolism was the only pathway affected by all three conditions with Vac perturbation (**Supplementary Tables 1**, **2**). These results suggested that although gut microbiota composition and function were partly restored from antibiotic exposure, recovery on HFD, but not on CD, significantly increased the impact of early Vac exposure on host metabolism.

DISCUSSION

Gut microbiota composition and diversity exert profound effects on host physiology and metabolism. Mounting evidences have shown that exposures to antibiotics in both animals and humans have transient and prolonged effects on host metabolism homeostasis. As summarized in **Figure 8**, the current study systematically investigated gut microbiota composition and host metabolism after short-term antibiotic intervention and at post-antibiotic recovery state and found that the impacts of antibiotic exposure on host metabolism were long-lasting, antibiotic-specific, and diet-dependent. These findings suggest that dietary

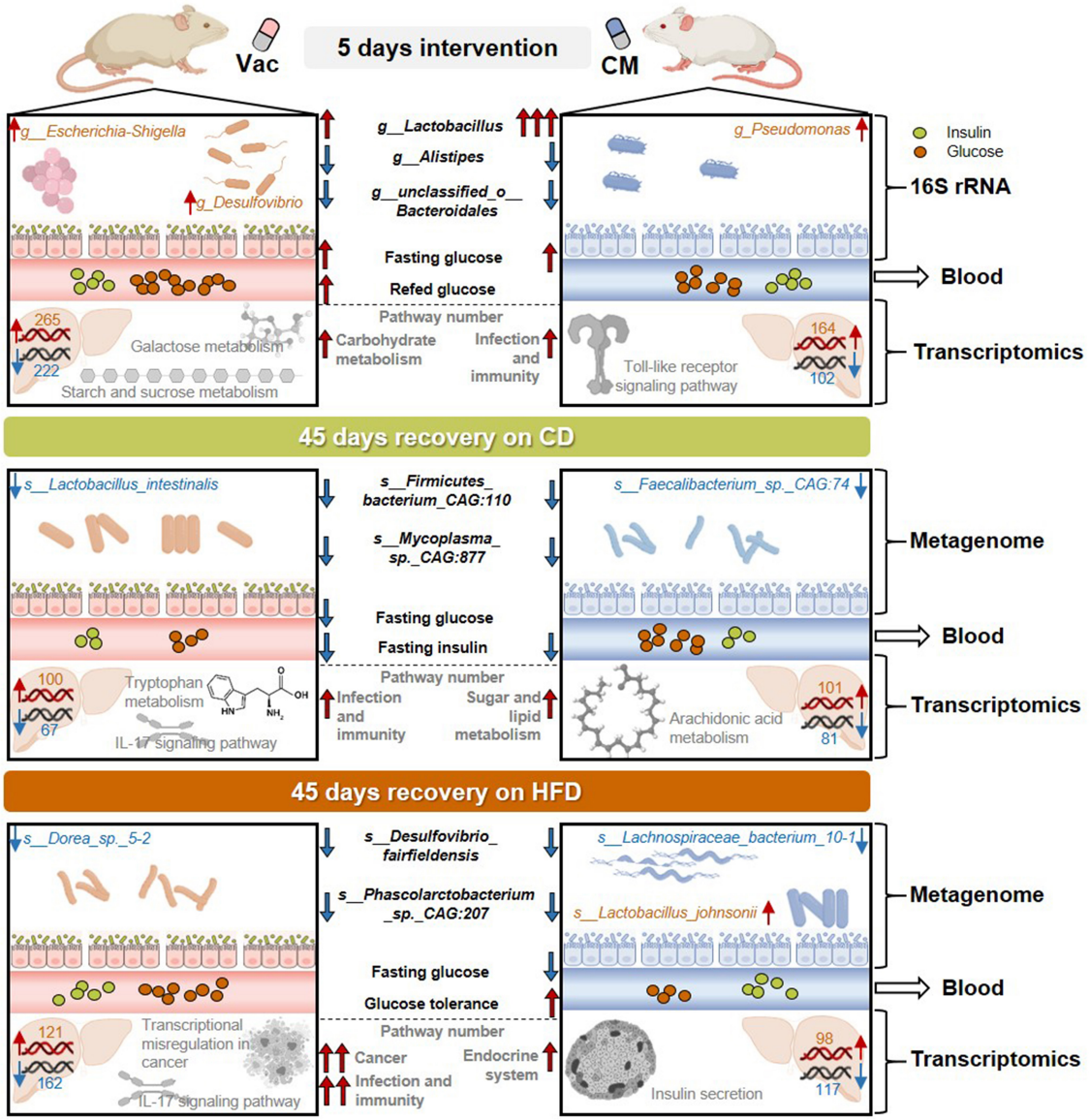

FIGURE 8 | The effects of short-term antibiotic intervention on gut microbiota composition and host metabolism were long-lasting, antibiotic-specific, and diet-dependent. The 5-day intervention with Vac and CM dramatically influenced gut microbiota composition, in which Vac and CM had unique or common effects on regulating the relative abundance of certain gut bacteria. In addition, short-term Vac and CM intervention differential affected blood glucose homeostasis and liver transcriptome, by mainly regulating carbohydrate metabolism-related pathways and infection- and immunity-related pathways, respectively. After a 45-day recovery on either CD or HFD, gut microbiota composition and function, as well as blood glucose imbalance, were partly restored. However, when rats were recovered on CD, certain species in *Lactobacillus* genus and in *Faecalibacterium* genus were reduced at post-Vac and post-CM recovery states, respectively. Differences in infection and immunity-related pathways were apparent after recovery from Vac treatment, while sugar and lipid metabolism-associated pathways were shifted after recovery from CM treatment as revealed by hepatic transcriptome. When rats were recovered on HFD, the relative abundance of species in *Desulfovibrio* and *Lachnospiraceae* genera was reduced, whereas *Lactobacillus johnsonii* was elevated significantly at post-CM recovery state. Moreover, HFD feeding worsened the extent of post-antibiotic recovery in Vac-treated rats including the alterations of infection, immunity, and cancer-related pathways and in CM-treated rats including endocrine system-associated pathways. CD, chow diet; CM, ciprofloxacin and metronidazole; HFD, high-fat diet; Vac, vancomycin.

management during post-antibiotic recovery should particularly be given more attention.

Exposure to antibiotics is closely linked with the changes of glucose metabolism in both human and animal studies. A randomized double-blinded study showed that 7-day Vac treatment reduced peripheral insulin sensitivity in obese males with metabolic syndrome (Vrieze et al., 2014), whereas another study found that it did not affect tissue-specific insulin sensitivity in obese, prediabetic males (Reijnders et al., 2016). In an animal study, short-term or long-term Vac intervention reduced fasting blood glucose in obese mice (Fujisaka et al., 2016; Aron-Wisnewsky et al., 2020). It has been reported that not only

Vac but also CM combination can improve noncaloric artificial sweetener-induced glucose intolerance in mice (Suez et al., 2014). However, in contrast with several previous studies in rodents, our results showed that 5-day exposure of Vac and CM increased the fasting blood glucose level and Vac alone elevated blood glucose under a re-fed state. In particular, short-term Vac decreased the insulin-induced p-AKT/AKT ratio, suggesting impaired hepatic insulin signaling after 5-day Vac intervention. Inconsistent with our findings, Fujisaka et al. (2016) found that Vac could increase the p-AKT/AKT ratio in the liver and muscle in HFD-fed mice without insulin supplementation, while metronidazole increased p-AKT level in the liver and adipose tissue only in response to insulin. These inconsistent results suggest that the interaction between gut microbiome and insulin signaling is very complex and many factors, such as animal model, diet, and host health conditions, will affect the outcomes. Additionally, changes of bacterial metabolites after antibiotic intervention, such as secondary bile acids and short-chain fatty acids, might play an important role in causing hepatic insulin dysregulation through the gut–liver axis, which warrants further investigation.

Since antibiotic treatment dramatically impacted glucose homeostasis and insulin signaling, to our surprise, little is known about how glucose levels change at post-antibiotic recovery state and whether diet will affect the recovery. Fu et al. (2018) have reported that treatment with antibiotic cocktail for 12 days reduced blood glucose in db/db mice, and this difference disappeared 24 days after antibiotic withdrawal. This result suggested that post-antibiotic recovery reversed antibiotic-induced changes of glucose homeostasis. However, another clinical trial showed that host metabolism, such as whole-body insulin sensitivity, remained unchanged by 7-day Vac perturbation as well as 8 weeks post-intervention (Reijnders et al., 2016). Our data revealed impaired insulin signaling after short-term Vac treatment, while recovery on CD could decrease fasting blood glucose level and insulin level, suggesting enhanced insulin activity at post-Vac recovery state on CD. More importantly, our further investigation found that HFD feeding during recovery increased the fasting insulin level in post-antibiotic groups (1.7 ng/ml in the Vac_CD group and 2 ng/ml in the CM_CD group, 5.6 ng/ml in the Vac_HFD group, and 5.7 ng/ml in the CM_HFD group) but not in the vehicle group (3.8 ng/ml in CD and 4.1 ng/ml in the HFD group), suggesting that early antibiotic exposure enhanced rat susceptibility to HFD-induced changes of fasting insulin level.

Short-term antibiotic perturbations have a long-term effect on gut microbial composition and function. Based on the KEGG pathway analysis, compared with the effect of CD on post-Vac recovery, HFD intake during post-Vac recovery led to functional changes in bacteria which related to glucose homeostasis. This result was in line with the phenotype changes that HFD reversed the CD-induced decrease in fasting insulin level during post-antibiotic recovery. The most significant change of gut microbiota composition induced by 5-day CM treatment was the 127-fold induction of the abundance of the *Lactobacillus* genus. Interestingly, when rats recovered under CD, the abundance of three species from *Lactobacillus* genus, *Lactobacillus intestinalis*, *Lactobacillus reuteri*, and *Lactobacillus* sp. *ASF360*, was reduced in the Vac_CD group, while there was no difference in the *Lactobacillus* species between the CM_CD and CD groups. Previous study reported that *L. intestinalis* was increased in Zucker diabetic fatty rats and can be a potential biomarker for the progression and complications of T2DM (Wang et al., 2020). However, some strains of *L. reuteri* are used as probiotics to improve insulin sensitivity (Mobini et al., 2017; Kolodziej and Szajewska, 2019). These findings suggested that *Lactobacillus* might play an important role in post-Vac recovery of glucose homeostasis. In contrast to the findings in the Vac_CD group, when rats were fed with HFD, the most significant long-term effect of Vac intervention was the reduction of the *Desulfovibrio* genus, which can be boosted by HFD and can produce hydrogen sulfide leading to acute inflammation (Attene-Ramos et al., 2006; Kushkevych et al., 2019; Rohr et al., 2020). Among the top 10 changed bacteria, four species in the *Desulfovibrio* genus were reduced in the Vac_HFD group compared with the HFD group. *Desulfovibrio* has been reported to positively correlate with the AUC of OGTT, insulin, and HOMA-IR (Zhou et al., 2018). Therefore, short-term Vac exposure might affect post-Vac recovery of host glucose homeostasis by regulating *Lactobacillus* abundance and *Desulfovibrio* abundance during long-term CD feeding and HFD feeding, respectively.

Early CM intervention also affected gut microbiota recovery based on diet. Compared with their respective control groups, among the top 10 changes species, two species that belong to the *Faecalibacterium* genus were reduced in the CM_CD group, while three species that belong to the unclassified Lachnospiraceae genus were reduced in the CM_HFD group. *Faecalibacterium prausnitzii*, which is a butyrate-producing bacteria, has anti-inflammatory properties and plays a crucial role in maintaining host physiology (Lopez-Siles et al., 2017). Certain strains of Lachnospiraceae, which also produce butyrate, can regulate host metabolism, immune response, and colonocyte growth (Meehan and Beiko, 2014). Additionally, higher levels of Lachnospiraceae have been negatively associated with the risk of some types of cancer (Flemer et al., 2018). These findings suggested that the long-term effect of CM exposure might be correlated with decreased beneficial butyrate production. Surprisingly, the abundance of *L. johnsonii* was the only significantly increased species in the CM_HFD group than in the HFD group. Because many studies have shown that *L. johnsonii* modulates host immune responses (Fonseca et al., 2017; Marcial et al., 2017; Xia et al., 2020), the elevated *L. johnsonii* might lead to better immune response in the CM_HFD group. In sum, different short-term antibiotic exposures have divergent effects on regulating host physiology and metabolism, which depend on the diet used during the recovery state.

Although few studies have reported altered expressions of hepatic genes and shifted functional pathways long after antibiotic exposure (Cho et al., 2012; Nobel et al., 2015), to our knowledge, little is known about the changes of liver biological functions after short-term antibiotic treatment and post-antibiotic recovery, especially with different diet feedings. Our data showed that short-term Vac exposure, but not CM exposure, induced more profound effects in later life on HFD than on CD, including altered infection, immunity, and

cancer-related pathways. Plenty of evidences have shown that HFD-driven dysbiosis was accompanied by a vast expansion of pathogen infection (Zeng et al., 2018; Las Heras et al., 2019; Mefferd et al., 2020). It has been shown that 2 weeks after Vac treatment, mice were still susceptible to pathogen colonization (Isaac et al., 2017). Therefore, our results of hepatic transcriptomics demonstrated that HFD-induced long-lasting effects of Vac treatment on liver function, including altered infection and immunity pathways as well as cancer-related pathways, might be partly due to enhanced susceptibility to pathogen intestinal colonization under HFD feeding.

In conclusion, long-lasting effects of antibiotic exposure on host metabolism are antibiotic-specific and diet-dependent. Our current study reveals the interplay between antibiotic-driven dysbiosis and biological functions of liver, and more importantly, we demonstrate that HFD intake during recovery could worsen Vac-induced long-term detrimental consequences. Although the role of dietary fiber in post-antibiotic recovery of gut microbiome is reported previously (Ng et al., 2019; Tanes et al., 2021), our study evaluates how high-fat diet affects post-antibiotic recovery of host metabolism, which will add our knowledge of the dietary effect on host health. Our finding highlights the importance of dietary management after antibiotic exposure. Further investigations are warranted to find out better dietary types that can reduce antibiotic-induced detrimental consequences, which might be of importance to benefit clinical use for post-antibiotic recovery.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/sra, PRJNA719272, PRJNA713932, and PRJNA702866.

ETHICS STATEMENT

The animal study was reviewed and approved by Shanghai University of Traditional Chinese Medicine Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

BL conducted the *in vivo* experiments, data analysis, and manuscript writing. HQ, YH, and JM helped in H&E staining of tissues and hepatic steatosis evaluation. NZ, GW, YG, and JZ helped in the animal experiment. WZ helped in the project design. LS and HL co-supervised the study and wrote the manuscript. HL conceptualized the project. 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/fcell.2021.680174/full#supplementary-material

Supplementary Figure 1 | Differential bacteria between antibiotic intervention group and control group at genus level ($p < 0.01$ under Mann–Whitney U test).

Supplementary Figure 2 | The impact of antibiotic intervention on rat phenotype and liver transcriptome. **(A)** Serum ALT, AST, LDL, and HDL levels after Vac and CM innervation. **(B)** Western blot analysis of protein level in gastrocnemius. Rats were injected with insulin 8 min before tissues collected. **(C)** Venn diagram of the overlap of the differential enriched gene sets between Vac vs. Con and CM vs. Con based on GSEA (NES > 1 or <-1, $p < 0.05$, p-adjust < 0.25). **(D)** The common gene sets that were regulated by both Vac and CM based on GSEA result. **(E)** The gene sets that were regulated by either Vac or CM based on GSEA result. **(F)** Heatmaps show the different regulatory effects of Vac and CM on insulin related genes. $n = 4$ per group for transcriptome.

Supplementary Figure 3 | The long-lasting impact of antibiotics treatment on rat phenotype and liver transcriptome when rats were fed with chow diet. **(A)** Serum LPS, serum TC, hepatic TG level. **(B)** H&E-stained liver sections and HE score. **(C)** Venn diagram of the overlap of the differential enriched gene sets between Vac_CD vs. CD and CM_CD vs. CD based on GSEA (NES > 1 or <-1, $p < 0.05$, p-adjust < 0.25). The gene sets that were regulated by either Vac or CM based on GSEA result. **(D)** Heatmaps show the different regulatory effects of Vac and CM on insulin related genes. $n = 3$ per group for transcriptome.

Supplementary Figure 4 | HFD affects post-antibiotic recovery of rat phenotype and hepatic transcriptome. **(A)** Serum LPS, serum TC, hepatic TG level. **(B)** H&E-stained liver sections and HE score. **(C)** Venn diagram of the overlap of the differential enriched gene sets between Vac_HFD vs. HFD and CM_HFD vs. HFD based on GSEA (NES > 1 or <-1, $p < 0.05$, p-adjust < 0.25). **(D)** The common gene sets that were regulated by both Vac_HFD and CD_HFD. **(E)** The gene sets that were regulated by either Vac_HFD or CM_HFD based on GSEA result. **(F)** Heatmaps show the different regulatory effects of Vac and CM on insulin related genes. $n = 3$ per group for transcriptome.

Supplementary Figure 5 | Shared bacterial functional pathways among three conditions. **(A)** The 11 shared pathways between Vac vs. Con, Vac_CD vs. CD and Vac_HFD vs. HFD on KEGG pathway level 3 were shown with heatmap (Log_2 Fold change). **(B)** The 48 shared pathways between CM vs. Con, CM_CD vs. CD and CM_HFD vs. HFD on KEGG pathway level 3 were shown with heatmap (Log_2 Fold change).

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The Lipopolysaccharide-Sensing Caspase(s)-4/11 Are Activated in Cirrhosis and Are Causally Associated With Progression to Multi-Organ Injury

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Background and Aims: The development of multi-organ injury in cirrhosis is associated with increased intestinal permeability, translocation of gut-derived bacterial products [e.g., lipopolysaccharide (LPS)] into the circulation, and increased non-apoptotic hepatocyte cell death. Pyroptosis is a non-apoptotic, lytic form of cell death mediated by the LPS-sensing caspase(s)-4/11 (caspase-4 in humans, caspase-11 in mice), which leads to activation of the effector protein Gasdermin D (GSDMD) and subsequent formation of pores in the plasma membrane. Endoplasmic reticulum (ER) stress, a feature of cirrhosis, has been identified as a factor promoting the activation of caspase-11, thus increasing sensitivity of the cell to LPS-mediated pyroptosis. The aim of this study was to determine the role of bacterial LPS in the activation of hepatic caspase(s)-4/11 and progression of hepatic and extra-hepatic organ injury in cirrhosis.

Materials and Methods: Human liver samples from patients with stable cirrhosis (SC) or acutely decompensated cirrhosis (AD) were analyzed for caspase-4 activation by immunohistochemistry. Wild-type and *Casp11*$^{-/-}$ mice underwent CCl_4 treatment by gavage to induce advanced liver fibrosis, and subsequently low-dose injection of LPS to mimic bacterial translocation and induce multi-organ injury. Liver, kidney, and brain function were assessed by plasma ALT/creatinine and brain water respectively. The activity of inflammatory caspases was assessed by fluorometric assay and the occurrence of pyroptosis and overall cell death in liver tissue by GSDMD cleavage and TUNEL assay, respectively. Primary human hepatocytes were cultured according to standard techniques.

Results: Human liver samples demonstrated increased caspase-4 activation in AD cirrhosis. Caspase-4 activation was associated with MELD score and circulating

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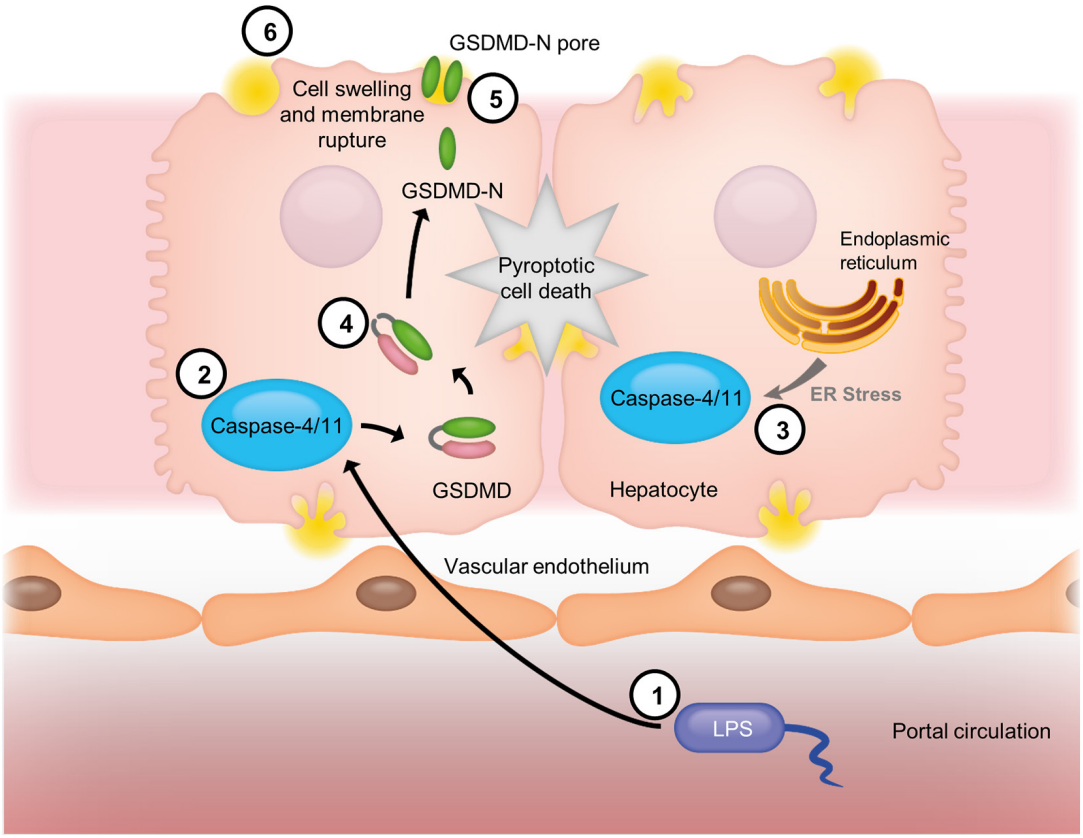

GRAPHICAL-ABSTRACT | Model of caspase-4/11 activation in cirrhosis. Gut-derived bacterial LPS (1) enters the portal circulation after translocation across the gut epithelium and is internalized by hepatocytes. Cytoplasmic LPS is recognized by caspase-4/11 (2), which undergoes self-activation upon ligand binding. The activity of caspase-4/11 is enhanced by endoplasmic reticulum stress (3), which occurs in fibrosis/cirrhosis, leading to "sensitization" of this pathway. Active caspase-4/11 cleaves the dimeric protein Gasdermin D (GSDMD) (4), and freeing the N-terminal domain to migrate to the plasma membrane and form GSDMD N-terminal octameric pores (5). GSDMD pores insert themselves into the plasma membrane, allowing the deregulated passage of molecules and causing cell swelling and membrane rupture, eventually resulting in pyroptotic cell death (6).

levels of LDH. Wild-type mice treated with CCl_4 developed significant multi-organ injury (increased ALT, creatinine, and brain water) upon LPS injection, and showed increased hepatic GSDMD cleavage compared to mice treated with CCl_4 alone. Primary human hepatocytes could be sensitized to pyroptosis by pre-treatment with the ER-stress inducer tunicamycin and LPS. *Casp11*$^{-/-}$ mice treated with CCl_4 + LPS were significantly protected from multi-organ injury compared to wild-type CCl_4 + LPS.

Conclusion: These data demonstrate for the first time a causal relationship between LPS-mediated activation of caspase(s)-4/11 and development of hepatic and extra-hepatic injury in cirrhosis.

Keywords: cirrhosis, lipopolysaccharide, caspase, liver failure, endotoxin, dysbiosis, pyroptosis

INTRODUCTION

Cirrhosis is responsible for over 1 million global deaths each year, equating to 2% of deaths worldwide (Mokdad et al., 2014). The natural history of cirrhosis is considered to be a progression through an asymptomatic compensated phase, to a decompensated state following the development of acute complications such as jaundice, variceal bleeding, ascites or hepatic encephalopathy (HE). Amongst patients with acutely decompensated cirrhosis (AD), a subgroup of 1 in 3 patients develop severe hepatic and extra-hepatic organ injury – a condition that has been termed acute-on-chronic liver failure (ACLF), and is associated with high short-term mortality (Moreau et al., 2013).

The mechanisms determining susceptibility to multi-organ injury in cirrhosis remain undetermined, but clear associations have been shown with systemic inflammation and altered host response to injury (Clària et al., 2016). The most common precipitants of multi-organ injury in cirrhosis are bacterial infection and alcoholic hepatitis (Moreau et al., 2013). In patients without an identified precipitating event, it is considered likely that translocation of bacterial products from the intestinal lumen contributes to systemic inflammation and multi-organ dysfunction (Markwick et al., 2015). Indeed, the presence of circulating bacterial products is a predictor of multi-organ injury and prognosis in cirrhosis (Michelena et al., 2015).

The major consequence of bacterial translocation (BT) in cirrhosis is systemic inflammation, through the action of pathogen-associated molecular patterns (PAMPs) such as bacterial lipopolysaccharide (LPS). Our group, and others, have shown circulating levels of LPS to be elevated in patients with severe alcoholic hepatitis, and to be a predictor of multiple organ failure and mortality (Markwick et al., 2015; Michelena et al., 2015). Additionally, increased host sensitivity to bacterial PAMPs has been noted in cirrhosis, with exaggerated *ex vivo* pro-inflammatory cytokine responses to LPS in humans and animal models (Tazi et al., 2007; Gandoura et al., 2013).

In parallel, there is accumulating evidence to support a role for excessive cell death in development of multi-organ failure in cirrhosis. We have previously shown that acute decompensation in cirrhosis is associated with elevated circulating markers of cell death, which progressively increase with disease severity (Macdonald et al., 2018). Moreover, the mode of cell death also changes with disease severity, from predominantly apoptotic to non-apoptotic cell death with development of multi-organ dysfunction. This may partly explain the negative clinical results from efforts to inhibit apoptosis in acute decompensation of cirrhosis using pan-caspase inhibitors (Mehta et al., 2018).

A direct link between LPS-sensing and non-apoptotic cell death has recently been described. Pyroptosis is a non-apoptotic, lytic mode of cell death mediated through inflammatory caspases, also termed the "non-canonical inflammasome." Intracellular LPS is directly sensed by caspase-4 (human)/caspase-11 (mouse) which directly leads to activation of this pathway through cleavage of the cytoplasmic protein Gasdermin-D (GSDMD). Subsequently, the N-terminal fragment of GSDMD forms pores in the plasma membrane which may precipitate lytic cell death, release of damage-associated molecular patterns (DAMPs), and amplification of local inflammatory responses (Ding et al., 2016). Unlike apoptosis, which is immunologically silent, pyroptosis is an immunogenic form of cell death. This is of potential benefit to the host in the elimination of intracellular pathogens. However, in the context of cirrhosis, high levels of hepatocyte pyroptosis in response to circulating LPS may lead to greater liver dysfunction, increased systemic inflammation and multi-organ failure. A link between endoplasmic reticulum stress, a feature of cirrhosis, and activation of LPS-sensing caspases has also been described, providing a potential mechanism for sensitization of pyroptosis pathways in cirrhosis (Endo et al., 2006).

The aim of the present study was to evaluate the role of LPS-sensing caspase(s)-4/11 in hepatic and extra-hepatic organ injury in cirrhosis. Cirrhosis is associated gut bacterial dysbiosis, the degree of which correlates with the severity of the disease (Bajaj, 2019). Moreover, cirrhosis is also associated with increased intestinal permeability, and it has been recently demonstrated that translocation of bacterial products to the circulation underpins the sensitization of hepatocytes to cell death that is observed in chronic liver disease (Isaacs-Ten et al., 2020). The hypothesis of this study was that activation of caspase(s)-4/11 and high levels of hepatic pyroptosis are causally related to the development of multi-organ injury in cirrhosis. To interrogate this hypothesis, we characterized activation of caspase-4 in liver tissue from patients with stable compensated and acutely decompensated cirrhosis, and explored the activation of caspase-11 and GSDMD in a mouse model of liver fibrosis with multi-organ injury. Further, we used *Casp11*$^{-/-}$ mice to dissect the role of this pathway in hepatic and extra-hepatic organ injury.

Abbreviations: ACLF, acute-on-chronic liver failure; AD, acutely decompensated cirrhosis; ALT, alanine aminotransferase; ATF4, activating transcription factor 4; CCl_4, carbon tetrachloride; CHOP, C/EBP homologous protein; CPA, collagen proportionate area; DAMP, damage-associated molecular pattern; DC, decompensated cirrhosis; DDIT3, DNA damage-inducible transcript 3 (gene coding for CHOP protein); ER, endoplasmic reticulum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSDMD, gasdermin-D; HC, healthy control; HE, hepatic encephalopathy; HSPA5, heat shock protein family A (Hsp70) member 5 (gene coding for BiP); LDH, lactate dehydrogenase; LPS, lipopolysaccharide/bacterial endotoxin; MELD, model for end-stage liver disease; PAMP, pathogen-associated molecular pattern; PPIB, peptidylprolyl isomerase B; SC, stable cirrhosis; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; XBP1, X-box binding protein 1.

MATERIALS AND METHODS

Human Liver Samples

Liver biopsy specimens formalin-fixed and paraffin-embedded (FFPE) were available from the Institute for Liver and Biliary Sciences (ILBS) Biobank, from an established cohort of 79 hospitalized patients presenting with acutely decompensated (AD) alcohol-related cirrhosis, and a further 10 outpatients with stable, compensated (SC) alcohol-related cirrhosis. Clinical data from these patients was collected from the time of biopsy, and Child-Pugh (CP) and Model for End Stage Liver Disease (MELD) scores calculated. Ethical approval was granted by the ILBS IRB (Ref: IEC/2019/71/MA07, November 02, 2019).

Mouse Model of Advanced Fibrosis and Multi-Organ Dysfunction

Male C57BL/6J mice, wild type (Charles Rivers, United Kingdom) or Casp11$^{-/-}$, were used for all experiments. Casp11$^{-/-}$ mice have a targeted deletion of 16 amino acids from exon 5 of *casp-11*, including the QACRG enzymatic active site (Wang et al., 1998). All mice were housed in a temperature and light controlled (12 hours light/dark cycle) facility at the Comparative Biology Unit, UCL, and received standard chow and water *ad libitum*. All procedures were performed in accordance with United Kingdom Home Office Animals (Scientific Procedures) Act 1986 (updated 2012). The study was approved by the University College London Animal Welfare

and Ethical Review Board (AWERB) and conducted with a United Kingdom Home Office project license.

The model of advanced fibrosis and multi-organ dysfunction was previously described by Sanyal and colleagues (Carl et al., 2016). Briefly, advanced fibrosis was induced by gavage of carbon tetrachloride (CCl_4 0.5ml/kg, 1:1 olive oil, 20 doses over 10 weeks). Control mice were treated with olive oil alone. Subsequently, low-dose *Klebsiella pneumoniae* lipopolysaccharide (LPS) (Merck, United Kingdom) was injected intraperitoneally (i.p) at 2–4 mg/kg to mimic bacterial translocation and induce ACLF, or equivalent volume of 0.9% saline as control. For some experiments, a high-dose of LPS (12 mg/kg) was injected in naïve mice. All experiments were terminated at 4 h following intervention.

Histopathological Assessment and Immunostaining

Human and mouse samples were formalin fixed, paraffin embedded and sections cut according to standard techniques. Human samples underwent immunostaining for Caspase-4 (Caspase-4 polyclonal antibody raised against AA: 95–137, AMS Bio, United Kingdom) according to manufacturer's protocols. Slides were reviewed by two investigators (CB and AS) blind to clinical characteristics. Under high magnification, 20 consecutive high-power fields (hpfs) were selected and the positive staining cells (dark brown cytoplasmic staining) were counted. The mean scores of both investigators were taken, and data expressed as average positive cells/hpf. Mouse sections were stained with hematoxylin and eosin (H&E) or picro-Sirius red, and collagen proportionate area (CPA) calculated as previously described (Hall et al., 2013). Additionally, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed on mouse liver sections (*In Situ* Cell Death Detection Kit, POD – Roche Diagnostics, United Kingdom) according to manufacturer's protocols. Degree of cell death was quantified by analysis of immunohistochemical positive areas measured by FIJI Image J software as described previously (Hall et al., 2013).

Characterization of Organ Dysfunction in Mouse Models

Mouse plasma alanine aminotransferase (ALT) and creatinine concentration were measured by Cobas Integra 400 automated analyzer (Roche Diagnostics, Burgess Hill, United Kingdom) using the relevant kits according to the manufacturer's instructions. Plasma lactate dehydrogenase (LDH) was also measured as a circulating marker of non-apoptotic cell death, using the LDH-Glo Cytotoxicity Assay (Promega, United Kingdom). Brain tissue water content was measured according to a previously described gravimetric technique (Marmarou et al., 1982). Circulating levels of LPS were measured by end-point chromogenic endotoxin detection assay based on the amebocyte lysate method (Thermo Fisher Scientific, United Kingdom).

Cell Culture

Cryopreserved primary human hepatocytes (Lonza Biologics, United Kingdom) were cultured with HCM Thawing Medium, Hepatocyte Plating Medium and HCM Hepatocyte Culture Medium (Lonza Biologics, United Kingdom) according to supplier's instructions. For some experiments cells were exposed to LPS from *Klebsiella pneumoniae* (Merck, United Kingdom) or tunicamycin (Merck, United Kingdom) according to the doses stated.

Protein Expression Analysis

Proteins were isolated from snap frozen human and mouse tissue samples and cell culture samples, by standard techniques and analyzed by Western blot. In brief, frozen tissues were aliquoted (50–100 mg) into screw cap tube (Starlab, United Kingdom) containing 1mm glass beads (Merck, United Kingdom) and homogenized in PBS using a Precellys 24 Tissue Homogenizer (Bertin Instruments, France). 2× RIPA buffer (Merck, United Kingdom) and protease inhibitor cocktail (Roche Diagnostics, Burgess Hill, United Kingdom) was added to the homogenate and incubated at 4°C with agitation for 10 min. Tubes were then centrifuged at 15.000*g* × 15 min and the supernatant collected aliquoted and stored at −80°C for future analysis. Cell culture samples were processed on-plate. Briefly, culture medium was removed and 20 $\mu l/cm^2$ RIPA buffer (protease inhibitor added to each well or flask. Cells were detached with a rubber policeman (Thermo Fisher scientific, United Kingdom), transferred to microcentrifuge tubes, incubated at 4°C with agitation for 10 min, then centrifuged at 15.000*g* × 15 min. Supernatant were collected, aliquoted, and stored at −80°C for future analysis. Blots were probed using the primary antibodies described in **Supplementary Table 1A**. Immune complexes were detected using horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology, United States) and enhanced chemiluminescence (ECL) reagents (BioRad, United Kingdom). Densitometric quantification was performed using ChemiDoc imaging stem and Image Lab software (BioRad, United Kingdom).

Messenger RNA Expression Analysis

Total RNA was extracted from snap frozen mouse liver and cell samples using TRI reagent (Merck, United Kingdom), and retrotranscribed using AffinityScript cDNA synthesis kit (Agilent, United Kingdom). Subsequently, gene expression was analyzed according to manufacturer's protocols, using the primers described in **Supplementary Table 1B**.

Measurement of Caspase Activity

Caspase-1 and Caspase-11 activity was measured in mouse liver homogenate using a fluorometric assay (Abcam, United Kingdom) as previously described (Khanova et al., 2018). Caspase-1 activity was measured by cleavage of the motif WEHD, and Caspase-11 by cleavage of the motif LEVD.

Statistics

Variables are presented as mean ± standard error, or median and interquartile range, depending on normal or non-normal distribution. Data were analyzed by *t*-test (with Welch correction where necessary), Mann-Whitney test, one-way ANOVA (with Tukey's *post hoc* test), Kruskal-Wallis (with Dunn's *post hoc* test), Pearson's or Spearman correlation as appropriate, using GraphPad Prism (version 5.03 for Windows; GraphPad Software, San Diego, CA, United States) and Minitab17 (Minitab, Inc. State College, PA, United States).

RESULTS

Caspase-4 Expression Is Increased in Liver Tissue of Patients With Acutely Decompensated Cirrhosis and Correlates With Disease Severity

Clinical characteristics for outpatients with stable compensated cirrhosis (SC) and hospitalized patients with acutely decompensated cirrhosis (AD) are presented in **Table 1**. The expression of caspase-4 in the liver was measured by immunohistochemistry, employing an antibody raised against the central portion of caspase-4 (AA range: 95–137) and quantified as number of positively stained cells per high power field. Abundance of caspase-4 in hepatocytes was significantly increased in AD patients, compared to stable cirrhotic controls (**Figure 1A**), and circulating LDH levels, a marker of non-apoptotic cell death, were significantly correlated with hepatic expression of caspase-4 in AD ($r = 0.3287$, $p = 0.026$). In AD, the expression of caspase-4 was also significantly correlated with disease severity by MELD score at time of biopsy ($r = 0.2700$, $p = 0.011$). By contrast, no significant correlation of caspase-4 expression with MELD score was noted in stable compensated patients ($r = 0.01972$, $p = 0.666$). In hospitalized patients with AD cirrhosis, further laboratory tests were available at 14-days and 28-days following liver biopsy; hepatic caspase-4 expression was also significantly correlated with MELD score at day 14 post-biopsy ($r = 0.2587$, $p = 0.027$), and more strongly correlated with MELD score at day 28 post-biopsy ($r = 0.4800$, $p < 0.001$). To explore the influence of hepatic caspase-4 expression on disease trajectory, we examined change in MELD score at day 28 (delta MELD) in AD cirrhosis patients. In patients with

TABLE 1 | Patient characteristics for liver tissue characterization.

	Stable compensated cirrhosis (SC) *n* = 10	**Acutely decompensated cirrhosis (AD) *n* = 79**
Age [mean (SEM)]	44.9 (3.2)	45.5 (1.0)
Male [n (%)]	10 (100)	78 (99)
Cause of acute decompensation [n (%)]		
Infection	N/A	15 (18.9)
Alcohol	N/A	26 (67.0)
GI bleeding	N/A	4 (5.0)
Unknown	N/A	7 (8.8)
Aetiology [n (%)]		
Alcohol	10 (100)	79 (100)
Clinical data at time of biopsy		
Bilirubin [median (IQR)]	0.75 (0.60–0.95)	20.2 (13.45–28.37)
INR [median (IQR)]	1.00 (0.93–1.00)	1.99 (1.75–2.29)
Creatinine [median (IQR)]	0.60 (0.50–0.70)	0.54 (0.34–0.78)
MELD score [median (IQR)]	6.00 (6.00–6.75)	19.66 (16.08–23.79)
Child-Pugh Class		
A	10 (100)	18 (23)
B	0 (0)	59 (75)
C	0 (0)	2 (2.4)
Clinical data at 28 days		
Bilirubin [median (IQR)]	N/A	10.6 (5.77–20.70)
INR [median (IQR)]	N/A	1.62 (1.14–2.00)
Creatinine [median (IQR)]	N/A	1.00 (0.59–1.50)
MELD score [median (IQR)]	N/A	19.16 (13.31–27.38)
Comorbidities [n (%)]		
None	9 (90)	75 (95)
T2DM	0 (0)	2 (2.4)
Cardiomyopathy	0 (0)	1 (1.2)
Hypertension	1 (10)	1 (1.2)
Caspase-4 positive cells [mean (SEM)]	10.2 (1.3)	25.8 (1.2)

decompensated cirrhosis, a change in MELD score of 5 points at 1 month has been shown to predict short-term mortality (Merion et al., 2003). Accordingly, AD patients were classified into three groups based on delta MELD [improved (< −5; $n = 22$), stable (−5 to + 5; $n = 30$), and worsened (> + 5; $n = 25$)]. The level of caspase-4 expression across the three groups was significantly different by multivariate analysis (**Figures 1B,C**), with *post hoc* testing showing a significant increase in caspase-4 expression between the 'improved' group and both the "stable" and "worsened" (improved vs. stable: 20.02 vs. 26.17, $p = 0.017$; improved vs. worsened: 20.02 vs. 30.48; $p < 0.001$). Taken together, these data demonstrate an association of hepatic caspase-4 expression with severity of liver disease in cirrhosis, and a further association with disease trajectory in hospitalized patients with decompensated cirrhosis.

Mice With Advanced Liver Fibrosis Treated With LPS Develop Multi-Organ Injury Associated With Activation of Hepatic Caspase-11 and GSDMD Cleavage

In order to study the relationship between acute decompensation of cirrhosis with activation of the caspase-4/11 pathway, we utilized an established mouse model of liver fibrosis with multi-organ injury. Mice were treated with carbon tetrachloride (CCl_4) over 10 weeks to establish advanced liver fibrosis (**Supplementary Figure 1A**), and subsequently injected intraperitoneally (i.p.) with LPS (2 mg/kg) to precipitate multi-organ injury. Mice treated with CCl_4 + LPS developed features of ACLF, with exaggerated liver injury and extra-hepatic organ injury compared to CCl_4 control (**Figure 2A**).

In accordance with the findings in humans, mice treated with CCl_4 + LPS showed increased abundance of active caspase-11 in the liver compared to naïve mice and mice treated with CCl_4 alone, as measured by abundance of the p26 fragment (**Figure 2B**). Further to that, the CCL_4 + LPS group also displayed increased caspase-11 enzymatic activity, as measured by fluorometric assay on liver lysates (**Figure 2C**). consistent with activation and processing of caspase-11 following LPS exposure. Accordingly, circulating LPS levels, a marker of intestinal permeability and translocation of gut-derived bacterial products, showed a trend toward increased levels in the CCl_4 and CCl_4 + LPS groups compared to naïve (**Supplementary Figure 1B**). In contrast to caspase-11, no changes in the activity of caspase-1 were observed between naïve, CCl_4 and CCl_4 + LPS treated mouse liver samples (**Supplementary Figure 1C**). Taken together, these findings are consistent with increased activity, or 'sensitization', of the caspase-4/11 pathway in liver tissue in cirrhosis, predisposing to increased responsiveness to gut-derived bacterial products such as LPS.

Since caspase-11 acts upstream of GSDMD, we measured the abundance of active GSDMD by quantification of the GSDMD N-terminal. Mice treated with CCl_4 + LPS showed increased levels of hepatic GSDMD N-terminal compared to the CCl_4 control group (**Figure 2D**), which was accompanied by a significant increase in overall cell death in liver tissue, as assessed by TUNEL staining (**Supplementary Figure 1D**). In a separate experiment, naïve mice were treated with high-dose LPS (12 mg/kg) and showed no significant increase in hepatic

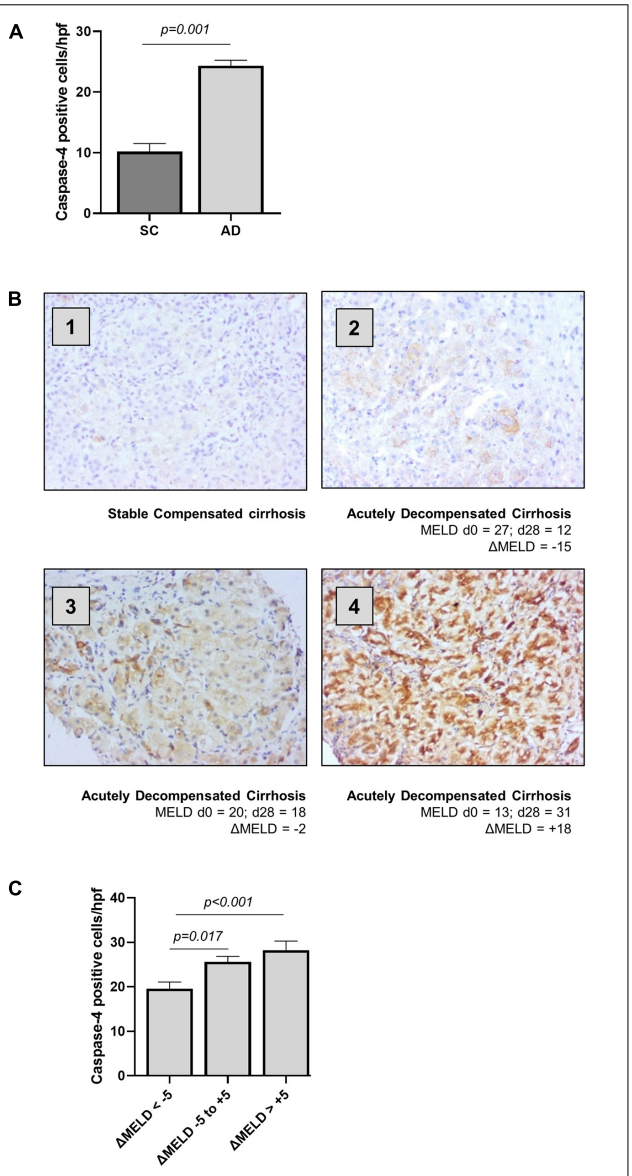

FIGURE 1 | Hepatic caspase-4 expression correlates with the severity of liver disease. The expression of caspase-4 in the liver was measured by immunohistochemistry and quantified as number of positive cells/high power field. **(A)** Patients with acutely decompensated cirrhosis were found to have elevated expression of caspase-4 in the liver compared to patients with stable, compensated cirrhosis (Mann-Whitney *U* test, $p < 0.001$). **(B)** Patients were divided into three categories, based on ΔMELD score (MELD d28 – MELD d0): 'Improved' patients with ΔMELD below −5, "stable" with ΔMELD between −5 and + 5, and "worsened" with an increase in MELD score greater than + 5 points. Panels 1–4 show representative caspase-4 staining for (1) stable cirrhosis (SC), (2) 'improved' AD, (3) "stable" AD, and (4) "worsened" AD. **(C)** Caspase-4 expression in the liver was significantly different among the three groups (Kruskal-Wallis test $p < 0.001$), being significantly higher in "stable" and "worsened" groups, compared to 'improved' patients (Dunn's test, $p = 0.017$ and $p < 0.001$ respectively).

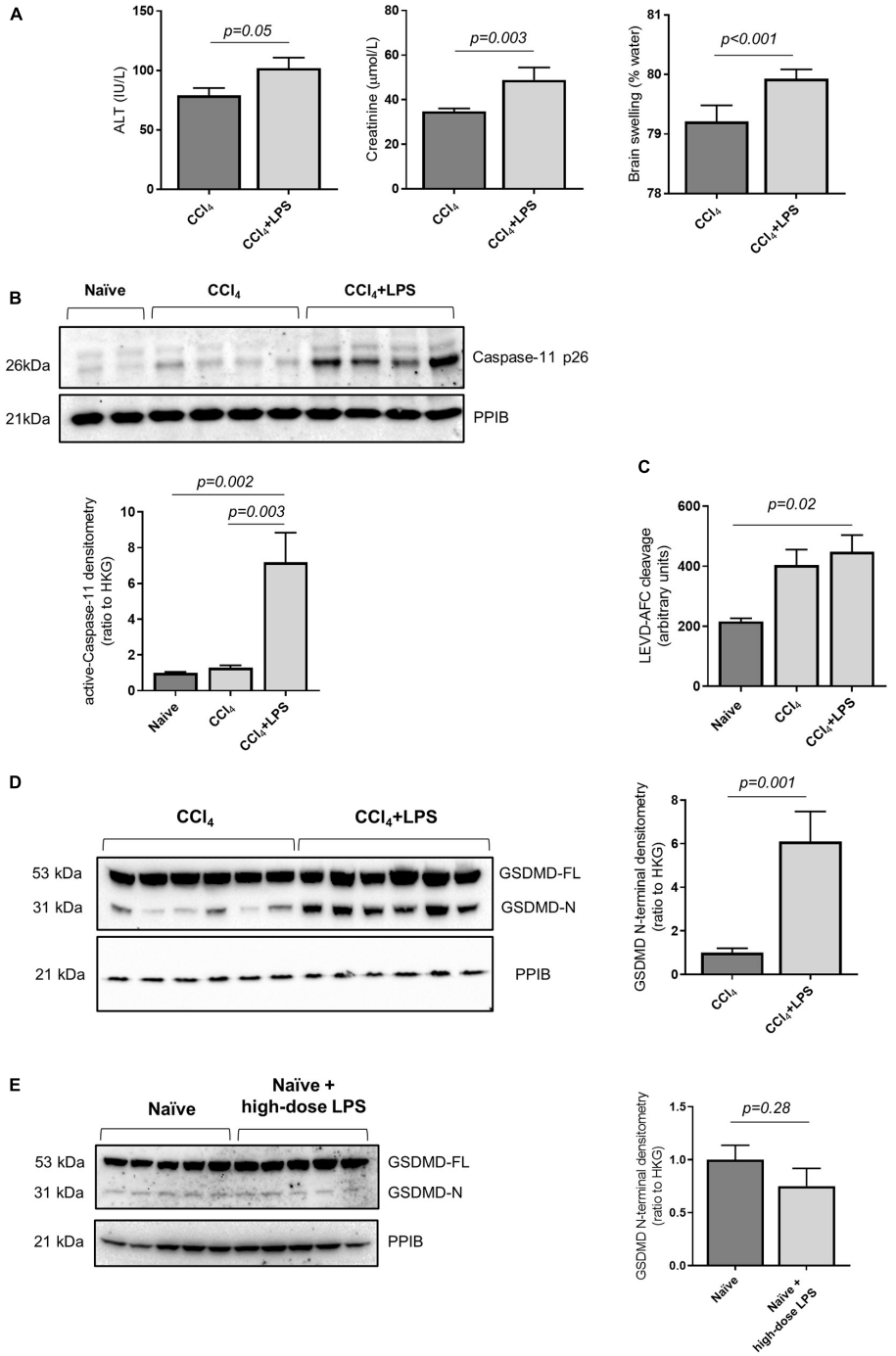

FIGURE 2 | Mouse model of decompensated liver cirrhosis displays activity of Caspase-11 and GSDMD cleavage. **(A)** Mice were treated with CCl_4 to develop advanced hepatic fibrosis. Following intraperitoneal injection of low-dose LPS (2 mg/kg), mice developed features of ACLF at 4 h (n = 7/group). The CCl_4 + LPS group demonstrates elevated plasma ALT (left panel; Student's t-test, p = 0.05), creatinine (center panel; Student's t-test, p = 0.003) and brain swelling (right panel; Student's t-test, $p < 0.001$). **(B)** Activation of caspase-11 was measured by relative abundance of active caspase-11 p26 compared to the housekeeping gene PPIB. CCl_4 + LPS treated mice (n = 7; n = 6 showed in the image) showed higher levels of caspase-11 p26 than naïve mice (n = 5) and mice treated with CC_4 alone (one-way ANOVA with Tukey *post hoc* test; p = 0.002 and p = 0.003, respectively). **(C)** The enzymatic cleavage of LEVD-AFC substrate in snap-frozen liver extracts showed a trend toward increase in the CCl_4 group, and was significantly elevated in CCl_4 + LPS-treated mice (n = 7) compared to naïve animals (n = 5) (one-way ANOVA with Tukey *post hoc* test p = 0.02). **(D)** Compared to animals treated with CCl_4 alone, the CCl_4 + LPS group shows higher expression of active GDSMD, measured as abundance of cleaved GSDMD N-terminal (Student's t-test, p = 0.001). **(E)** There is no apparent increase in hepatic GSDMD activation in naïve mice or naïve mice treated with high-dose LPS (12 mg/kg) groups (Student's t-test, p = 0.28).

GSDMD cleavage over baseline level in naïve mice (**Figure 2E**), thus suggesting that prior sensitization of the caspase-11 pathway is required to trigger hepatic GSDMD cleavage in response to LPS exposure.

Importantly, no increase in GSDMD cleavage was seen in the kidney or brain of the CCl_4 + LPS group compared to CCl_4 alone despite the increase in plasma creatinine and brain tissue water content noted in the CCl_4 + LPS group (**Supplementary Figure 2A**). Representative liver and kidney H&E sections from naïve, CCl_4, and CCl_4 + LPS groups are presented in **Supplementary Figure 2B**.

Endoplasmic Reticulum Stress Is Associated With the Upregulation in Caspase-11 Activity in Liver Tissue From Mice With Advanced Fibrosis

Prior work has demonstrated that hepatocyte ER stress is associated with liver fibrosis and cell death (Lebeaupin et al., 2015; Iracheta-Vellve et al., 2016). ER stress occurs as a consequence of disrupted intracellular homeostasis and accumulation of misfolded proteins, and has been associated with induction of caspase-11 activity through a direct interaction with the ER stress protein C/EBP homologous protein (CHOP)

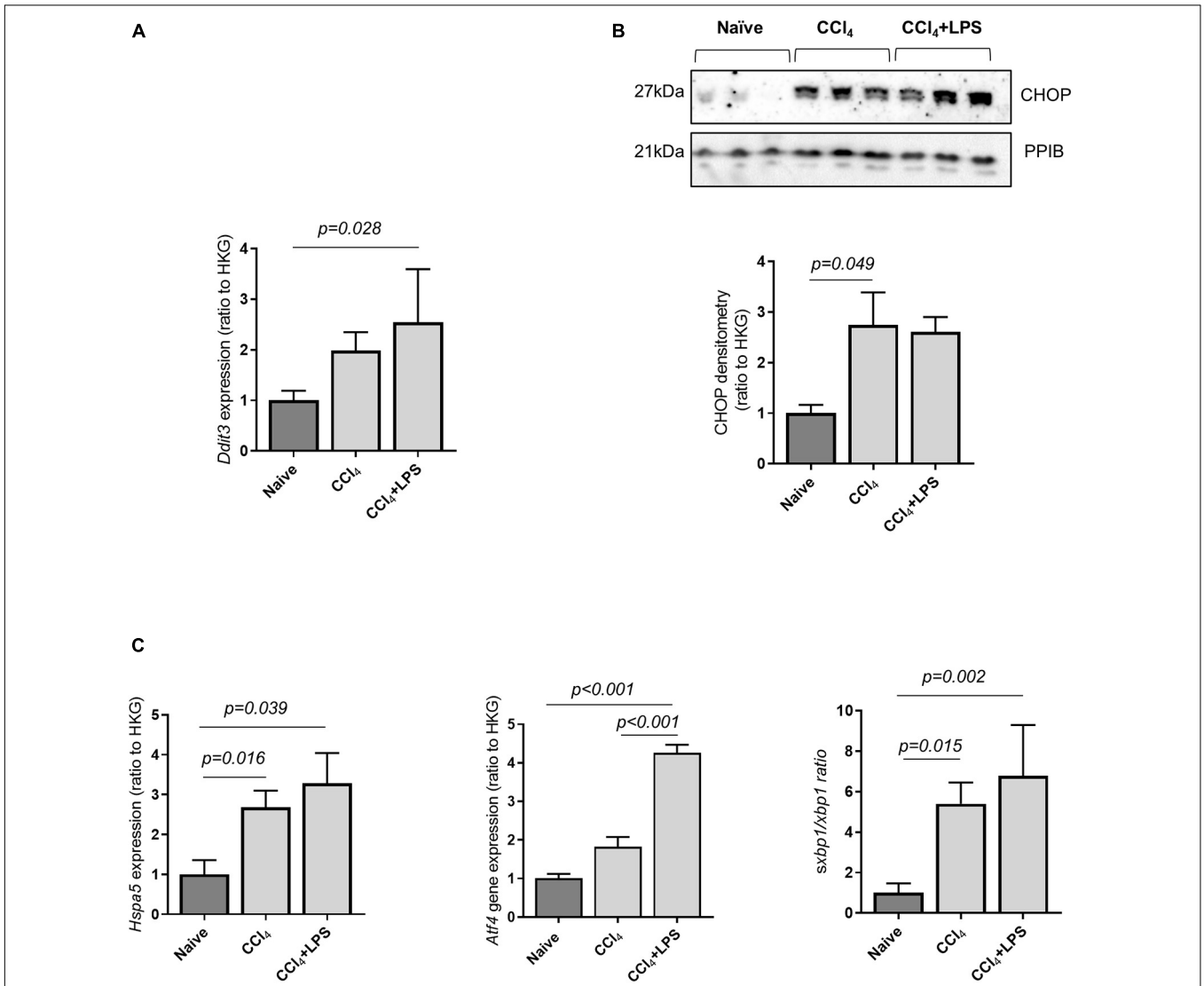

FIGURE 3 | Cirrhotic mice display increased hepatic endoplasmic reticulum stress. The expression of key genes involved in ER stress were measured by qPCR and/or Western blot from snap-frozen liver extract in CCl_4-treated (n = 7), and naïve (n = 5) mice. The housekeeping gene ppib was used for normalization across experiments. **(A)** Gene expression of the ER-stress marker *Ddit3*, coding for the protein CHOP, was increased in mice treated with CCl_4 + LPS (Dunn's test, p = 0.028). **(B)** Protein expression of mature CHOP was significantly increased in the CCl_4-treated group, compared to control (Dunn's test p = 0.049). **(C)** Gene expression of *Hspa5, Atf4, and ratio of spliced to unsplice xbp1* were significantly different across groups (one-way ANOVA: *Hspa5*, p = 0.039; *Atf4*, $p < 0.001$; s*Xbp1/Xbp1*, p = 0.002).

(Endo et al., 2006). Accordingly, CHOP expression was measured in liver tissue from naïve and CCl_4-treated mice, and a significant upregulation of D*dit3* mRNA and CHOP protein expression was found in mice with advanced liver fibrosis compared to control (**Figures 3A,B**). ER stress was further confirmed by measurements of other markers genes: Hspa5, coding for the ER chaperone protein GRP78, Atf4, a stress-induced transcription factor, and spliced Xbp1, which were found to be elevated in the liver of CCl_4-treated mice (**Figure 3C**). These results demonstrate an association between hepatic ER stress and upregulation of the caspase-11 pathway in CCl_4-treated mice.

Caspase-11 Deficient Mice Are Protected From Hepatic and Extra-Hepatic Organ Injury in ACLF

To explore the specific role of caspase-11 in the onset of multi-organ injury in response to LPS insult, we employed caspase-11 deficient mice (*Casp11*$^{-/-}$ mice). Upon treatment with CCl_4, *Casp11*$^{-/-}$ mice developed a similar level of advanced fibrosis compared to wild-type (wt) mice (collagen proportionate area measurement: 5.3 ± 0.3 vs. 5.8 ± 0.5%, $p = 0.36$; result not shown), but were significantly protected from hepatic and extra-hepatic organ injury following i.p. injection of LPS (4 mg/kg) (**Figure 4A**). This was associated with a significant reduction in circulating LDH (**Figure 4B**) and in the number of TUNEL-positive cells on liver immunostaining (**Figure 4C**), consistent with a reduction in hepatocyte cell death. Absence of caspase-11 protein in the liver of *Casp11*$^{-/-}$ mice following treatment with CCl_4 and LPS was confirmed by western blotting (**Supplementary Figure 3**).

Hepatocytes Undergo Pyroptosis in a Dose-Dependent Manner in Response to LPS, and Are "Sensitized" by Prior Low-Dose LPS Exposure and ER Stress

Prior data has shown that hepatocytes are the key cells undergoing apoptotic and non-apoptotic cell death in rodent models of ACLF (Adebayo et al., 2015). This observation was supported by TUNEL staining of liver tissue from CCl_4 and CCl_4 + LPS mice, which demonstrated primarily hepatocyte

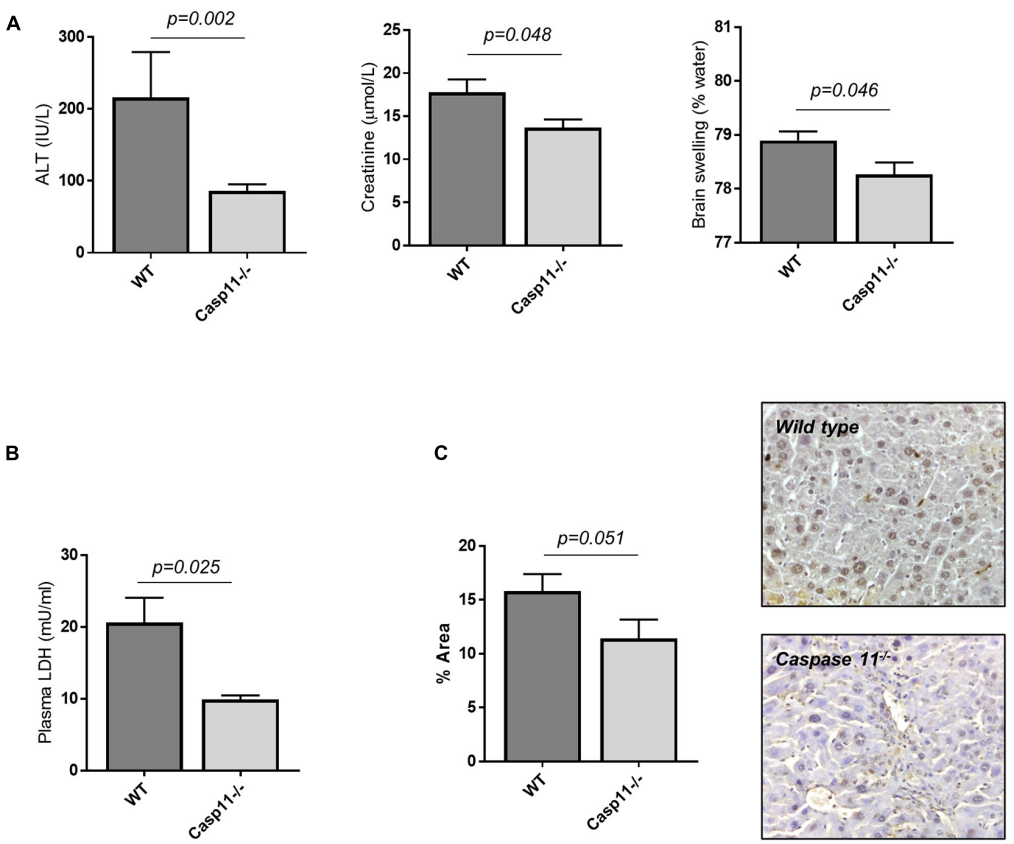

FIGURE 4 | Caspase-11 deficiency protects from hepatic and extra-hepatic organ injury in ACLF. Wild type and *casp11*$^{-/-}$ type mice were treated with CCl_4 (0.5ml/kg, 20 doses) to develop advanced hepatic fibrosis, and subsequently injected i.p., with LPS (4 mg/kg). **(A)** 4 h following LPS injection, *Casp11*$^{-/-}$ mice ($n = 6$) displayed significantly lower ALT (Student's *t*-test, $p = 0.002$), creatinine (Student's *t*-test, $p = 0.048$), and brain tissue water content (Student's *t*-test, $p = 0.046$) than wild type ($n = 8$). **(B)** Lower levels of cell death were also reflected in reduced levels of plasma LDH (Student's *t*-test with Welch correction, $p = 0.025$). **(C)** Hepatocyte cell death was assessed by TUNEL assay and quantified by measuring positively stained areas. *Casp11*$^{-/-}$ mice displayed lower levels of hepatocyte cell death than wild type (Student's *t*-test, $p = 0.051$).

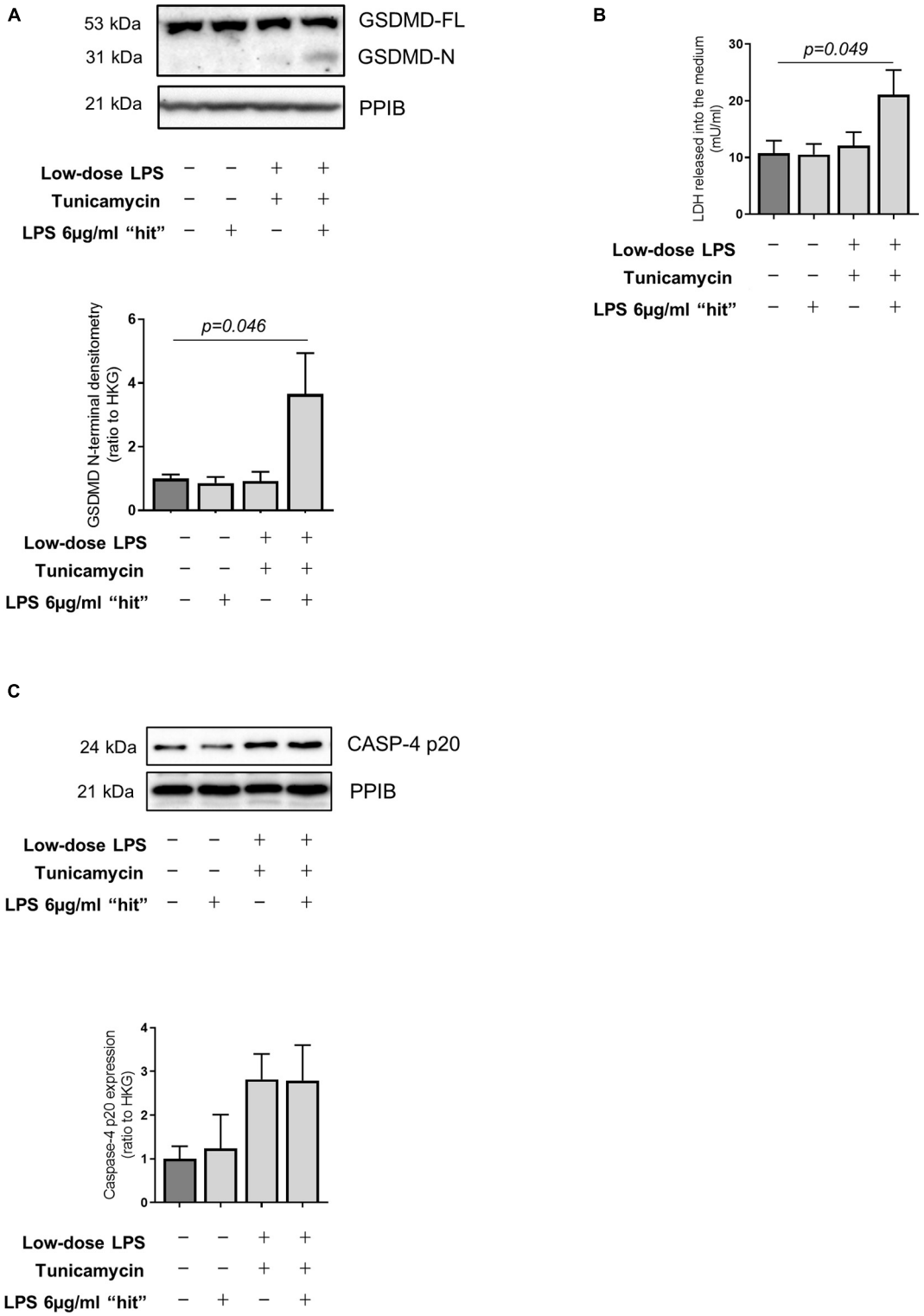

FIGURE 5 | LPS-dependent pyroptosis in hepatocytes and sensitization by ER stress. **(A)** Primary human hepatocytes treated for 24 h with very low-dose LPS (100 ng/ml) and the ER-stress inducer tunicamycin (1 μM) prior to subsequent LPS 'hit' (6 μg/ml) show increased GSDMD cleavage compared to control (one-way ANOVA with Tukey *post hoc* test, $p = 0.046$, $n = 3$ experiments/group). **(B)** Susceptibility to induced pyroptosis was confirmed by increased release of LDH upon LPS "hit," by hepatocytes treated with low-dose LPS (100 ng/ml) and tunicamycin (1 μM) (one-way ANOVA with Tukey *post hoc* test, $p = 0.049$, $n = 3$ experiments/group). **(C)** Primary human hepatocytes showed a trend toward increased active Caspase-4 upon tunicamycin treatment, as measured by the abundance of the p20 fragment.

death following LPS injection, and a significant increase in overall cell death in the CCl_4 + LPS group (**Supplementary Figure 1D**). Prior work has also demonstrated that hepatocytes can actively internalize LPS through carrier mechanisms, and that hepatocytes play a key role in the clearance of LPS during endotoxemia (Deng et al., 2013; Topchiy et al., 2016).

Recent work has utilized a model of exposure of hepatocytes to low-dose LPS to mimic low-level gut-derived translocation of bacterial products in cirrhosis (Isaacs-Ten et al., 2020). Accordingly, we pre-treated primary human hepatocytes with low-dose LPS (100 ng/ml) alongside the ER stress inducer tunicamycin for 24 h to reflect the hepatocyte microenvironment in cirrhosis, prior to a subsequent LPS 'hit' (6 μg/ml). These experiments demonstrate that hepatocytes are resistant to pyroptosis in the baseline state but can be sensitized to pyroptosis in an environment of ER stress and low-dose LPS exposure, displaying increased abundance of GSDMD N-terminal (**Figure 5A**) and LDH release (**Figure 5B**). Treated hepatocytes also showed a trend toward increased activation of caspase-4, measured as abundance of the active (p20) fragment (**Figure 5C**), recapitulating the data collected *in vivo*.

DISCUSSION

In this study we demonstrate, for the first time, a role for LPS-sensing inflammatory caspases and the pyroptosis pathway in the progression to multi-organ injury in cirrhosis. The novel findings of this study are: (i) increased hepatic expression of caspase-4 is a feature of acutely decompensated cirrhosis and correlates with disease severity, (ii) hepatic expression of caspase-11 is also upregulated in a mouse model of advanced liver fibrosis, (iii) hepatic expression of the cleaved fragment of GSDMD, the effector protein of the pyroptosis pathway, is increased in mice with liver fibrosis and multi-organ injury, (iv) *Casp11*$^{-/-}$ mice with advanced liver fibrosis are protected from excess hepatocyte death and multi-organ injury. These findings are of particular relevance, since cirrhosis is associated with progressive changes in the composition of the microbiome as well as translocation of bacterial-derived products (Bajaj, 2019). The work presented here provides a mechanistic link between bacterial dysbiosis and LPS translocation in cirrhosis, and LPS-mediated liver and multi-organ dysfunction (see graphical abstract). Moreover, these findings have translational importance through the potential utility of pyroptosis inhibitors as novel therapeutic strategies for acutely decompensated cirrhosis and ACLF.

Two groups have recently implicated activation of the pyroptosis pathways as a feature of fatty liver disease and alcohol-related liver disease. Xu et al. (2018) demonstrated increased expression of capases-1/4/5 and resulting GSDMD cleavage in patients with non-alcoholic steatohepatitis (NASH), with degree of cleavage correlating with hepatic inflammation. Further, they demonstrated protection from NASH in *Gsdmd*$^{-/-}$ mice, and additionally used a hepatocyte-directed vector expressing GSDMD N-terminus to exacerbate hepatic injury. Khanova and colleagues also investigated the caspase-4/11-GSDMD pathway in alcoholic hepatitis and identified hepatic GSDMD cleavage as a feature of alcoholic hepatitis in mice and patients (Khanova et al., 2018). Additionally, they also used hepatocyte-specific over-expression of GSDMD N-terminus to exacerbate the alcoholic hepatitis phenotype, and demonstrated protection using *Casp1/Casp11* double knockout mice. Both these studies demonstrate a role for hepatocyte pyroptosis in steatohepatitis, although the mechanisms responsible for activation of caspase(s)-4/11 in liver disease remain ill-defined. The present study extends these previous observations and demonstrates a mechanistic link between translocation of gut-derived LPS and hepatocyte cell death in cirrhosis. Specifically, our study is the first to explore this pathway in advanced liver disease and to use mice with selective deficiency in caspase-11, thereby confirming a causal role for this pathway in multi-organ injury associated with decompensated cirrhosis. Importantly, these data are also consistent with human data, which demonstrate much higher levels of cell death in decompensated cirrhosis than in stable cirrhosis (Macdonald et al., 2018). Additionally, prior data supports a shift in mode of cell death, from apoptotic to non-apoptotic, with development of multi-organ failure in cirrhosis. Thus, although pyroptosis may be a feature of steatohepatitis, high levels of pyroptotic cell death are likely to play a pivotal role in the development of severe liver injury and multi-organ failure.

The data presented here also demonstrate that despite the occurrence of kidney injury and brain swelling in ACLF, there is no significant GSDMD cleavage in the kidney or brain in our mouse model of ACLF in response to LPS injection, suggesting that the injury observed in these organs is caused by mechanisms other than GSDMD-dependent pyroptosis. Indeed, the marked protection of the extra-hepatic organs in *Casp11*$^{-/-}$ mice suggests that the primary event is hepatic pyroptosis subsequently leading to extra-hepatic organ injury, rather than direct LPS-induced injury in these extra-hepatic organs. The recently described roles of extracellular vesicles in inter organ communication and the correlation between liver injury and increased levels of extracellular vesicle release from hepatocytes (Malhi, 2019), warrant investigation into whether they might be conduit of the signal leading to multi-organ failure in response to liver injury in cirrhosis.

Currently, there are no specific therapies for acutely decompensated cirrhosis and ACLF, and previous attempts to modify cell death pathways in this group, through pan-caspase inhibition, have been unsuccessful (Mehta et al., 2018). The data presented here suggest that a more targeted approach to inhibit inflammatory caspases or downstream pyroptosis pathways would be preferable. Recent work from Hu et al. demonstrates that disulfiram, a licensed drug with an excellent safety record, is an inhibitor of pyroptosis (Hu et al., 2020). Consequently, it is clear that disulfiram is an attractive candidate for repurposing as a potential therapeutic for cirrhosis and ACLF, and clinical trials are warranted.

The limitations of this work are the skewed distribution of patients between the AD and SC groups, which is however justified by the limited variability among the SC patients, and that we cannot exclude a role for pyroptosis in hepatic phagocytic cells, such as infiltrating bone-marrow derived

monocytes or Kupffer cells, in the development of ACLF. The immunostaining data, in mice and humans, presented here demonstrate primarily hepatocyte staining in liver tissue samples, although the involvement of monocyte-macrophage cells cannot be absolutely excluded histologically. The data presented here are also consistent with the findings from Khanova et al. (2018) and Xu et al. (2018) in previous studies who noted primarily hepatocyte pyroptosis in disease models, as well as clinical data from the CANONIC cohort study which demonstrate that circulating markers of cell death in ACLF are correlated with markers of hepatic injury (bilirubin, alanine aminotransferase) rather than markers of extra-hepatic organ dysfunction (Moreau et al., 2013; Khanova et al., 2018; Xu et al., 2018). Fundamentally, these findings build upon an existing body of work supporting a specific role of hepatocytes in handling circulating LPS, as distinct from the innate immune compartment which plays a primary role in responding to microorganisms and DAMPs (Mimura et al., 1995; Deng et al., 2013; Topchiy et al., 2016). Our data further suggests that the handling of circulating LPS in cirrhosis is aberrant due to sensitization of the hepatocyte caspase-4/11 pathway.

CONCLUSION

In this study we demonstrate for the first time upregulation of inflammatory caspase(s)-4/11 and increased hepatocyte pyroptosis in acutely decompensated cirrhosis, and a causal link between translocation of gut-derived LPS and liver- and multi-organ injury in mouse models of liver fibrosis. This work highlights pyroptosis as a potential novel target for therapy in patients with acutely decompensated cirrhosis and ACLF.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institute of Liver and Biliary Sciences, Institutional Review Board (IRB) – 02/11/2019. Written informed consent for participation was not required for this study in accordance with the National Legislation and the institutional requirements. The animal study was reviewed and approved by UCL Animal Welfare and Ethical Review Body (AWERB).

AUTHOR CONTRIBUTIONS

US and NB contributed to experimental design, data collection and analysis, and drafted the article. EW, SB, CB, AbH, AS, ViP, VaP, AnH, TL, and AN contributed to data collection and drafted the article. RW, SC, BP, RM, and RJ contributed to experimental design and drafted the article. GM supervised the study, contributed to experimental design, data collection and analysis, and drafted the article. 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/fcell.2021.668459/full#supplementary-material

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Conflict of Interest: RJ has research collaborations with Takeda and Yaqrit, consults for Mallinckrodt and Yaqrit and has received speaking fees from Grifols, and founder of Yaqrit Limited and Thoeris GmBh, which is developing UCL inventions for treatment of patients with cirrhosis.

The remaining 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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REVIEW
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The Role of Short-Chain Fatty Acids and Bile Acids in Intestinal and Liver Function, Inflammation, and Carcinogenesis

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During the past decade, researchers have investigated the role of microbiota in health and disease. Recent findings support the hypothesis that commensal bacteria and in particular microbiota-derived metabolites have an impact on development of inflammation and carcinogenesis. Major classes of microbial-derived molecules such as short-chain fatty acids (SCFA) and secondary bile acids (BAs) were shown to have immunomodulatory potential in various autoimmune, inflammatory as well as cancerous disease models and are dependent on diet-derived substrates. The versatile mechanisms underlying both beneficial and detrimental effects of bacterial metabolites comprise diverse regulatory pathways in lymphocytes and non-immune cells including changes in the signaling, metabolic and epigenetic status of these. Consequently, SCFAs as strong modulators of immunometabolism and histone deacetylase (HDAC) inhibitors have been investigated as therapeutic agents attenuating inflammatory and autoimmune disorders. Moreover, BAs were shown to modulate the microbial composition, adaptive and innate immune response. In this review, we will discuss the recent findings in the field of microbiota-derived metabolites, especially with respect to the molecular and cellular mechanisms of SCFA and BA biology in the context of intestinal and liver diseases.

Keywords: liver, microbiome and dysbiosis, intestine, immunology, short-chain fatty acid, bile acids, T cell, myeloid cells

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INTRODUCTION

The triangular interdependency between gut microbiota, diet and immune cells is substantially connected to the functionality of a symbiotic cellular network and therefore to the host's health status. The gut as residence for a highly dense microbial community harbors a unique diversity of non-mammalian genes required for the synthesis of various bioactive molecules. These soluble messengers are bridging the gap between host cells as well as commensal bacteria and are required for the maintenance of energy homeostasis, shaping the mucosal immune system and even influencing host behavior (Blumberg and Powrie, 2012; Levy et al., 2016). Changes in the microbiota have been shown to be involved in pathophysiological processes. While microbial diversity is associated with a beneficial outcome in allogenic stem cell transplantation, the impact

of the gut microbiota on checkpoint blockade in cancer therapy showed opposing effects (Coutzac et al., 2020; Peled et al., 2020).

The production of short-chain fatty acids (SCFAs), a major class of microbial metabolites, requires bacterial fermentation of both water-soluble dietary fiber (e.g., pectin, guar gum, and inulin) and insoluble fiber (e.g., resistant starch) in the gut lumen by members of the human microbiome (**Figure 1**). Upon food intake, indigestible complex carbohydrates pass the upper part of the gastrointestinal tract, where they become metabolized under anaerobic conditions with peak concentration of SCFAs in the cecum and proximal colon (Cummings et al., 1987). Acetate, propionate and butyrate are the most abundant SCFAs in the gut of conventionally raised mice. Additionally, pentanoate, formate and branched-chain fatty acids (BCFAs) have been identified at much lower levels in the intestine of rodents and humans (Koh et al., 2016). In contrast to SCFAs and lactate, which is another product of the carbohydrate metabolism, BCFAs are derived from fermentation of branched amino acids such as valine, leucine and isoleucine (Smith, 1998; Yang et al., 2010; Rios-Covian et al., 2020). SCFAs are absent in germ-free (GF) animals and were shown to affect different aspects of human health. These implicate, besides autoimmunity and inflammation, the maintenance of gut homeostasis, an equilibrium of interactions between the intestinal epithelium, the host immune system, commensal bacteria and regulatory mechanisms (Kim et al., 2014; Luu et al., 2019).

Notably, besides dietary components, commensal bacteria are able to modify host-derived molecules such as bile acids (BAs). Upon food uptake, stimulation of the gallbladder leads to influx of primary liver-derived BAs into the duodenum being responsible for emulsification of dietary fat (Ridlon et al., 2006). Although most BAs become reabsorbed in the ileum and transported to the liver via the enterohepatic circulation, a smaller fraction is transformed into secondary BAs by bacterial conversion in the colon (Schaap et al., 2014). It has been described that both primary and secondary BAs interact with a family of nuclear (FXR) and G-protein-coupled receptors (GPRs) agonistically or antagonistically, collectively known as BA-activated receptors (BARs), thereby modulating cellular signaling as well as immune response (Chen et al., 2011; Carr and Reid, 2015). Recently, it has been shown that secondary BAs such as 3β-hydroxydeoxycholic acid (isoDCA) were able to increase differentiation of regulatory T cells (Tregs) by interaction with the farnesoid X receptor on dendritic cells (DCs) highlighting a potential for novel therapeutics (Campbell et al., 2020b).

In this review, we examine recent work investigating the modes of action by which two major groups of bacterial metabolites, SCFAs and BAs, impact on liver- and gut-associated inflammatory and cancerous diseases.

MECHANISMS OF SCFA-MEDIATED MODIFICATION OF HOST CELLS

The research focus on SCFAs as a major class of bacterial-derived metabolites has revealed various of their modes of action as well as different cellular modifications (**Figure 2A**) depending on the respective cell type (**Figure 3**). The diffusible molecules have been shown to be agonists for eukaryotic GPRs which are involved in diverse signaling pathways. Previous studies have demonstrated that binding of acetate and propionate to GPR41 and GPR43 expressed on colonocytes induces p38 and ERK/MAPK activation contributing to the inflammatory response (Kim et al., 2013). Apart from colonocytes, enteroendocrine cells were shown to sense SCFAs via GPR41 and GPR43 (Nøhr et al., 2013). SCFA binding to GPR43 on regulatory T cells (Tregs) mediated protection against colitis in mice (Smith et al., 2013). Similarly, SCFA interaction with GPR109a on dendritic cells (DCs) promoted Treg differentiation and tolerance in the intestinal tissue (Singh et al., 2014).

Due to their small size, either passive diffusion across the cell membrane or active transport via sodium-coupled transporters enable SCFAs to enter the cytoplasma or even the nucleus of eukaryotic cells where they elicit a histone deacetylase (HDAC)-inhibitory activity of a distinct magnitude. A rather weak HDAC-inhibitory activity has been observed in experiments using acetate, while propionate and especially butyrate show stronger enzyme inhibition. SCFAs regulate the expression of genes associated with cell proliferation, differentiation, epithelial integrity, and immune response (Kim et al., 2014, 2016; Schilderink et al., 2016; Goverse et al., 2017; Luu et al., 2018). Recent studies have shown that not only enhanced histone H3 acetylation at the *Foxp3* locus but also increased acetylation of the Foxp3 protein itself can be modulated by butyrate, stabilizing the genetic integrity of Tregs (Arpaia et al., 2013; Furusawa et al., 2013).

Besides their HDAC-inhibitory properties, SCFAs are able to increase the activation of mammalian target of rapamycin (mTOR), a central regulator of cell growth and energy homeostasis (Sengupta et al., 2010). Consequently, mTOR-mediated enhancement of glycolysis contributes to the pool of acetyl-CoA. Balmer and colleagues showed that excess acetyl-CoA enters the tricarboxylic acid cycle (TCA) where it becomes converted into citrate. The pharmacologic inhibition of the ATP-citrate lyase (ACLY), an enzyme involved in the conversion of TCA-derived citrate into acetyl-CoA in the nucleus, strongly reduced the IFN-γ production in acetate-treated $CD8^+$ memory T cells (Balmer et al., 2016). The nuclear acetyl-CoA served as a substrate for histone acetyltransferases (HATs) which facilitate the conjugation of acetyl groups to histones, thereby regulating gene expression and consequently the production of cytokines such as IL-10 and IFN-γ (Wellen et al., 2009; Zhao et al., 2016; Bantug et al., 2018; Luu et al., 2019). These data strongly support the concept of a metabolic-epigenetic crosstalk in which cellular metabolism-derived molecules serve as source for posttranslational modifications (PTMs) (**Figure 2A**).

As one of the most frequent PTMs, acetylation of proteins, such as histones, has been investigated intensively. Recent studies identified even longer alkyl motifs derived from SCFAs as substrates for histone modification (Kebede et al., 2017; Fellows et al., 2018). Kebede and colleagues described the propionylation and butyrylation of histone H3 as a novel mark of active chromatin in HeLa cells. In accordance with the concept that microbiota-derived metabolites act on the epigenetic state of

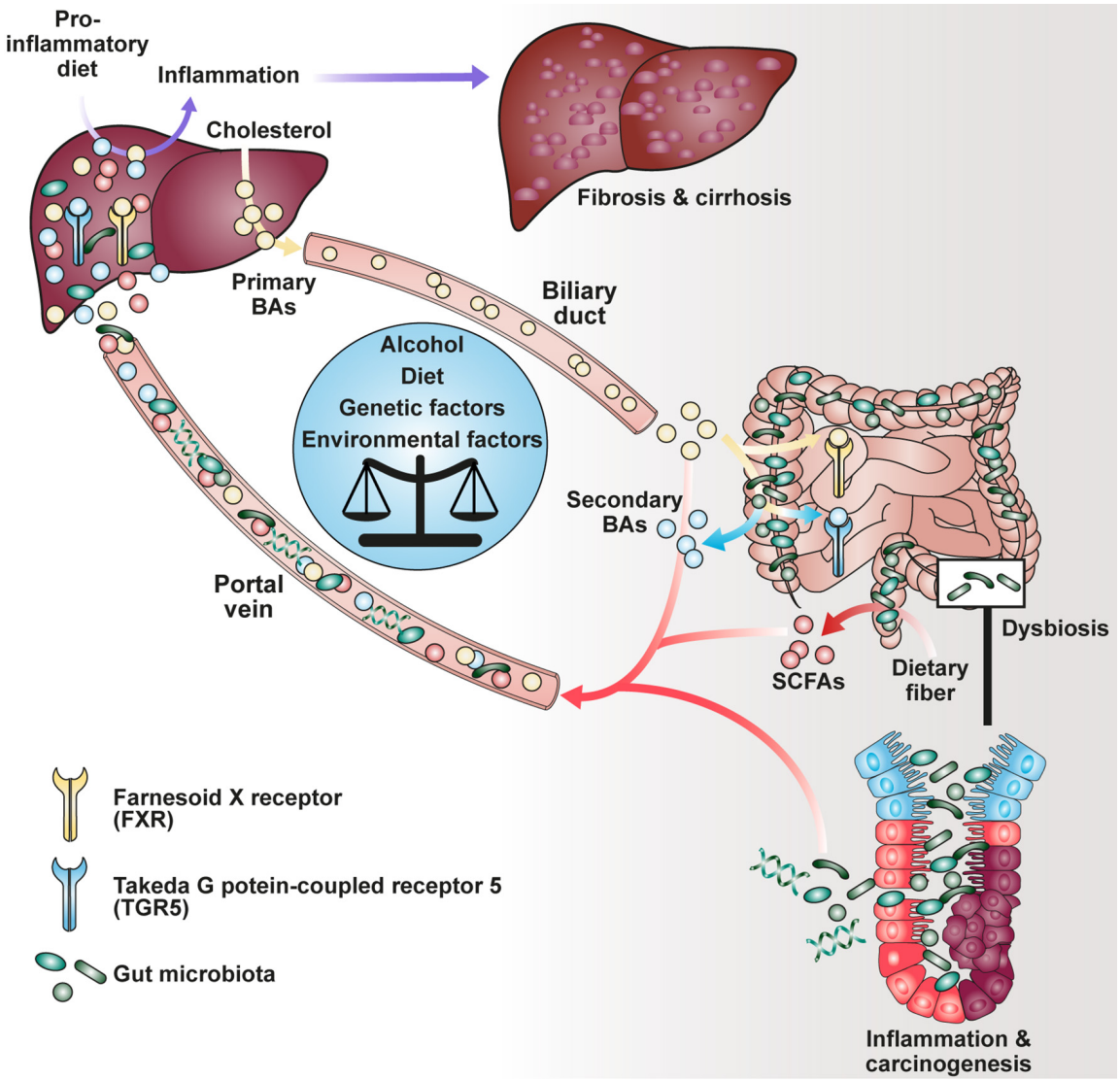

FIGURE 1 | Impact of BAs and SCFAs on the gut-liver-axis.

host cells, it has been shown that antibiotic treatment reduces the microbial SCFA-mediated histone crotonylation in intestinal crypts (Fellows et al., 2018). These findings suggest a link between microbiota and epigenetic regulation opening the venue for investigating new PTMs based on microbial metabolites.

SCFAS IMPACT ON INTESTINAL HOMEOSTASIS AND INFLAMMATION

The gut is home to a diverse and dense bacterial community, a unique site of interaction between host and microbiota. Disturbance of this finely regulated balance was shown to be involved in inflammatory diseases such as inflammatory bowel disease (IBD) and colitis-associated carcinogenesis (CAC) (Kamada et al., 2013). Maintenance of the intestinal immune system requires an equilibrium between defense against pathogens as well as tolerance to commensals and food antigens. Therefore, various mechanisms are involved in regulating the immunological response, impacting on intestinal epithelial cells (IECs), induction of anti-inflammatory cells and suppression of inflammatory cells.

Recent studies have identified several effects of SCFAs on epithelial cells. SCFA administration was shown to stimulate retinoic acid (RA) production in the intestinal epithelium, a vitamin A derivate converted by aldehyde dehydrogenases, which is associated with signaling and expansion of peripheral Tregs (pTregs) in the context of a immunosuppressive response (**Figure 3**; Benson et al., 2007; Hill et al., 2008; Schilderink et al., 2016). Further, butyrate treatment of epithelial cells increased the production of IL-18 via a GPR109a-mediated mechanism which contributes to intestinal homeostasis and protects against

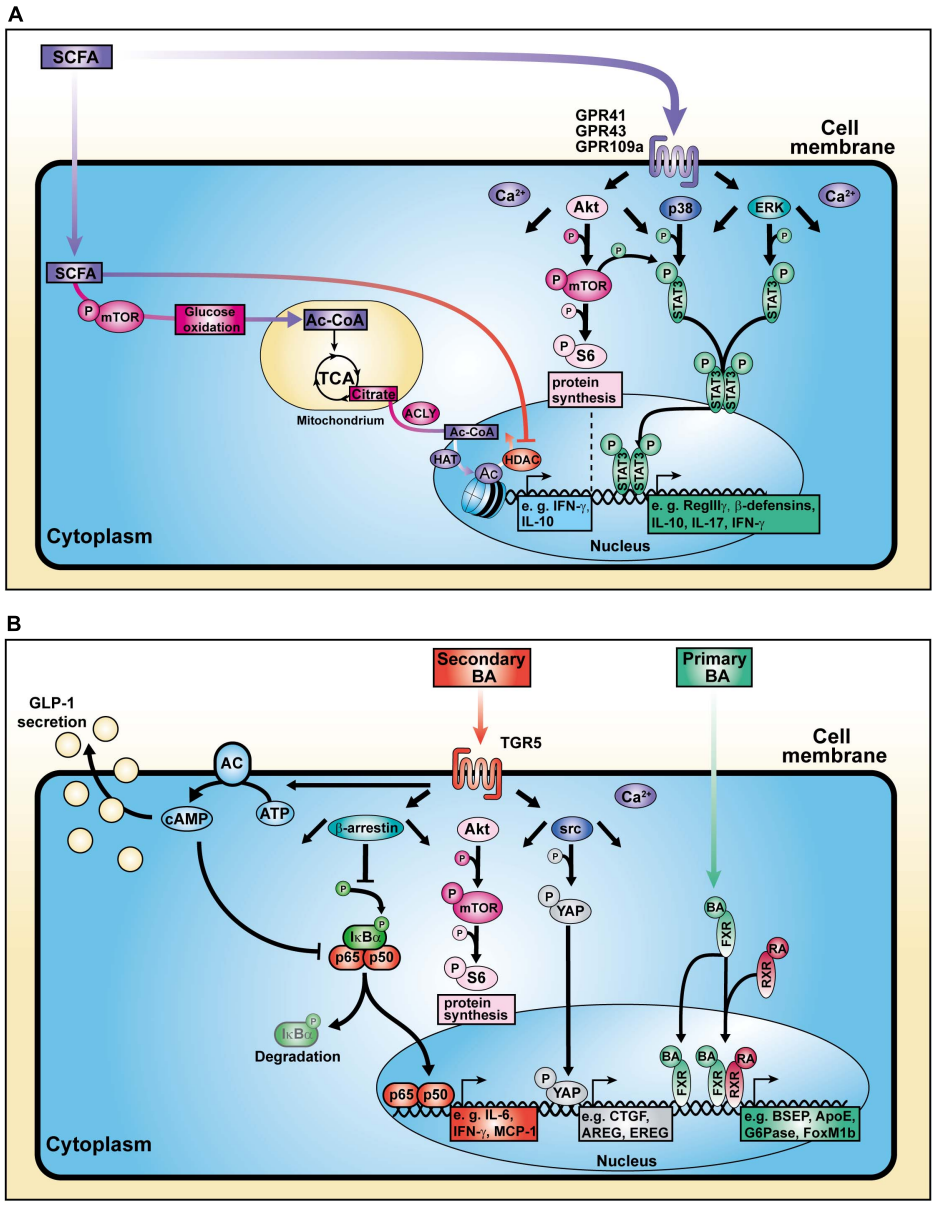

FIGURE 2 | Cellular mechanisms of action of SCFAs and BAs. **(A)** SCFAs influence cell signaling, metabolic activity, and histone modification. **(B)** BAs bind to surface and nuclear receptors.

colorectal carcinogenesis (Kalina et al., 2002; Zaki et al., 2010; Singh et al., 2014). Similarly, binding of SCFAs to GPR41 and GPR43 enhanced both expression of anti-microbial factors such as RegIIIγ and β-defensins in IEC by enhancing mTOR and STAT3 signaling, whereas mice deficient for the receptors suffered from impaired immune response against *C. rodentium* infection (Kim et al., 2013; Zhao et al., 2018). Besides regulation of anti-microbial molecules in epithelial cells, the increase in metabolic input and consequently acetyl-CoA upon SCFA or dietary fiber administration also regulates genes involved in plasma cell differentiation and IgA antibody production, important factors in maintaining gut homeostasis as well (Kim et al., 2016). The relevance of bacterial-derived SCFAs to gut homeostasis has been investigated in GF mice suffering from reduced mucosal integrity and IgA (Moreau et al., 1978; Duboc et al., 2013; Zeng et al., 2016).

SCFAs also mediate immunosuppression by either inducing IL-10 in different immune cells or repressing inflammatory macrophages in the lamina propria causing hyporesponsiveness to commensal bacteria (Hayashi et al., 2013; Sun et al., 2018). The importance of this aspect in IBD was highlighted by antibiotics-treated mice deficient for SCFAs and suffering from hyperresponsive macrophages and inflammation (Scott et al., 2018). A link between SCFA-mediated mTOR activation and IL-10 production in T cells and regulatory B cells (Bregs) has recently been shown by our lab investigating

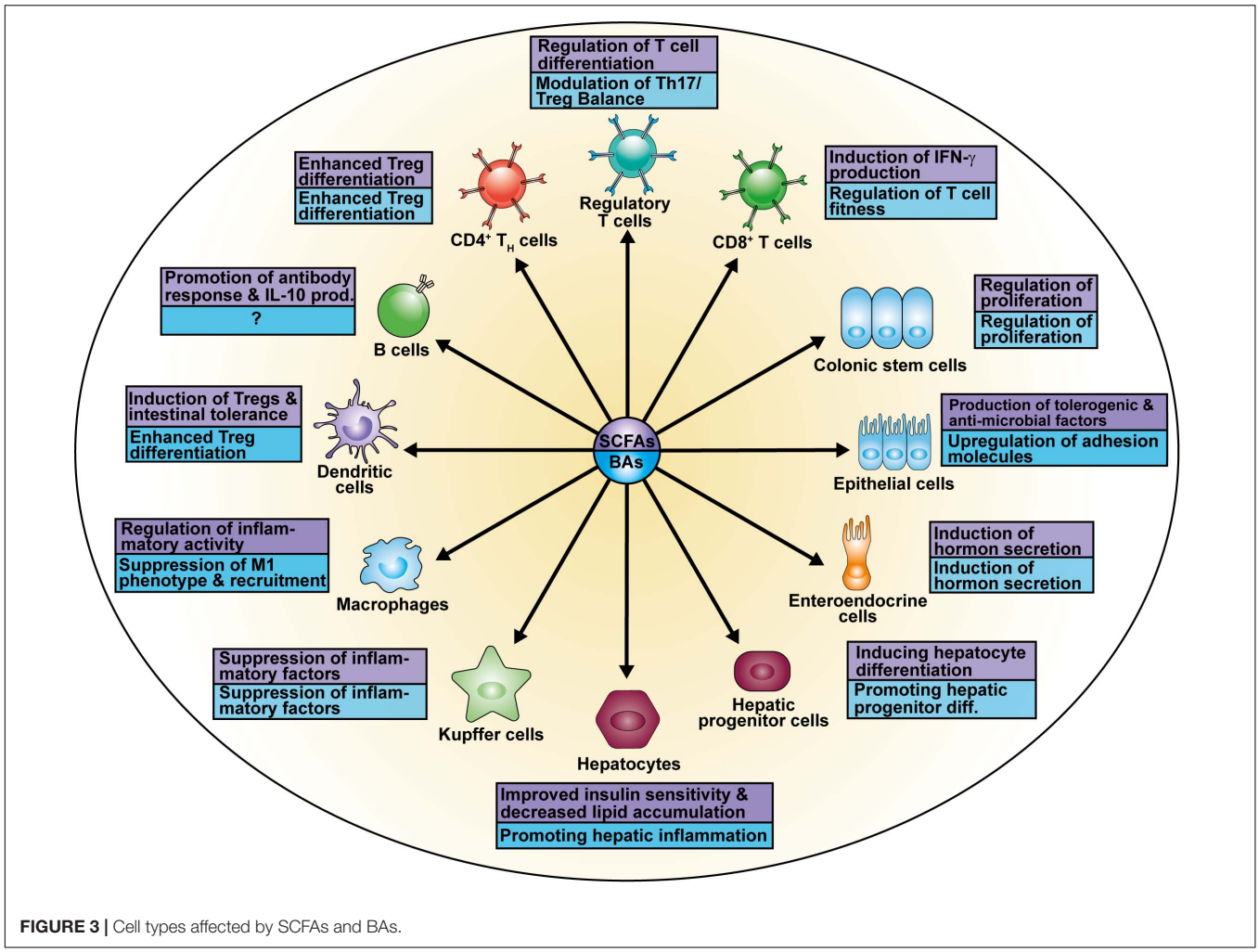

FIGURE 3 | Cell types affected by SCFAs and BAs.

the subdominant microbiota-derived pentanoate (also known as valerate). Pentanoate enhanced glycolysis and consequently intracellular acetyl-CoA levels as HAT substrate, suggesting a regulation of the *Il10* locus by this mechanism (Luu et al., 2019).

The research on Tregs as crucial mediators of gut homeostasis and oral tolerance has identified acetate, propionate and butyrate as central molecules bridging the gap between commensals and the mucosal immune system. Their importance was emphasized by restoration of the colonic Treg population in GF mice, lacking both microbiota and commensal-derived metabolites, by SCFA supplementation (Smith et al., 2013). Different mechanisms underlying colonic Treg expansion have been proposed. Inhibition of HDACs leads to hyperacetylation at histone H3 and H4, particularly the acetylation in the promoter and CNS regions of the *Foxp3* locus which causes an increased expression of the Treg master regulator (Furusawa et al., 2013). Furthermore, enhanced acetylation of the Foxp3 protein itself upon butyrate treatment was shown to stabilize this transcription factor, protecting it from degradation (Arpaia et al., 2013). Apart from acting as HDAC inhibitors, Smith and colleagues described GPR43 to be exclusively expressed on colonic Tregs but not on those of other tissues, thereby pointing out the receptor-mediated Treg induction by SCFAs (Smith et al., 2013). Recent studies have identified several *Clostridium* strains among the commensal species, shown to facilitate colonic Treg maturation (Atarashi et al., 2011, 2013). Colonization of mice with 17 *Clostridium* strains producing SCFAs isolated from healthy humans resulted in a TGF-β-rich environment which supported Treg expansion and differentiation (Atarashi et al., 2013). Moreover, spore-forming *Clostridia* are involved in the fermentation of indigestible dietary fiber in the colon fueling the pool of SCFAs as key metabolites (Koh et al., 2016). In conclusion, these results demonstrate the complexity of microbiota-mediated regulation of gut homeostasis.

SCFAS IN DEVELOPMENT OF COLORECTAL CANCER AND STEM CELL RENEWAL

Although the contribution of SCFAs to maintenance of gut homeostasis has been investigated extensively, there is an increasing body of evidence that commensal bacteria and bacterial metabolites have opposing roles in inflammatory

responses and carcinogenesis depending on the cell type and the environment. Park and colleagues revealed that HDAC inhibition and mTOR activation rather than interaction with GPR43 is functionally important for the impact of SCFAs on T cells (Park et al., 2014). Further, they suggested that this main class of microbial metabolites boosts differentiation of naïve T cells into Th1 and Th17 cells during encounter with pathogens. Since these T cell subtypes were also described to be involved in colitis and inflammation-associated carcinogenesis in the colon, an involvement of SCFAs in colon carcinogenesis has been investigated recently (Grivennikov et al., 2010).

In a steady-state situation, butyrate is present in the mM range in the gut lumen and serves as the primary energy source for colonocytes (Flint et al., 2012). Butyrate's HDAC-inhibitory properties have been pointed out by various studies (Park et al., 2014; Kespohl et al., 2017; Luu et al., 2018, 2019). Further, its impact on the cellular metabolism of colonocytes was investigated by the analysis of colonic tissue derived from GF mice. Colonocytes from these mice showed an energy-deprived state accompanied by reduced levels of enzymes involved in intermediary metabolism such as the TCA cycle, oxidative phosphorylation and ATP. Reconstitution with butyrate restored mitochondrial respiration in GF colonocytes (Donohoe et al., 2011). However, in the context of cancerous colonocytes, butyrate was shown to act paradoxically. While low-dose butyrate stimulates the proliferation of cancerous colonocytes not undergoing the Warburg effect in a low-glucose environment, comparably to non-cancerous ones, butyrate inhibits the proliferation of colonocytes utilizing the Warburg effect in a glucose-rich environment (Donohoe et al., 2012). The anti-proliferative effect of butyrate on glycolytic cells was attributed to histone hyperacetylation and changes in expression of genes involved in proliferation and apoptosis. High-dose butyrate caused histone hyperacetylation via its HDAC-inhibitory properties, whereas low-dose butyrate provided acetyl groups for HATs through its metabolization (Donohoe et al., 2012). These results emphasize a differential utilization of butyrate depending on the metabolic demand of the respective cell. In contrast to the potentially beneficial inhibition of cancerous colonocytes, the metabolization of butyrate by normal colonic epithelial cells was shown to mediate protection from its rather detrimental effect on colonic stem cells. Kaiko and colleagues exposed stem/progenitor cells *in vivo* to butyrate either by mucosal injury or application to zebrafish, naturally crypt-less host organisms, resulting in inhibited cell proliferation as well as impaired wound repair. These results suggested that the crypt structure anatomy might have co-evolved with metabolic pathways reacting to the microbiome (Kaiko et al., 2016). In contrast to the inhibitory effect of butyrate on colonic stem cells, high butyrate concentrations promoted differentiation of embryonic stem cells into hepatic progenitor cells (Ren et al., 2010). Interestingly, conversion of phytate into inositol-1,4,5-triposphate by commensals was identified as HDAC3 modulator countering the inhibition of epithelial growth by butyrate (Wu et al., 2020). In addition to that, butyrate is capable of promoting carcinogenesis in a genetic mouse model based on mutations in the *Apc* and the mismatch repair gene *Msh2* ($Apc^{Min/+}$;$Msh2^{-/-}$) (Belcheva et al., 2014). The authors showed an inflammation-independent contribution of butyrate to tumor development which was likely associated with an increased proliferation of epithelial stem cells and was reduced by feeding low-carbohydrate diet, linking the impact of microbiota and nutrition on tumorigenesis.

SCFAS AND BAS IMPACT ON LIVER FUNCTION, INFLAMMATION AND CARCINOGENESIS

The fermentation of dietary soluble fiber into SCFAs has been considered broadly as beneficial, promoting studies investigating the effect of various diets on host immunity and pathophysiology (Trompette et al., 2014; Tan et al., 2016; Zou et al., 2018). Although most of the interactions between diet, immune system and microbiota have been observed in the gut tissue, effects on organs in the periphery were described (Trompette et al., 2014; Haghikia et al., 2016). Recently, the profile of gut microbiota derived from feces of cirrhotic patients with hepatocellular carcinoma (HCC) showed an increase in *E. coli* pointing out that liver function is influenced by gut microbes (Grąt et al., 2016). It has been hypothesized that detection of the microbiota via surface receptors on hepatocytes such as TLR4 or TLR9 contributes to chronic liver injury, thus promoting cholestasis and HCC (Dapito et al., 2012; Cai et al., 2017). In contrast, TLR5 on hepatocytes mediated protection in a mouse model of high-fat-diet (HFD)-induced liver steatosis being important for bacterial clearance (Etienne-Mesmin et al., 2016). Emerging evidence has implicated that HFD enhances intestinal permeability, inflammation, and disease risk (Murphy et al., 2015). In mouse models, HFD induced alteration of the microbiota composition and reduced microbial diversity (Liu et al., 2021). Interestingly, clinical trials associated diet-induced obesity with decrease of microbial gene richness as well as increase of low-grade inflammation (Cotillard et al., 2013). Dysbiosis was further shown to cause reduction of intestinal integrity leading subsequently to bacterial translocation and endotoxemia (Cani et al., 2008; Dapito et al., 2012). Hence, HFD links diffusion of lipopolysaccharide from the gut to systemic inflammatory response as an exemplary factor involved in the gut-liver-axis (Cani et al., 2007, 2008; Laugerette et al., 2012).

A growing body of evidence suggests that SCFAs are key molecules of the gut-liver-axis with the capacity to either indirectly or directly impact on physiological liver function. On the one hand, they trigger the secretion of gut hormones such as GLP-1 by enteroendocrine cells improving glucose tolerance (Tolhurst et al., 2012). These effects have been attributed to GPR41 and GPR43 signaling in L cells which showed increased surface expression of both receptors. Ablation of these beneficial effects on liver function was observed in *Gpr41*-/ *Gpr43*-deficient mice (Tolhurst et al., 2012; Shimizu et al., 2019). On the other hand, the portal circulation enables the direction of gut-derived SCFAs toward the liver. Based on experiments with physiological amounts of isotope-labeled SCFAs, den Besten and colleagues demonstrated that 62% of the infused propionate in the

murine cecum was involved in whole body glucose production, accounting for 69% of total glucose synthesis (den Besten et al., 2013). Consistently, hepatocyte-like cells in a coculture system with epithelial cells were treated with propionate and showed increased glycogen synthesis as well as storage (El Hage et al., 2020). Further, oral administration of HFD supplemented with acetate and propionate in different ratios was associated with increased insulin sensitivity and reduced triglyceride content in the liver (Weitkunat et al., 2016). Mice fed either HAc or HPr showed lower blood glucose levels 240 min after glucose administration as compared to HFD feeding. At the same time, decreased levels of plasma insulin were detected in the HAc and HPr groups. These results highlighted a more efficient glucose uptake with a lower demand for insulin upon SCFA treatment (Weitkunat et al., 2017). The beneficial effects on insulin resistance might be attributed to the previous observation that SCFAs promote GLP-1 secretion (Tolhurst et al., 2012).

It has been found that SCFAs are able to stimulate hormone secretion by enteroendocrine cells but their effect on hepatic responsiveness was investigated to lesser extent. A recent study showed similar levels of GLP-1 in the serum of NAFLD patients and healthy controls but detected a downregulation of the hepatic GLP-1 receptor (GLP-1R) in NAFLD patients. In a mouse model of NAFLD, administration of butyrate reversed the reduction of GLP-1R and led to upregulation of hepatic AMPK phosphorylation and insulin receptor expression in treated mice. Moreover, increase in GLP-1R expression levels in HepG2 cells was mediated by butyrate's HDAC-inhibitory activity, acting indepently of GPR43 and GPR109a (Zhou et al., 2018). Interestingly, binding of propionate to GPR43 on hepatic tumor cells inhibited their growth (Bindels et al., 2012). Early studies investigating the effect of butyrate on a myeloid subset of hepatic cells, Kupffer cells, showed a significant decrease in TNF-α as well as an increase in prostaglandin E2 (PEG2) production (Perez R. et al., 1998; Perez R.V. et al., 1998). These observations highlighted a potential role of butyrate in Kupffer cell immunoregulation which might protect from HCC by alleviating inflammatory responses as prerequisite.

In addition, the role of SCFAs, BAs and diet in HCC development was investigated resulting in opposing observations. Singh and colleagues have found that the addition of the soluble fiber inulin to the diet induced HCC in a microbiota-dependent manner in dysbiotic mice but not in germ-free or antibiotics-treated mice (Singh et al., 2018). Moreover, inulin-enriched HFD promoted dysbiosis and HCC in WT mice which was associated with liver inflammation, neutrophil influx and cholestasis (**Figure 1**). These pathologies were ameliorated by either depleting butyrate-producing bacteria or excluding soluble fiber from diet as source of SCFA generation. Of note, inhibition of the enterohepatic recycling of BAs reduced liver carcinogenesis suggesting their involvement in hepatic inflammation (Singh et al., 2018). The nourishment of mice with fermentable dietary fiber guar gum altered the gut microbiota composition and elevated the bile acid levels in the liver (Janssen et al., 2017). Although diet-induced obesity was reduced, guar gum-related BA levels enhanced liver inflammation and fibrosis. Consistently, administration of taurocholic acid led also to hepatic inflammation which could be reduced by antibiotics treatment (Janssen et al., 2017). In agreement with these findings, analysis of gut microbiome from stool of non-alcoholic fatty liver disease (NAFLD) patients, who suffer from adipokine dysregulation, insulin resistance and fat accumulation in the liver, revealed elevated levels of propionate and BAs (Lee et al., 2020).

While various mechanisms have been proposed for SCFA activity, the effects of BAs on liver injury remain controversial. Treatment of murine and human hepatocytes with pathophysiologic levels of BAs induced the expression of pro-inflammatory cytokines which recruit neutrophils to the hepatic tissue in a CCL2-dependent manner. In addition, TLR9 in hepatocytes was identified as an important mediator of BA-induced liver inflammation (Cai et al., 2017).

Yamada and colleagues demonstrated that secondary BAs promote HCC development in a model of non-alcoholic steatohepatitis (NASH), a progressive form of NAFLD characterized by liver inflammation and fibrosis with the potential to develop into HCC (Yamada et al., 2018). The group fed a new class of steatohepatitis-inducing high fat diet (STHD-01), inducing NASH within 9 weeks post administration consequently progressing into HCC after 41 weeks in WT mice. Accumulation of both cholesterol and BAs in liver and feces were observed after STHD feeding. Interestingly, antibiotics treatment reduced the accumulation of secondary BAs suggesting an impact on bacterial conversion of primary BAs. The group hypothesized that secondary BA-induced mTOR activation in the liver might be responsible for hepatic carcinogenesis in NASH. Another study depicted a connection between circulating BAs and mTOR signaling via the Takeda G protein-coupled receptor 5 (TGR5), emphasizing the role of BA receptors in modulating cellular processes (**Figure 2B**; Zhai et al., 2018).

BA RECEPTORS HAVE AN AMBIGUOUS ROLE IN SHAPING THE MICROBIOME, LIVER FUNCTION AND INFLAMMATORY RESPONSE

Researchers have focused on the regulation of BA and host metabolism mediated by the farnesoid X receptor (FXR) and TGR5 (**Figure 1**). The FXR is a nuclear receptor with the highest expression in liver and ileum functioning as a transcription factor which regulates various target genes either as monomer or upon dimerization with the retinoid X receptor (RXR) and subsequent promoter binding (**Figure 2B**; Kassam et al., 2003; Lefebvre et al., 2009; Teodoro et al., 2011). The target genes are related to BA, lipoprotein and glucose metabolism (e.g., BSEP, ApoE, G6Pase) but also to liver regeneration as demonstrated for the expression of transcription factor FoxM1b (Huang et al., 2006; Wang et al., 2008). With respect to liver regeneration, BA signaling was shown to promote stem cell differentiation toward hepatocytes (Sawitza et al., 2015).

The generation of *Fxr*-deficient mice was a prerequisite for mechanistic studies which showed partially contradictory results, attributed to differences in diet, genetic background

as well as microbiota changes among animal facilities. These mice fed chow diet were susceptible to hyperglycemia and hypercholesterolemia (Lambert et al., 2003; Ma et al., 2006). On the one hand, *Fxr*-deficient mice on *Ldlr*$^{-/-}$ background were protected against HFD-induced obesity and atherosclerosis (Zhang et al., 2012). On the other hand, *Fxr*-deficient mice on *Apoe*$^{-/-}$ background showed elevated atherosclerosis scores (Hanniman et al., 2005). However, in the context of HFD and genetically obese backgrounds (ob/ob), *Fxr*-deficient mice prevalently showed beneficial effects with regards to glucose homeostasis and obesity (Prawitt et al., 2011; Zhang et al., 2012; Parséus et al., 2017). Similar discrepancies were observed in studies investigating the effect of intestinal and hepatic *Fxr*-deficiency on liver steatosis. FXR expression in the gut was shown to mediate HFD-induced NAFLD, whereas liver-specific FXR activity protected against hepatic steatosis (Li et al., 2013; Jiang et al., 2015; Schmitt et al., 2015).

The gut microbiota has been identified as another crucial factor impacting FXR signaling. Sayin and colleagues revealed that the primary BA tauro-β-maurichолic (TβMCA) can be metabolized by gut bacteria (Sayin et al., 2013). Hence, reduction of the natural FXR antagonist improved FXR signaling in mice. Additionally, microbial activity provides secondary BAs as TGR5 ligands by conversion of primary ones (Kuipers et al., 2014). Experimental approaches with gnotobiotic animals revealed that microbiota influences diet-induced obesity in a FXR-dependent manner (Li et al., 2013; Jiang et al., 2015). Further, transfer of microbiota derived from HFD-fed *Fxr*-deficient into GF mice inhibited weight gain compared to bacterial colonization from WT mice (Parséus et al., 2017). *Fxr*-deficient mice on HFD not only showed enhanced levels of the primary BAs βMCA and TβMCA. At the same time, BA profiles of GF *Fxr*-deficient and WT animals were comparable. These findings suggest that the altered microbiota has reduced conversion of primary to secondary BAs due to FXR deletion.

While gut bacteria contribute to the pool of available BAs, vice versa, these are able to shape the microbial composition by either supporting the growth of BA-metabolizing bacteria or growth inhibition of bile sensitive bacteria. Early studies have observed that blockade of bile flow into the gut as result of a biliary obstruction led to bacterial translocation (Clements et al., 1996). Interestingly, experiments in rats showed that bacterial expansion can be reduced by oral bile acid treatment (Lorenzo-Zúñiga et al., 2003). Besides the intrinsic bactericidal properties of BAs, stimulation of FXR induces the production of antimicrobial molecules by immune cells, additionally shaping the microbial colonization (Inagaki et al., 2006).

The membrane-bound G protein-coupled receptor TGR5 is ubiquitously expressed in various tissues such as intestine, liver and gallbladder (Cipriani et al., 2011; Bidault-Jourdainne et al., 2021). In contrast to FXR, TGR5 mainly binds secondary BAs. Therefore, *Tgr5*-deficient mice have served as models to investigate its impact on BA and microbial composition. Although breeding of these mice resulted in healthy offspring, a reduction of the bile acid pool suggested a role of TGR5 in BA homeostasis (Maruyama et al., 2006).

In a model of diet-induced obesity, Thomas and colleagues found that TGR5 signaling leads to glucagon-like peptide-1 (GLP-1) secretion by enteroendocrine cells (**Figure 2B**; Thomas et al., 2009). Thereby, improvement of both pancreatic and liver function as well as tolerance of glucose were observed in obese mice. In addition to that, TGR5 targeting with the specific agonist INT-777 inhibited hepatosteatosis, offering a treatment option for metabolic diseases. Moreover, TGR5 stimulation is involved in the expression of junctional adhesion molecule A (JAM-A) by biliary epithelial cells (Merlen et al., 2020). While JAM-A was downregulated as well as hypophosphorylated in BA ducts and gallbladder from *Tgr5*-deficient mice, administration of a specific TGR5 agonist in WT mice stabilized the adhesion molecule via JAM-A Ser28 phosphorylation. Additionally, TGR5-agonist-treated mice were less susceptible to choleostasis-induced liver damage due to reduced bile leakage, in contrast to JAM-A-KO mice. Hence, hepatic TGR5 signaling mediates liver protection. Interestingly, also in the context of intestinal inflammation, TGR5 deletion was associated with increased intestinal permeability leading to higher severity during colitis (Cipriani et al., 2011). Additionally, TGR5 activation was shown to activate intestinal stem cells inducing Src/YAP-driven regeneration in response to tissue injury (Sorrentino et al., 2020).

Similarly, it was shown that TGR5 deletion in a model of alcohol-induced liver disease causes even greater liver damage as a result of steatosis and inflammation (Spatz et al., 2021). This phenotype was related to enhanced recruitment of inflammatory macrophages to the liver. Furthermore, deficiency in the BA receptor resulted in dysbiosis as demonstrated by microbiota transfer from *Tgr5*-deficient mice into their WT counterparts, worsening alcohol-mediated hepatic inflammation. Of note, the pool of secondary BAs was reduced in these animals attributed to a lower abundance of bacterial genes related to BA transformation. The importance of BA transformation was further demonstrated in the work by Ma and colleagues which showed that the conversion of primary into secondary BAs by *Clostridium* species repressed production of CXCL16 in liver sinusoidal endothelial cells. Antibiotic depletion of BA-transforming bacteria increased the levels of primary BAs and CXCL16 production. Subsequent recruitment of natural killer T cells controlled growth of liver cancer (Ma et al., 2018).

Recently, investigations of inflammatory macrophages in chronic liver disease pointed out that TGR5 expression was reduced in liver samples from humans and mice suffering from NASH (Shi et al., 2020). The group described that macrophages derived from *Tgr5*-deficient were prone to M1 polarization accompanied by pro-inflammatory cytokine production. Mechanistically, TGR5 inhibits the NLRP3 inflammasome activation as well as caspase-1 cleavage, protecting against liver steatosis. Data supporting the contribution of TGR5 deletion to inflammation revealed that *Tgr5*-deficient macrophages and Kupffer cell enhanced the expression of pro-inflammatory factors such as IL-6 and MCP-1 in response to LPS. This phenotype was associated with reduced β-arrestin2-dependent suppression of the NF-κB pathway (**Figure 2B**; Witherow et al., 2004; Wang et al., 2011). Importantly, it was pointed out that the

inflammatory activity of Kupffer cells promotes the progression of HCC (Maeda et al., 2005; He and Karin, 2011).

While the mentioned studies have focused on inflammatory myeloid cells in the onset of BA-dependent liver disease, a recent study has identified a role of BAs in inducing immunosuppressive Treg response via DCs. The secondary BA 3β-hydroxydeoxycholic acid (isoDCA) was shown to reduce the immunostimulatory activity of DCs and to enhance Treg differentiation (Campbell et al., 2020b). Genetic deletion of FXR in DCs mimicked the effects of isoDCA administration suggesting an antagonistic mechanism involved in Treg generation. Furthermore, the design of a bacterial consortium comprised of isoDCA-producing *Bacteroides* strains induced the differentiation of colonic RORγt$^+$ Tregs. Another study revealed that the Treg-intrinsic BA receptor VDR contributes to the pool of extrathymic Tregs which elicited anti-inflammatory response during colitis (Song et al., 2020). Interestingly, Hang and colleagues (2019) suggested another mechanism by which the BAs 3-oxolithocholic acid (3-OxoLCA) and isoallolithocholic acid (isoalloLCA) act directly on T cells. Binding of 3-OxoLCA to RORγt inhibited development of TH17 cells, whereas induction of mitochondrial reactive oxygen species by isoalloLCA enhanced Treg differentiation. Thereby, BAs are able to modulate the Th17/Treg balance which is an example of how they contribute to immunoregulatory mechanisms of gut homeostasis, rather than acting pro-inflammatory, as part of the gut-liver-axis.

CONCLUSION AND FUTURE DIRECTIONS

Various studies have highlighted microbiota-derived SCFAs and BAs as factors impacting host physiology, development of diseases and outcome of treatment strategies. The identification of microbial metabolites and their respective modes of action might be crucial for the development of new therapeutic approaches and identification of biomarkers.

Especially with respect to SCFAs, different mechanisms were identified by which these small aliphatic molecules influence cellular signaling, metabolism and epigenetics (Kim et al., 2013, 2016; Luu et al., 2018). However, most studies have investigated these independently, not covering the potential crosstalk between the pathways. Recently, a connection between the induction of the mTOR pathway and the provision of acetyl-CoA as substrate for HAT-mediated histone acetylation in lymphocytes was pointed out (Luu et al., 2019; Qiu et al., 2019). Additionally, Schulthess and co-workers described a link between butyrate-mediated inhibition of HDAC3 and the decrease in mTOR activity by macrophages (Schulthess et al., 2019). These results emphasize a bidirectional connection between the epigenetic and metabolic pathways and that more research should be invested into unraveling pathway inter-connectivity to understand cell-specific regulatory networks.

Considering the utilization of acetate as source for PTMs, it is conceivable that even longer SCFA-derived histone alkylations will be identified as new biomarkers (Kebede et al., 2017; Fellows et al., 2018). More experimental evidence is needed to evaluate their biological role in transcriptional regulation of cells such as colonocytes and intestinal immune cells and their potential as therapeutic or diagnostic markers.

Although SCFAs are considered for therapeutic administration, studies have reported contradictory observations with regards to the effects of SCFAs. Their effects have been described as both beneficial and adverse depending on the disease model (Smith et al., 2013; Singh et al., 2014, 2018; Trompette et al., 2014; Kim et al., 2016; Kespohl et al., 2017). Although the research on these small molecules was focused on their immunosuppressive capacities, recent data propose a role in inflammatory responses (Smith et al., 2013; Trompette et al., 2014; Kespohl et al., 2017; Luu et al., 2018, 2019; Bachem et al., 2019). For instance, it has been reported that SCFAs are able to boost Th17 differentiation as benefical effect upon pathogen encounter but similar mechanisms were demonstrated to repress Th17 cells in a mouse model of autoimmune encephalomyelitis (Park et al., 2014; Luu et al., 2019). We have recently shown that SCFAs are also capable of promoting the cytotoxic phenotype of tumor-specific CD8$^+$ T cells and chimeric antigen receptor (CAR) T cells, thereby enhancing their anti-tumor activity (Luu et al., 2021). These findings suggest that the impact of microbial metabolites is highly context-dependent. Hence, future work should investigate both their immunostimulatory and immunosuppressive capacities for a comprehensive analysis.

Likewise, studies have reported contradictory observations with regards to the effects of BAs in genetically modified animals and different disease models which need further investigation (Hanniman et al., 2005; Zhang et al., 2012; Li et al., 2013; Schmitt et al., 2015; Parséus et al., 2017).

As an example, the work with *Fxr*-deficient animals has identified the genetic background as crucial factor influencing the experimental outcome. *Fxr*-deficient mice on *Ldlr*$^{-/-}$ and ob/ob background were protected against HFD-induced obesity and atherosclerosis in contrast to mice on *Apoe*-/- background (Hanniman et al., 2005; Ma et al., 2006; Zhang et al., 2012). Even work on organ-specific FXR-deletion in the liver and intestinal tissue showed conflicting results underlining the need for a systematic comparison between these models (Li et al., 2013; Schmitt et al., 2015). The development of next generation *in vivo* tools might comprise tissue- or cell-specific FXR-deletion combined with *Ldlr*- or *Apoe*-deficiency in the same tissue rather than systemic knockouts, thereby reducing incidental effects.

Moreover, the reviewed studies were dependent on administration of fiber-rich diet or HFD to either shape the microbial composition or to stimulate inflammatory response. Due to a wide range of commercially available diets and supplements, the exact composition might differ between the studies (Pellizzon, 2016). Standardization of diet composition for *in vivo* experiments could help to unify newly developed models.

With regards to standardization, the microbial composition is another crucial aspect impacting on disease outcome. A prominent example is the *Il10*-deficient mouse model for spontaneous colitis. Different groups have reported varying histopathology scores depending on the SPF condition in their respective animal facility and the colonization with commensal strains (Burich et al., 2001; Balish and Warner, 2002; Schultz et al., 2002; Keubler et al., 2015). Moreover, Ivanov and

colleagues (2009) identified segmented filamentous bacteria (SFB) in the murine gut as inducer of intestinal Th17 cells which were demonstrated to promote central nervous system autoimmmunity (Luu et al., 2019). Of note, SFB were present in the gut of WT mice derived from Taconic Farms, while there were not detected in those raised at Jackson Laboratory (Ivanov et al., 2009). Therefore, not only uniformity of bacterial colonization in WT mice but also of its variations in genetically modified strains needs to be considered to assure reproducible results and will additionally improve the monitoring of changes within the microbiota composition over the course of the experiment.

Although the impact of BAs on various immune cell types has been investigated, their effect on B cells has not been described yet. Recent studies demonstrated that microbiota-derived serotonine-derivates and SCFAs can act as aryl-hydrocarbon receptor ligands or metabolic enhancer in regulatory B cells (Bregs), respectively (Rosser et al., 2020). Also the finding that BAs impact on intestinal Treg differentiation underlines their immunosuppressive capacities (Hang et al., 2019; Song et al., 2020). As BAs are capable of inducing either tolerogenic or inflammatory responses, it remains to be clarified whether B cells are prone to become Bregs, antigen-presenting or plasma cells upon BA treatment. Also the interaction between BAs and $CD8^+$ T cells has been investigated insufficiently. Both the involvement of $CD8^+$ T cells in controlling the BA synthesis by inducing cholangitis and the effect of FXR deletion on T cell fitness were described previously (Glaser et al., 2019; Campbell et al., 2020a). In addition, a recent study has revealed accumulation of $CXCR6^+$ auto-aggressive $CD8^+$ T cells in the liver of mice and patients suffering from NASH (Dudek et al., 2021). Yet, the underlying mechanisms linked to BAs as well as the assessment of *Fxr*- and *Tgr5*-deficient $CD8^+$ T cell in the context of NASH or HCC remain to be subject of future work.

Finally, there are gaps in our knowledge regarding the interconnectivity between SCFA and BA biology. Although many mechanistic insights were provided by work on either SCFAs or BAs, their simultaneous impact on the same pathways, synergistic or opposing effects have not been elucidated in depth. For instance, the activity of SCFAs as GPR41/GPR43 agonists might influence the BA-induced signaling via TGR5. Moreover, limited data is available on the interaction between SCFA producers, BA-sensitive and BA-transforming bacteria. A first attempt of analyzing the interaction between SCFAs and BAs was analyzed by Sheng and colleagues. The group has demonstrated that lack of butyrate-producing bacteria enhanced hepatitis in *Fxr*-deficient mice fed a western diet, while administration of butyrate reversed inflammation caused by the *Fxr*-deficiency-derived BA dysregulation (Sheng et al., 2017). This work highlighted the potential of the joint expertise from the SCFA and BA biology field which might enable future research to fill the gaps within our knowledge with respect to the complex inter-kingdom crosstalk between commensals and eukaryotic cells.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Next Generation Microbiome Research: Identification of Keystone Species in the Metabolic Regulation of Host-Gut Microbiota Interplay

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The community of the diverse microorganisms residing in the gastrointestinal tract, known as the gut microbiota, is exceedingly being studied for its impact on health and disease. This community plays a major role in nutrient metabolism, maintenance of the intestinal epithelial barrier but also in local and systemic immunomodulation. A dysbiosis of the gut microbiota, characterized by an unbalanced microbial ecology, often leads to a loss of essential functions that may be associated with proinflammatory conditions. Specifically, some key microbes that are depleted in dysbiotic ecosystems, called keystone species, carry unique functions that are essential for the balance of the microbiota. In this review, we discuss current understanding of reported keystone species and their proposed functions in health. We also elaborate on current and future bioinformatics tools needed to identify missing functions in the gut carried by keystone species. We propose that the identification of such keystone species functions is a major step for the understanding of microbiome dynamics in disease and toward the development of microbiome-based therapeutics.

Keywords: microbiome, dysbiosis, keystone, metagenomics, bioinformatics, inflammation, immunity, metabolism

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INTRODUCTION

Animals are superorganisms composed of eukaryotic and prokaryotic cells in a similar proportion along an even larger number of viruses (Sender et al., 2016). The reason for this intricate mixture of organisms spanning all kingdoms of life is that every living animal is the result of a long co-evolution between all of these organisms. Hence, within every gut lies a complex community of microorganisms composed of trillions of prokaryotic and eukaryotic microbial cells, including bacteria, fungi and archaea along a multitude of viruses (Hillman et al., 2017; Moissl-Eichinger et al., 2018). As a result, we humans carry within our microbiomes an immense reservoir of genes that perform numerous functions for our own benefit, many of which are still unknown.

There is currently no consensus about the definition of a healthy gut microbiome because of high inter-individual variability, which is influenced by numerous external factors (Rinninella et al., 2019). Nevertheless, it is generally considered that a healthy gut microbiome is a rich and diverse ecosystem acting in symbiosis with its host (van de Guchte et al., 2018). Even if there is a lack of evidence to identify a robust universal set of core healthy microbial taxa, there is a remarkable

stability of microbial functions that maintain symbiosis with the host (Human Microbiome Project Consortium, 2012). Conversely, we have learned over the past 20 years that some chronic disorders are consistently associated with a shift in microbial patterns, often referred to as "dysbiosis" (Hooks and O'Malley, 2017). For example, obesity has been reported to be associated with a low Bacteroidetes/Firmicutes ratio (Turnbaugh et al., 2009). Even if the Firmicutes phylum regroups a large number of potentially beneficial bacteria, this ratio has progressively been established as a hallmark of the obese dysbiotic gut microbiome (Crovesy et al., 2020). Another feature of obesity-associated dysbiosis is a reduced microbiome diversity, as illustrated by the high proportion of fecal samples from obese individuals that fall within the "low gene count" category (Le Chatelier et al., 2013). Similarly, several chronic diseases have been associated with reduced gut microbiome diversity, such as Crohn's disease (Manichanh et al., 2006), hypertension (Li et al., 2017) and non-alcoholic steatohepatitis (NASH) (Astbury et al., 2020).

THE KEYSTONE SPECIES CONCEPT AS A DRIVER OF MICROBIAL DIVERSITY

An important ecological concept is that every complex ecosystem is structured by a few important species dubbed "keystones." This term was coined in 1966 by the American ecologist Paine (1966) who identified specific sea stars as important predators that regulate the biodiversity of seashores. Since, this term has been used in various ways and with different meanings. For the purpose of this review, we adopt the definition of keystone taxa proposed by Banerjee et al. (2018): *where "microbial keystone taxa are highly connected taxa that individually or in a guild exert a considerable influence on microbiome structure and functioning irrespective of their abundance across space and time. These taxa have a unique and crucial role in microbial communities, and their removal can cause a dramatic shift in microbiome structure and functioning*" (Banerjee et al., 2018). This is a crucial concept as it shapes our understanding of the regulation of complex ecosystems, how they establish, how they remain stable over long periods of time and how they adapt to environmental changes.

Translated to the gut environment, we must first appreciate that mammals harbor not just one gut ecosystem, but a variety of ecosystems, each roughly corresponding to a different section of the gastrointestinal tract from the mouth to the rectum. Each ecosystem is regulated by a set of environmental factors such as pH, bile acid concentration and peristalsis, which have long been thought of as a barrier that segregates ecosystems from one another. However, this view has been recently challenged, as it is now proposed that oral bacteria act as a reservoir of microorganisms that pass through the gut to replenish the downstream ecosystems (Schmidt et al., 2019). Within each ecosystem, microbes interact with each other through numerous mechanisms, such as secretion of quorum sensing molecules, cross-feeding and synthesis of antimicrobial compounds. Of particular interest, quorum sensing is a cell-cell interaction mechanism used by bacteria to regulate their own population. Usually in biofilms, some bacterial cells stimulate their own growth and those of their neighboring kin, through secretion of autoinducer molecules (Mukherjee and Bassler, 2019). In the gut microbiota, it has been shown that Firmicutes use this strategy to maintain their population level (Thompson et al., 2015). Interestingly, there is emerging evidence that host cells can interfere with these bacterial signals to shape the microbial community (Mukherjee and Bassler, 2019). Yet, most of our knowledge of quorum sensing is derived from the study of pathogens and there remains numerous gaps in our understanding of its use by commensal bacteria. Inter-species syntrophy or cross-feeding occurs when a species depends on the availability of nutrients (e.g., sugars, amino acids, and vitamins) that are produced by other species. For instance, this typically involves degradation of complex molecules such as carbohydrates by specialized species that release monosaccharides in the environment. The latter are then taken up by non-degrading species for their own benefit. These mechanisms have been recently thoroughly reviewed by D'Souza et al. (2018). Inter-species cross-feeding interactions within an ecosystem cause reliance on specific microbes that carry essential functions for other species. Hence many keystone species have been described based on the identification of enzymes involved in cross-feeding interactions (Centanni et al., 2018; **Table 1**). These are only a few examples of the diversity of possible microbial interaction routes. For a thoughtful review of the topic, we refer the reader to the review by Pacheco and Segrè (2019).

Together, these mechanisms depict a high level of inter-dependencies between bacterial species within an ecosystem. These interactions lead the ecosystem to structure around clusters of microbes that co-develop into a guild of co-abundant species. This concept was well illustrated in a recent study aimed at identifying gut bacterial species involved in post-antibiotic recovery in human cohorts. In a metagenome-wide association study, Chng et al. (2020) demonstrated that a succession of primary colonizers set the stage for late dominant species, which feed on the breakdown products of the pioneer species (Gibbons, 2020). This study identified 7 bacterial species acting as primary colonizers, with a metabolic capacity to extract carbon and energy from mucin and complex dietary carbohydrates, thus acting at the bottom of the food chain. Even if in this example most of the identified primary colonizers were abundant species, low abundance bacteria (<0.1% relative abundance) should not be neglected as they may carry essential functions that support growth of other dominant species. This concept has been very well illustrated in a study of "*Candidatus Desulfosporosinus infrequens*," a sulfate-reducing organism found in wetlands (Hausmann et al., 2019). Although it remained in a seemingly dormant state at zero-growth over more than 7 weeks, it was reported to be in fact highly metabolically active, contributing to regulate methane production and therefore to sustain a diverse ecosystem (Hausmann et al., 2019).

In view of the intricate interplay between the gut microbiome function and its host metabolism, it is now established that loosing part of these functions is associated with a number of modern non-communicable diseases. As a consequence, techniques designed to manipulate gut dwelling microbiomes

TABLE 1 | Non-exhaustive list of prominent keystone taxa of the human gut microbiome.

Keystone species (in alphabetical order)	Function	Method of identification	Example of reported disease association in humans
Akkermansia muciniphila	Mucin degrader	Empirical (Belzer et al., 2017)	Intestinal inflammation, obesity and metabolic diseases (Yassour et al., 2016)
Bacteroides thetaiotaomicron	Degradation of complex carbohydrates (arabinogalactan); selective BSH activity (Yao et al., 2018)	Empirical (Cartmell et al., 2018)	Unclear – Controversial association with IBD (Sitkin and Pokrotnieks, 2019)
Bifidobacterium longum	Degradation of complex carbohydrates, particularly Human Milk Oligosaccharides; BSH activity (Tanaka et al., 2000)	Empirical (Yu et al., 2013; Gotoh et al., 2019)	Highly prevalent in healthy newborns (Favier et al., 2003)
Bifidobacterium pseudolongum	Degradation of complex carbohydrates	Empirical (Centanni et al., 2018)	Highly prevalent human breast milk (Lugli et al., 2020)
Christensenella minuta	Stimulate ecosystem diversity (Mazier et al., 2021); acetate producer (Morotomi et al., 2012); BSH activity (Déjean et al., 2021)	Co-occurrence networks (Goodrich et al., 2014; Kumpitsch et al., 2020 ahead of publication) Empirical (Ruaud et al., 2020; Mazier et al., 2021)	Obesity and metabolic diseases (Goodrich et al., 2014); Crohn's disease (Pascal et al., 2017)
Faecalibacterium prausnitzii	Butyrate producer	Presence/absence (Leylabadlo et al., 2020)	Crohn's disease (Sokol et al., 2008), Ulcerative Colitis (Varela et al., 2013)
Methanobrevibacter smithii	Produces methane from H_2 and acetate	Empirical and co-occurrence networks (Goodrich et al., 2014; Kumpitsch et al., 2020 ahead of publication)	Obesity (Goodrich et al., 2014), Crohn's disease (Pascal et al., 2017)
Ruminococcus bromii	Resistant starch degrader; Butyrate producer	Empirical (Ze et al., 2012)	Highly prevalent microbe in healthy individuals (Beghini et al., 2021)

are gaining increasing attention, and several microbiome-based biotherapies are currently in development (Doré et al., 2017; Valencia et al., 2017). Hence, a deep understanding of gut microbiome ecology and how it can be durably restored is crucial for effective clinical translation. In this regard, a recent study evaluated bacterial dispersal strategies of human gut-associated microbes and classified them in five categories that may provide a guide for appropriate restoration strategies: (i) "tenacious" strains that are highly persistent among human communities, (ii) "spatiopersistent" strains that tend to be associated with specific geographical locations but colonize at a later developmental stage (i.e., not in infants), (iii) "heredipersistent" strains that tend to persist within closely related individuals such as within families and have broad geographical presence, (iv) average persistent strains, and (v) non-persistent strains (Hildebrand et al., 2021). Interestingly, the authors propose that fecal microbial transplantation may be most efficient to target tenacious and spatiopersistent taxa while heredipersistent taxa may require regular reinfections, which may therefore be best targeted through chronic single strain exposure.

IMPORTANT METABOLIC PATHWAYS UNDER THE GUT MICROBIOME INFLUENCE

Beyond microbiota classification, insights on the functional impact of the microbiome are emerging from metagenomic analyses, integration with omics data sets, particularly metabolomics and *in vivo* validation studies. In this section, we review recent studies highlighting the key contribution of microbial metabolites and associated pathways in controlling host physiology. Among the multitude of metabolic activities harbored by human gut microbiomes, we focus on the roles of short chain fatty acids (SCFA), tryptophan- and cholesterol-derived metabolites, and their crosstalk with host factors, e.g., histone modifying enzymes, G proteins-coupled receptors, aryl hydrocarbon receptor (AhR), indoleamine 2,3-dioxygenase 1 (IDO1) and tryptophan hydroxylase 1 (TpH1) in barrier maintenance, immune regulation, and the gut-brain axis.

SCFAs

Short chain fatty acids are the primary end products of bacterial fermentation of dietary fibers (but can also be derived from proteins and peptides in a lesser extent) and are important regulators of gut microbial ecology as well as host physiology. The main SCFAs are acetate, propionate, and butyrate (Cummings et al., 1987). Fiber-derived monosaccharides, such as hexoses, deoxyhexoses, and pentoses are converted by several bacterial metabolic enzymes to pyruvate which is then further metabolized to acetyl-CoA, succinate or lactate that primarily feed SCFA production. Acetate is derived from acetyl-CoA generated from pyruvate directly or through the Wood-Ljungdahl pathway. Butyrate is also produced from acetyl-CoA, but through the condensation of two acetyl-CoA molecules into acetoacetyl-CoA that is metabolized to butyryl-CoA and then butyrate. Some gut

bacteria can also convert lactate to butyrate. Propionate is derived from lactate or succinate in the acrylate and succinate pathways, respectively. It can also be produced by the propanediol pathway that converts deoxyhexoses to proprionyl-CoA. The concentration of SCFAs is highest in the proximal colon reaching ∼130 mmol/kg of luminal content (Cummings et al., 1987). However, the effective concentration reaching the intestinal cells is presumably lower due to the thick mucus layer and intestinal peristalsis. Among the SCFAs, butyrate constitutes an important energy source for colonocytes and is mostly consumed in the colon. Propionate and acetate are further metabolized in the liver, but taken the high concentration of acetate in the gut, it is the main SCFA that remains in the systemic circulation. Nonetheless, butyrate and propionate can also impact host systemic physiology indirectly through hormonal and nervous system signals. SCFAs can enter the cells though diffusion or via the transporter SLC5A8 and exert their effects through three reported mechanisms: (a) epigenetic control of gene expression via inhibition of histone deacetylases (HDAC), e.g., in intestinal epithelial cells (IECs) and immune cells, (b) by acting as ligands of G-protein coupled receptors (GPCRs), primarily GPR43 and GPR41, also called free fatty acid receptors 2 (FFAR2) and FFAR3, respectively, and GPR109A, also known as niacin receptor 1 or Hydroxycarboxylic Acid Receptor 2 (HCA2), and/or (c) by acting as an AhR agonist, as has been shown for butyrate in IECs (Marinelli et al., 2019).

Because of the energetic reliance of colonocytes on butyrate, it is not surprising that this SCFA is a critical regulator of intestinal barrier integrity and mucosal immune homeostasis. Butyrate confers a protective role in experimental mouse models of colitis [e.g., with dextran sodium sulfate (DSS)], *Il10*$^{-/-}$ mice (Wang et al., 2016) or *Clostridium difficile* infection (Fachi et al., 2019). These effects were also noted in ulcerative colitis patients, as shown early on by Scheppach et al. (1992). Butyrate also protects against colitis-associated colorectal cancer (CRC) as has been reported using the *APC*$^{min/+}$ mice (Singh et al., 2014) or with the azoxymethane (AOM)-DSS model (Singh et al., 2014). In contrast, in the *APC*$^{min/+}$*MSH2*$^{-/-}$ mouse model with stem-like CRC characteristics, butyrate was shown to promote tumorigenesis (Belcheva et al., 2014), presumably through enhancing stem cell regeneration. Mechanistically, butyrate signals through GPR43 and GPR109A on IECs to stimulate inflammasome-dependent IL-18 production (Macia et al., 2015), which is required for intestinal epithelial integrity (Dupaul-Chicoine et al., 2010; **Figure 1**). It also protects from colonic inflammation through HDAC inhibition that blunts lamina propria macrophages inflammatory signaling (Chang et al., 2014) and dendritic cells differentiation (Millard et al., 2002; Wang et al., 2008; Singh et al., 2010), and promotes regulatory T cells (T_{reg}) generation (Arpaia et al., 2013; Furusawa et al., 2013), through acetylation of the *FoxP3* locus (**Figure 1**). More recently, butyrate, in addition to propionate and acetate, was shown to induce IL-22 expression in CD4^{+} T cells and innate lymphoid cells (ILC) through GPR41 and HDAC inhibition; the latter enabling enhanced binding of HIF1α to the *Il22* gene promoter (Yang et al., 2020). In cancer cells, which favor glucose metabolism (Warburg effect), butyrate was shown to accumulate in the nuclei leading to effective inhibitory concentrations of HDACs (Donohoe et al., 2012). As a result, butyrate can epigenetically deregulate the expression of key genes involved in cell proliferation, cell death and differentiation in cancer cells but not normal colonocytes (Donohoe et al., 2012). Propionate, but not acetate, similarly promotes these processes through HDAC inhibition. A metagenomic-based approach was able to identify the main butyrate producers of the gut microbiome as *Eubacterium rectale*, *Faecalibacterium prausnitzii,* and *Anaerostipes coli* S22/1 (Louis et al., 2010; Muñoz-Tamayo et al., 2011). Yet, only *F. prausnitzii* was formally identified as a keystone species (**Table 1**). Interestingly, *E. rectale* was identified in another study using metagenomic time series as a bacterium benefiting from the presence of putative keystone species such as *Bacteroides fragilis and Bacteroides stercosis* (Fisher and Mehta, 2014).

In metabolism, dietary fibers and SCFAs are generally associated with lean weight and improved glycemic index. For example, an improvement in insulin sensitivity was reported in a trial in which individuals were given a diet supplemented with a resistant starch for 4 weeks (Robertson et al., 2005). This beneficial effect can be attributed to SCFA. In a randomized controlled trial, administration of an inulin-propionate ester to overweight adult humans over 24 weeks reduced body weight gain, abdominal adiposity and hepatic lipid accumulation compared to the inulin-control group (Chambers et al., 2015). Similarly, colonic infusion of SCFA mixtures in overweight/obese men, at concentrations comparable to those reached after dietary fibers intake, increased fat oxidation and energy expenditure (Canfora et al., 2017). Mechanistically, SCFAs may act by stimulating the production of the anorexigenic gut hormones peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) (**Figure 1**), as has been shown in humans with acetate (Freeland and Wolever, 2010) and propionate (Venter et al., 1990) or through intestinal gluconeogenesis (IGN), where both propionate and butyrate were shown to enhance IEC *de novo* synthesis of glucose, stimulating increased insulin sensitivity through gut-brain communication (De Vadder et al., 2015). GPR41 mediates improved energy metabolism through its expression on neurons of the enteric nervous system (ENS) and on sympathetic neurons that promote enhanced energy expenditure (**Figure 1**). Consistently, *Gpr41*$^{-/-}$ mice were shown to be leaner than wild-type controls (Samuel et al., 2008). On the other hand, the results with *Gpr43*$^{-/-}$ mice are controversial as these mice were described to be obese even on normal diet in one study (Kimura et al., 2013), but lean with improved metabolic parameters in another (Bjursell et al., 2011). Nonetheless, these mouse phenotypes were lost under germ-free conditions or with antibiotic treatment, demonstrating microbiota-mediated metabolic effects.

Tryptophan Metabolism

Microbial metabolism of dietary tryptophan (enriched in cruciferous green leaf vegetables, e.g., parsley, cauliflower, kale, broccoli, etc.) has recently emerged as an important pathway by which the microbiota regulates intestinal homeostasis, particularly through AhR activation. Since its cloning in 1992 (Burbach et al., 1992), AhR has gained much interest for

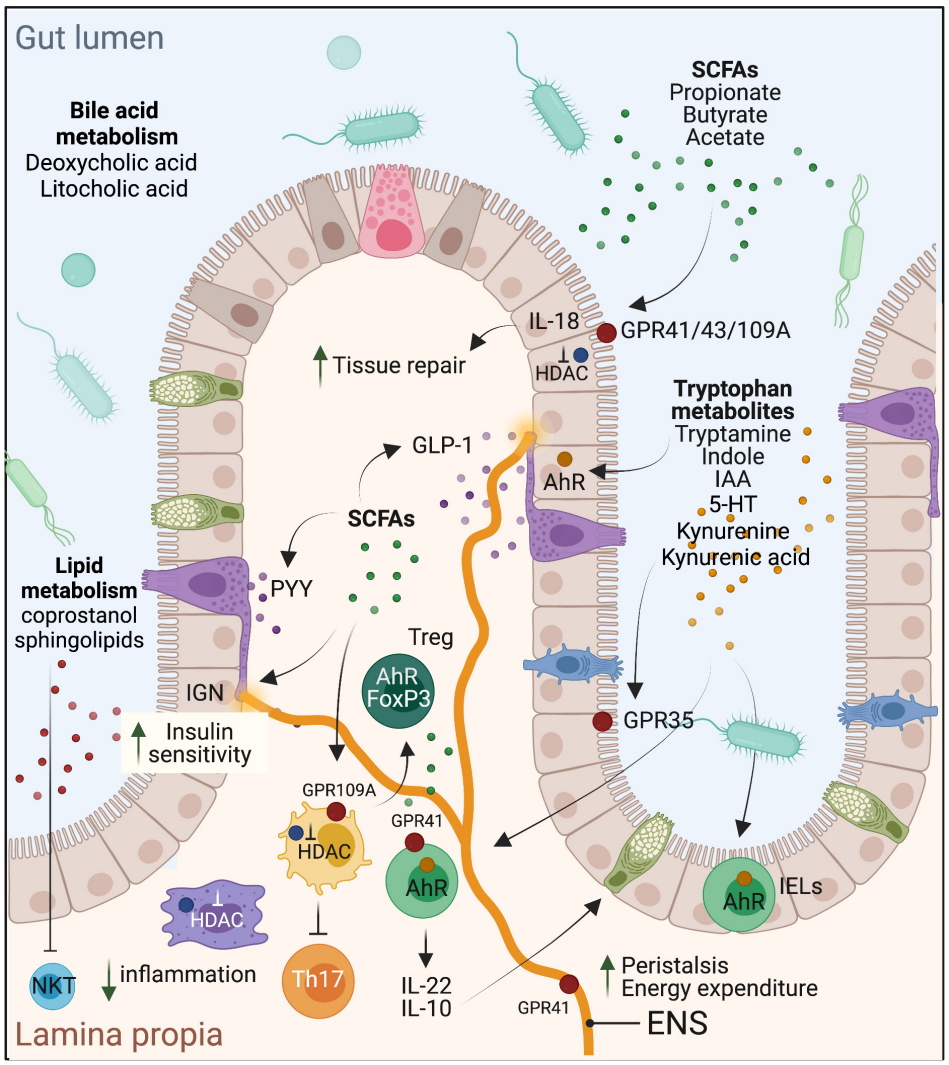

FIGURE 1 | Schematic representation of the effects of select microbial metabolites on intestinal homeostasis, mucosal immune regulation, and metabolic health of the host.

its role as an environmental sensor. Beyond its activation by xenobiotics, tryptophan-derived ligands catabolized by the microbiota, including indole, indolo[3,2-b]carbazole, indole acetic acid (IAA), 3-methylindole and tryptamine, to name a few, have been demonstrated to act as potent high-affinity AhR ligands (Zelante et al., 2013; Hubbard et al., 2015). AhR is expressed in different intestinal cell types, including IECs, immune cells, particularly intraepithelial lymphocytes (IELs), innate lymphoid cells (ILC)3 (Gomez de Agüero et al., 2016), more recently ILC2 (Li et al., 2018), Th17 and T_{reg} (Quintana et al., 2008; Veldhoen et al., 2008), and neurons of the ENS (Obata et al., 2020). Through this collective expression, AhR exerts physiologically crucial roles in barrier integrity and intestinal and immune homeostasis, notably through regulation of IEC tight junctions (Yu et al., 2018; Singh et al., 2019), generation and survival of IELs (Cervantes-Barragan et al., 2017), production of IL-22 (Zelante et al., 2013) and IL-10 (Aoki et al., 2018; Powell et al., 2020), and regulation of peristalsis and microbiota density (**Figure 1**). In IECs, AhR has been recently implicated in the regulation of goblet cells differentiation, particularly in preventing goblet cell depletion in the colon during aging (Powell et al., 2020). This process is mediated by IL-10 and induced by AhR in response to microbiota-derived indoles. IL-22 or type I IFN, which are involved in intestinal tissue repair following acute injury, do not seem to be required in this case (Powell et al., 2020). In parallel to IECs, AhR activation in CD4 + T cells was shown to regulate their differentiation into CD4 + CD8αα + double-positive immunoregulatory IELs. These cells are absent in germ-free mice but restored with *Lactobacillus reuteri,* a species with tryptophan catabolizing capacity (Cervantes-Barragan et al., 2017). AhR activity is similarly required for the generation and maintenance of ILC3 (Gomez de Agüero et al., 2016), and patients with Crohn's disease exhibit decreased AhR expression in their inflamed ileum accompanied by a conversion of ILC3

to ILC1 (Li et al., 2016). Last, AhR expression is elevated in intestinal T_{reg} and seems to be required for their gut homing as well as for suppression of Th1 inflammatory gene expression (Ye et al., 2017). AhR is also expressed in colonic neurons, in a microbiota-dependent manner, and this neuronal-specific expression is central in the control of intestinal peristalsis, positioning AhR as a nexus of intestinal neural circuitry (Obata et al., 2020). Collectively, AhR protects the epithelial barrier, promotes intestinal immune tolerance and protects from intestinal inflammation. Consequently, deregulation of its activity is associated with inflammatory and metabolic diseases, and microbiome stimulation of this pathway, particularly through tryptophan metabolism, has been demonstrated as an "environmental" mean to counter these pathologies. For instance, individuals with inflammatory bowel diseases (IBD) (Lamas et al., 2016), the metabolic syndrome (Natividad et al., 2018) or celiac disease (Lamas et al., 2020) have decreased fecal concentrations of AhR ligands and reduced AhR activity. Interestingly, supplementation of experimental mice modeling these pathologies with a high-tryptophan diet, AhR ligands or with bacterial species that metabolize tryptophan such as *L. reuteri,* improved the mice health status (Marafini et al., 2019; Chen et al., 2020). In a randomized controlled clinical trial, administration of AhR ligands in the form of the traditional Chinese medicine indigo naturalis to ulcerative colitis patients for 8 weeks showed clinical benefit, including a decrease in the Mayo score, mucosal healing and remission in some cases (Naganuma et al., 2018). Nonetheless, caution is warranted prior to considering the development of AhR ligands for therapeutics taken toxicity issues with deregulated AhR responses.

Besides AhR ligands, tryptophan is additionally metabolized into kynurenine and serotonin (Clarke et al., 2012; Yano et al., 2015). In the kynurenine pathway (KP), IDO1 is mainly responsible to convert tryptophan to kynurenine and downstream end products including niacin, nicotinamide adenine dinucleotide (NAD), quinolinic acid and kynurenic acid (Cervenka et al., 2017; Kennedy et al., 2017). The latter exerts immunoregulatory effects and protects the intestine by signaling through GPR35, expressed on IECs and immune cells (Gao et al., 2018). The serotonin pathway converts tryptophan into the neurotransmitter 5-hydroxytryptamine (5-HT), i.e., serotonin, via TpH1 expressed in a specialized IEC type known as enterochromaffin cells in the gut, and TpH2 in the brain. While peripheral 5-HT, which constitutes 90% of all serotonin produced in the body, does not cross the blood–brain barrier (BBB), it regulates several intestinal processes including stimulation of ENS neurons, peristalsis and nutrient absorption, to name a few (Mawe and Hoffman, 2013). Moreover, both tryptophan and 5-HT precursor (5-HTP) cross the BBB impacting central serotonin effects on host physiology. The commensal microbiota plays important roles in tryptophan metabolism to kynurenine and serotonin as demonstrated in GF or antibiotics-treated mice [reviewed in Agus et al. (2018)]. Several commensal bacteria express enzymes related to KP enzymes and can thus produce kynurenine metabolites, e.g., the neurotoxic 3-hydroxyanthranilic acid (O'Farrell and Harkin, 2017). Further, through SCFA and BA metabolism, the microbiota can induce TpH1 and stimulate 5-HT biosynthesis (Reigstad et al., 2015; Yano et al., 2015). Gut-derived kynurenines and 5-HT are additionally implicated in the pathogenesis of chronic inflammatory, metabolic and neuropsychiatric diseases. For instance, $IDO1^{-/-}$ mice are more susceptible to 2,4,6-trinitrobenzene sulfate-induced colitis (Takamatsu et al., 2013) whereas $TpH1^{-/-}$ mice show enhanced protection in response to DSS- or dinitrobenzene sulfonic acid-induced colitis (Ghia et al., 2009), suggesting that while kynurenine is protective in the gut, 5-HT might be deleterious. However, more recent findings indicate that 5-HT could exert pro- or anti-inflammatory effects in the gut depending on the respective engagement of 5-HT7 versus 5-HT4 receptors (Spohn and Mawe, 2017). Kynurenines and 5-HT play contrasting roles in obesity and insulin resistance. The KP is overactivated in obesity and its activity correlates with BMI and the metabolic syndrome, presumably through the action of kynurenine derivatives such as xanthurenic acid (Oxenkrug, 2013). In contrast, 5-HT levels are decreased in obese individuals, which is consistent with the role of 5-HT in promoting satiety (Voigt and Fink, 2015), lipolysis in white adipose tissue and hepatic gluconeogenesis (Sumara et al., 2012).

Cholesterol and Lipid Metabolism

Cholesterol and lipid metabolism by the intestinal microbiota is an additional facet by which the microbiota influences host physiology. On one hand, microbial components, specifically *Peptostreptococcus anaerobius,* have been identified as inducers of cholesterol biosynthesis in colonocytes, mediated by SREBP2 activation downstream of TLR signaling, which supports dysplasia and CRC development in a mouse model, and is consistent with elevated levels of this bacteria in the stool of CRC patients (Tsoi et al., 2017). On the other hand, microbial metabolism of cholesterol into coprostanol, a poorly absorbed sterol, has been recently demonstrated as a mechanism reducing host serum cholesterol levels (Kenny et al., 2020). Notably, this function was attributed to a clade of uncultured bacteria harboring ismA genes encoding cholesterol dehydrogenases (Kenny et al., 2020). Besides cholesterol, bacterial metabolism of sphingolipids (SL), lipids with a long-chain amino alcohol backbone, also contributes to immune homeostasis in the gut and to the host metabolic health. Bacteria of the Bacteroidetes phylum, which constitutes ∼30–40% of the healthy human intestinal microbiota, have the capacity to synthesize sphingolipids (SL), owing to their expression of the enzyme serine palmitoyltransferase (Heaver et al., 2018). Bacterially derived SL promote immune homeostasis in the gut, acting early in life to tame invariant natural killer T (iNKT) cells proliferation (An et al., 2014). Consistently, in a model of oxazolone-induced colitis, treatment of mice with *B. fragilis* SL lessened the colitis phenotype by reducing iNKT cell numbers (An et al., 2014). Notably, the stools of IBD patients contain an elevated signature of host SL including ceramides, but are depleted of bacterially derived SL which protect the intestine. Indeed, colonization of germ-free mice with *Bacteroides thetaiotaomicron* deficient in SL elicited intestinal inflammation (Brown et al., 2019). To address how the gut microbiota influence host metabolic pathways and ceramide levels, a recent study explored this question in a

model of diet-induced obesity (Johnson et al., 2020). The authors showed that bacterially derived SL can enter colonocytes and reach the liver through the portal vein circulation, impacting metabolic parameters, e.g., insulin resistance, primarily through liver ceramides (Johnson et al., 2020). Together, these indicate that cholesterol and lipid metabolism by gut bacteria significantly influence host metabolism and physiology. However, these pathways are still poorly understood and require to be fully investigated before further therapeutic exploitation.

Bile Acid Metabolism

Bile acid metabolism by the gut microbiota has gained considerable interest in the recent years as they are being recognized as crucial microbiome-derived metabolites that regulate multiple important functions involved in health and disease (Hylemon et al., 2018). Primary bile acids are mostly synthesized by the liver hepatocytes from cholesterol following irreversible 7alpha-hydroxylation by the cytochrome P450 CYP7A1 (Chávez-Talavera et al., 2017). In humans, these are cholic acid (CA) and chenodeoxycholic acid (CDCA), while in mice, CDCA is further metabolized into beta-muricholic acid (betaMCA) (Pandak and Kakiyama, 2019). These hydrophobic primary bile acids are then made amphipathic through conjugation with glycine and taurine before being secreted in the gall bladder along phospholipids to make up the bile that will primarily serve as detergent upon release into the duodenum during digestion (Chávez-Talavera et al., 2017). In the small intestine, conjugated bile acids are deconjugated by microbial bile salt hydrolases (BSH) that release the hydrophobic moieties that are reabsorbed mostly through passive diffusion along the epithelium and through active reabsorption in the terminal ileum. In total, 95% of the initial bile acid pool is reabsorbed through this enterohepatic cycle. Yet, 5% of bile acids escape reabsorption and travel down the colon where they undergo further metabolism by gut bacteria such as 7alpha-dehydroxylation, which leads to formation of secondary bile acids such as deoxycholic acid and litocholic acid from the metabolism of CA and CDCA, respectively, (Ridlon et al., 2016). This is a brief overview of the complex metabolism of bile acids. For an exhaustive review of the role of gut microbes on bile acid metabolism, we refer the reader to the work by Ridlon et al. (2016). Gut microbial taxa with documented BSH activity include *Lactobacillus* spp. (Foley et al., 2021), *Bifidobacterium longum* (Tanaka et al., 2000), *Enterococcus faecalis* (Chand et al., 2018), *B. thetaiotaomicron* (Yao et al., 2018), and *Christensenella minuta* (Déjean et al., 2021), some of which have been classified as keystone species (**Table 1**).

Beyond their role in lipid absorption, bile acids have systemic functions as they also regulate hepatic energy metabolism, adipocyte differentiation and dampen immune activation through their interaction with bile acid receptors Farnesoid X Receptor (FXR) and Takeda G protein Receptor 5 (TGR-5) (Foley et al., 2021). Because of these multiple effects, they were recently suggested to form gut microbiota-derived hormones (Kliewer and Mangelsdorf, 2015; Hylemon et al., 2018). Both conjugated and unconjugated bile acids participate in this host-microbiota crosstalk. Interestingly, new bile acid conjugates specifically produced in the small intestine, were recently discovered. These involve bacterial conjugation with phenylalanine, tyrosine, and leucine, three hydrophobic amino acids, which had never been described associated with these molecules. Unsurprisingly, we still ignore the physiological role of these novel microbially derived compounds (Quinn et al., 2020).

Bile acids have been associated with a number of chronic disorders including obesity, NASH, IBD (Schirmer et al., 2019), Primary Biliary Cholangitis (formerly known as Primary Biliary Cirrhosis) and Primary Sclerosing Cholangitis. Interestingly, some disorders have been specifically associated with a defect in gut bacterial metabolism of bile acids such as *Clostridioides difficile* infections, which have been shown to be corrected by restoring gut microbial BSH activity (Mullish et al., 2019). Indeed, bile acid deconjugation releases free unconjugated bile salts that are toxic to most bacteria and thus act as a regulator of the microbial ecosystem. Hence, this is one of the key functions carried by gut bacteria that impacts significantly on the composition of the gut microbiome community.

BIOINFORMATIC TOOLS TO IDENTIFY KEYSTONE SPECIES AND FUNCTIONS OF THE GUT MICROBIOME

In light of these findings, restoring key metabolic pathways carried by gut microbiota could open a wide range of therapeutic perspectives. In particular, the identification of keystone species carrying these functions in the gut microbiome appears as an essential step for the development of future biotherapies targeting the gut microbial ecosystem. Keystone species of the human microbiome have often been identified using empirical evidence (Banerjee et al., 2018). Nevertheless, bioinformatics is increasingly used to identify keystone species and several methods have been developed to exploit next-generation sequencing (NGS) data.

"Presence or Absence" Associated With Health and Disease

One of the most common methods to characterize the human gut microbiome has been the use of amplification and sequencing of marker genes in stool samples. The most used marker gene is the 16S rRNA gene for the detection of bacteria, but other housekeeping genes are occasionally used to capture bacterial diversity (Case et al., 2007; Ogier et al., 2019). 16S rRNA can be used to compare the abundance of microorganisms at the genus or species level in different states (e.g., healthy versus diseased, different food sources, etc.) by assigning the reads to clusters of organisms grouped by taxonomic marker gene similarity, called Operational Taxonomic Units (OTUs). A widely used technique to then assess variations between microbial communities is to use UniFrac beta-diversity metric coupled with unsupervised multivariate statistics using Principal Component Analysis (PCA) or its derivatives (Lozupone and Knight, 2005; Ramette, 2007). However, the main hurdle associated with the calculation of the UniFrac method is the high computer

power required, although this has been largely improved in the latest Striped UniFrac algorithm (McDonald et al., 2018). More complex probabilistic methods, such as Dirichlet multinomial mixtures, have been developed to improve the analysis of metagenomics samples by clustering and classifying microbial communities based on a probability distribution. This method especially considers the discrete nature, the sparsity and the variability of the sequencing depth, and has been applied to the analysis of samples from obese and lean twins and to IBD patients (Holmes et al., 2012; Ding and Schloss, 2014).

Even if evaluating the presence or absence of a genus or species between two states using marker gene sequencing in stool samples seems promising, a key part of the keystone species definition is the interspecies interaction (Banerjee et al., 2018). Hierarchical clustering of bacterial communities correlated with disease association have been extensively used to attempt identification of important bacterial taxa (Jackson et al., 2018). Correlation with taxon presence or absence is often confirmed using metrics such as Jaccard's index: between two species, this index is defined by the ratio between the number of samples where both species are present out of the number of samples tested. The values of this index range between 0 and 1, from no correlation to a strong correlation, respectively, (Mainali et al., 2017). Nevertheless, an extensive study on microbial community modeling showed that indexes selected to evaluate correlations between species should be adapted to the specific dataset being studied. It is noteworthy that Jaccard index has been reported to have a relatively low sensitivity compared to other metrics such as the Pearson index when applied to co-occurrence networks (Berry and Widder, 2014). Therefore, even if the use of Jaccard's index is reliable to identify the correlations using taxon presence or absence, the use of Pearson or Spearman indexes should be preferred to assess correlations when using network-based methods.

To overcome the issues associated with taxon-based correlation analysis that may lead to conflicting results due to spurious associations, it is noteworthy that Wu and co-authors have recently proposed to exploit the concept of bacterial guild to reduce metagenomic data dimensionality into ecologically meaningful functional units (Wu et al., 2021). Although this approach is still limited by the use of relative abundance data, it proposes an interesting approach to refine data analysis of 16S-based datasets to identify relevant disease associations.

Prediction of Interspecies Interactions by Network-Based Methods

The need to consider interspecies interactions to identify keystone species in a community led to the development of new network-based methods. The most common methods are co-occurrence or co-abundance networks applied to 16S rRNA gene (or other marker gene) sequencing or metagenomic data. These networks are often produced by calculating a pairwise correlation coefficient between each pair of OTUs but other methods are being developed to build interaction networks (Berry and Widder, 2014).

Co-occurrence networks have been extensively used to identify keystone species. An exhaustive study by Berry and Widder (2014). evaluated the performance of these networks in interaction with several correlation metrics. They used generalized Lotka-Volterra modeling (gLVM) to simulate competitive and cooperative interactions between species (Berry and Widder, 2014). This study revealed that high mean degree (the average number of edges a node has in the network), high closeness centrality (the average distance of a node to any other node), high transitivity and low betweenness centrality (the betweenness centrality of the node A is the number of shortest paths between a pair of nodes B and C passing through the node A) can predict the keystone nature of species with 85% accuracy (Berry and Widder, 2014). These parameters produce highly interconnected nodes (i.e., keystone species) called "hub" patterns corresponding to a number of species interacting together directly and indirectly (Berry and Widder, 2014; Layeghifard et al., 2017; Banerjee et al., 2018). This enables identification of the guilds of bacteria that depend on the presence of keystone species. For example, this method was used by Zhang et al. (2014) on 454-pyrosequencing 16S rRNA sequencing data from human intestinal biopsy samples. It allowed to identify *Ruminococcus gnavus*, *Faecalibacterium prausnitzii*, *Prevotella copri,* and *Anoxybacillus flavithermus* as potential keystones of a healthy human intestinal mucosal microbiota because they displayed the highest number of linkers (Zhang et al., 2014). As already mentioned, only *F. prausnitzii* has been so far empirically validated as a keystone species for its ability to produce butyrate (**Table 1**). Its loss has been associated with the development of IBD in several studies (Sokol et al., 2008; Varela et al., 2013).

Another study from Fisher and Mehta (2014) used discrete-time Lotka-Volterra models to simulate the abundance variations of 10 species for 1000 timesteps and 100 initial conditions. After demonstrating that correlations in species abundance were not predictive of interactions between species, they used this simulated dataset to prove that LIMITS, an algorithm that uses sparse linear regression corrected by bootstrap aggregation, can be relevant to identify keystone species in two time-series samples from the gut microbiome (Fisher and Mehta, 2014). They concluded that *B. stercosis* and *B. fragilis* showed more interactions than other bacteria and that these interactions were mainly beneficial, since the growth of these two bacteria has been correlated with an increased abundance of *B. thetaiotaomicron* and *E. rectale*, the latter being a well-known butyrate producer (Fisher and Mehta, 2014). These results are coherent with other studies identifying *Bacteroides sp.* as key members of the gut microbiome (Loftus et al., 2021) involved in the degradation of complex carbohydrates (Cartmell et al., 2018). Nevertheless, the major drawback of this method is the need of time-series samples which are difficult and time-consuming to obtain. Furthermore, only a few abundant species are studied, thus the identification of low abundant keystone species is fairly unlikely using this method.

Other network-based methods, such as association rule-mining, are being applied to microbiome sequencing data in order to identify keystone species. Briefly, this method allows to estimate whether the presence of a species is required for

the presence of other species. In a study by Chng et al. (2020), using the "efficient_apriori" Python package, they inferred binary association rules between species on 782 microbiome profiles. This method allowed to identify "primary" species, which presence is required for other species to thrive. Furthermore, they showed that some of these "primary" species, such as *Bacillus uniformis*, *F. prausnitzii* or *Ruminococcus torques*, were associated with the recovery of the gut microbiota after antibiotic exposure and carried specific metabolic functions such as mucin and carbohydrates degradation (Chng et al., 2020). As mentioned in **Table 1**, other members of the *Ruminococcus* genus have been identified as resistant-starch degraders (Ze et al., 2012) essential to maintain a healthy gut microbiome (Beghini et al., 2021).

Co-occurrence networks between members of the microbiota can also be applied using generalized boosted linear models (GBLMs), as exemplified in a study where it was implemented to investigate the Human Microbiome Project cohort (Faust et al., 2012). More recently, a co-occurrence network using the Random Matrix Theory (RMT) method was applied to murine gut microbiome data in order to identify putative keystone species. In this study, 32 were identified as highly connected species linked to other OTUs (Liu et al., 2019).

Although these methods are useful to predict putative keystone species, the identification of the keystone functions carried by these species is essential to understand the interactions between the keystone bacteria and its guild. However, the use of amplicon sequencing does not allow the precise identification of the strains or the metabolic functions they carry within the gut microbiome because the assignation of OTUs only allows the reconstruction of metabolic pathways based on reference genomes, thus inducing a possible loss of strain-specific metabolic functions (Frioux et al., 2020). Therefore, a few recent methods have been developed to overcome these issues using metagenomic sequencing that reconstruct strain-specific metabolic pathways.

Reconstruction of Metabolic Pathways at Ecosystem Level

The recent development of metagenomics has provided a clearer view of the diversity of the gut microbiota, especially by allowing access to yet-uncultured bacteria (Almeida et al., 2019). The reconstruction of genome-scale metabolic networks and models (GSMNs) using Metagenome-Assembled Genomes (MAGs) or the inference of functional categories to single genes allows to precisely predict the metabolic capabilities of the gut microbiome (Frioux et al., 2020). A good example of such tools is the Metage2Metabo algorithm developed by Belcour et al. (2019) that enables the analysis of metabolic pathways at the ecosystem level using both reference genomes and MAGs. As keystone species carry essential metabolic functions in the gut ecosystem, this tool was used to detect putative keystone bacteria. In order to accurately predict the metabolic capabilities of the communities, both available nutrients and genome information (from reference databases or metagenomic samples) are combined to predict minimal communities of bacteria that can produce target metabolic compounds. The bacteria encountered in all predicted minimal communities are considered essential symbionts, whereas bacteria encountered in at least one of the predicted minimal communities, but not necessarily all of them, are considered alternative symbionts (Belcour et al., 2019). This method has been applied to a set of over 1,500 reference genomes from the human gut, allowing the identification of 11 essential symbionts, namely *Propionibacterium* sp. KPL2009, *Paenibacillus polymyxa*, *Bacillus licheniformis*, *Lactococcus lactis*, *Enterococcus casseliflavus*, *E. faecalis*, *Hungatella hathewayi*, *Dorea longicatena*, *R. torques*, *Burkholderiales bacterium,* and *Citrobacter portucalensis*, and 194 alternative symbionts (Belcour et al., 2019). As the reference genomes were assembled from 155 fecal samples, bacterial communities from each individual were mixed, maybe explaining the large amount of alternative symbionts (Zou et al., 2019). In addition, the fairly reduced number of predicted keystone species compared to the input dataset of genomes could be due to the non-exhaustive list of initial nutrients and target metabolic compounds that need to be provided to the software in order to predict minimal communities. Nonetheless, this tool is the first tool to the best of our knowledge that is specifically designed to identify both putative keystone species and their associated metabolic functions in complex microbial communities.

FINAL CONSIDERATIONS

Modern sequencing technologies enable broad mapping of virtually any microbial ecosystem. Nevertheless, it is important to keep in mind that the quality of microbiome data and the information we derive from them highly rely on the quality of the original sampling. Indeed, environmental factors strongly influence bacterial communities that adapt to any variation, being the time of day, temperature, pH, food supply (i.e., diet for gut microbiomes), and the age of their host for host-associated microbiomes. In this regard, the keystone species *C. minuta* has been reported to be increased with aging (Waters and Ley, 2019). Hence, repeated sampling can be recommended in order to account for temporal variations and obtain more accurate pictures of bacterial ecosystem compositions (Ji et al., 2019).

Another important consideration about sampling is that most large human studies focus on easily accessible locations with non-invasive tools, analyzing oral and fecal samples for instance. As a consequence, the analyzed microbiomes poorly represent the inner ecosystems located deep within the gut. Typically, fecal samples reflect the microbiome of the distal colon, largely dominated by Clostridiales, while it has been shown that the gut microbiota follows a specific spatial distribution along the gastrointestinal tract, which is even further complicated by regional specificities as illustrated by the differences observed between luminal and mucosa-associated species (Donaldson et al., 2016). Hence, most studies of human cohorts are limited to the study of the distal gastrointestinal tract. Despite this limitation, NGS methods and bioinformatics have been effectively supporting the search for keystone species in the gut microbiome. The rapid improvement of NGS techniques that generates increasingly large datasets allowing for deep

characterization of the gut microbiome community also calls for new bioinformatics tools to analyze NGS datasets in a more effective and complete way.

For metagenomics studies and as summarized in **Figure 2**, two methodology decisions drastically influence the results: (i) the DNA sequencing technology and (ii) the bioinformatic methods that will be applied to analyze these datasets.

The choice of a DNA sequencing strategy determines the information retrieved from a sample. Amplicon sequencing, especially 16S rRNA gene sequencing, allows to have an overview of the bacterial content of the samples by assigning the obtained reads to taxonomies. Metabolic networks can then be inferred using the reference genomes of the species or genus identified in the samples (Frioux et al., 2020). Although this approach has some merits, several technological biases can result in partial taxonomic assignation, thus reducing the completeness of the analysis. For instance, 16S rRNA gene sequencing is often partial because it mostly targets a couple of hypervariable regions, although it is technically possible to target a nearly complete 16S rRNA sequence, depending on chosen primers and sequencing technology. Indeed, an extensive study by Johnson et al., revealed that a full 16S rRNA gene sequencing significantly improves the taxonomic resolution. Out of all of the partial sequencing tested, the V4 region performed worst, and the relative number of OTUs produced using the different sub-regions was not consistent depending on the identity threshold (Johnson et al., 2019). It has also been noted that the development of long-read sequencing platforms enhanced the accuracy of the sequencing thus improving the detection of single nucleotide polymorphisms (SNPs) in the complete 16S rRNA gene. Multiple copies of the 16S rRNA genes carrying unique SNPs can even be detected using this technique, allowing a strain-level identification (Johnson et al., 2019).

The thrive of metagenomic sequencing also benefited from the use of NGS to perform shotgun sequencing. A study by Ranjan et al. (2016) concluded that the use of shotgun sequencing compared to 16S sequencing significantly improved the diversity of bacterial species detected, thus allowing a finer prediction of the genes carried by a bacterial community. For instance, their study showed that more than 1,000 species of proteobacteria were only detected by shotgun sequencing performed on a stool sample and twice the amount of genes were predicted on average using one of the shotgun short-reads metagenomic technologies. The comparison of different short-read sequencing

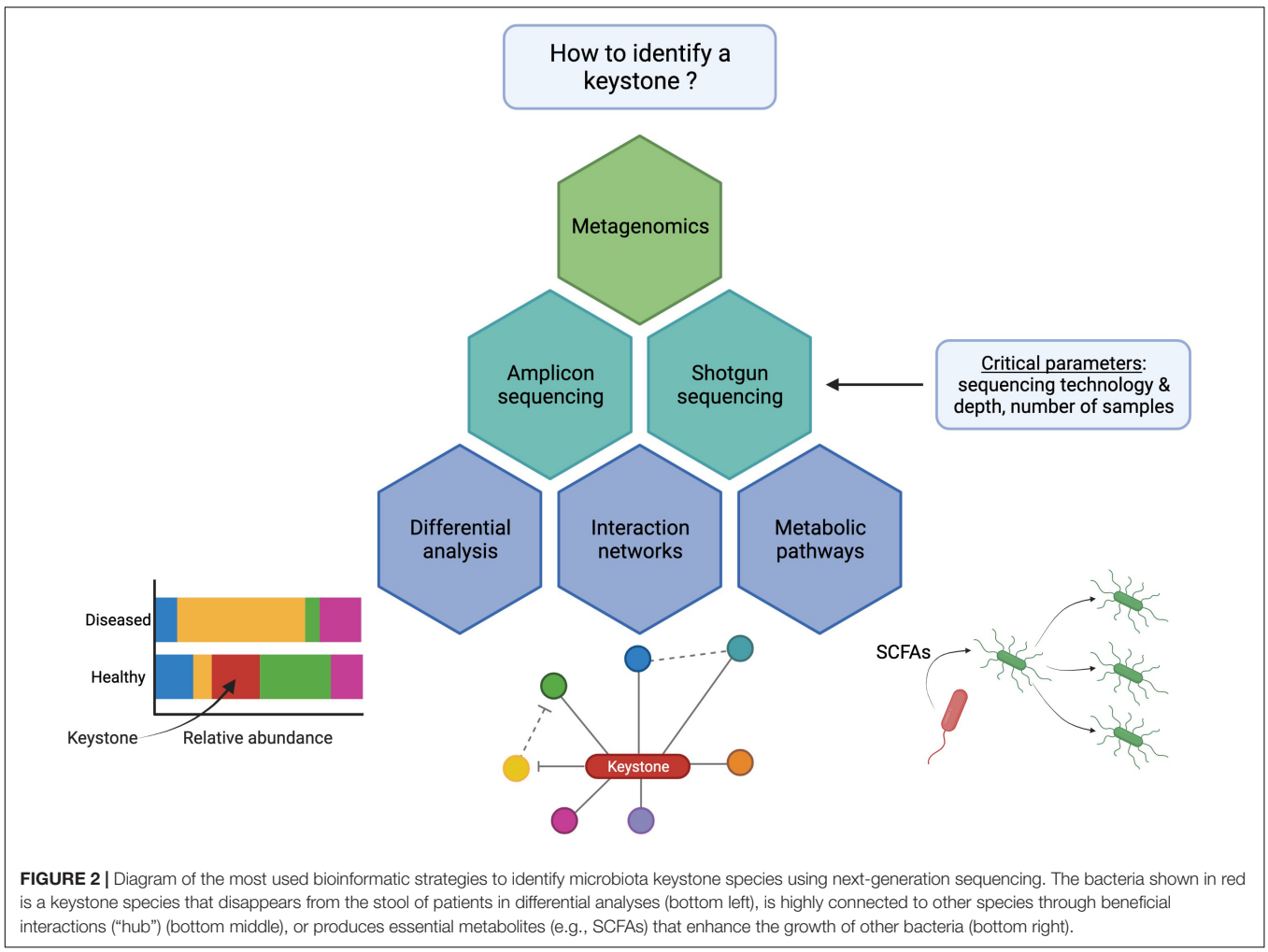

FIGURE 2 | Diagram of the most used bioinformatic strategies to identify microbiota keystone species using next-generation sequencing. The bacteria shown in red is a keystone species that disappears from the stool of patients in differential analyses (bottom left), is highly connected to other species through beneficial interactions ("hub") (bottom middle), or produces essential metabolites (e.g., SCFAs) that enhance the growth of other bacteria (bottom right).

technologies, namely MiSeq and HiSeq, showed that the extra length produced by MiSeq sequencing (150–300 bp compared to 100 bp) enables a more specific identification of bacterial species and improves accuracy and completeness of MAGs that are essential to reliably predict the metabolic potential of the ecosystem (Ranjan et al., 2016; Frioux et al., 2020). A more recent study showed that the use of long-read technologies (e.g., PacBio) improved drastically the completeness of the MAGs and associated predicted genes: more than 98% of the genes predicted from PacBio-assembled MAGs were complete compared to only 40% using HiSeq-assembled MAGs (Xie et al., 2020). Indeed, the use of long reads enables the detection of repeated elements often found in ribosomal RNA genes or bacteriophage-related insertions that are frequently missed with short read sequencing (Derakhshani et al., 2020).

Once sequencing data have been acquired, the identification of species in the gut microbiome requires the assignment of the reads to taxonomies. At this step, not only is the quality of the reads important, but the accurate assignment to taxonomies also depends on the reference catalog used. For the assignment of 16S rRNA genes, two factors strongly influence results. First, there is high reliance on the diversity contained in the reference catalog and second, the amplified regions can skew the identification of OTUs. Indeed, 16S rRNA gene catalogs are often built from complete 16S sequences whereas the amplification and sequencing of the 16S rRNA gene usually targets hypervariable regions (V-regions), leading to biased performances and challenges when comparing different studies. To overcome this hurdle, a new tool called OTUX has been proposed, that combines several custom datasets of OTUs defined by 16S rRNA V-regions retrieved from full-length 16S rRNA sequences (Yadav et al., 2019). This new method was challenged against conventional full-length 16S databases and revealed an improved assignment of reads for all of the V-regions targeted, except for the V1–V3 region (Yadav et al., 2019). For the assignment of shotgun reads, using MAGs could significantly improve identification accuracy of bacterial species. Recent research efforts produced a comprehensive catalog containing more than 200,000 reference genomes from the human gut microbiome referenced as the UHGG database (Almeida et al., 2020). This work also revealed that 81% of the species in the catalog lacked a cultured representative, indicating the huge potential for discovering new keystone species in the future (Almeida et al., 2020).

Another critical parameter in the analysis of microbial communities using NGS is the sequencing depth. Several keystone species from the human gut microbiome, such as *C. minuta,* which presence in stool samples is significantly associated with BMI, are low abundance microbes that can be missed when using a reduced sequencing depth (Waters and Ley, 2019). Thus, an increased depth is required to ensure that the whole diversity of species from the gut microbiome is identified (Berry and Widder, 2014).

Considering data processing using bioinformatic tools, one of the main issues regarding the use of sequencing data to identify correlations between taxa within the gut microbiota is the compositional nature of these datasets. The analysis of read counts begins with their normalization by the total number of sequenced reads. As explained by Friedman and Alm, the estimation of correlation between parameters (e.g., species) is biased by the relationship between the fractions: because they must sum to 1, they are not independent (Friedman and Alm, 2012). Thus, a study can be artificially biased by the disappearance of highly abundant species due to technical or assignment issues: the relative abundance of low-abundant species will increase as the variables are dependent even if their absolute abundance is constant. Quantitative PCR (qPCR) is the predominant method to quantify biomass using 16S rRNA gene amplification, however, this method could significantly influence the models. Li et al. (2019) noticed a high variation between replicates when quantifying biomass from stool samples. Another flaw of this technique is due to the variable number of 16S rRNA gene copies in several microbial phyla such Firmicutes and Bacteroidetes, which results in an over-representation of such species. Friedman and Alm (2012) demonstrated that standard Pearson correlation estimation can falsely predict negative correlations between one dominant and several low abundant species because of the dependence between abundances. This issue has also been pointed by Berry and Widder (2014): they noted a loss of specificity in the co-occurrence networks when relative abundances were used. As a consequence, the SparCC method was developed to estimate the linear Pearson correlation between transformed variables: the log transformation of the ratio of abundances between a pair of OTUs because the ratio between abundances is independent from other OTUs included. Using SparCC, it was demonstrated that the bias observed in standard correlation studies that is induced by dominant species, is greatly reduced in simulated data of varying diversity. Applied to the HMP dataset, SparCC revealed new positive correlations between highly abundant and low abundant species, instead of the spurious negative correlations usually observed when using standard correlations (Friedman and Alm, 2012).

Recently, another method was developed by Li et al., to overcome the compositional bias when using generalized Lotka-Volterra (gLV) models. As explained above, gLV models are one of the most common approaches for modeling microbial interactions. These models also suffer from the compositional bias induced by the relative abundances. Indeed, absolute biomass values are needed in order to accurately fit the gLV differential equations model of each organisms' growth rate to the data (Li et al., 2019). Therefore, Li et al. (2019) developed an algorithm called BEEM that estimates biomass *in silico* before inferring interactions when total biomass cannot be estimated experimentally. This algorithm introduces relative abundances in the equation modeling the growth rate of each species, resulting in two parameters that can be estimated using longitudinal datasets. This method was applied to diverse synthetic or existing datasets and accurately estimated biomass and gLVM parameters. It also allowed the identification of *F. prausnitzii* and *B. uniformis* as putative keystone species sharing numerous positive interactions with other bacteria (Li et al., 2019). Yet, this method is limited by available growth information for common species. Hence, only common dominant bacteria for which the information exists, may be accurately predicted.

Finally, recent developments have proposed to apply inferential statistics to network-based models in order to gain confidence in result interpretation. A study by Röttjers et al. (2021) recently proposed the use of null models to identify network properties that can be used to compare networks. They showed that among 20 networks built from time-series stool samples collected from 20 women, the new tool called anuran, could identify patterns that were found in 20–25% of the networks but only 3 associations were found in 10 networks or more, suggesting that associations between species or taxa may greatly vary from one individual to another (Röttjers et al., 2021). Although this is a proof-of-concept work applied to a limited population, this is an interesting approach to robustly identify keystone species based on stable interaction networks.

CONCLUSION

In this review, we discussed the role of key gut microbes-derived metabolites in intestinal homeostasis, metabolic health, immune regulation, and gut-brain interactions. We next described current bioinformatic tools used in microbiome studies and highlighted weaknesses associated with some of these approaches for the identification of keystone species and their missing functions. We point for instance that commonly used bioinformatic tools that provide "presence or absence" information are not suitable in this regard as they fail to provide a view of species interactions. Network-based methods that calculate co-occurrence or co-abundance of species are thus more suited to predict keystoneness. However, these methods need to be complemented with approaches that reconstruct species-specific metabolic pathways, such as the Metage2Metabo algorithm, but in a system where nutrient, genomic information and metabolites output are simultaneously analyzed.

Our review has provided a snapshot of recent discoveries on gut microbiome metabolic activities and the current state of the field with respect to bioinformatics analysis of the microbiome. We propose that in the quest for keystone species, future studies should consider harmonization of sample and data processing and the integration of additional variables including age, gender, nutrition, and other environmental cues. It is also particularly important to provide mechanistic evidence supporting the functions of keystone species in modulating the microbiome ecosystem for instance through quorum sensing, cross-feeding, bacteriocins or through other as of yet unknown mechanisms. Such studies will set the stage to design microbiota-based therapeutic interventions to counter chronic diseases, by restoring keystone species and their beneficial effects on microbiome balance to support a healthy symbiotic interaction with their host.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Bacterial Translocation as Inflammatory Driver in Crohn's Disease

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Crohn's disease (CD) is a chronic inflammatory disorder of the gastrointestinal tract responsible for intestinal lesions. The multifactorial etiology attributed to CD includes a combination of environmental and host susceptibility factors, which result in an impaired host–microbe gut interaction. Bacterial overgrowth and dysbiosis, increased intestinal barrier permeability, and altered inflammatory responses in patients with CD have been described in the past. Those events explain the pathogenesis of luminal translocation of bacteria or its products into the blood, a frequent event in CD, which, in turn, favors a sustained inflammatory response in these patients. In this review, we navigate through the interaction between bacterial antigen translocation, permeability of the intestinal barrier, immunologic response of the host, and genetic predisposition as a combined effect on the inflammatory response observed in CD. Several lines of evidence support that translocation of bacterial products leads to uncontrolled inflammation in CD patients, and as a matter of fact, the presence of gut bacterial genomic fragments at a systemic level constitutes a marker for increased risk of relapse among CD patients. Also, the significant percentage of CD patients who lose response to biologic therapies may be influenced by the translocation of bacterial products, which are well-known drivers of proinflammatory cytokine production by host immune cells. Further mechanistic studies evaluating cellular and humoral immune responses, gut microbiota alterations, and genetic predisposition will help clinicians to better control and personalize the management of CD patients in the future.

Keywords: Crohn's disease, bacterial translocation, intestinal permeability, dysbiosis, NOD2, inflammatory response, anti-TNF-α

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INTRODUCTION

Crohn's disease (CD) is a type of chronic idiopathic inflammatory bowel disease (IBD) that may affect any part of the gastrointestinal (GI) tract and causes inflammatory, stricturing, or penetrating intestinal lesions (Torres et al., 2017). The prevalence of CD has increased worldwide in the past 50 years, and it imposes a considerable economic burden on health systems as it requires new and costly treatment options and trained specialists to manage CD patients (Ng et al., 2017;

Windsor and Kaplan, 2019). The etiology of CD includes several aspects involving environmental factors, genetic susceptibility, and the impaired immune interaction of the host with the intestinal microbiota (Chang, 2020).

The immune response in CD is induced by different cell types such as neutrophils and macrophages that act together with epithelial cells in promoting and generating inflammatory phenomena by releasing soluble factors as tumor necrosis factor-alpha (TNF-α) and antimicrobial peptides like defensins and cathelicidins (Ramasundara et al., 2009; Gutiérrez et al., 2011). Intestinal bacteria are key contributors to the onset, perpetuation, and pathogenesis of chronic intestinal inflammation suggesting a disturbed immune gut response to bacterial antigens. This hypothesis is supported by several lines of evidence: (1) CD clinical lesions are mainly located in the distal ileum and colon, which are regions of high microbial concentration, (2) several studies demonstrate that luminal bacteria are necessary for the development of disease in murine models (Harper et al., 1985; Elson et al., 2005), and (3) CD patients present dysbiosis or reduced biodiversity in the composition of their gut microbiota associated with increased bacteria with proinflammatory properties and less anti-inflammatory bacterial species (Willing et al., 2010; Chassaing and Darfeuille-Michaud, 2011; Manichanh et al., 2012).

Our gut epithelial cells act as a physical barrier between the lumen of the GI tract and the inner mucosa contributing to nutrient and fluid absorption and impeding intact bacteria penetration. Genetic polymorphisms affecting barrier function or chronic inflammation may contribute to an abnormal intestinal permeability and, therefore, favor bacterial translocation (BT) and aggravate the course of disease. Genes associated with intestinal homeostasis involve autophagy, innate and adaptive immune regulation, microbial defense, or barrier function, among others (Franke et al., 2010; Khor et al., 2011). Some risk loci might influence immunological function such as nucleotide-binding oligomerization domain-containing 2 (NOD2), which encodes an intracellular receptor for muramyl dipeptide (MDP), a component in bacterial cell walls (Inohara et al., 2003). In this regard, three common variants of NOD2 loci apparently confer susceptibility to CD (Hugot et al., 2001) suggesting that an impaired response to bacterial antigens may contribute, and further studies indicate that low Foxp3+ regulatory T-cell (Treg) counts and a variant NOD2 genotype can be markers of loss of response to anti-TNF-α in CD patients (Juanola et al., 2014).

In this review, we integrate the effect of bacterial antigen translocation, the host immunologic response, and the genetic background in the inflammatory response observed in CD.

GUT-DERIVED BACTERIAL ANTIGEN TRANSLOCATION

Bacterial translocation is known as the passage of bacteria or its products, such as endotoxins, from the GI tract to mesenteric lymph nodes and systemic circulation (Alexander et al., 1990). This event has been demonstrated in CD by several studies in which the presence of bacteria in the lymph nodes (Takesue et al., 2002; Peyrin-Biroulet et al., 2012; O'Brien et al., 2014) or bacterial genomic fragments in the blood (Gutiérrez et al., 2009, 2011, 2014) are detected. Bacterial passage from the bowel lumen is a common phenomenon in CD, and it is involved in the pathogenesis by inducing, perpetuating, or exacerbating an inflammatory state (Swank and Deitch, 1996). The risk factors influencing BT are mainly intestinal bacterial overgrowth or dysbiosis, increased intestinal permeability, and local and systemic immunological alterations and can be followed in **Table 1**.

Human species have evolved with the symbiotic intestinal microbiota, which is composed of 10 (Franke et al., 2010) to 10 (Inohara et al., 2003) microorganisms including bacteria, fungus, archaea, and viruses whose total genome represents 100 times our own genome (Gill et al., 2006). GI microbiota is mainly composed of four bacterial divisions: *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria*, but the composition and the luminal concentrations of bacterial species may vary in the different GI tract regions (Eckburg et al., 2005). These commensal bacteria provide an abundant source of antigens that can activate pathogenic immune responses resulting in chronic intestinal inflammation and clinical manifestations of CD-susceptible patients.

An increase in the number and/or altered composition of microbial species in the small bowel is known as small intestinal bacterial overgrowth (SIBO), which is potentially caused by fistulae, strictures, a slowed intestinal transit, low acid gastric secretion, and altered local immune activity such as common variable immunodeficiencies associated with low IgA (Pignata et al., 1990; Husebye, 2005; Klaus et al., 2009). Patients with CD show some of these features, so they are predisposed to develop SIBO and a complicated clinical course of CD. Bacterial overgrowth or abnormal microflora (dysbiosis) within the gut have been described in the past in CD patients (Manichanh et al., 2006; Lupp et al., 2007; Sartor, 2008), and they are present in the early stages of CD being further amplified by antibiotic treatment (Gevers et al., 2014; Kowalska-Duplaga et al., 2019). Also, healthy first-degree relatives of CD patients show an altered microbiota suggesting a genetic predisposition to develop this condition (Joossens et al., 2011). In addition, there are differences in the diversity of microbiota depending on disease activity: between inflamed areas in different IBD phenotypes, and in microbial metabolism (Forbes et al., 2016; Franzosa et al., 2019; Lloyd-Price et al., 2019). Overall, the dysbiotic profile in CD patients is characterized by a decrease in *Bacteroidetes* and *Firmicutes*, and an increase in *Enterobacteriaceae* microbial populations (Frank et al., 2007; Kostic et al., 2014; Khan et al., 2019). The decrease in *Faecalibacterium prausnitzii* has been widely observed (Hedin et al., 2016), which is a bacterium with an important role in the regulation of Th17 cells (Zhang M. et al., 2019). In fact, this species, together with *Lactobacillus* and *Bifidobacterium*, among others, display a protective role in intestinal inflammation (Hrdý et al., 2020; Singh et al., 2020). Additionally, reduced abundance of *Lachnospiraceae* and *Ruminococcaceae* families producing short-chain fatty acids (SCFAs) are associated with poor response to TNF-α biologic therapy and frequent relapses

TABLE 1 | Risk factors for bacterial translocation in Crohn's disease include intestinal dysbiosis, altered intestinal integrity, and immune dysfunction.

	Altered parameter	Study	Observation
Dysbiosis	↓ *Bacteroidetes* ↓ *Firmicutes* ↑ *Enterobacteriaceae*	Kostic et al., 2014	The authors give an overview of the microbial changes observed in IBD.
	↓ *Faecalibacterium prausnitzii*	Hedin et al., 2016	CD patients show intestinal dysbiosis associated with reduced diversity of microbiota and lower counts of *F. prausnitzii*. This bacterial species regulates Th17 responses.
	↓ *Lactobacillus* ↓ *Bifidobacterium*	Hrdý et al., 2020; Singh et al., 2020	Treatment with specific *Bifidobacterium* and *Lactobacillus* probiotic strains reduce experimental intestinal inflammation and induce tolerogenic dendritic cells.
	↓ *Lachnospiraceae* ↓ *Ruminococcaceae*	Yilmaz et al., 2019	Reduced abundance of *Lachnospiraceae* and *Ruminococcaceae* families is associated with poor response to anti-TNF-α and frequent relapses.
	↑ *Desulfovibrio*	Metwaly et al., 2020	Enrichment of sulfate-reducing bacteria in active CD patients and mice with microbiota from CD patients with active disease.
	↑ *Mycobacterium avium paratuberculosis*	Schwartz et al., 2000; Naser et al., 2004	Increased counts of the intracellular pathogen MAP have been detected in tissue and blood samples from CD patients.
	↑ Adherent-invasive *Escherichia coli*	Darfeuille-Michaud et al., 2004	High prevalence of adherent-invasive *E. coli* in the ileal mucosa of CD patients.
	↑ *Clostridiodes difficile*	Razik et al., 2016	IBD patients have increased *Clostridiodes difficile* infections.
	↑ *Debaryomyces hansenii*	Jain et al., 2021	Expansion of *D. hansenii* fungi in inflamed intestinal tissue is associated with reduced mucosal healing in IBD.
	↑ *Caudovirales*	Norman et al., 2015	Increased richness of *Caudovirales* bacteriophages in fecal samples obtained from IBD patients.
Intestinal permeability	↓ MUC-1, 3, 4, 5B	Buisine et al., 1999	Reduced gene expression of mucin genes in healthy and inflamed mucosa of CD patients.
	↓ E-cadherin ↓ β-catenin	Kosovac et al., 2010	Patients with CD and NOD2 polimorphisms show increased BT associated an altered composition of the epithelial barrier.
	↑ Claudin-2 ↓ Claudin-3, 5, 8 ↓ Occludin	Zeissig et al., 2007	The authors describe a barrier dysfunction in active CD patients due to altered epithelial tight junction structure.
Immune response	Paneth cells	Wehkamp et al., 2005	Reduced secretion of antimicrobial peptides in intestinal mucosal extracts of CD patients.
	Macrophages	Kamada et al., 2008; Smith et al., 2009	Macrophages contributing to chronic intestinal inflammation in CD produce IL-6, IL-23, TNF-α and INF-γ proinflammatory cytokines and display an impaired clearance of bacteria.
	Neutrophils	Hayee et al., 2011	Neutrophils from CD patients show reduced respiratory burst and release extracellular traps which impair intestinal integrity.
	Eosinophils	Yantiss, 2015; Click et al., 2017	Increased eosinophilia in mucosal biopsies and peripheral blood in patients with CD.
	Dendritic cells	Middel et al., 2006; Baumgart et al., 2009; Senhaji et al., 2015	Patients with CD have increased amount of mature dendritic cells expressing high levels of co-stimulatory molecule CD40 and excessive inflammatory response.
	ILC1	Bernink et al., 2013	High frequency of IFN-γ-producing ILC1 cells in inflamed intestines of CD patients.
	ILC2	Bailey et al., 2012	IL13-producing ILC2 cells contribute to collagen deposition in fibrotic intestinal samples of CD patients.
	ILC3	Geremia et al., 2011	Accumulation of ILC3 cells that produce IL-17, IL-22, and IFN-γ in response to IL-23 in inflamed intestines of CD patients.
	Th1	Fuss et al., 1996; Monteleone et al., 1997; Parronchi et al., 1997; Pizarro et al., 1999	Crohn's disease is associated with type 1 T-helper lymphocyte intestinal responses and secretion of IFN-γ, IL-12 and IL-18.
	Th17	Fujino et al., 2003; Cummings et al., 2007; Geremia et al., 2011	Immunity mediated by type 17 T-helper responses is relevant in CD as demonstrated by increased expression of Th17-related cytokines and increased susceptibility to CD in patients with IL-23R gene variants.
	Treg	Chamouard et al., 2009; Hovhannisyan et al., 2011; Qiao et al., 2013; Ueno et al., 2013	Reduced tolerogenic regulatory T cell response in CD patients is associated with reduced expression of regulatory-associated transcription factors and a Th17-like cytokine profile observed in Tregs.

in CD (Yilmaz et al., 2019). Also, the altered gut microbiome is associated with enrichment of sulfate-reducing bacteria in active CD patients and mice with microbiota from CD patients with active disease (Metwaly et al., 2020).

Decreased complexity and diversity of commensal bacteria that promote intestinal homeostasis play a critical role in CD due to the disrupted capacity of the microbiota to exclude pathogens, which can favor inflammatory responses. *Mycobacterium avium paratuberculosis* (MAP) is an obligate intracellular pathogen detected in intestinal samples of CD patients by different molecular biology and cell culture techniques (Mishina et al., 1996; Schwartz et al., 2000). The contribution of MAP in the pathogenesis of CD was further confirmed by the detection of cultivable MAP in the blood of patients with CD (Naser et al., 2004). Therefore, MAP has been proposed as a potential etiologic infectious agent of CD, although this hypothesis remains to be validated (McNees et al., 2015). Another microbial pathogen such as adherent/invasive *E. coli* has been detected in biological samples of the ileum in patients with CD (Darfeuille-Michaud et al., 2004), and more interestingly, there is an increased severity of CD in those patients with high levels of serum antibodies detecting porin C present in the outer membrane of *E. coli* (Mow et al., 2004). *Clostridiodes difficile* is also an opportunistic pathogen in IBD patients frequently causing symptoms ranging from diarrhea to fulminant colitis and death (D'Aoust et al., 2017). IBD patients have a higher risk of *Clostridiodes difficile* infection, which is associated with longer hospitalization periods and increased resource costs (Nguyen et al., 2008; Razik et al., 2016). Despite literature showing a clear perturbation in IBD microbiota, it is not a clear cause–effect link. It is known that the inflammatory state in CD affects the microbial composition (Craven et al., 2012) but also that IBD microbiota can induce intestinal inflammation (Nagao-Kitamoto et al., 2016). In this regard, treatments addressed to restore healthy gut microbiota in IBD, such as administration of prebiotics and probiotics (Limketkai et al., 2020), antibiotic therapy (Castiglione et al., 2003), and fecal microbiota transplantation (Britton et al., 2020) need further investigations.

Antibiotics used on patients with inflammatory intestinal disease are targeted toward bacteria that, in turn, favor the colonization of intestinal niches by other members of the intestinal microbiota. Relevance of fungal microbiota dysbiosis have been described in patients with CD (Liguori et al., 2016), and antibodies to anti-*Saccharomyces cerevisiae* have been detected in CD (Seow et al., 2009). The interaction between intestinal fungi and host immune system occurs through receptors of the host innate immune system such as Dectin-1 (Iliev et al., 2012). Recently, poor mucosal healing in CD has been associated with overgrowth of *Debaryomyces hansenii* underlying that not only bacteria but also fungi species may modulate the intestinal inflammatory disease (Jain et al., 2021). Relevance of fungi in mucosal healing was evidenced in the study of Jain et al. (2021) by detecting the presence of *D. hansenii* in intestinal wounds with impaired healing after antibiotic treatment, whereas the administration of antifungal amphotericin B reduced fungi detection and increased wound regeneration. Oral gavage of *D. hansenii* altered crypt regeneration in conventional mice not treated with antibiotics and increased the severity of experimental colitis. The authors confirmed that macrophages were recruited in the areas colonized by *D. hansenii* and that CCL5 and type I IFN secreted by myeloid cells are required to alter mucosal healing, supporting CCL5 as a potential target in CD. Additionally, changes in the enteric virome associated with an expansion of *Caudovirales* bacteriophages have been described in patients with CD (Norman et al., 2015). Viral infection by the enteric murine norovirus in experimental models carrying the CD susceptibility gene ATG16L1 is associated with multiple pathologic abnormalities in the intestine (Cadwell et al., 2010). Even if increasing knowledge is required to understand the interactions existing between intestinal microbiota and the host during CD, we can assume that intestinal microbes play an active role in the progression of intestinal inflammatory disease.

The intestinal epithelium acts as a physical and antimicrobial barrier against pathogenic bacteria and environmental antigens (Okamoto and Watanabe, 2016). When the intestinal barrier is disrupted, commensal microbiota, which in physiological conditions exist in a symbiotic relationship with humans, can cross the epithelium and contribute to intestinal inflammation. The intestinal mucosal barrier is composed of both the outer mucus layer, which is comprised by secreted mucinous and antibacterial components, and the inner subepithelial elements involving the immune system (Salim and Söderholm, 2011). Epithelial cells together with M-cells, mucus-secreting globet cells and Paneth cells form a polarized monolayer structure linked by apical junctions which are formed by tight junctions and subadjacent adherens junctions (Turner, 2009). The junctional complex is composed of transmembrane and peripheral proteins including actin, claudins, occludins, zonula occludens (ZO)-1, and junctional adhesion molecules. Enteric glial cells located in the intestinal mucosa also regulate the permeability of the intestinal epithelial barrier in CD by producing 15-hydroxyeicosatetraenoic acid, a polyunsaturated fatty acid that increases the expression of ZO-1 (Pochard et al., 2016). Crucial functions of the intestinal barrier include maintenance of intestinal homeostasis by allowing the absorption of essential nutrients, as well as tolerance to commensal bacteria, and prevention of the entry of injurious bacterial components. A disturbance in one of the components that are involved in the epithelial barrier function can increase its permeability leading to an impaired ability to avoid BT. Altered expression of mucins 1, 3, 4, and 5B in the ileal mucosa of patients with CD favor the binding of microbes to the intestinal surface (Buisine et al., 1999). Additionally, the protein composition of tight and adherens junctions on intestinal cell–cell contacts is altered on CD patients (Zeissig et al., 2007; Kosovac et al., 2010). Disturbances in the permeability of the intestinal barrier associated with a derangement of the tight junction were also probed by freeze–fracture electron microscopic analysis (Marin et al., 1983a,b). Reduced integrity of the intestinal barrier leads to an increased absorption of luminal microbial antigens and serum concentrations of endotoxins, lipopolysaccharide-binding protein (LBP), and CD14s, which are markers of disease activity in CD (Pastor Rojo et al., 2007; Lakatos et al., 2011).

The importance of genetic background as a contributing factor to the impaired barrier function in CD comes from

studies with first-degree relatives of patients with CD showing that NOD2 3020insC mutation is associated with increased mucosal permeability (Irvine and Marshall, 2000; Buhner et al., 2006). Also, a gene polymorphism in adherens junction protein E-cadherin (CDH1 gene) was observed in some patients with CD resulting in a cytoplasmic mis-localization of the protein pointing to a defect in the intestinal barrier structure (Muise et al., 2009). From a clinical point of view, increased intestinal permeability has been reported to predict an increased risk of relapse in CD patients on remission (Arnott et al., 2000; Tibble et al., 2000) and is considered as a risk factor for CD onset (Turpin et al., 2020). Serum proteins and antibodies related to immune responses to intestinal microbiota can predict the development of CD up to 5 years before the diagnosis (Torres et al., 2020). Therefore, leaky gut in patients with CD may allow the passage of intestinal microbes across the intestinal epithelium and drive local and systemic proinflammatory responses that worsens the prognosis in patients with CD.

As a consequence of the impaired intestinal integrity, CD patients need to respond to the frequent bacterial challenges to which they are exposed to and ensure the clearance of translocating bacteria. A competent intestinal antimicrobial peptide response is required to protect host from pathogens and to provide tolerance to normal flora (O'Neil et al., 1999; Ramasundara et al., 2009). Several studies have shown that Paneth cells in CD patients display alterations in the production and the activity of different antimicrobial peptides such as cathelicidin (Schauber et al., 2006; Tran et al., 2017), α-defensins (Wehkamp et al., 2005; Elphick et al., 2008), and β-defensins (Kocsis et al., 2008; Schroeder et al., 2011), which are detrimental in the control of BT. Mutations in ATG16L1 and NOD2 in Paneth cells are associated with abnormalities in packaging and secretion of antimicrobials (Liu et al., 2014; VanDussen et al., 2014), therefore, affecting the antibacterial activity of the intestinal barrier by reduced secretion of mucosal α-defensins observed in CD (Wehkamp et al., 2004, 2005; Kobayashi et al., 2005; Petnicki-Ocwieja et al., 2009). Intriguingly, serum levels of α-defensins, but not β-defensins, are increased in patients with CD and they have been associated with serum C-reactive protein and TNF-α (Yamaguchi et al., 2009), while in healthy donors, peripheral α-defensins remain constitutively expressed and β-defensins are induced by bacterial-derived products (Fang et al., 2003). We have demonstrated that bactDNA can modulate the expression of β-defensin (DEFB) 2 and cathelicidin LL-37 through the mediation of NOD2 status by the signaling pathway of nuclear factor (NF)-κB in CD (Gutiérrez et al., 2011). This evidence suggests that the NOD2 gene regulates signaling pathways linked to defensins and cathelicidins through the nuclear factor (NF)-κβ (Wehkamp et al., 2004; Voss et al., 2006). Consequently, patients with a NOD2 mutation have an increased likelihood of developing ileal CD, and it is commonly accepted that an impaired NOD2 function can lead to a poor host clearance of bacteria, which can promote and perpetuate intestinal inflammation. A reduction in bacterial clearance has also been related to polymorphisms in ATG16L1 and IRGM genes, autophagy genes related to CD susceptibility (Hampe et al., 2007; Parkes et al., 2007; Rioux et al., 2007). A mutation on ATG16L1 and IRGM genes induces an injured autophagy pathway, resulting in a defective elimination of damaged cellular organelles and long-lived proteins as well as an altered degradation of intracellular bacteria.

Consequently, increased BT burden and altered microbial clearance in CD patients will induce sustained intestinal inflammatory responses that will be the topic addressed in the following section.

INFLAMMATORY RESPONSE TO BACTERIAL TRANSLOCATION IN CROHN'S DISEASE

The GI tract represents the largest surface area exposed to a wide and heterogeneous community of bacterial antigens. The gut is strictly regulated by innate and adaptive defense mechanisms, which altogether interact with commensal bacteria to promote the maintenance of intestinal homeostasis. Since CD is an immune-mediated condition triggered by environmental factors that imbalance the gut microbiota, perturb the intestinal barrier, and abnormally stimulate the gut immune response, an alteration in any of these compartments determines how the inflammatory immune response develops and may predispose to a disturbance of the bowel, leading to chronic inflammation. Here, we will describe in each one of the components involved in the process of BT and its role in the gut immune response and inflammation, which are summarized in **Figure 1**.

Intestinal barrier permeability increases the bacterial pressure to which the immune system needs to respond. When BT occurs, the first line of defense against microbial pathogens in the gut is composed of germline-coded pattern-recognition receptors (PRRs), which belong to the innate immune system (Medzhitov and Janeway, 2002). These receptors are located on both the extracellular or the intracellular side, and they recognize molecular patterns that are conserved in bacteria: pathogen-associated molecular patterns (PAMPs). PRRs are composed of transmembrane Toll-like receptors (TLRs), which have a key role in microbial recognition and induction of antimicrobial genes, and cytosolic NOD receptors whose main activity relies on bacterial clearance (Cario, 2005). Bacterial antigens such as endotoxin, forming complexes with LBP or DNA can sense and activate monocytes and macrophages via TLR receptors triggering the release of proinflammatory cytokines and chemokines TNF-α, IL-6, IL-8, IL-21, or IFN-γ through (NF)-κβ pathway (Hemmi et al., 2000; Wagner, 2002), similar to what MDP does via NOD2 (Lala et al., 2003; Eckmann and Karin, 2005) contributing to microbiota dysbiosis and tissue damage.

NLRs are important mediators in the control of intestinal inflammation, since the presence of gene polymorphisms in these molecules confers susceptibility to CD (Cummings et al., 2010). The activation of NLRs by PAMPs or danger-associated molecular patterns (DAMPs) result in downstream NF-kB signaling or caspase-1-mediated formation of inflammasomes (Rubino et al., 2012). NLRP3 inflammasome is activated in CD (Lazaridis et al., 2017), and its inhibition suppress the release of proinflammatory mediators (Liu et al., 2017). However,

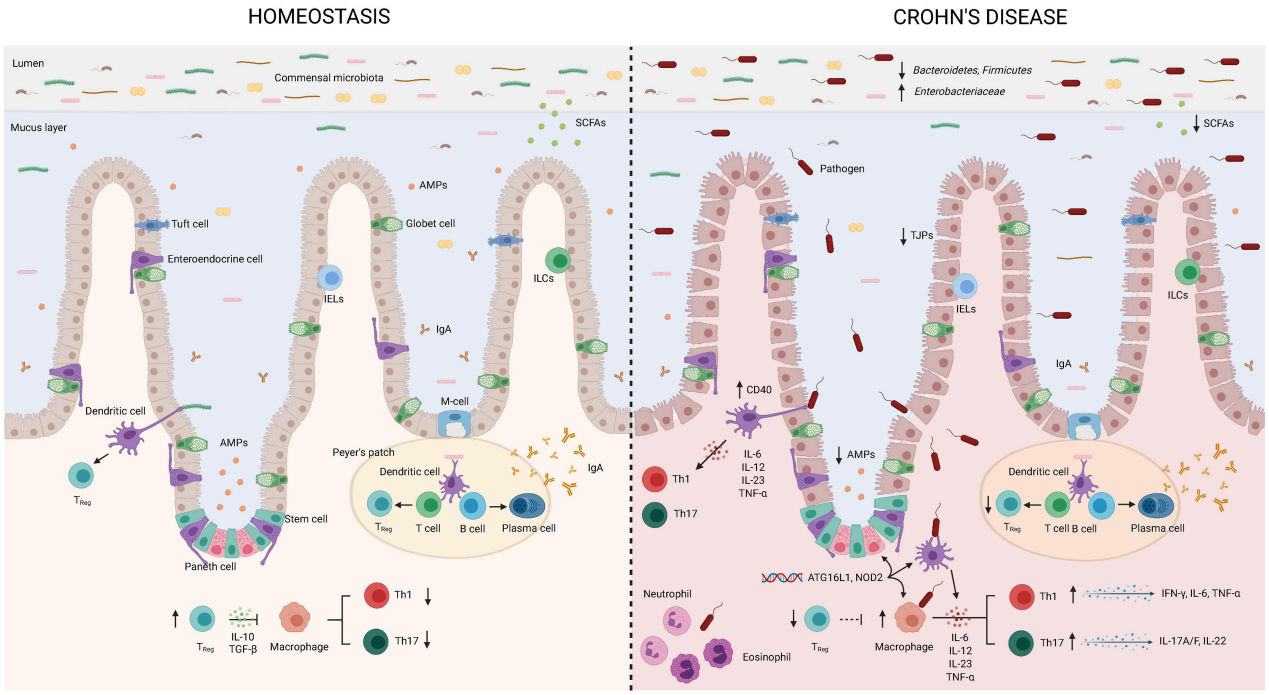

FIGURE 1 | Bacterial translocation in Crohn's disease. Intestinal tolerogenic mechanisms are altered in Crohn's disease leading to sustained inflammatory status. Integrity of the epithelial barrier is altered due to reduced expression of tight junction proteins. Increased populations of *Enterobacteriaceae* and pathogens, as well as reduced *Bacteroidetes*, *Firmicutes*, and populations of bacteria producing short chain fatty acids define intestinal dysbiosis in CD. Paneth cells display alterations in the production and secretion of antimicrobial peptides that can be explained by the gene status (NOD2). Translocating bacteria or its products can activate antigen presenting cells as macrophages and dendritic cells. Gene variants in ATG16L1 and NOD2 are associated with abnormalities in the secretion of antimicrobial peptides by Paneth cells and altered function of intestinal DCs and macrophages. Dendritic cells express higher levels of CD40 leading to increased interactions with T-lymphocytes and production of proinflammatory cytokines. Low regulatory T-lymphocyte differentiation will favor Th1 and Th17 subsets that will further produce proinflammatory cytokines. Neutrophils and eosinophils will be recruited to the site of infection and further contribute to induce an inflammatory environment in the attempt to eliminate translocating bacteria. AMPs, antimicrobial peptides; IgA, Immunoglobulin A; IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor; IELs, intraepithelial lymphocytes; ILCs, innate lymphoid cells; SCFAs, short chain fatty acids; TJPs, tight-junction proteins; T_{REG}, regulatory T-cells. This figure has been created using the BioRender platform.

results from experimental models show controversial results since adverse and protective roles for NLRP3 have been reported. Attenuated colitis was described in both NLRP3-deficient mice (Bauer et al., 2012) and after selective blockade of NLRP3 (Perera et al., 2018) in different animal models of intestinal inflammation, whereas inflammatory progression associated with altered intestinal integrity and increased mortality have also been outlined in NLRP3 knockout mice with experimental colitis (Zaki et al., 2010). It seems that the contribution of NLRP3 to the pathogenesis of IBD is highly influenced by the environment, including intestinal microbiota, as this molecule not only controls potential invading pathogens (Song-Zhao et al., 2014) but also participates in an inflammatory lytic cell death of innate immune cells mediated by caspase-1, known as pyroptosis (Fink and Cookson, 2006). NLRC4 is another relevant member of the NLR family able to detect flagellin and components of the type III bacterial secretory apparatus. NLRC4 inflammasome expressed in intestinal phagocytes seems to become relevant in the discrimination of pathogen and commensal microbiota through the production of IL-1β (Franchi et al., 2012). Additionally, NLRC4-deficient mice were more susceptible to experimental colitis associated with increased mortality following flagellated-*Salmonella* infection (Carvalho et al., 2012). Recent studies demonstrated that NLRP6 inflammasome can be activated either by lipoteichoic acid from *Listeria monocytogenes* (Hara et al., 2018) or via interaction with LPS and ATP (Leng et al., 2020). NLRP6 not only becomes relevant in the host immune response to microbial infections through the production of IL-18 but also mediates the secretion of mucins by globet cells (Wlodarska et al., 2014). Indeed, NLRP6-deficient mice showed more severe experimental colitis associated with a thinner mucus layer, susceptibility to bacterial infections, and altered intestinal microbiota (Elinav et al., 2011; Wlodarska et al., 2014). AIM2 belongs to the innate immune receptors sensing self or foreign cytosolic double-stranded DNA that results in the activation of caspase-1 mediated by the AIM2 inflammasome and consequent release of processed IL-1β and IL-18 (Hornung et al., 2009). AIM2 protects against intestinal inflammation induced by experimental colitis by limiting the growth of *E. coli* and by affecting to the production of antimicrobial peptides (Hu et al., 2015).

In this first-line defense system, macrophages and dendritic cells (DC) play a key role. Alterations in these cell populations

have been widely studied in the context of human IBD. Macrophages derived from the peripheral blood monocytes from CD patients showed impaired secretion of cytokines after *E. coli* insult and TLR ligation, contributing to a defective bacterial clearance (Smith et al., 2009). In addition, these cells showed an altered expression of surface markers, abundant secretion of IFN-γ, IL-6, IL-23, and TNF-α (Kamada et al., 2008). TNF-α and IFN-γ are major contributors to intestinal permeability (Cao et al., 2013; Xu et al., 2019). Intestinal DC directly samples luminal bacteria and transfers bacterial antigens to the mesenteric lymph nodes and Peyer's patches to recruit neutrophils and eosinophils, and to modulate the subsequent T-cell responses (Rescigno et al., 2001; Hart et al., 2005).

Neutrophils recruited to the site of infection phagocyte and kill invading pathogens through reactive oxygen species (ROS) production, neutrophil extracellular traps (NETs), and generation of lytic proteins (Wéra et al., 2016). Neutrophils can also orchestrate local immune responses by releasing cytokines and chemokines such as IL-8, CXCL1, CCL3, CCL4, and CCL20 among others, that can interact and recruit leukocytes from innate and adaptative immune populations including other neutrophils, basophils, eosinophils, macrophages, monocytes, DCs, and T cells (Tecchio and Cassatella, 2016). The proper functioning of neutrophils is crucial to resolve the inflammation induced by translocating pathogens since the lack of function or an increased neutrophil activity may be the origin of intestinal inflammation. While accumulation of neutrophils in the lamina propria correlates with the activity of the disease in UC (Bressenot et al., 2015), several research lines report deficient neutrophil activity in CD. Some studies suggest that neutrophils from CD patients show impaired ROS generation (Hayee et al., 2011); however, it is not clear if there exists an intrinsic failure in neutrophils activity in CD or if this is due to defective macrophage signaling and consequent reduced neutrophil recruitment within the inflammatory area (Segal and Loewi, 1976). Reduced production of the neutrophil chemokine IL-8 support the abnormal neutrophil chemotaxis observed in inflammatory lesions during CD (Marks et al., 2006). The reduced activity of neutrophils against luminal microbes may partially explain the chronic local and systemic inflammation underlying CD induced by a permanent activation of macrophages and T cells (Segal, 2018). On the other hand, eosinophilia is present in CD mucosal biopsies, and it is specially abundant in mucosal nerves (Yantiss, 2015). Some clinical studies suggest peripheral blood eosinophilia as a marker of worse outcome in CD patients (Click et al., 2017).

Under physiological conditions, DC ensures homeostasis inducing a tolerogenic intestinal state (Kretschmer et al., 2005; Tsuji and Kosaka, 2008; Raker et al., 2015). However, the proinflammatory intestinal milieu in CD hinders the tolerogenic profile of these cells (Iliev et al., 2009). During inflammation, there is an increase in the number, maturation, and retention of DC, contributing to inflammation (Middel et al., 2006; Verstege et al., 2008). In CD, DC express higher levels of CD40 leading to increased interactions with T-lymphocytes and the production of great amounts of proinflammatory cytokines (Senhaji et al., 2015) such as IL-6 and IL-12, which are related to microbial changes (Ng et al., 2011) and dysregulation in T-cell apoptosis (Atreya et al., 2000) and, also, IL-8 and TNF-α (Baumgart et al., 2009). TNF-α is the key effector cytokine driving tissue injury during intestinal inflammation (Garrett et al., 2007); it can modulate intestinal mucus secretion and composition (McElroy et al., 2011) and the epithelial barrier function (Al-Sadi et al., 2016; Grabinger et al., 2017). In addition, NOD2 variants, which are most widely detected genetic risk variants associated with CD pathogenesis, disturb DC bacterial sensing, cytokine production, and antigen presentation pathways (Cooney et al., 2010).

Innate lymphoid cells (ILC) cells are also involved in the innate immune response in CD. Its biological relevance lies in their capacity to sense environmental signals and to respond with the secretion of cytokines, producing a profound impact on epithelial cells (Maloy and Powrie, 2011; Sonnenberg and Artis, 2012), and conditioning T-cell responses (von Burg et al., 2015). In CD patients, there is an expansion of an intraepithelial ILC1 subset that produces IFN-γ in response to stimulation with IL-12 and IL-15 (Bernink et al., 2013; Fuchs et al., 2013), possible implication of ILC2 in the development of intestinal fibrosis through IL-13 secretion (Bailey et al., 2012), and ILC3 accumulation in inflamed areas, where they contribute to inflammation through increased IL-17 production and the recruitment of other immune cells (Geremia et al., 2011).

Mucosal $CD4^+$ T-cells are central players in maintaining a proinflammatory cytokine response by pushing a predominantly T-helper type 1 (Th1)-mediated inflammatory state in environments where IL-12 is released by antigen-presenting cells (APCs). For many years, it was accepted that CD was mainly mediated by Th1 cells (Brand, 2009), based on the fact that an elevation of the Th1 cytokines was observed in CD patients (Fuss et al., 1996; Monteleone et al., 1997; Parronchi et al., 1997; Pizarro et al., 1999). However, further studies had led to the identification of another subset of $CD4^+$ T characterized by the production of IL-17A, IL-17-F, and IL-22, which mediate T-helper type 17 (Th17) cells responses in CD (Strober and Fuss, 2011). An increase in Th17 cytokines produced by Th17 cells in inflamed gut mucosa (Fujino et al., 2003; Nielsen et al., 2003) as well as isolation and characterization of Th17 cells from gut mucosa of patients with CD (Annunziato et al., 2007) has supported the role of this cell population in IBD pathogenesis. CD displays a complex frame where Th1 and Th17 responses shift and depend on disease progression (Friedrich et al., 2019).

The differentiation of naïve T cells to Th17 cells is induced primarily by IL-6 and transforming growth factor (TGF)-β (Bettelli et al., 2006; Ivanov et al., 2006) and further reinforced by IL-1β and IL-23 (Langrish et al., 2005; Chung et al., 2009). IL-23 displays a central role in the maintenance and terminal commitment of naïve cells (Stritesky et al., 2008; McGeachy et al., 2009) and is implicated on the proliferation and expansion of Th17 cell populations (Veldhoen et al., 2006; Bettelli et al., 2007). IL-23R signaling in T cells drives the accumulation of intestinal Th17 cells while reducing the differentiation of tolerogenic FoxP3+ T-cells, as well as a reduced production of IL-10 by T-cells (Ahern et al., 2010). IL-23 induces T-cell expression of IL-17A, IL-17F, TNF-α, and granulocyte macrophage colony-stimulating

factor (GM-CSF) (Langrish et al., 2005; Tait Wojno et al., 2019). Increased expression of IL-17A and IL-17F has been detected in the mucosa of patients with active CD (Fujino et al., 2003; Nielsen et al., 2003; Hölttä et al., 2008; Seiderer et al., 2008; Geremia et al., 2011). In addition, genome association analysis has revealed many IL-23R variants linked with CD (Cummings et al., 2007; Cotterill et al., 2010), and some IL-23R loss of function mutations are protective in both UC and CD (Kim et al., 2011). Therapies targeted to IL-23 and its signaling pathways are promising approaches in CD treatment as observed in other inflammatory disorders such as psoriasis or multiple sclerosis (Neurath, 2017; Visvanathan et al., 2018). Antibodies targeting the IL-23 signaling are classified in those recognizing the p40 subunit shared by IL-12 and IL-23 or the p19 subunit unique in IL-23. Ustekinumab is an anti-p40 antibody with a favorable safety profile due to low rate of adverse events that shows a high rate of response and induce remission in moderate to severe CD patients (Feagan et al., 2016). Results from a phase 2 clinical trial in CD patients who failed in anti-TNF-α showed that selective IL-23 blockade using brazikumab, an anti-p19 antibody, was associated with clinical improvement at weeks 8 and 24, and higher serum levels of IL-22 (Sands et al., 2017). Similarly, another anti-IL-23-specific antibody, risankizumab, induced clinical remission in CD patients with active disease at week 12 (Feagan et al., 2017). Biological treatments targeting the IL-17 signaling are effective in psoriasis (Langley et al., 2014). Nevertheless, antibody therapy against IL-17, secukinumab, and its receptor IL-17R, brodalumab, have demonstrated unexpected results in CD, since two different clinical trials reported that the administration of secukinumab and brodalumab in moderate to severe CD patients were not effective and reported more adverse events and worsening of CD (Hueber et al., 2012; Targan et al., 2016).

IL-22 is another Th17-derived cytokine whose implication in IBD has been controversial. Some studies point to a protective role in the intestinal epithelium, stimulating the production of antimicrobial peptides (Okumura and Takeda, 2017), mucus secretion (Sugimoto et al., 2008), intestinal cell proliferation and survival (Zhang X. et al., 2019), and mucosal healing (Patnaude et al., 2021), while others mark that IL-22 may drive intestinal inflammation and gut epithelial cell death (Zha et al., 2019). These data suggest that its role during intestinal inflammation is highly context dependent. In fact, in the presence of eosinophilia, which is common during intestinal inflammation, IL-22 protective actions could be insufficient due to an increase in IL-22-binding protein (IL-22BP) (Martin et al., 2016).

Intensive research aiming to elucidate the contribution of Th17 responses to IBD have reported that IL-17 may exacerbate (Zhang et al., 2006) or protect (Yang et al., 2008; O'Connor et al., 2009) against intestinal inflammation depending on the experimental model studied. Results from Zhang and colleagues showed that IL-17R knockout mice presented reduced activity of experimental colitis induced by trinitrobenzenesulfonic (TNBS) acid. In line with this, the treatment with a soluble IL-17 receptor IgG fusion lessened intestinal inflammation induced by TNBS. On the other hand, studies conducted in animal models of colitis induced either by dextran sulfate sodium in IL-17 knockout mice or by $CD45RB^{hi}$ adoptive transfer using IL-17 or IL-17R-genetically deficient T-cells revealed an accelerated disease, therefore suggesting a protective role of IL-17 in those experimental systems. In order to determine the contribution of both Th1 and Th17 responses in CD, Sakuraba et al. isolated dendritic cells and lymphocytes from mesenteric lymph nodes of patients with CD. The authors observed that isolated CD4+ T-cells were producing increased levels of IFN-γ and IL-17, but isolated dendritic cells were activating CD4+T-lymphocytes toward the production of IFN-γ (Sakuraba et al., 2009). Taken together, these evidences suggest that BT might contribute to modulate the inflammatory response in CD via enhancing a Th1/Th17 response associated with the presence of bacteria or their products, which perpetuates the progression of the disease in a subgroup of patients.

The intestinal Treg population is relevant in the inflammatory responses to BT in CD, as they oversee tissue repair and immunological tolerance toward food antigens and microbiota in the gut, contributing to intestinal homeostasis (Kim et al., 2016; Tanoue et al., 2016; Xu et al., 2018). They belong to CD4+ lymphocytes and can suppress the immune response interacting with different components of the innate and adaptive immune response. Treg cells are highly heterogenous and express different lineage-specific transcription factors and cellular markers in different scenarios (Zhang et al., 2020). Treg cell populations produce IL-10 and TGF-β, and they can be naturally synthesized through thymic selection or induced after antigenic stimulation outside the thymus (Roncarolo et al., 2006; Sakaguchi et al., 2010), also in the gut by mucosal $CD103^{+}$ dendritic cells via a TGF-β and retinoic acid-dependent mechanism (Coombes et al., 2007). Treg secretion of IL-10 is important to control the gut balance. In fact, intestinal Th1-mediated inflammatory responses result in spontaneous colitis in IL-10-deficient mice (Davidson et al., 1996), and polymorphisms in the human IL-10R result in exacerbated intestinal immune responses (Glocker et al., 2009).

Changes in the percentage of Treg cells in patients with IBD have been reported (Maul et al., 2005), and a decreased number of CD4+ CD25+ FoxP3+ Treg cells have been observed in the lamina propria of patients with CD-related NOD2 variants (Rahman et al., 2010). Also, mutations in FOXP3 gene are related to the development of IBD (Okou et al., 2014). In the inflammatory milieu of CD, some groups have reported an enhanced recruitment of Treg cells in mucosal areas, suggesting a deficient suppressive activity during inflammation (Chamouard et al., 2009). These could be explained through changes in its cytokine profile similar to Th17 cells in the context of IBD (Hovhannisyan et al., 2011; Ueno et al., 2013) and also a diminished expression of transcription factors involved in Treg regulation in CD (Qiao et al., 2013). On the other hand, recently, a subset of Treg CD161+ cells has been found highly enriched in the mucosa of CD patients, which are involved in wound healing and associated with reduced inflammation (Povoleri et al., 2018). All of these suggest that different Treg subsets could behave differentially in IBD. Due to its immunomodulatory capacity, therapies targeting this cell population are being assessed with promising results (Desreumaux et al., 2012; Trotta et al., 2018; Clough et al., 2020).

The microbiome is key in the equilibrium between Treg and Th17 in the gut (Lochner et al., 2011; Ohnmacht et al., 2015;

Sefik et al., 2015). Microbiota from IBD donors into germ-free mice reduced the presence of RORγt+ Treg cells but increased Th17 and Th2 populations (Britton et al., 2019). On the other hand, commensal microbiota can promote CD4+ CD25+ FoxP3+ Treg cells *in vivo*, which control the innate inflammatory cascade to translocating microbes by reducing proinflammatory cytokine production, reducing T-cell proliferation, reducing dendritic cell co-stimulatory molecule expression, and attenuating (NF)-κβ activation (O'Mahony et al., 2008). SCFAs produced by *Bifidobacteria* and *Clostridia*, like butyrate, cause inhibition of histone deacetylase (HDAC), promoting FoxP3 expression (Arpaia et al., 2013) and production of retinoic acid (Smith et al., 2013; Schilderink et al., 2016) polysaccharide A of *Bacteroides fragilis* induces an intestinal tolerogenic environment by promoting the IL-10-producing Foxp3+ Treg population (Round and Mazmanian, 2010), and indole-3-aldehyde, produced by *Lactobacillus reuteri*, is a tryptophan precursor involved in the plasticity of T cells (Lamas et al., 2016). In addition, *Clostridiodes* spp. mixture transplantation is also associated with increased counts of intestinal Treg cells in mice (Atarashi et al., 2013; Narushima et al., 2014).

EFFICACY OF ANTI-TNF-α TREATMENT IN PATIENTS WITH BACTERIAL TRANSLOCATION

Biologic treatments including anti-TNF-α, adhesion molecule inhibitors, and p-40 IL-12/23 inhibitor, ustekinumab, are effective therapies for patients with moderate to severe IBD (Katsanos et al., 2019). Anti-TNF-α monoclonal antibodies were the first biologic agents that demonstrated effectiveness in the treatment of CD (Rutgeerts et al., 1999; Hanauer et al., 2002; Sands et al., 2004; Xiao et al., 2016) as TNF-α is increased in the intestinal mucosa of IBD patients (Breese et al., 1994; Dionne et al., 1997). Increased intestinal TNF-α could be directly involved in BT, as it can disrupt intestinal epithelial integrity (Al-Sadi et al., 2016; Grabinger et al., 2017) and mediate tissue injury (Garrett et al., 2007). However, it is known that 30–40% of patients with IBD under anti-TNF therapy show a primary non-response, and up to 50% may present adverse events or develop secondary non-response over time (Ben-Horin and Chowers, 2011; Papamichael et al., 2017). Focusing on the efficacy of anti-TNF-α therapy, further research has also found that undetectable serum through concentration of anti-TNF-α levels (Maser et al., 2006) and decreased free TNF-α binding capacity of anti-TNF-α drugs (Ainsworth et al., 2008) are predictors of poor response to anti-TNF-α treatment of patients with CD. Even if serum levels of TNF-α have also been proposed to predict the efficacy of anti-TNF-α in CD patients (Martínez-Borra et al., 2002), several studies have reported that serum TNF-α is not a good predictor of clinical response to anti-TNF-α therapy (Ogawa et al., 2012). Besides clinical factors and the development of antibodies against anti-TNF-α agents (Baert et al., 2003), several other factors such as BT and a susceptible genotype, intestinal dysbiosis, and even the Treg population may have a role in this loss of response.

In the past, we investigated the effects of different gene variants and BT in the efficacy of anti-TNF-α therapy in CD. We identified a subgroup of CD patients characterized by the presence of a variant NOD2 genotype, in combination or not with a variant ATG16L1 genotype, who may need an intensified anti-TNF-α drug schedule since they showed increased bactDNA translocation, augmented inflammatory response, and increased risk of relapse. In detail, the presence of a variant NOD2 genotype, either alone or combined with ATG16L1 variant genotype, was associated with increased bactDNA translocation, and the presence of serum bactDNA was associated with relapse at 6 months. Patients with bactDNA showed increased proinflammatory cytokines response that was further augmented in patients who were also carrying combined NOD2/ATG16L1 variants. A variant NOD2 genotype correlated with reduced phagocytic and bactericidal activities in neutrophils and exacerbated *in vitro* TNF-α secretion in response to *E. coli*, suggesting that neutrophils from CD patients carrying a variant NOD2 genotype have altered bacterial clearance. Evaluation on anti-TNF-α therapy on patients carrying NOD2/ATG16L1 combined genotypes revealed that most of these patients were on an intensified anti-TNF-α drug schedule. Moreover, free anti-TNF-α levels were significantly decreased in the serum of patients with bactDNA translocation and a variant NOD2 genotype and, especially, in patients with a combined NOD2/ATG16L1 variant, suggesting that increased drug consumption is necessary on these patients to promote an adequate tolerogenic response (Gutiérrez et al., 2014). We further demonstrated that the presence of bactDNA in CD patients is a significant independent risk factor of short-term relapse in those in remission, especially in the ones with mucosal lesions, suggesting that the presence of mucosal damage is not essential for BT, but it contributes to it, in synergy with bactDNA (Gutiérrez et al., 2016). In line with this, we observed that the increase in bactDNA and TNF-α in CD patients could be related with a variant in IL-26 gene. This variant was associated with an impaired antibacterial clearance, increased inflammatory cytokines, and an increment in anti-TNF-α consumption in CD patients (Piñero et al., 2017). This also contributes to explain why SNPs in IL-26 gene confer genetic susceptibility to CD (Silverberg et al., 2009). All these findings suggest that BT aggravates the inflammatory response and predisposes to risk of relapse and need of intensified anti-TNF-α drug therapies in susceptible CD patients.

It is well-known that levels of anti-TNF-α determine the treatment response (Moore et al., 2016), but recent studies manifest that intestinal dysbiosis might also play a role in the efficacy of the biologic therapy. Therefore, initial gut microbial composition and cytokine profile before anti-TNF-α therapy, as well as anti-TNF-α-induced microbial changes during the treatment are key in the achievement of clinical remission (Jones-Hall and Nakatsu, 2016; Franzin et al., 2021) and IBD patients with greater gut dysbiosis achieve clinical remission later (Aden et al., 2019). The treatment with anti-TNF-α improves the intestinal dysbiosis in CD by increasing SCFAs producing bacteria like *Anaerostipes*, *Blautia*, *Coprococcus*, *Faecalibacterium*, *Lachnospira*, and *Roseburia* (Kowalska-Duplaga et al., 2020;

Seong et al., 2020) and decreasing bacterial species associated with mucosal damage (Busquets et al., 2015; Ribaldone et al., 2019). The relevance of intestinal microbiota in the efficacy of current IBD treatments is certainly an open research field that deserves more in-depth investigations.

Finally, the Treg population has been shown to actively participate in the loss of response to anti-TNF-α. An increased peripheral blood Treg cell population after anti-TNF-α therapy administration is related with increased serum levels of TGF-β and IL-10 and with the clinical improvement observed in patients with CD (Di Sabatino et al., 2010; Guidi et al., 2013). Indeed, we have also reported that Treg population is susceptible to significantly increase after anti-TNF-α administration in CD patients bearing a wild-type NOD2 genotype. Nevertheless, CD patients carrying a polymorphism in NOD2 have lower available serum levels of anti-TNF-α and an impaired capacity to induce the Treg population. Altogether, these results suggest an impaired immunological function in this subgroup of CD patients, as demonstrated by increased serum levels of TNF-α. Accordingly, most of these patients were on anti-TNF-α intensified therapy and showed a more aggressive CD phenotype. Furthermore, we found that CD patients showing perianal lesions had lower circulating Treg population. Thus, immunophenotyping Treg cells in blood of patients with CD can be a fast and helpful methodology to anticipate not only the clinical response to biological therapy but also a more aggressive phenotype of CD (Juanola et al., 2014).

FUTURE DIRECTIONS

To predict CD behavior is a topic of strong interest that would greatly improve the welfare of patients. The multifactorial etiology of the disease makes it necessary to consider several aspects from genetic to environmental factors in an attempt to determine the risk of relapse (Timmer et al., 1998; Tibble et al., 2000; Beaugerie et al., 2006; Takeuchi et al., 2006). However, the clinical value, so far, is limited due to lack of specificity.

As shown in this review, many lines of evidence point to the translocation of bacterial products as an important player leading to uncontrolled inflammation in CD patients. Even if the question still arises about BT as the cause or the consequence of intestinal inflammation, it is widely accepted that host–bacterial interactions influence CD. Therefore, evaluating the presence of gut bacterial antigens at a systemic level may constitute a new marker for increased risk of relapse among CD patients. Of particular interest is the combination of BT and CD-related susceptibility genes such as NOD2, which probably facilitates the translocation of bacterial antigens; this is worth exploring in the context of response to TNF-α antagonists and risk of relapse.

Further studies aimed at understanding the interaction between the immune system, both at systemic and mucosal level, gut microbiota, and genetic predisposition will help clinicians to better control and individually treat CD patients in the future.

AUTHOR CONTRIBUTIONS

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

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Low-Intensity Exercise Modulates Gut Microbiota to Fight Against Radiation-Induced Gut Toxicity in Mouse Models

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Radiation-induced gastrointestinal (GI) tract toxicity halts radiotherapy and degrades the prognosis of cancer patients. Physical activity defined as "any bodily movement produced by skeletal muscle that requires energy expenditure" is a beneficial lifestyle modification for health. Here, we investigate whether walking, a low-intensity form of exercise, could alleviate intestinal radiation injury. Short-term (15 days) walking protected against radiation-induced GI tract toxicity in both male and female mice, as judged by longer colons, denser intestinal villi, more goblet cells, and lower expression of inflammation-related genes in the small intestines. High-throughput sequencing and untargeted metabolomics analysis showed that walking restructured the gut microbiota configuration, such as elevated *Akkermansia muciniphila*, and reprogramed the gut metabolome of irradiated mice. Deletion of gut flora erased the radioprotection of walking, and the abdomen local irradiated recipients who received fecal microbiome from donors with walking treatment exhibited milder intestinal toxicity. Oral gavage of *A. muciniphila* mitigated the radiation-induced GI tract injury. Importantly, walking did not change the tumor growth after radiotherapy. Together, our findings provide novel insights into walking and underpin that walking is a safe and effective form to protect against GI syndrome of patients with radiotherapy without financial burden in a preclinical setting.

Keywords: radiotherapy, radiation-induced gastrointestinal tract toxicity, intestinal inflammation, low-intensity exercise, walking, gut microbiota, *Akkermansia muciniphila*

INTRODUCTION

As non-infectious diseases, cancers have been attributed as the leading cause of death globally. It is estimated that there will be at least 10,000,000 new cases of cancer and 6,800,000 cancer deaths from 2018 to 2040[1]. Radiation therapy serves about 4,70,000 patients per year in the United States, and up to 50% of cancer patients will undergo radiotherapy for either curative or adjuvant purposes (Citrin, 2017). Radiotherapy is the first-line treatment for multiple cancers, including head and neck tumors and abdominopelvic tumors. Although ionizing radiation kills

[1]https://gco.iarc.fr/tomorrow/home

malignant tumor cells effectively, early and late adverse side effects are common and grievously intertwined with the remedy (De Ruysscher et al., 2019; Romesser et al., 2019). Iatrogenic local irradiation for abdominopelvic cancers aligns with varying degrees of gastrointestinal (GI) tract complications, covering nausea, malabsorption, diarrhea, and intestinal obstruction, which halt radiotherapy prematurely and degrade the life quality of patients (Hauer-Jensen et al., 2014). To date, safe and effective therapeutic options have been scarce to fight against these complications in clinical application (Vozenin et al., 2019; Whelan et al., 2019).

Bad living habits, such as smoking, drinking, and sedentary lifestyle, emerge as potential pitfalls propelling nearly half of cancers (Islami et al., 2018). The sedentariness has been proved to precipitate the occurrence and development of several cancers (Kerr et al., 2017). Lately, mounting evidence corroborates that physical activity is beneficial for health, including adjusting metabolism, preventing cancer occurrence (Hespanhol Junior et al., 2015; Geng et al., 2019; Rezende et al., 2019; Takahashi et al., 2019), and even synergizing cancer treatment as an immune adjuvant or chemosensitizer (Betof et al., 2015; Febbraio, 2017; Duggal et al., 2019). Heretofore, most researches focus on vigorous exercise, which carries potential cardiovascular risk and is not suitable for frail patients with surgery or radiotherapy (Marijon et al., 2011). As a form of low-intensity exercise, walking has been proved to improve mental health and sleep quality and to prolong the progression-free survival of patients with locally advanced or metastatic colorectal cancer (CRC; Hamer and Steptoe, 2008; Oja et al., 2018; Guercio et al., 2019; Tang et al., 2019). However, whether walking can be employed as a rehabilitation strategy for cancer patients with radiotherapy remains unidentified.

Neonatal gut is colonized by microorganisms immediately following birth. The gut microbiome of infant is modified with development and participates in the formation and improvement of innate and adaptive immunity (Milani et al., 2017). Recently, studies on gut microbiota have experienced a renaissance and explored the tight relationships between enteric flora and host's health. On the one hand, the gut microbiota modulates metabolism and maintains energy balance of hosts (Lim et al., 2017). On the other hand, intestinal flora dysbiosis precipitates a broad range of intra-intestinal diseases, such as inflammatory bowel disease (IBD) and CRC, and extra-intestinal diseases, covering cardiovascular disorders, diabetes, obesity, and neurodegenerative diseases (Gérard, 2016; Pistollato et al., 2016; Tang et al., 2017; Leiva-Gea et al., 2018; Ostojic, 2018; Fattorusso et al., 2019). Our previous studies have identified the vital roles of intestinal microbes in rehabilitation of radiation-induced GI tract toxicity (Cui et al., 2017). Notwithstanding that heredity, diet, and antibiotic usage are key determinants for gut flora configuration, lifestyle, stress, and exercise have been proven to educate and tune the gut microbiota community as well (Conlon and Bird, 2014; Zhong et al., 2019). Notably, physical exercise modifies the gut bacteria configuration to benefit the host's haleness (Monda et al., 2017; de Sire et al., 2020). In this study, we reported that short-term walking reshaped the gut microbiota and mitigated radiation-induced GI toxicity in both male and female mice without accelerating the proliferation of cancer cells. Further exploration demonstrated that the radioprotective effects of walking were partly dependent on gut microbiota, such as *Akkermansia muciniphila*. In brief, our findings provide new insights into the function and underlying protective mechanism of walking in the context of intestinal radiation toxicity in a preclinical experimental setting.

MATERIALS AND METHODS

Mice

Male/female 6- to 8-week-old C57BL/6J mice or 4-week-old male BALB/c athymic nude mice were purchased from Beijing Huafukang Bioscience Co., Inc. (Beijing, China). Mice were housed in the specific pathogen-free (SPF) level animal facility at the Institute of Radiation Medicine (IRM), the Chinese Academy of Medical Sciences (CAMS), and maintained in an enriched environment with a temperature-controlled room in a 12-h light–dark cycle, with food and water available. Before the experiment, the mice were adapted to the experimental environment for a week. Animal experiments were performed according to the institutional guidelines approved by the Animal Care and Ethics Committee of IRM-PUMC (the Ethical Approval number is IRM-DWLL-2019096), which complied with the Guide for the Care and Use of Laboratory Animals and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Walking Protocol

The speed of walking (defined as less than 6 m/min in mouse models) was determined firstly according to the previous studies (Bellardita and Kiehn, 2015; Caggiano et al., 2018). Analysis of the movement behavior and the breath measurement of mice showed that compared with 6 m/min, 35 min of 1 m/min walking is a sustainable way of exercise for mice under pathological conditions, which ensures the quality and quantity of the walking (**Supplementary Figure 1**). Taking the physical exercise ability of the irradiated mice or patients with radiotherapy, 1 m/min walking was finally selected for intervention in the mice. In order to synchronize the segmented radiotherapy strategy for clinical cancer patients, walking strategy was adjusted to 6 days per week. Due to the physical fitness of the BALB/c athymic nude mice, the daily time and speed of walking were reduced on the basis of walking in C57BL/6J mice (30 min of 1 m/min). All mice completed the walking with quality and quantity.

Radiation Study and Experimental Group

A Gammacell-40 ^{137}Cs irradiator (Atomic Energy of Canada Limited, Chalk River, ON, Canada) at a dose rate of 0.88 Gy/min was used for all experiments (Con, control; TAI, total abdominal irradiation; W, walking; FMT, fecal microbiota transplantation; ABX, antibiotic cocktail; ABXW, antibiotic cocktail and

walking). The C57BL/6J mice were divided into Con, TAI, TAI + W, TAI + FMT, ABX, and ABXW five groups; and mice in each group except for Con group received 12 Gy of γ-ray TAI when the body weight reached 19–20 g (Li et al., 2020a). The mice in TAI + W group maintained walking on the treadmill (Beijing Zhongshidichuang Science and Technology Development Co., Ltd., Beijing, China) for 15 days (1 m/min, 35 min/day, 6 days/week) following radiation exposure, while TAI group was maintained on fasting and water deprivation for 35 min to eliminate the influence of food and water. For TAI + FMT group, abdominal local irradiated mice were administrated with fecal microbiome via oral route (Cui et al., 2017). The donors were abdominal local irradiated male C57BL/6J mice with walking treatment (based on the "Walking protocol"). The mice in ABX group were housed with drinking water supplemented with an antibiotic cocktail (ABX) (vancomycin, metronidazole, ciprofloxacin, ampicillin, and streptomycin) that can subvert existing gut microbes (Xiao et al., 2020), while ABXW group maintained the walking treatment for 15 days during ABX treatment. During the non-experimental period, the water and food were available *ad libitum*.

Culture of *Akkermansia muciniphila* and *Akkermansia muciniphila* Transplantation Treatment

A. muciniphila MucT (ATCC BAA-835) was cultured in brain heart infusion broth containing 10 mg/L of resazurin (an oxidation–reduction indicator) under strict anaerobic conditions. A representative culture stock was used to determine the CFU/ml under anaerobic conditions by plate counting using mucin media containing 1% agarose. This culture was diluted with anaerobic phosphate-buffered saline (PBS) to a final concentration of 1.5×10^8 CFU/100 μl. To explore the effects of *A. muciniphila* on radiation-induced intestinal injury, the irradiated mice (12 Gy of TAI) were treated with an oral administration of *A. muciniphila* (1.5×10^8 CFU) suspended in sterile PBS for 15 days, while the contrast mice were given sterile PBS with equivalent volume. For the quantitative analysis of *A. muciniphila* in colon, the colons of mice were cut lengthwise; and few feces and mucus layer were scraped off with sterile cotton brush. Then the DNA was extracted by using TIANamp Stool DNA kit (TIANGEN, Beijing, China) and used for q-PCR.

Cell Culture

Human CRC cell line HCT-8 or human lung cancer cell line A549 were obtained from the American Type Culture Collection (ATCC) and certified to be mycoplasma-free. The cells were cultured with 10% fetal bovine serum (Gibco, Grand Island, NY, United States), 100 U/ml of penicillin, and 100 mg/ml of streptomycin and grown at 5% CO_2 and 37°C (Van Hoorde et al., 2000).

Tissues Collection

After 15 days of walking treatment or *A. muciniphila* supplementation treatment, the C57BL/6J mice were sacrificed to assess the inflammation of the intestine. The length of colon was measured (Xiao et al., 2020), and small intestine tissue was removed for RNA isolation, protein extraction, and histological staining.

Quantification of the Expression of IL-1β, IL-6, TNF-α, and Reactive Oxygen Species by ELISA

Small intestine tissues in each experimental group were ground with 200 μl of saline, followed by centrifugation for 10 min at 6,000 rpm and 4°C. Protein levels were measured from the clear supernatant using ELISA kit (Mlbio, Shanghai, China) according to the manufacturer's instructions. Optical density was read at 450 nm (Rayto, Shenzhen, China).

RNA Isolation and Quantitative Reverse Transcription Real-Time PCR

Total RNA was extracted from intestine tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's protocol. Complementary DNA was synthesized from total RNA using poly(A)-tailed total RNA and reverse transcription primer with ImPro-II Reverse Transcriptase (Promega, Madison, WI, United States), according to the manufacturer's protocol. The qRT-PCR was performed according to the instructions of Fast Start Universal SYBR Green Master (Rox) (Roche Diagnostics GmbH, Mannheim, Germany). Experiments were conducted in duplicate in three independent assays. Relative transcriptional folds were calculated as $2^{-\Delta\Delta Ct}$. GAPDH was used as an internal control for normalization. All primers are listed in **Supplementary Table 1**.

Histology

Following euthanasia, small intestine tissues of C57BL/6J mice were fixed in 4% buffered formalin overnight at room temperature and then embedded in paraffin. Tissues were sectioned at 5-μm thickness and co-stained with hematoxylin and eosin (H&E) using HE Staining Kit (Solarbio, Beijing, China) (Cardiff et al., 2014). For periodic acid–Schiff (PAS) staining, the small intestines of mice were fixed in Carnoy's solution (absolute ethanol:chloroform:glacial acetic acid = 6:3:1) for 3 h. Dewaxed sections were hydrated and incubated in 1% periodic acid for 10 min followed by incubation in Schiff's reagent for 10 min. Sections were counterstained with Mayer's hematoxylin for 30 s and then washed and dehydrated before mounting with Pertex. Immunohistochemical (IHC) staining of tumor samples from BALB/c athymic nude mice were performed as previously reported (Xiao et al., 2020); and the primary antibody of rabbit anti-Ki-67 (Proteintech Group, Chicago, IL, United States) was used. Categorization of immunostaining intensity was performed by three independent pathologists. Sections were examined under light microscopy.

In vivo Tumor Xenograft Assay

HCT-8 (or A549) cells were harvested and suspended at 2×10^7 per ml with sterile normal saline. Groups of 4-week-old-male nude mice were subcutaneously injected in the armpit with 200 μl of cell suspensions (Xiao et al., 2020). When the tumor volume reached approximately 100 mm^3, the mice were divided into two groups randomly based on the sizes of the tumors (n = 8 per group) (Sack et al., 2011; Martín-Ruiz et al., 2020) and received 10 Gy of radiotherapy (2 Gy × 5 day) (Kogelnik and Withers, 1978; Xiao et al., 2020). For the irradiation, mice were positioned under a lead shield so that only the tumor area was exposed. The mice in TAI + W group maintained the walking treatment for 15 days following radiotherapy, while TAI group maintained fasting and water deprivation for the same time. Tumor growth was measured every 3 days. Tumor volume (V) was monitored by measuring the length (L) and width (W) with calipers and calculated with the formula $V = (L \times W^2) \times 0.5$. After 15 days, tumor-bearing mice were sacrificed, and the tumors were excised and weighed.

Bacterial Diversity Analysis

Stool samples were freshly collected from two independent experiments and stored at −80°C until use. DNA was extracted from the stool using the Power Fecal® DNA Isolation Kit (MoBio, Carlsbad, CA, United States). The DNA was recovered with 30 ml of buffer in the kit. PCR products were mixed in equidensity ratios. Then, mixture PCR products were purified with Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany). The 16S ribosomal RNA (rRNA) V4 gene was analyzed to evaluate the bacterial diversity using lonS5TMXL lon 530 Chip (Thermo Fisher, Waltham, MA, United States). Sequence analysis was performed by Uparse software (Uparse v7.0.1001)[2]. Sequences with ≥97% similarity were assigned to the same operational taxonomic units (OTUs). Representative sequence for each OTU was screened for further annotation. For each representative sequence, the Silva123 Database was used based on RDP classifier (Version 2.2)[3] algorithm to annotate taxonomic information. Briefly, each cohort contains 16 mice, and four mice share one cage. For gut microbiota analysis, we collected two fecal pellets from each cage to avoid cage effects. The primers are listed in **Supplementary Table 1**.

Untargeted Metabolomics–Metabolite Extraction

Feces were individually grounded with liquid nitrogen, and the homogenate was suspended with prechilled 80% methanol and 0.1% formic acid (FA) by well vortexing. The samples were incubated on ice for 5 min and then were centrifuged at 15,000 rpm, 4°C for 5 min. Some of supernatant was diluted to final concentration containing 60% methanol by liquid chromatography–MS (LC-MS)-grade water. The samples were subsequently transferred to a fresh Eppendorf tube with 0.22-μm filter and then were centrifuged at 15,000 *g*, 4°C, for 10 min. Finally, the filtrate was injected into the LC-MS/MS system analysis (Xiao et al., 2020).

Untargeted Metabolomics—Ultra-High-Performance Liquid Chromatography–MS/MS Analysis

LC-MS/MS analyses were performed using a Vanquish UHPLC system (Thermo Fisher, Waltham, MA, United States) coupled with an Orbitrap Q Exactive HF-X mass spectrometer (Thermo Fisher, Waltham, MA, United States). Samples were injected into a Hyperil Gold column (100 × 2.1 mm, 1.9 μm) using a 16-min linear gradient at a flow rate of 0.2 ml/min. The eluents for the positive polarity mode were eluent A (0.1% FA in water) and eluent B (methanol). The eluents for the negative polarity mode were eluent A (5 mM of ammonium acetate, pH 9.0) and eluent B (methanol). The solvent gradient was set as follows: 2% B, 1.5 min; 2%–100% B, 12.0 min; 100% B, 14.0 min; 100%–2% B, 14.1 min; and 2% B, 16 min. Q Exactive HF-X mass spectrometer was operated in positive/negative polarity mode with spray voltage of 3.2 kV, capillary temperature of 320°C, sheath gas flow rate of 35 arb, and aux gas flow rate of 10 arb.

Untargeted Metabolomics—Data Analysis

The raw data files generated by UHPLC-MS/MS were processed using the Compound Discoverer 3.0 (CD 3.0, Thermo Fisher, Waltham, MA, United States) to perform peak alignment, peak picking, and quantitation for each metabolite. The main parameters were set as follows: retention time tolerance, 0.2 min; actual mass tolerance, 5 ppm; signal intensity tolerance, 30%; signal/noise ratio, 3; and minimum intensity, 100,000. After that, peak intensities were normalized to the total spectral intensity. The normalized data were used to predict the molecular formula based on additive ions, molecular ion peaks, and fragment ions. And then peaks were matched with the mzCloud[4] and ChemSpider[5] database to obtain the accurate qualitative and relative quantitative results.

Statistical Analysis

Each experiment was repeated at least three times. Data were assessed with normal distribution using the Kolmogorov–Smirnov test. The data are presented as the means ± SD with respect to the number of samples (n) in each group. Significance was assessed by comparing the mean values using Student's t-test and Wilcoxon rank sum test for independent groups, as follows: $^*p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.005$. Results with $p < 0.05$ were considered statistically significant.

[2]http://drive5.com/uparse/

[3]http://sourceforge.net/projects/rdpclassifier/

[4]https://www.mzcloud.org/

[5]http://www.chemspider.com/

RESULTS

Walking Alleviates Radiation-Induced Gastrointestinal Tract Injuries in Male Mice

All experimental mice were exposed to abdomen local irradiation. Then, one cohort of mice was enforced to walk (35 min of 1 m/min, **Supplementary Figure 1**) for 2 weeks. As shown in **Figure 1A**, walking did not change the body weight loss of irradiated mice. To address the effects of walking on radiation-induced GI toxicity, we assessed the colon length and histological structure of the small intestines. Intriguingly, mice with walking treatment had longer colon (**Figures 1B,C**), denser small intestinal villi, and more goblet cells (**Figure 1D**) than did the mice with irradiation only. In addition, abdominal radiation stimuli elicited the syndromes for enteritis, as judged by the upregulation of inflammatory factors expression in the small intestine; however, short-term walking erased the elevation (**Supplementary Figure 2**; **Figures 1E–I**). Meanwhile, walking reduced the level of intestinal oxidative stress following ionizing radiation (**Figures 1I,J**). Together, our findings demonstrate that walking as a form of low-intensity exercise protects against radiation-induced GI tract toxicity in male mice.

Walking Reshapes Gut Microbiota Configuration Following Radiation Challenge

Given the close relationship between the gut microbiota and the radiation-induced GI tract toxicity, we performed 16S rRNA gene amplicon surveys to analyze the bacterial composition in fecal pellets from abdomen local irradiated mice with or without short-term walking treatment. As shown in **Figures 2A–C**, 2 weeks of walking treatment decreased the alpha diversity of gut flora in the irradiated mice. Reversely, the beta diversity of microorganism in droppings was increased from the mice with walking treatment (**Figure 2D**; **Supplementary Figures 3A–C**). Weighted principal coordinates analysis (PCoA) and non-metric multidimensional scaling (NMDS) further exhibited an obvious separation of enteric bacteria between the two cohorts, indicating that walking indeed remolds the radiation-shifted intestinal bacterial profile (**Figures 2E,F**). In detail, the mice with short-term walking treatment showed a predominance of *Dubosiella*, *Bacteroides*, *Akkermansia*, and *Lactobacillus* at the genus level (**Figures 2G,H**; **Supplementary Figures 3D–F**). Next, the analysis of metabolome of gut microbiome showed that walking propelled a variation in metabolite profile (**Figures 2I,J**; **Supplementary Figures 3G,H**), indicating that walking not only alters the gut microbiota structure but also impacts the function of gut flora. Together, our observations demonstrate that short-term walking restructures the gut microbiome after radiation exposure.

Walking Mitigates Intestinal Radiation Toxicity Depending on Gut Microbiota

To find out whether walking mitigating radiation-induced GI tract toxicity depends on gut microbiota, an antibiotic cocktail (ABX) was added in the drinking water to remove the gut microbes of the irradiated mice. Intriguingly, ABX treatment hindered the radioprotection of walking toward GI injuries, as judged by shortening colon, loss of intestinal villi, reduced goblet cells, and elevated inflammatory status (**Supplementary Figure 4**), implying that gut microbiota might contribute to the radioprotection of short-term walking.

Next, FMT was performed to further validate the roles of gut microbiota in the system. The donor mice walked for 2 weeks after abdomen local irradiation (**Supplementary Figure 5**). Same with the donors, the alpha diversity of gut bacteria in irradiated recipients declined as compared with that of the mice with irradiation only (**Figures 3A,B**). Although weighted_unifrac analysis showed no change of the enteric microbiota statistically (**Figure 3C**), PCoA and NMDS plot was conducted to visualize differences in bacterial taxa composition between the two groups (**Figures 3C–E**; **Supplementary Figure 6D**). In parallel, FMT caused an enrichment on *Dubosiella*, *Bacteroides*, *Akkermansia*, and *Lactobacillus* at the genus level in recipients compared with saline-treated controls (**Figure 3F**; **Supplementary Figures 6E–G**), indicating the gut microbiota community of recipients is shifted and similar to that of the donors after FMT. Consistent with the aforementioned results, recipients shared the same dynamic changes of body weight to irradiated controls (**Supplementary Figure 6H**) and had longer colon (**Figure 3G**), denser intestinal villi, more goblet cells (**Figure 3H**), lower proinflammatory cytokine levels, and fewer reactive oxygen species (ROS) production than in saline treatment (**Figures 3I–L**; **Supplementary Figures 6I–K**). Together, our observations corroborate that walking fights against radiation-induced GI tract injuries at least partly depending on modulating gut microbiota.

Akkermansia muciniphila Mitigates Radiation-Elevated Inflammatory Status in Digestive Tract

The 16S rRNA sequencing analysis showed a predominance of *A. muciniphila* at the genus level following walking and FMT experiments. In addition, *A. muciniphila* has been reported to be negative correlation with numerous diseases including IBDs, and the frequency of the bacteria was increased in dextran sodium sulfate (DSS) mice with running treatment. Thus, we speculated that as a potential probiotic, *A. muciniphila* might be the key element for the radioprotection of walking. The abdominal irradiated mice were treated with *A. muciniphila* (1.5×10^8 CFU) via oral route for 15 days. As shown in **Figure 4A**, the relative abundance of *A. muciniphila* in feces increased after the treatment, validating that *A. muciniphila* was colonized in the intestinal tract of mice successfully. Although oral gavage of *A. muciniphila* did not change the body weight of irradiated mice (**Figure 4B**), the length of colon and the integrity of intestinal villi were improved (**Figures 4C,D**; **Supplementary Figure 7A**). In addition, *A. muciniphila* treatment reduced the levels of inflammatory markers in the small intestine (**Figures 4E–K**; **Supplementary Figure 7B**), suggesting that the enrichment of *A. muciniphila* in lower digestive tract ameliorates radiation-induced enteritis. Together, our observations demonstrate

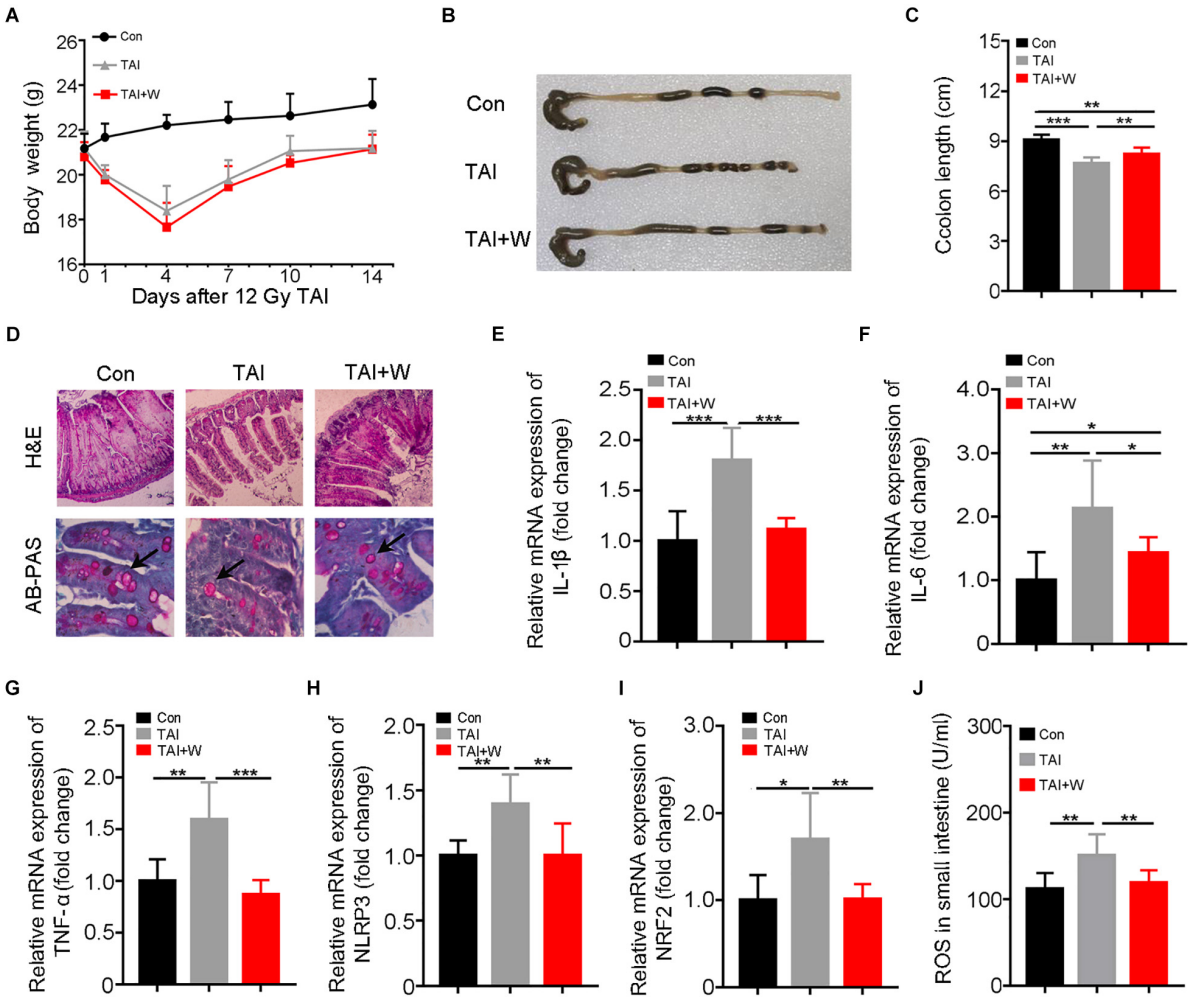

FIGURE 1 | Walking as a non-pharmacological regimen alleviates radiation-induced GI tract injuries in male mice. Male mice were exposed to 12 Gy of TAI, and TAI + W group maintained walking for 15 days, and TAI group had no treatment. Then the colon and small intestine tissues were obtained at day 16, $n = 10$ per group. **(A)** The body weight of male mice in the three groups. **(B)** Photographs of dissected colon from male mice in the three groups. **(C)** Statistical results of colon length between two groups. **(D)** The morphology of the small intestine from male mice in the three groups is shown by H&E and PAS staining. The black arrows point to the goblet cells. **(E–I)** The expression levels of *IL-1*β, *IL-6*, *TNF-*α, *NLRP3*, and *NRF2* were examined in small intestine tissues from male mice by qRT-PCR. **(J)** The levels of ROS in the small intestine of male mice were measured by ELISA. Significant differences are indicated: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.005$ by Student's *t*-test between two cohorts. GI, gastrointestinal; TAI, total abdominal irradiation; W, walking; PAS, periodic acid–Schiff; ROS, reactive oxygen species.

that *A. muciniphila* might bolster the radioprotection of walking treatment.

Walking Fights Against Radiation-Induced Gastrointestinal Tract Toxicity in Female Mice

Given that sexual dimorphism affected the treatment efficacy in radiation syndrome (Cui et al., 2019), we elucidated whether walking as a non-pharmacological remedy can be used to protect females against GI tract toxicity following irradiation. Same to the male counterparts, abdomen local irradiated female mice with short-term walking treatment did not show further weight loss (**Figure 5A**). As expected, the female mice with walking treatment had longer colons, denser small intestinal villi, and more goblet cells (**Figures 5B–D**). ELISA and qRT-PCR assays revealed that walking reduced the expression of inflammatory factors (**Figures 5E–H**; **Supplementary Figures 8A,B**) and the level of oxidative stress (**Figures 5I,J**). Together, our findings indicate that walking is an efficacious strategy to mitigate radiation-induced GI tract toxicity in both male and female mice.

Walking Does Not Alter the Proliferation of Cancer Cells Following Radiation Exposure

To find out the safety of walking for cancer patients with radiotherapy, we injected HCT-8 (or A549) cells into nude mice subcutaneously and recorded the tumor growth after local radiation stimuli with or without walking treatment. Intriguingly, walking did not change the proliferation of HCT-8 (or A549) cells and the terminal weight of the xenografts (**Figures 6A–C,E–G**).

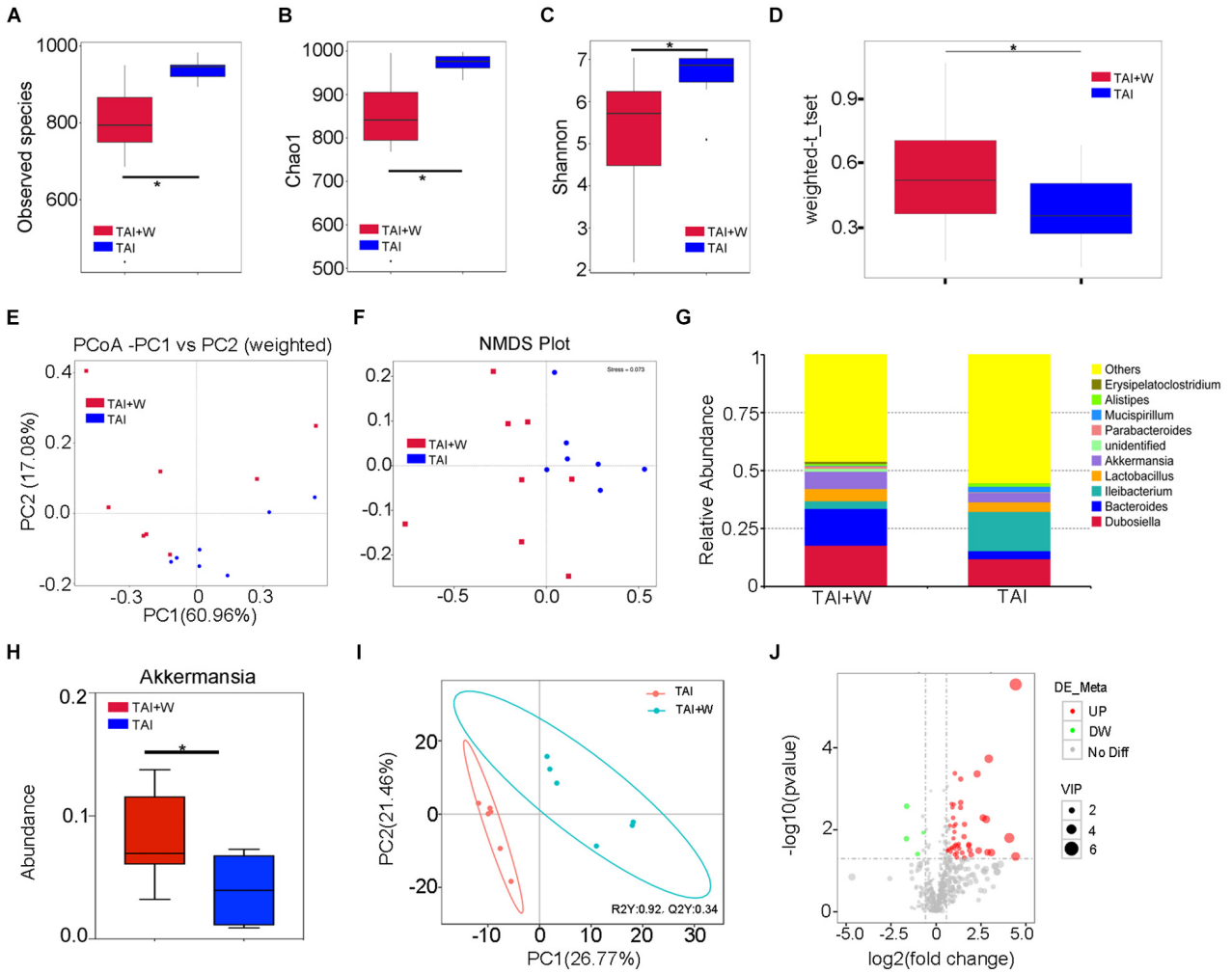

FIGURE 2 | Walking reshapes gut microbiota configuration following radiation challenge. The gut bacterial composition structures in male mice of TAI (radiation alone) and TAI + W (walking after radiation) groups were measured by 16S rRNA high-throughput sequencing at 16 days following TAI exposure, $n = 7$ (TAI group) or $n = 8$ (TAI + W groups). The gut metabolite composition was detected by untargeted metabolomics at 16 days following TAI exposure, $n = 6$ (TAI group) or $n = 6$ (TAI + W groups). **(A–C)** Alpha diversity was measured: **(A)** the observed species number, **(B)** Chao1 diversity index, and **(C)** Shannon diversity index. **(D)** The beta diversity of intestinal bacteria was compared by weighted *t*-test analysis. **(E,F)** PCoA (weighted) and NMDS were performed to assess the alteration of gut bacteria taxonomic profile from male mice in two groups. **(G)** The relative abundances of the top 10 varied strain bacteria at the genus level in male mice of two groups. **(H)** The abundance of *Akkermansia* in male mice of two groups. **(I)** The PLSDA score of positive metabolites in feces in the two groups. **(J)** The volcano diagram of positive metabolites in feces. **(A–C)** Significant differences are indicated: Wilcoxon rank sum test. **(D,H)** Significant differences are indicated: $^*p < 0.05$ by Student's *t*-test between two cohorts. TAI, total abdominal irradiation; W, walking; PCoA, principal coordinates analysis; NMDS, non-metric multidimensional scaling; PLSDA, partial least-squares discriminant analysis.

Then we tested the expression of Ki-67, a cell proliferative marker, in the tumor tissues from the two groups. IHC assay revealed that walking did not alter the expression of Ki-67, further indicating that walking does not interfere with the tumoricidal effects of radiation therapy (**Figures 6D,H**).

DISCUSSION

Radiotherapy as a milestone for oncotherapy is applied to more than 50% of the global cancer patients, but the radiation toxicity of the whole body, especially for adjacent organs, is urgently worrisome (Citrin, 2017; De Ruysscher et al., 2019). For example, abdomen local irradiation, as a common means for treating abdominopelvic tumor, always leads harm to the hematopoietic system, GI tract, and even reproductive system, which is near the exposed area (Rappleye et al., 1975; Zhang et al., 2016; Oktem et al., 2018). As an organ within the treatment field for all intra-abdominal, retroperitoneal, and pelvic tumors, the intestine gets interfered unavoidably during or after radiotherapy, manifesting in acute (or chronic) inflammation, apoptosis, and fibrosis (Wei et al., 2016). In the United Kingdom, about 90% of patients receiving pelvic radiation reported alterations in their bowel function, which leads to negative effects on daily activity

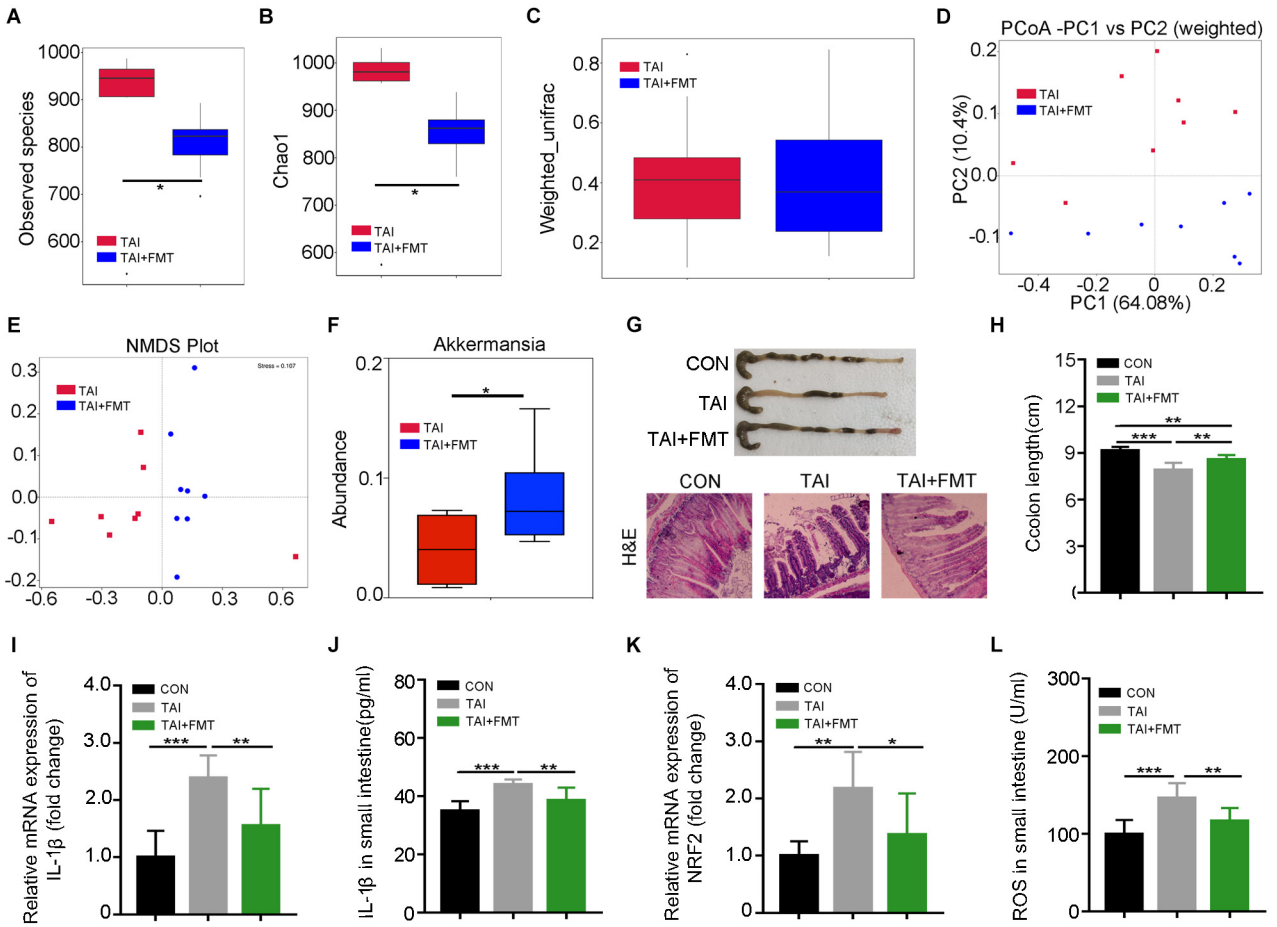

FIGURE 3 | Walking mitigates intestinal radiation toxicity depending on gut microbiota. Male mice were exposed to 12 Gy of TAI, and for TAI + FMT group, irradiated mice were administrated with fecal microbiota via oral route from male donors that maintained walking following radiation exposure. The gut bacterial composition structures in male mice of TAI and TAI + FMT groups were measured by 16S rRNA high-throughput sequencing at 16 days after TAI exposure, $n = 8$ per group. The colon and small intestine tissues were obtained at day 16, $n = 10$ per group. **(A,B)** Alpha diversity was measured: **(A)** the observed species number and **(B)** Chao1 diversity index. **(C)** The beta diversity of intestinal bacteria. **(D,E)** PCoA (weighted) and NMDS were performed to assess the alteration of gut bacteria taxonomic profile from male mice in two groups. **(F)** The abundance of *Akkermansia* in male mice of two groups. **(G)** Photographs of dissected colon and morphology of the small intestine shown by H&E from male mice in the two groups. **(H)** Statistical results of colon length between two groups. **(I,K)** The expression levels of *IL-1*β and *NRF2* were examined in small intestine tissues from male mice by qRT-PCR. **(J,L)** The levels of *IL-1*β and ROS in the small intestine of male mice were measured by ELISA. **(A–C)** Significant differences are indicated: Wilcoxon rank sum test. **(D–L)** Significant differences are indicated: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.005$ by Student's *t*-test between two cohorts. TAI, total abdominal irradiation; FMT, fecal microbiota transplantation; PCoA, principal coordinates analysis; NMDS, non-metric multidimensional scaling; ROS, reactive oxygen species.

in up to 50% (Andreyev, 2007; Takemura et al., 2018). Despite great advancement in delivery technology of radiotherapy [e.g., FLASH-RT and accelerated partial breast irradiation (APBI)], these adverse side effects remain an overwhelming medical challenge (Vozenin et al., 2019; Whelan et al., 2019). In addition, mounting evidence proves that proinflammatory cytokines, such as interleukin-1β, interleukin-18, and inflammatory CC chemokines, are associated with carcinogenesis (Vetrano et al., 2010; Chen and Núñez, 2011). The chronic proinflammatory state of intestine hijacking immune system precipitates tumor outgrowth (Biragyn and Ferrucci, 2018; Olafsson et al., 2020). All the reports highlight the ill effects of intestinal inflammation in tumorigenesis and oncotherapy. Importantly, there are no safe and effective therapeutic approaches to overcome intestinal radiation injury currently, and some studies show that the secondary reactions of the corresponding drugs reduce patient tolerability and even interrupt the treatment (Rios et al., 2014; Lawrie et al., 2018). In this study, we observed that short-term walking, a low-intensity physical activity, drove milder GI tract toxicity, especially lower level of intestinal inflammation. The findings suggest that walking might be a rehabilitation maneuver for cancer patients with radiotherapy. Physical activities are identified as low, moderate, and vigorous intensities on the basis of those metabolic equivalents (Martínez et al., 1997). Different intensities of exercise elicit different responses (Marijon et al., 2011; Schnohr et al., 2015). Running as a form of vigorous exercise receives more attention; however, the intertwined adverse effects on knee and heart are still an

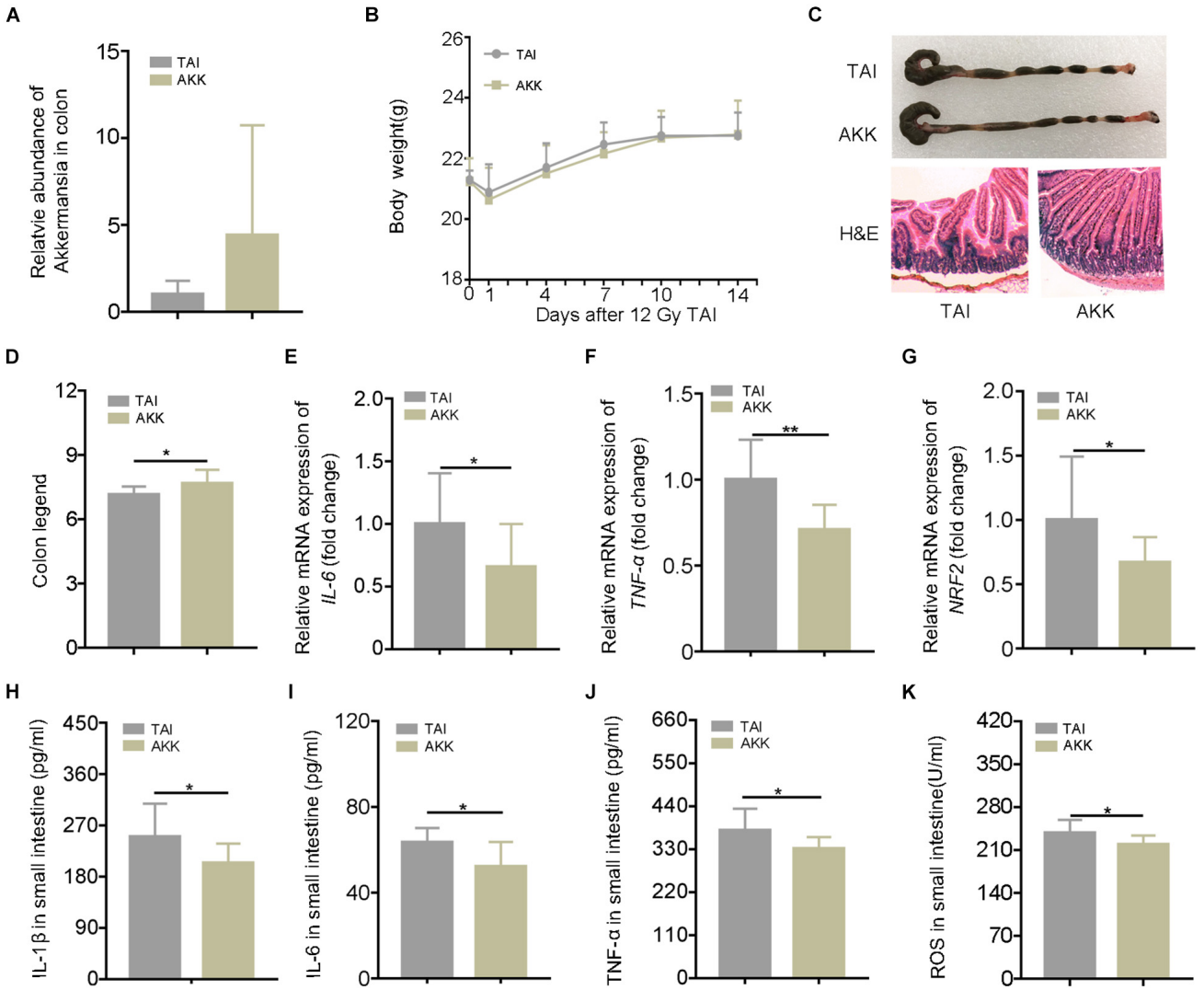

FIGURE 4 | *Akkermansia muciniphila* (AKK) mitigates radiation-elevated inflammatory status in digestive tract. Male mice were exposed to 12 Gy of TAI; and for AKK group, the male mice were orally administrated with *A. muciniphila* ($1.5 * 10^8$ CFU) for 15 days following radiation challenge, $n = 10$. **(A)** The relative abundance of *A. muciniphila* in the colon was detected by q-PCR. **(B)** The body weight of male mice in the two groups. **(C)** Photographs of dissected colon and morphology of the small intestine shown by H&E from male mice in the two groups. **(D)** Statistical results of colon length between two groups. **(E–G)** The expression levels of *IL-6*, *TNF-α*, and *NRF2* were examined in small intestine tissues from male mice by qRT-PCR. **(H–K)** The levels of *IL-1β*, *IL-6*, *TNF-α*, and ROS in the small intestine of male mice were measured by ELISA. Significant differences are indicated: $^*p < 0.05$, and $^{**}p < 0.01$ by Student's *t*-test between two cohorts. TAI, total abdominal irradiation; ROS, reactive oxygen species.

issue (Whyte et al., 2008; Alentorn-Geli et al., 2017). Compared with running, walking is a more comfortable and feasible form of exercise and can be acceptable to weak patients undergoing radiotherapy. Many meta-analyses have revealed that walking reduces cardiovascular risk, governs body mass index, and regulates blood glucose and lipid (Hamer and Steptoe, 2008; Oja et al., 2018). In this study, walking did not cause weight loss in experimental mice, indicating that walking is a kind of safe exercise for cancer patients with radiotherapy. Importantly, due to the low-intensity and short exercise time per day, walking is suitable for almost all mobile cancer patients without economic burden. Sexual dimorphism impacts the curative effects and the prognosis of cancer patients (Cui et al., 2019). Thus, we collected the data from male and female mouse models in this study and reported that walking is applicable to cancer patients in both sexes to improve the prognosis following radiotherapy.

National Comprehensive Cancer Network (NCCN) Guidelines point out that radiotherapy is the optional remedy for pelvic and abdomen tumors including prostate cancer (early, middle, and late stages) and cervical cancer (invasive cancer of various stages) and thoracic tumors such as breast cancer (early, locally advanced and metastatic breast cancer). In this study, we identified that walking did not accelerate the proliferation of cancer cell in tumor xenograft models. Although the model

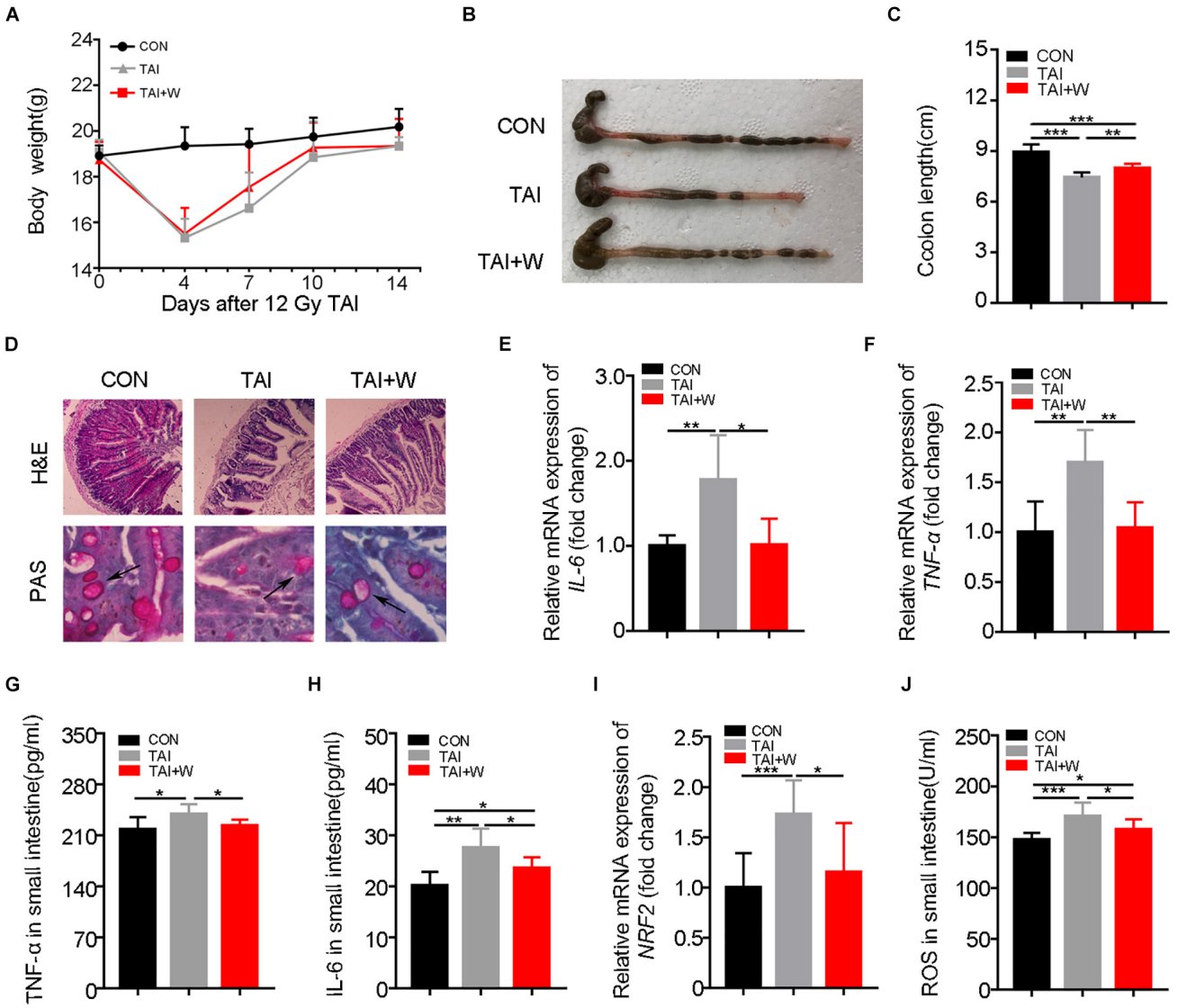

FIGURE 5 | Walking protects against radiation-induced GI tract toxicity in female mice. Except for female mice in Con group, female mice of the other two groups were exposed to 12 Gy of TAI, TAI + W group maintained walking for 15 days, and TAI group had no treatment. **(A)** The body weight of male mice in the three groups. **(B)** Photographs of dissected colon from male mice in the two groups. **(C)** Statistical results of colon length between two groups. **(D)** The morphology of the small intestine from male mice in the two groups was shown by H&E and PAS staining. The black arrows point to the goblet cells. **(E–G)** The expression levels of *IL-6*, *TNF-α*, and *NRF2* were examined in small intestine tissues from male mice by qRT-PCR. **(H–J)** The levels of IL-6, TNF-α, and ROS in the small intestine of male mice were measured by ELISA. Significant differences are indicated: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.005$ by Student's *t*-test between two cohorts. GI, gastrointestinal; Con, control; TAI, total abdominal irradiation; W, walking; PAS, periodic acid–Schiff.

cannot be used to evaluate the stage of cancers, we focused on the ameliorating effect of walking on radiotherapy-intertwined intestinal toxicity. Therefore, all the patients with radiotherapy suffering from GI tract syndrome could perform walking to mitigate the complications.

Millions of commensal microbes inhabit the GI tract of mammals and are involved in immune regulation and energy metabolism. Intestinal microorganism imbalance propels multiple diseases. More and more studies suggest that gut microbiota plays vital roles in intestinal radiation injury (Touchefeu et al., 2014; Huang et al., 2019). Clinical trials identify that exercise indeed improves the prognosis of cancer patients; however, the underlying mechanism remains confusing (Wang et al., 2011; Bjerre et al., 2019; Lundt and Jentschke, 2019). In light of the close relationship between exercise and gut microbiota, the irradiated mice were treated with antibiotic cocktail or FMT in the present study. Gut flora deletion erased the radioprotection of walking, and recipients harboring gut microbes from donor with walking treatment exhibited milder GI tract injuries. All the results indicated that radioprotection of walking might be partly dependent on gut bacterial structure reorganization. Walking spurred an enrichment on some

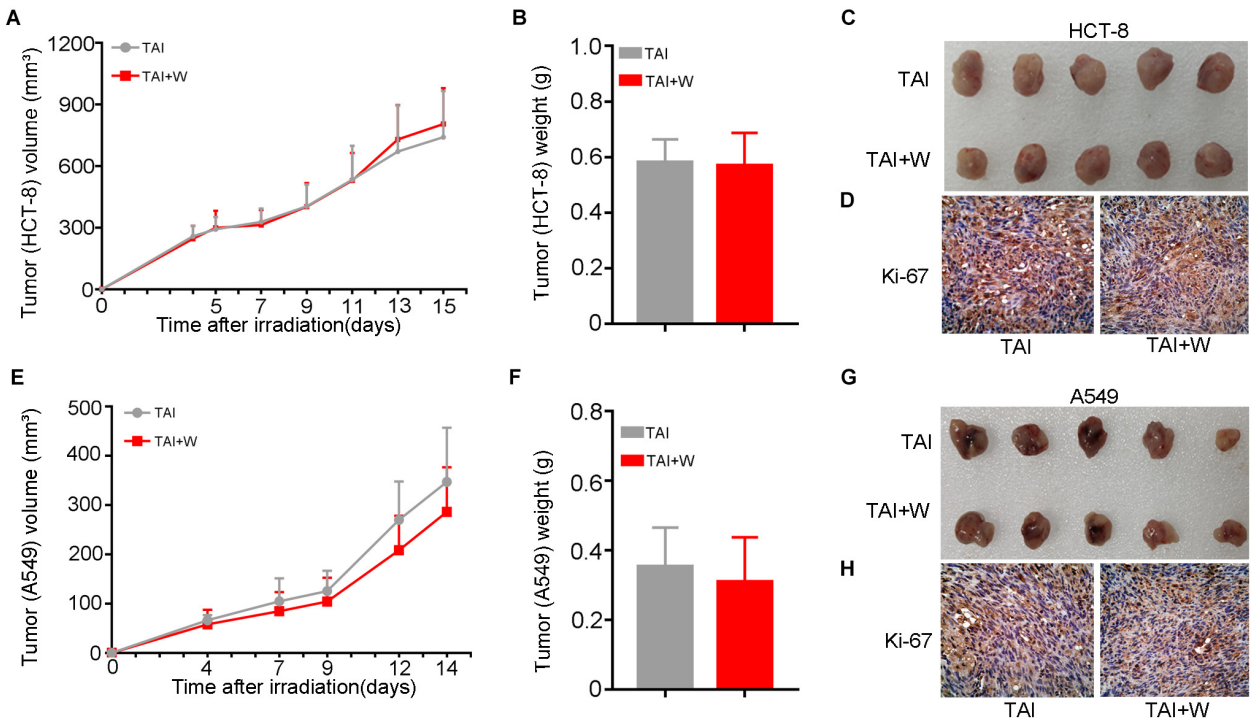

FIGURE 6 | Walking did not alter the proliferation of cancer cells following radiation exposure. We subcutaneously injected HCT-8 or A549 tumor cells into the armpit of nude mice; then the mice received 10 Gy of radiotherapy (2 Gy * 5 days) when the tumors reached 100 mm^3. The growth of tumors was observed in mice with walking (TAI + W) or not (TAI). The tumor volume was recorded during the tumorigenicity process every 2 or 3 days, $n = 8$ per group. **(A–C)** The growth curves of tumors, tumor weights, and representative image of excised tumors of HCT-8 group. **(D)** The expression levels of Ki-67 in HCT-8 tumor tissues were examined by IHC staining. **(E–G)** The growth curves of tumors, tumor weights, and representative image of excised tumors of A549 group. **(H)** The expression levels of Ki-67 in A549 tumor tissues were examined by IHC staining. GI, gastrointestinal; Con, control; TAI, total abdominal irradiation; W, walking; PAS, periodic acid–Schiff; IHC, immunohistochemical.

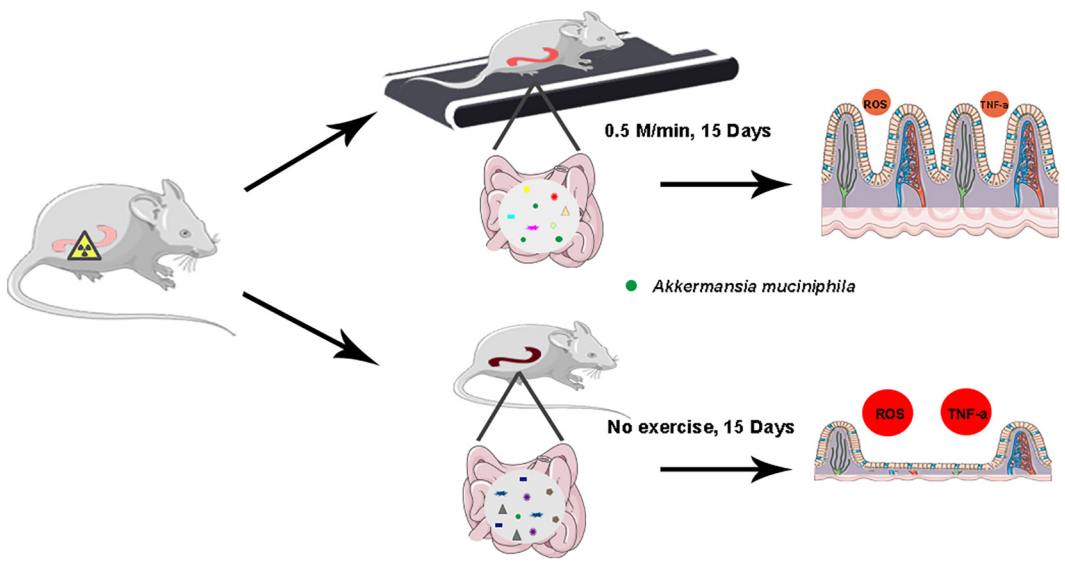

FIGURE 7 | Illustration of walking for the alleviation of radiation-induced intestinal inflammation.

intestinal bacteria, such as *Akkermansia*, *Bacteroides*, *Dubosiella*, and *Lactobacillus*. *Bacteroides* shows an enrichment at the early stage after irradiation, implying that the bacteria might be a driver for radiation toxicity (Wang et al., 2019; Li et al., 2020b). Yet the effect of *Dubosiella* on enteritis is still controversial (Sheng et al., 2020). *Lactobacillus* as well-known probiotics have been proved to be associated with improvement in patients suffering from radiation syndrome in clinical trials (Linn et al., 2019). A recent study has reported that *A. muciniphila* improves intestinal radiation injury following whole body irradiation (Kim et al., 2021). Notably, the experimental mice in the present study were exposed to abdominal local irradiation, which is more similar to iatrogenic irradiation such as radiotherapy for pelvic and abdominal tumors. In addition, the published study focuses on the relationship between *A. muciniphila* supplement and the function of intestinal stem cells. Given that radiation-induced gastroenteritis and colitis are common adverse side effects in cancer patients with systemic therapy or radiotherapy (Jairam et al., 2019), we wondered whether *A. muciniphila* might be employed to protect against radiation intestinal inflammation in this study. We treated irradiated mice with *A. muciniphila* via oral route. *A. muciniphila* replenishment reduced inflammation levels and increased integrity of the small intestine after local radiation stimuli. The results bolster that *A. muciniphila* might be a potential probiotic for cancer patients with radiotherapy. However, the optimal use method of *A. muciniphila* in clinical application required further study.

There are still some limitations requiring further study to pave the way for walking to be integrated into clinical application. Firstly, although the walking protocol ensures the quality and quantity of exercise, involuntary walking cannot be completely avoided. Secondly, the frequency and intensity of walking for patients with radiotherapy need to be further explored based on clinical trials. Finally, long-term low-intensity exercise has been proved to reduce the occurrence of CRC and stimulate tumor cell apoptosis (Kim et al., 2020), and our findings identify that 15-day walking treatment does not accelerate the growth of tumors. Therefore, long-term walking might be necessary to explore its effects on metastasis of cancers. In conclusion, walking as a low-intensity physical activity alleviates intestinal radiation toxicity in both male and female mice. Mechanistically, walking remolded the gut microbiota configuration and reprogrammed the intestinal microbial metabolome of abdomen local irradiated mice. *A. muciniphila*, a potential probiotic, might be employed to fight against radiation-induced GI tract injuries (**Figure 7**). Together, our observations provide new insights into the function of walking and underpin that walking is a safe and effective form to improve the prognosis of cancer patients with radiotherapy suffering from GI tract syndrome without financial burden in a preclinical setting.

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: MetaboLights, and the accession number is MTBLS2826.

ETHICS STATEMENT

The animal study was reviewed and approved by animal experiments were performed according to the institutional guidelines approved by the Animal Care and Ethics Committee of IRM-PUMC.

AUTHOR CONTRIBUTIONS

BW, Y-XJ, and MC designed the experiments, analyzed the data, and wrote the manuscript. BW and Y-XJ performed the experiments and wrote the manuscript. MC provided writing assistance. S-QZ, H-WX, J-LD, Y-XJ, and YL proofread the article. MC, X-DY, and S-JF oversaw the entire project. 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/fcell.2021.706755/full#supplementary-material

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REVIEW
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Dietary Patterns and Associated Microbiome Changes that Promote Oncogenesis

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The recent increases in cancer incidences have been linked to lifestyle changes that result in obesity and metabolic syndrome. It is now evident that these trends are associated with the profound changes that occur in the intestinal microbiome, producing altered microbial population signatures that interact, directly or indirectly, with potentially pro-carcinogenic molecular pathways of transcription, proliferation, and inflammation. The effects of the entire gut microbial population on overall health are complex, but individual bacteria are known to play important and definable roles. Recent detailed examinations of a large number of subjects show a tight correlation between habitual diets, fecal microbiome signatures, and markers of metabolic health. Diets that score higher in healthfulness or diversity such as plant-based diets, have altered ratios of specific bacteria, including an increase in short-chain fatty acid producers, which in turn have been linked to improved metabolic markers and lowered cancer risk. Contrarily, numerous studies have implicated less healthy, lower-scoring diets such as the Western diet with reduced intestinal epithelial defenses and promotion of specific bacteria that affect carcinogenic pathways. In this review, we will describe how different dietary patterns affect microbial populations in the gut and illustrate the subsequent impact of bacterial products and metabolites on molecular pathways of cancer development, both locally in the gut and systemically in distant organs.

Keywords: microbiome, dietary pattern, Western diet, plant-based diet, colorectal cancer, alcohol, oncogenesis

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INTRODUCTION

Cancer is one of the leading causes of mortality amongst all ages and ethnic groups worldwide. The incidence of cancer has been increasing every year, with a particularly dramatic increase in developing countries. In fact, over the last two decades, 55% of total cancer incidences were documented in developing countries. It is predicted that by the year 2050, this number will reach to 70% (Bray et al., 2018; Sung et al., 2021). With this current trend, GLOBOCAN predicts the cancer burden to rise to 27.5 million new cases per year by 2040 (Sung et al., 2021). Globally, lung, breast, and colon cancers are the most frequently occurring cancers. This recent increase in cancer incidence has been linked to changes in lifestyles that have resulted in an increase in obesity and the metabolic syndrome, which are among the leading risk factors for cancer (Hulvat, 2020).

With emerging novel technologies, there is an abundance of information and new knowledge of cancer biology each year. Among those, recent advancements in metagenomics have allowed for better characterization of the human gut microbiome diversity and its impact on the host organism's predisposition to various cancers (Yu et al., 2017). Approximately half of the cells in the human body are those of commensal bacteria, not human cells (Sender et al., 2016). Nevertheless, the interplay between the microbiome and disease has only recently begun to be illuminated. The human gut microbiome is an extremely dynamic "organ", with multiple factors constantly affecting the diversity and composition of this microenvironment. These factors include diet, lifestyle, drugs such as antibiotics, delivery method at childbirth and the genetic makeup of the individual (Wen and Duffy, 2017). It is now evident that diet is a major contributor to the variation in gut microbiota, producing population signatures that interact, both directly and indirectly, with molecular pathways affecting key biological processes including transcription, proliferation, and inflammation, that can have pro-carcinogenic effects (Cheung et al., 2017). Evolutionarily, the human diet has always been shaped by many different factors, including cultural, geographical, economical, and seasonal. However with the dramatic increase in the rate of globalization in recent decades, the boundaries between various dietary patterns and lifestyles have blended (Arkan, 2017). Within the context of this global trend, the broad assumption—supported by progressively emerging evidence—is that diet can make us susceptible to certain diseases through alteration of our microbiome composition.

Of late, research on the microbiome has garnered enormous attention from the scientific world, as well as from the public. Although microbiome research is still in its infancy, it has already been established that the microbiome has a direct influence on almost all the pathophysiological processes in the human body (Asnicar et al., 2021; George et al., 2021). The composition of the gut microbiome and its interaction with the cellular processes in the gut epithelium have been shown to predispose an individual to certain diseases, including but not limited to colorectal, liver, and other cancers, inflammatory bowel disease (IBD) and other autoimmune and neurologic diseases including Alzheimer' (Tedelind et al., 2007; Nosho et al., 2016; Vogt et al., 2017). However, due to the tremendous diversity of gut microbiome, and the heterogeneity of cancer pathophysiology, the direct link between microbiome composition and cancer pathogenesis is yet to be established (Scott et al., 2019). The purpose of this review is to summarize recent research into the relationship between dietary patterns and the gut microbiome and associated metabolome, and how they directly regulate pro-carcinogenic pathways. We look at the effect of altered gut microbiome locally in the gut colonic tissue and environment and systemically, where microbiome-derived toxins and metabolites affect distant organs via portavenous and arterial circulation. For comparison, this review also focuses on the beneficial effects of a healthier, "plant-rich" diet.

DIETARY PATTERNS AND CANCER RISK

It is challenging to define an absolute dietary pattern, particularly as most observational and research studies traditionally focus on key components of certain types of diet, excluding the overall effects and the synergy between dietary components (Klement and Pazienza, 2019). However, in the real-world, and as direct consequence of globalization making every type of diet accessible in nearly every country on-demand, people do not consume isolated products. Moreover, due to the global coverage of the food manufacturing industry, consumers in many countries are not given the information necessary to understand what the exact constituents and additives of their foods are. Today, nutritional epidemiology is trying to observe the changes in human health due to the overall dietary composition, since the predominance and the trend of specific diets is more important than consumption of certain isolated foods (Hosseinkhani et al., 2021). Researchers studying nutrition and health often use food frequency questionnaires (FFQs) to track the frequency of foods consumed over a fixed period of time. The FFQs are then evaluated using different dietary indices, that quantitatively measures an individual diet's adherence to dietary guidelines in order to correlate a person's diet history with various health outcomes such as obesity or biomarker concentrations (**Supplementary Table S1**). In this review, the term "dietary pattern", will be used to segregate and draw the line between "Western type of diet" and "Plant-based diet".

The dietary pattern labeled "Western diet" is characterized by relatively high fat content, particularly saturated fats, highly processed carbohydrates, and lesser amounts of fiber (**Table 1**). The main dietary signature of the western type of diet is the overconsumption of processed food that has undergone chemical treatment and has a high level of emulsifiers and other synthetic additives (Cordain et al., 2005). Moreover, increased consumption of refined sugar, dietary salt and animal-based products, especially red meat, are also key attributes of the Western diet, along with dramatically decreased consumption of dietary fiber (Zhong et al., 2021). The term Western diet has predominantly come from the correlation of a set of diseases that mostly occur in the western world (Cordain et al., 2005). Interestingly, the term Western diet is also used to describe a specific high-fat diet used in some animal studies, however this does not apply to this review. An increasing number of quantitative studies have demonstrated positive links between continuous overconsumption of red meat and ultra-processed food (UPF) as one of the key drivers of developing different types of tumors (Chen et al., 2020a). Adherence to the Western diet is thus considered as one of the main risk factors in developing cancer (van den Berg et al., 2021).

Numerous cohort and case control studies have been designed to interrogate the correlation between processed food consumption and various health outcomes. A recent cohort study, amongst the French population from 2009 to 2017, determined that the risk of developing cancer directly correlates with the increased consumption of UPF (Fiolet et al., 2018). The same French cohort study has also observed a direct association between UPF intake and weight gain, as well

TABLE 1 | Different dietary patterns and components.

Broadly classified as (in this review)	Diet name	Dietary components	Less frequent
		Frequent	
PLANT-BASED	Vegan (Craig, 2009)	Fruits and Vegetables Whole Grains Legumes and Beans Nuts and seeds	Dairy and eggs Animal products Fish Meat products
	Vegetarian (Melina et al., 2016)	Fruits and Vegetables Whole Grains Legumes and Beans Nuts/Seeds Dairy and eggs	Meat products Fish
	Mediterranean (Di Daniele et al., 2017)	Fruits and Vegetables Whole Grains Legumes and Beans Nuts/Seeds Fish Unsaturated fats such as olive oil	High intake of red meat Dairy
	DASH (Dietary Advances to Stop Hypertension) (Vogt et al., 1999)	Fruits and Vegetables Low-fat dairy products	High-salt, high-sugar, highly-processed foods Refined carbohydrates
WESTERN	Omnivore (Cordain et al., 2005)	Fruits and Vegetables Whole Grains Fish Meat	
	Western pattern diet (Clarys et al., 2013)	Red meat/Processed meat Pre-packaged and fried foods Butter Candy and sweets High-fat dairy products Refined grains High-fructose corn syrup drinks	Vegetables, Fruits Whole grains

as increased risk of developing obesity (Beslay et al., 2020). A German population-based case-control study has established strong correlation between increased intake of not only processed meat but also red meat with increased risk of developing colorectal cancer (CRC) in mixed age groups (Carr et al., 2017). A population-based case-control study in Israel, has also established the dose-dependent correlation between the concurrent intake of UPF and smoking on one hand and the severity of colorectal neoplasia on the other, with higher intake of UPF and smoking resulting in more advanced colorectal adenomas (Fliss-Isakov et al., 2020).

A systematic review, analyzing 11 meta-analysis of the effect of red meat in development of CRC, concluded that increased intake of red meat and processed meat elevated CRC risk by 20–30% (Aykan, 2015). Another systematic review, focusing on the broader effect of UPFs on the health outcomes, pulling from 12 recent cohort studies and 8 cross-sectional studies, demonstrated significant correlation between UPF intake and increased risk of developing obesity, type II diabetes and several types of cancer (Chen et al., 2020a).

In contrast to the Western diet, a plant-based dietary pattern, which includes the vegetarian, vegan, and Mediterranean diets, features a preponderance of the dietary components that are plant-based, such as whole grains, plant-derived oils, and legumes, with significantly reduced animal and fish products, as well as minimal to no processed food (**Table 1**). Although, geographically plant-based diet is mostly predominant in the regions of Mediterranean basin (Klement and Pazienza, 2019), in the recent years plant-based diet is acquiring more followers around the globe due to its established protective properties (Tsvetikova and Koshel, 2020). High number of recent epidemiological studies claim protective effects of a plant-based diet from a set of non-communicable diseases (NCDs), such as the metabolic syndrome and various types of cancer, especially CRC (Pasolli et al., 2019; Alexandrov et al., 2020). Furthermore, adherence to healthier plant-based dietary patterns results in a quick shift in microbial population towards more beneficial bacteria (Alexandrov et al., 2020; Kim et al., 2020). This shift in microbial populations has multiple consequences which have been linked with improved tight-junction homeostasis of the gut epithelium layer, better immune surveillance, and control over inflammatory processes (Tsvetikova and Koshel, 2020; Asnicar et al., 2021) as we will explore later.

The relationship between dietary patterns and the microbiome has been investigated by many studies, with the significant recent addition of the extensive Arivale/Institute for Systems Biology study (Manor et al., 2020) and the international multicenter PREDICT-1 study (Asnicar et al., 2021). The Arivale study

TABLE 2 | Microbial signatures of the gut microbiome, in respect to Western dietary (WD) pattern and plant-based (PD) dietary pattern (N/I non-identified).

	Bacterial signature of WD microbiome (De Filippis et al., 2016; Asnicar et al., 2021)	Function	Bacterial signature of PD microbiome (De Filippis et al., 2016; Asnicar et al., 2021)	Function
Increased Population	Clostridium bolteae	Increase cardiometabolic risk	Faecalibacterium prausnitzii	Butyrate production
	Atopobium parvulum	Hydrogen sulfide production	Roseburia intestinalis	Butyrate production
	Actinomycosis odontolyticus	Hydrogen sulfide production	Akkermansia muciniphila	Enhancement of mucin production
	Bilophila wadsworthia	Secondary bile acids production	Prevotella copri	Glucose homeostasis, improvement in postprandial glucose responses
	Streptococcus bovis/ gallolyticus	Nitric oxide production	Roseburia hominis	Butyrate production
	Clostridium saccharolyticum	Increase in cardiometabolic risk	Agathobaculum butyriciproducens	Butyrate production
	Clostridium innocuum	Increase cardiometabolic risk	Anaerostipes hadrus	Butyrate production
	Clostridium symbiosum	Increase cardiometabolic risk	Firmicutes bacterium	Postprandial lipoprotein remodeling
	Clostridium spiroforme	Increase cardiometabolic risk	Haemophilus parainfluenzae	Reduces GlycA levels, systemic inflammation, cardiometabolic risks
	Clostridium leptum	Increase cardiometabolic risk	Eubacterium eligens	N/I
	Flavonifractor plautii	Increase cardiometabolic risk	Lawsonibacter asaccharolyticus	Butyrate production
	Ruthenibacterium lactatiformans	N/I	Oscillibacter sp	N/I
	Escherichia coli	N/I	Streptococcus thermophilus	Probiotic
	Collinsella intestinalis	N/I	Bifidobacterium animalis	Probiotic
	Eggerthella lenta	N/I		
	Anaerotruncus colihomini	N/I		
	Clostridium spiroforme	Increase cardiometabolic risk		
	Ruminococcus gnavus	Increase cardiometabolic risk		
Decreased Population	Lactobacillus acidophilus (Prebiotic bacteria)	Reduces nitroreductase activity		
	Prevotella copri	Glucose homeostasis, improved postprandial glucose responses		
	SCFAs producers	SCFA production, immune homeostasis		

analysed 3,409 individuals enrolled in a wellness program with extensive characterization of metabolic markers, lifestyle, diet, and stool microbiome 16S amplicon sequencing. They found that specific microbial populations with increased diversity were associated with improved cardiometabolic markers and plant-based dietary patterns (health-related group). Conversely, they found that specific microbial populations with less diversity were associated with worse cardiometabolic and lifestyle markers (disease-related group). The health related group had increased genera *Coprococcus, Lachnospira, Faecalibacterium*, and unclassified genera from the Ruminococcaceae and Clostridiales family/order. The disease-related group had increased abundance of the genera *Bacteroides, Ruminococcus, Sutterella, Bilophila, Acidaminococcus*, and *Megasphaera*. PREDICT-1 investigators analyzed stool microbiome metagenomic sequencing data from 1,098 individuals from the UK and United States, and correlated the results with demographic variables, detailed dietary logs, and cardiometabolic blood markers (Asnicar et al., 2021). Major findings from the study included that the intrasample alpha diversity, or an estimate of the total number or richness of bacterial species in a sample, significantly correlated with 56 of 295 tested correlations with personal characteristics, habitual diet and metabolic markers. Microbiome species richness was positively correlated with favorable high-density lipoprotein levels, whereas body-mass index (BMI), visceral fat, and probability of fatty liver were inversely correlated with species richness. Data from individual food diaries were evaluated using validated dietary indices such as the alternate Mediterranean diet score (aMED), Healthy Eating Index (HEI) and the Plant-based Dietary Indices (PDI) that have previously been shown to correlate with reduced risk of chronic diseases (**Supplementary Table S1**). These indices showed a tight correlation with microbial composition, demonstrating how habitual diets influence the microbiome. Out of the 30 bacterial species that showed the strongest overall correlation with markers of nutritional and cardiometabolic health, 15 species were positively associated with healthy plant-based diets and negatively associated with visceral fat, liver fat probability, and high-risk metabolic markers. These included *F. prausnitzii, Proventella copri, Roseburia*, *Oscillibacter*, and several *Firmicutes* species. Conversely, the other 15 bacterial species were negatively associated with healthy diets, and positively associated with increased visceral fat, liver fat probability, and high-risk metabolic markers. These included *Clostridia* species, *R. gnavus*, and *F. plautil*. The repertoire of 30 bacterial species represents a novel composite quantitative marker of the link between dietary patterns and cardiometabolic

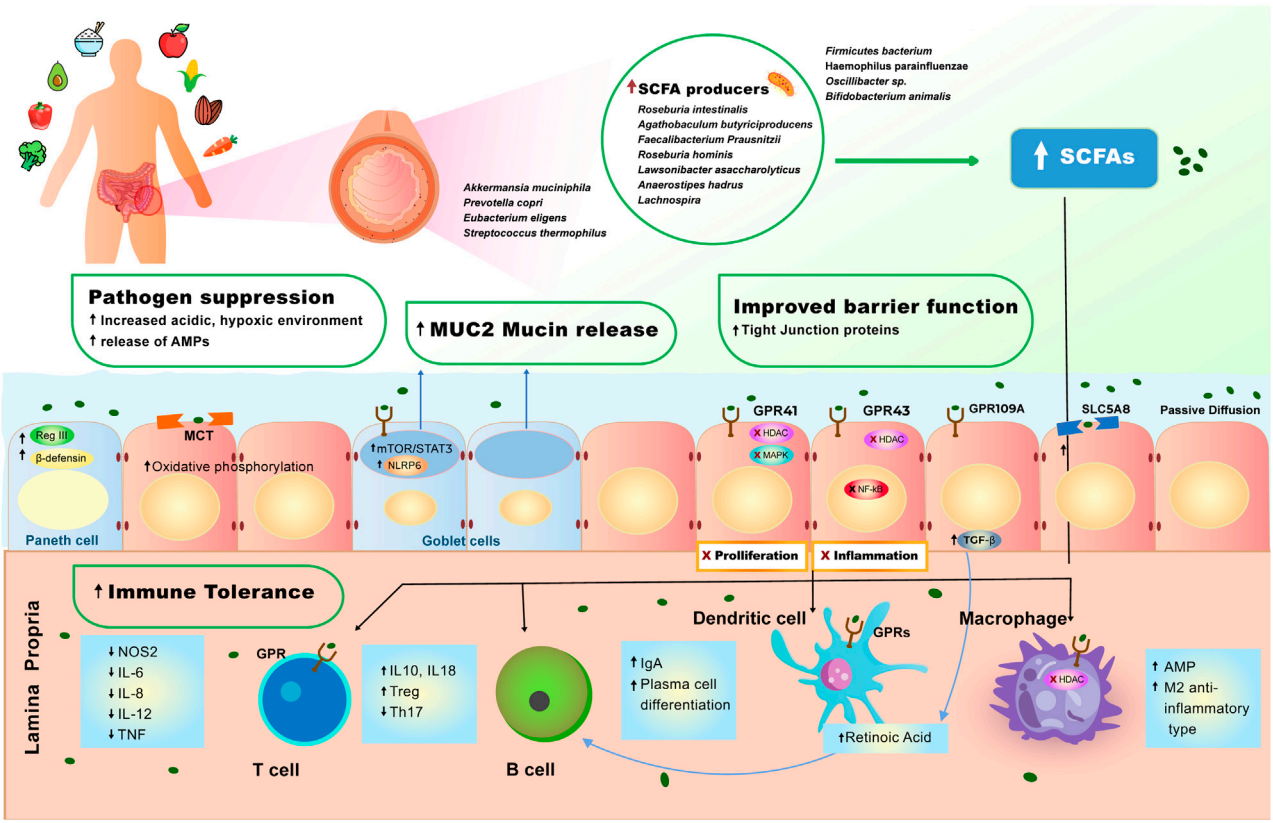

FIGURE 1 | Effect of plant-diet on the gut microbiome and intestinal homeostasis. A diet rich in plant-based foods results in high abundance of short-chain fatty acid (SCFA) producing bacteria in the gut lumen. High production of SCFAs by gut microbiome is strongly associated with pathogen suppression, mucus production, improved barrier function and immune tolerance (see *Molecular Mechanisms of the Role of Plant-Based Diet in Cancer Protection*). SCFAs induce a set of anti-inflammatory events via interaction with GPR41, GPR43, GPR109A receptors and MCT and SLC5A8 transporters that are expressed on the surface of intestinal epithelial cells and immune cells. 1) SCFAs bind GPR109A on the epithelial cell which leads to increase in TGF- β production, enhancing the differentiation of Treg cells, which leads to increase of immune tolerance in the gut. 2) SCFAs also bind to GPR43 inhibiting HDAC and NF- κB, resulting in decreased inflammation. 3) SCFAs bind to GPR41, inhibiting HDAC and MAPK signaling pathway, resulting in inhibition of proliferation. 4) SCFAs cross the epithelial cell barrier and reach lamina propria, where they induce cascade of anti-inflammatory reactions, regulating immune homeostasis in the lower bowel. In the lamina propria, SCFAs bind to macrophage (MQ cell) inhibiting HDAC, facilitating polarization of macrophages to M2-anti-inflammatory type, and increasing production of antimicrobial peptides (AMPs). 5) SCFAs bind to dendritic (DC) cell enhancing retinoic acid production, further facilitating production immunoglobulin A (IgA) and differentiation of Tregs. 6) SCFAs induce production of TGF- β and retinoic acid, causing B cells to stimulate production of IgA and facilitate plasma B cell differentiation. 7) SCFAs bind to GPR on T cells, initiating the production of IL-10, which increases Tregs pool, and inhibits expansion of T helper (Th) 17.

health, and broadly support a dichotomous clinically relevant separation of healthy "plant-based" and less healthy "western, high fat" dietary patterns with the associated "healthy" microbiome or eubiosis and the "unhealthy" microbiome or dysbiosis (**Table 2**) (Asnicar et al., 2021). Furthermore, diet and gut microbiome has been directly linked with the circulating metabolome of human serum (Bar et al., 2020). Bar et al. found that over 50% of the observed variance in 1,251 human serum metabolites was explained by diet and microbiome variables, in a study of 491 subjects who were carefully phenotyped according to genetics, gut microbiome, diet and lifestyle measures (Bar et al., 2020). Finally, a recent longitudinal study of 307 well characterized subjects correlated adherence to the plant-based Mediterranean diet with specific microbial and functional patterns which in turn correlated with favorable cardiometabolic markers (lipids, c-reactive protein, and hemoglobin A1C) (Wang et al., 2021). These studies strongly suggest the mechanisms whereby adherence to a Mediterranean diet results in reduced cardiovascular, metabolic, and cancer related outcomes demonstrated in the PREDIMED randomized clinical trial (Estruch et al., 2018; Toledo et al., 2015). More prospective randomized intervention trials of dietary components and specific bacterial communities are needed to further test the link between dietary composition, microbiome structure/function parameters, and cardiometabolic and oncogenic outcomes.

MOLECULAR MECHANISMS OF THE ROLE OF PLANT-BASED DIET IN CANCER PROTECTION

The gut microbiome and the human intestinal immune system have co-evolved over evolutionary time to stay in balance and to

regulate each other, the ideal status quo that corresponds to a healthy state. This balance is composed of four elements (**Figure 1**). First, the bacteria pathogens are suppressed and are kept compartmentalized from the intestinal epithelium by a thick mucus layer, the release of antimicrobial peptides (RegIIIg) and secreted IgA to protect the epithelial surfaces from invasion, and the presence of intraepithelial immune cells and neutrophils that can migrate into the intestinal lumen. Second, the intestinal epithelium has tight junctions that form the epithelial barrier that are strengthened by luminal metabolites (Biragyn and Ferrucci, 2018). Third, inputs from luminal antigens regulate intestinal macrophages to become hyporesponsive and exhibit tolerance, producing IL-10 and less inflammatory cytokines (Round and Mazmanian, 2010; Vinolo et al., 2011). Fourth, there is active suppression of microbe reacting effector T-cells by Foxp3b Treg cells and Roc-3-Tr1 cells via IL-10; and intraepithelial immune cells use MHC to present bacterial antigens and down regulates reactive CD4b T cells (Geuking et al., 2011; Zhang et al., 2019).

Maintenance of a healthy balance for the intestinal barrier and immune homeostasis depends on a balance of beneficial bacteria and dietary nutrients, especially dietary fiber. Fiber is a major component of the plant-based diet and is an indigestible carbohydrate for the mammalian gastrointestinal tract, consequently certain bacterial phyla in the gut are responsible for fiber fermentation and production of short-chain fatty acids (SCFAs) (Wilson et al., 2020), which is a key metabolite in a continuous dialogue between the host and the gut microbiome.

Homeostasis of the mucus layer is a key component in intestinal health (Cai et al., 2020; Paone and Cani, 2020). Mucus serves as a mechanical and chemical shield, protecting epithelial cells from the pathogenic attacks. Along with Goblet cells, the production of mucus is maintained by the commensal bacteria in the gut microbiome (Paone and Cani, 2020). Numerous studies have observed that high-fat Western diet promotes the deterioration of intestinal mucus layer, whereas plant-based diet enhances mucus layer thickness. One result of low fiber diets is the reduced delivery of fiber to these bacteria, resulting in a switch to metabolizing endogenous carbohydrates present on intestinal mucin glycoproteins. This results in a reduction in the quality of the protective intestinal mucous coat (Png et al., 2010; Ijssennagger et al., 2015). This was shown experimentally by Desai et al. using a gnotobiotic mice colonized with a synthetic human gut microbiota (Desai et al., 2016). When these mice were fed a fiber deficient diet, mucin degrading bacteria levels increased and the susceptibility to enteric pathogens increased (Desai et al., 2016). These data indicate the importance of adequate dietary fiber in maintaining epithelial barrier protection.

Due to the absence of mammalian enzymes that can degrade carbohydrates, especially resistant starch, certain *Firmicutes* and *Bacteroides* species are able to ferment indigestible carbohydrates leading to the production of SCFAs (Biragyn and Ferrucci, 2018). Evidently the predominant majority of SCFAs produced are acetate, propionate, and butyrate.

SCFAs are extremely bio-active molecules, primarily acting through interaction with G-protein coupled receptors: GPR41, GPR43, and GPR109A and by direct inhibition of histone deacetylase (HDAC). Uptake of SCFAs in colon epithelial cells occurs by multiple mechanisms. These include passive diffusion, specific monocarboxylate transporters (MCT), as well as through SLC5A8 receptors (Ulven, 2012). Increased production of SCFAs by gut microbiome is strongly associated with the improvement of barrier junctions, increase in protective mucosal layer, increase in immune tolerance and suppression of intestinal inflammation (Smith et al., 2013; Schwiertz et al., 2010). SCFAs are produced in the gut lumen where they interact with intestinal epithelial cells, however SCFAs are also able to cross the epithelial layer and reach the lamina propria, where they can then interact with the set of immune cells, and also enter into the systemic circulation (**Figure 1**) (Blacher et al., 2017; Zhang et al., 2019).

A recent study has shown that mice fed on low fiber intake resulted in depletion of butyrate production, which in turn directly caused disruption of the gut microbial diversity, leading to systemic inflammation and mortality from necrotizing pancreatitis. The mortality rate in these mice significantly decreases upon oral and systemic introduction of butyrate (van den Berg et al., 2021). Immune regulation and maintenance of anti-inflammatory environment in the gut lumen is extremely convoluted process, and its disturbance leads to accumulated mechanisms that may promote oncogenesis. It is now established that propionate and butyrate bind to GPR43 on the surface of Foxp3+ expressing Treg cell, and facilitate the differentiation of Treg cells as well as elevate the production of IL-10, hence controlling and decreasing level of intestinal inflammation, and increasing immune tolerance at a large scale (Smith et al., 2013; Mandaliya et al., 2021). Another mechanism by which SCFAs regulate Treg cell differentiation is through the production of TGF-β by the epithelial cells (Basson et al., 2016).

Similarly to butyrate, propionate acts as a ligand for SCFA-sensing receptors GPR43, GPR41, and GPR109A (Furusawa et al., 2015), inhibiting the expression of pro-inflammatory cytokines such as IL-6, IL-8, and TNF. Propionate inhibits the MAPK signaling pathway and prevents proliferation of CRC cells (Davido et al., 2001), while acetate dramatically reduces production of pro-inflammatory agents via inhibition of NF-κB signaling pathway in CRC cells, inhibiting the cancer progression. Thus, depletion of SCFAs can lead to colonic and extraintestinal inflammation, resulting in unfavorable outcomes like inflammatory bowel disease (IBD) and CRC (Davido et al., 2001). Mandaliya et al. introduced butyrate and propionate to diabetic mice that were fed a high fat diet (HFD), observing both T cell polarization and inhibition of pro-inflammatory IL-6 cytokine production dramatically minimizing inflammation grade in the gut (Mandaliya et al., 2021). A single-blind pilot study compared the contribution of dietary fiber towards SCFAs concentration and Treg cell population, showing the group with higher intake of dietary fiber demonstrated a dramatic increase in total CD4+T cells and Tregs. Moreover, upon activation of its receptor, GRP109A, butyrate can inhibit several key pro-inflammatory pathways that are highly involved in CRC, such as protein kinase B or Akt (PKB/Akt) and NF-κB signaling pathways (Chen et al., 2018).

SCFAs regulate epigenetic changes via their ability to inhibit histone deacetylase (HDAC). HDAC inhibition regulates the levels of Treg cells in the colon, though the mechanism is not fully understood (Verma and Shukla, 2013). Through HDAC inhibition, butyrate also initiates polarization of macrophages to M2 phenotype, hence leading to a decrease in inflammatory ability of macrophages cells (Li et al., 2019a). Butyrate also acts on the population of intestinal macrophages to promote production of anti-inflammatory IL-10 and through the inhibition of HDAC, initiates the production of antimicrobial peptides (AMPs), boosting pathogenic clearance in the intestine (Mohammadi et al., 2018). Moreover, HDAC inhibition in macrophages leads to the dramatic decrease in production of pro-inflammatory cytokines such as, IL-6, IL-12, nitric oxide (NO), and TNF (Scott et al., 2018). The inhibition of HDAC activity by propionate, reduced the level the tumorigenic lesions in the colon (Casanova et al., 2018).

Furthermore, interaction between SCFAs and intestinal epithelial cells leads to the elevated secretion of NLRP3 inflammasome that further results in increased secretion of IL-18, hence facilitating the improvement of tight junction's homeostasis (Macia et al., 2015; Nowarski et al., 2015). Moreover, SCFA-receptors are also present on Paneth, Goblet and L cells, hence binding of SCFAs to these receptors' triggers production of molecules of defending nature. When SCFAs bind GPCR on Goblet cells, it triggers the activation of NLRP6, as well as mTOR/STAT3 signaling pathway to increase mucus production in the gut lumen (Wlodarska et al., 2014; Li et al., 2019b). Simultaneously, butyrate acts via Paneth cell GRP43 receptor resulting in the production of key anti-microbial peptides, such as RegIIIγ and β-defensin (Birchenough et al., 2016). These two cascades of reactions, also lead to an improved barrier junction, as well as elevated innate response to the continuous flow of pathogens. Interestingly, enteroendocrine L cells, that are part of the colonic epithelium and express SCFA receptor on their surface, upon the interaction with acetate and butyrate, produce glucagon-like peptide-1 (GLP-1) and fasting peptide YY (PYY) peptides (Brooks et al., 2017; Zhao et al., 2018) These peptides are thought to increase energy intake while decreasing appetite, hence these peptide are involved in the gut-brain axis and are potential therapeutic agents in treating conditions like obesity. In summary, the majority of research studies support that SCFA derived from dietary fiber plays a key role in epithelial defenses and immune regulation in the colon, however further research is essential to better understand benefit-based stratification amongst various dietary fiber types. For instance, a recent study by Singh et al. has demonstrated that diet rich in soluble inulin fiber provoked icteric hepatocellular carcinoma (HCC) in dysbiosis mice models (Singh et al., 2018).

WESTERN DIETARY PATTERN—LOCAL PATHOPHYSIOLOGIC AND MOLECULAR EFFECTS

Direct effects of dietary constituents, microbiota, and microbial products are thought to play a causative role in colorectal carcinogenesis though multiple mechanisms, including genotoxic, inflammatory, immune mediated, and metabolic (Scott et al., 2019) In addition to being associated with a distinct microbial signature, western-type diets are characterized by certain dietary constituents (N-nitroso compounds, heterocyclic amines, and heme) and increased secondary bile acids and other metabolic products derived from enriched bacterial species that can directly promote a local pro-inflammatory and pro-carcinogenic environment in the colon. In addition, the lack of dietary fiber in western-type diets results in metabolic shifts that impact epithelial defense against inflammation (Bhaskaran et al., 2018).

The relative increase in specific bacterial genus and species in the microbiome found in patients with pre-cancerous adenomas and CRC has fueled investigations into the possible direct pro-carcinogenic or pro-inflammatory effects of these bacteria (Mima et al., 2016a; Yu et al., 2018). Initial studies focused on comparing microbiome and metabolome changes in patients with various stages of colorectal neoplasia. Yu et al. used shotgun metagenomic sequencing and identified 20 microbial gene markers that were significantly associated with CRC (Yu et al., 2017). A co-occurrence network, generated from the relative abundance of 20 bacterial species was significantly associated with CRC. Wirbel et al. conducted a meta-analysis of 8 geographically diverse fecal shotgun metagenomic studies of CRC patients (n = 768). They found a core set of 29 species that were enriched in CRC metagenomes. Functional characteristics of these core species indicated enrichment of protein and mucin catabolism genes and elevated production of secondary bile acids, likely reflecting diets high in fat and meat nutrients (Wirbel et al., 2019). Furthermore, Ng et al. used metagenomic sequencing to describe a specific virome and mycobiome signatures associated with CRC (Ng et al., 2019).

Other studies have interrogated the temporal framework changes in the microbiome during adenoma-carcinoma development sequence. Fecal samples from patients with CRC, advanced adenomas, non-advanced polyps, and normal subjects were studied to represent different stages of neoplastic evolution. Nakatsu et al. found significant differences in the mucosal bacterial communities found in normal mucosa, adenoma, and carcinoma samples (Nakatsu et al., 2015). Zhang et al. showed that the relative abundance of 24 bacterial species significantly changed in fecal samples between normal, non-adenomatous polyps, adenomas and carcinoma groups of patients, with relatively higher amounts of *Fusobacterium nucleatum* and pro-inflammatory periodontal bacteria, along with lower amounts of beneficial short-chain fatty acid (SCFA) producers in the CRC groups compared with the other groups (Zhang et al., 2018). This correlated with a trend for increasing C-reactive protein and STNFR-II in the adenoma and CRC groups (Zhang et al., 2018). Similarly, Hale et al. found modest but significant changes in the fecal 16S rRNA gene characterization of microbiota of 547 adenoma patients compared with 233 patients without adenomas (Hale et al., 2017). Taxa that were more abundant in patients with adenoma included *Bilophila*, *Desulfovibrio*, proinflammatory *Mogibacterium*, and Bacteroidetes species; whereas taxa that were increased in

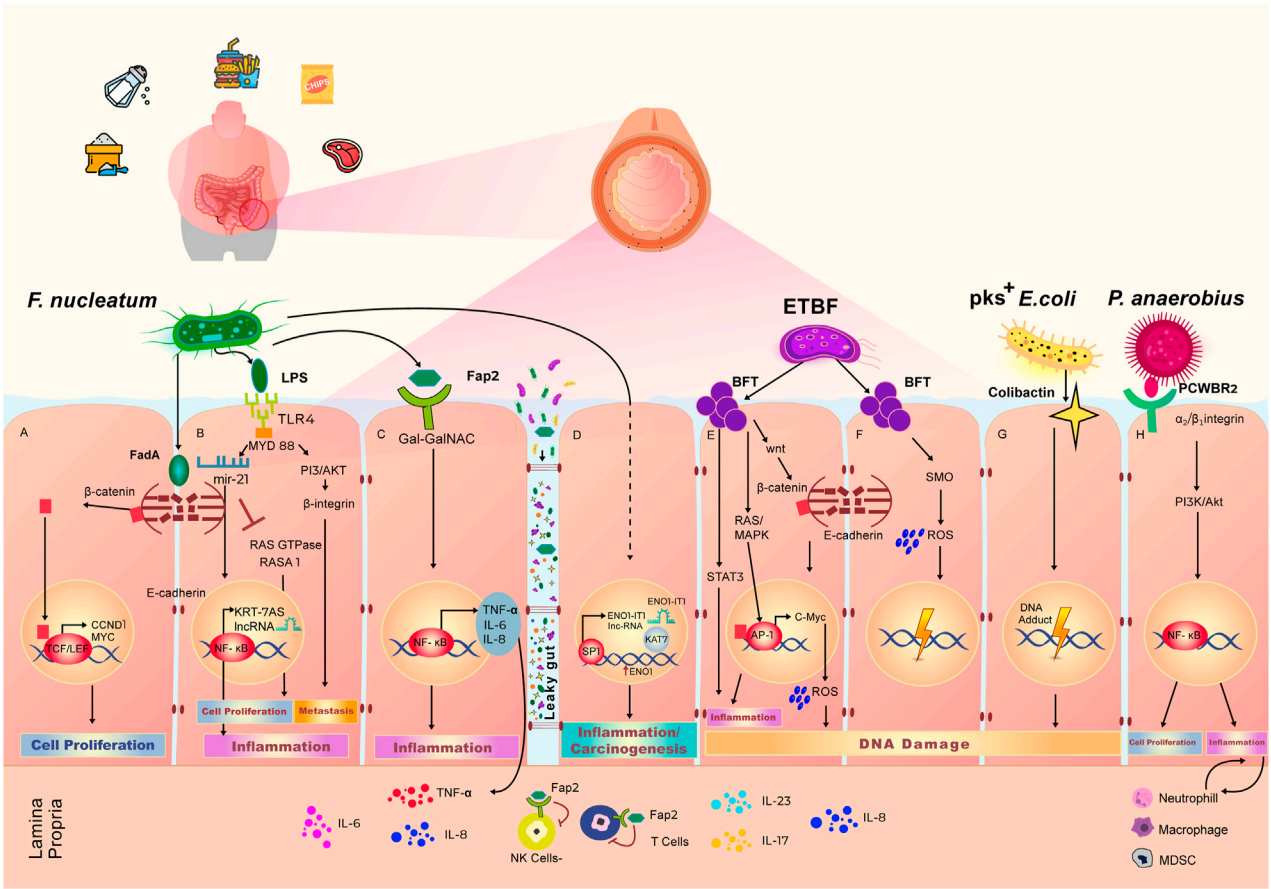

FIGURE 2 | Effect of Western diet on the gut microbiome and local pro-carcinogenic pathways. Adherence to Western diet results in the high abundance of *Fusobacterium nucleatum*, Enterotoxigenic *Bacteroides fragilis* (ETBF), pks$_+$ *Escherichia coli* and *Peptostreptococcus anaerobius*. Overpopulation of these bacteria in the gut microbiome leads to the increased cell proliferation, elevated grade of inflammation and accumulation of DNA damage, eventually resulting in an environment favorable to initiation or progression of colorectal cancer. **(A)** *F. nucleatum* secretes FadA toxin, which binds the E-cadherin complex on the wall of intestinal epithelial cells. Upon interaction with FadA, E-cadherin undergoes phosphorylation, which lead to cleavage and subsequent translocation of the β-Catenin component of the complex to the nucleus. β-Catenin binds with TCF/LEF transcription complex, triggering the expression of CCND1 and MYC, that govern cell proliferation. **(B)** LPS is a polysaccharide on the cell wall of *F. nucleatum*, that binds the TLR4 receptor which are expressed on the intestinal epithelial cell membrane. LPS-TLR4 complex activates MYD88 adaptor that results in inhibition of a Ras GTPase—RASA1. The inhibition of RASA1 leads to unwanted cell proliferation. LPS-TLR4-MYD88 complex also induces the production of microRNA miR 21, that in turn activates NF-κB transcription factor and leads to the production of pro-inflammatory agents including KRT-7AS long non-coding RNA. **(C)** Upon binding of microbial protein Fap2 with cell membrane receptor, surface expressed polysaccharide Gal-Gal-NAc modulates the activation of NF-κB transcription factor that leads to the increase in intestinal inflammation through the production of TNF-α, IL-6, and IL-8. Increased intestinal permeability also, known as "Leaky gut" phenomena, allows the bi-directional passage of bacteria and their metabolites, as well as immune inflammatory agents. Through the leaky gut, Fap2 is able to reach lamina propria and diminish the activity of Natural killer (NK) cells and T cells. **(D)** *F. nucleatum* interacts with intestinal epithelial cells to stimulate the production of long non-coding RNA (lncRNA) ENO1-IT1 via SP1 transcription factor. Production of ENO1-IT1 increases concurrently the expression of ENO1. This cascade of reactions leads to the increased level of carcinogenesis and inflammation. **(E)** ETBF produces BFT toxin, that interacts with epithelial cells to trigger intestinal inflammation via activation of STAT3 signaling pathway. BFT activates RAS/MAPK pathways to further initiate AP1 and start the production of IL-8 resulting in intestinal inflammation. BFT stimulates the translocation of β-Catenin to the nucleus initiating expression of C-Myc and production of reactive oxygen species (ROS), leading to DNA damage. **(F)** BFT also induce the production of spermine oxidase (SMO) and SMO-dependent ROS production, resulting in DNA damage. **(G)** Colibactin is secreted by of pks$_+$ *E. coli*. Interaction of colibactin with epithelial cells results in accumulation of DNA adducts and further DNA damage. **(H)** *P. anaerobius* surface protein PCWBR2 binds to α_2/β_1 integrin on the epithelial cells to initiate cell proliferation via activation of PI3K/Akt signaling pathway and NF-κB transcription factor. This interaction also leads to expansion of neutrophils, macrophages and myeloid derived suppressor cells driving chronic inflammation and tumor progression.

patients without adenomas included *Veillonella*, Firmicutes, Clostridia and *Actinobacteria*.

Kim et al. expanded these observations to focus on the differences in the fecal microbiome and metabolome signatures of 102 patients with advanced adenomas compared with 102 matched healthy controls without polyps and with 36 patients with CRC, adjusting for sex and age in the groups. They found that several bioactive lipid pathways were significantly associated with the adenoma group, including polyunsaturated fatty acids, secondary bile acid pathways, endocannabinoid metabolism, and sphingolipid pathways (Kim et al., 2020). The relative amounts of these lipids were not quantified, which could

determine the pro-vs anti-inflammatory effects of these lipids. These metabolomic pathways were correlated with genus-level microbiome sequencing, and they found positive correlations with *Bacteroides* and 10 or more sub pathways, whereas four genera from *Firmicutes* and one from *Actinobacteria* showed negative correlation with these pathways (Kim et al., 2020). The authors stated that the changes observed in the metabolic pathways are too small to be useful as diagnostic markers of adenomas, however these observations illustrate the possible biological pathways that are early events in the adenoma-carcinoma pathway. Overall, these studies indicate that there are specific fecal and mucosal microbiome signatures associated with the development of colonic neoplasia.

Directly supporting the direct carcinogenic effect of the gut microbiome is the pivotal observation that fecal samples from CRC patients promote intestinal tumorigenesis in colon carcinogenic mouse models induced by azoxymethane (Wong et al., 2017). To determine the specific mechanisms that can explain this, potentially pathogenic bacteria found to be enriched in colorectal adenomas and carcinomas have been investigated to determine if they have direct carcinogenic effects in the colon (**Figure 2**). These include *F. nucleatum*, Enterotoxigenic *Bacteroides fragilis* (ETBF), *pks*$^{+}$ *Escherichia coli*, and *Peptostreptococcus anaerobius* (**Figure 2**) (Dai et al., 2018; Haghi et al., 2019).

Although the review is focused on the microbial composition in the gut lumen, it is important to highlight arising areas of mycobiome and virome and how much they contribute to oncogenesis. Both fungal and viral composition in the gut, has been shown to be altered in patients with cancer in comparison to healthy individuals (Vallianou et al., 2021). It has also been found that CRC patients show virome dysbiosis, however more studies are required in order to fully elucidate the contribution of eukaryotic viruses in cancer development (Massimino et al., 2021).

Fusobacterium Nucleatum

F. nucleatum is a Gram-negative commensal anaerobe that in the past decade has drawn a lot of attention due to its strong association with the development of colorectal neoplasia (Abu-Ghazaleh et al., 2021). There are numerous studies linking *F. nucleatum* overpopulation in the gut microbiome to local and distant cancers in the human body. Recent population-based studies have demonstrated overpopulation and presence of *F. nucleatum* in biopsies of colorectal adenomas, and patient stool screening in comparison to the healthy individuals (Mima et al., 2016b; Mehta et al., 2017). To understand how *F. nucleatum* potentially causes carcinogenesis both locally in the colon and systemically, it is important to establish the interaction of metabolites produced by *F. nucleatum* and the cells of the colonic epithelium.

FadA is one of the virulence toxins produced by *F. nucleatum* in the gut microbiome, and it plays a crucial role in initiating carcinogenic processes in the colonic epithelium. The FadA protein has a helical form which helps it to bind to the extracellular domain of E-cadherin, an epithelial cell adhesion protein E-cadherin is internalized upon interacting with FadA, and immediately phosphorylated, which results in the release and accumulation of β-catenin molecules in the cytoplasm. β-catenin which is then subsequently translocate to the nucleus interacts with T-cell factor and lymphoid enhancer factor (TCF/LEF)-family transcription factors (Kostic et al., 2013; Rubinstein et al., 2013), initiating transcription of a set of pro-inflammatory, proliferative genes and oncogenes, such as CCND1 and MYC (Abu-Ghazaleh et al., 2021). Hence, in a situation with continuous production of FadA, the transcription machinery constantly drives a set of oncogenes along with pro-inflammatory genes, resulting in proliferation of CRC cell and favorable tumor microenvironment.

However, not all CRC cells have E-cadherin on their adherent tight junctions, therefore, not all cancer progression and inflammation events are triggered via FadA activity (Rubinstein et al., 2013; Gholizadeh et al., 2017). Accordingly, another *F. nucleatum* metabolite, Fap2, has been identified to be elevated in CRC patients. A dual mechanism of Fap2 activity leads to both immune suppression and establishment of tumor microenvironment (Abed et al., 2016; Hashemi Goradel et al., 2019). A host polysaccharide Gal-Gal-NAc, was identified to be predominant on all the types of CRC cells. Gal-Gal-NAc acts as a polysaccharide modulator for Fap2, and upon this interaction, Fap2 activates NF-κB signaling pathway that result in overproduction of TNF-α, IL-6, and IL-8 (Abed et al., 2016; Abed et al., 2017; Yachida et al., 2019). These immune chemokines are extremely pro-inflammatory, hence continuous enrichment of CRC cells via Fap2 leads to chronic inflammation in the colon and enhancement of the favorable tumor microenvironment. Moreover, Fap2 binds to the TIGIT receptor on the Natural Killer (NK) cells and T-cells, inhibiting immune T cells polarization, leading cancer cells to escape from immune surveillance (Gur et al., 2015; Mima et al., 2015).

Several studies have shown that *F. nucleatum* -infected CRCs have a higher rate of proliferation. Lipopolysaccharide (LPS) is an antigen found on the cell-wall of *F. nucleatum*. This key proinflammatory, immunogenic bacterial metabolite, a well-known inducer of the Toll-like Receptor (TLR) and NF-κB pathways that lead to inflammation and oncogenic changes both locally and systemically (Salcedo et al., 2010; Yang et al., 2017a). Activated NF-κB transcription factor, results in activation of MYD88 signaling pathway and promotes an increased expression of the microRNA 21 (miR-21), which in turn inhibits the activity of RAS GTPase—RASA1 (Ciesielska et al., 2021). miR-21 was found to significantly downregulate the production of RASA1, a member of RAS GTPases family that plays a key role in inactivating set of oncoproteins such as RAS (Salcedo et al., 2010). When RASA1 is downregulated, the MAPK signaling pathways is further activated and results in CRC cell proliferation and eventual metastasis. Moreover, apart from inhibiting the production of RASA1, miR21 also downregulates Pdcd4, which is a key tumor suppressor (Yang et al., 2017a).

Escherichia Coli

Escherichia coli is a symbiotic and commensal bacterium, widely occurring in oral, vaginal and intestinal microflora. Certain

strains of *E. coli* can induce carcinogenic changes on the cellular level (Arthur et al., 2012). An *E. coli* strain from the B2 phylogroup that possesses polyketide synthase island (pks^+), was found to colonizes a healthy gut microbiome in response to a shift and continuous adherence to a Western dietary pattern. Recent studies have confirmed an increased level of pks^+ *E. coli* strain in patients with advanced CRC (Kohoutova et al., 2014). pks^+ *E. coli* produces colibactin, an extremely virulent secondary genotoxin. Multiple *in vivo* studies have confirmed that colibactin introduces DNA double-strand breaks leading to genomic instability and thereby considerably elevates the risk of acquiring further mutations (Cougnoux et al., 2014; Wilson et al., 2019).

Although colonic inflammation is known to be one the key risk factors in developing CRC, and bacterial toxins drive continuous pro-inflammatory agents in the gut, there are also numerous mutational signatures that distinguish CRC patients, that are not related to the inflammatory pool. Distinctive mutational signatures were identified in 5,876 cancer patients, the majority of them with CRC (Alexandrov et al., 2020). A recent study by Pleguezuelos-Manzano et al., exposed human intestinal organoids to the genotoxic pks^+ *E. coli* via luminal injection for 5 months, comparing it to organoids injected with isogenic pks^- mutant bacteria and then performed whole genome sequencing. They found that organoids injected with genotoxic *pks* + *E. coli* resulted in the same subset of mutational signatures that had been deduced from a cohort of CRC patients (Pleguezuelos-Manzano et al., 2020).

Mechanistically, colibactin induces damage due to the presence of a cyclopropane ring within its structure. Wilson et al. have demonstrated *in vivo* evidence of colibactin activity causing DNA adducts and alkylation, leading to eventual DNA damage. The study demonstrated the strong link between colibactin structure and ability to cause DNA double stranded break, through creating DNA interstand cross-link, leading to genomic instability, and further accumulation of distinct mutation leading to CRC (Wilson et al., 2019; Xue et al., 2019).

Enterotoxigenic *Bacteroides fragilis*

B. fragilis is a commensal Gram-negative anaerobe, that exists in a symbiotic fashion with the host organism (Gagnaire et al., 2017). Recent studies have strongly linked adherence to the HFD Western diet to the elevated population of *Bacteroides fragilis* in the gut microbiome (Abu-Ghazaleh et al., 2021). Due to the sharp increase in CRC cases worldwide, numerous studies have been looking at the interaction of *B. fragilis* with the colonic epithelium. The colonization of colonic epithelium with *B. fragilis* is one of the key signatures of the microbiome in CRC patients. Although the causality of the events is yet to be fully determined, recent population-based studies have shown increased level of *B. fragilis* strains in patients with inflammatory bowel disease (IBD) and colitis (Dejea et al., 2018; Rashidan et al., 2018). *B. fragilis* normally comprises up to 2% of total microbiome volume (Wu et al., 2007), but only a certain strain, Enterotoxigenic *Bacteroides fragilis* (ETBF), is associated with the development of CRC and can be characterized as carcinogenic (Liu et al., 2020; Mohseni et al., 2020). ETBF releases the zinc-metalloprotease *B. fragilis* toxin (BFT), that binds a receptor on the colonocyte and induces favorable conditions for CRC progression. This occurs by establishing chronic inflammatory microenvironment with activating a set of oncogenes and initiating production of reactive oxygen species (ROS) (Hernández-Luna et al., 2019). Upon interaction between BFT and epithelial cell receptor, β-catenin molecule dissociates from the E-cadherin complex and travels to the nucleus (Chung et al., 2018). Abundant of unphosphorylated β-catenin in the nucleus initiates the NF-κB/AP1 transcription machinery, causing the overexpression of pro-inflammatory cytokine like IL-8 and oncogenes such as C-MYC (Cheng et al., 2020). Moreover, BFT triggers the STAT3 signaling pathway, giving rise to the continuous production of IL-17 and IL-23, significantly increasing the extent of inflammation locally in the gut (Thiele Orberg et al., 2017; Chung et al., 2018). This continuous production of BFT promotes proliferation of CRC cells and maintains chronic inflammation at the sites of colonic epithelium. Furthermore, ETBF infected colonic cells produce reactive oxygen species (ROS), leading to the progressive genomic instability, exponentially elevating risk of acquiring new mutations, and development of CRC (Cheng et al., 2020).

Peptostreptococcus anaerobius

Another example of a potentially pathogenic species enriched in CRC samples is *Peptostreptococcus anaerobius.* This species have been shown in cell culture to have direct inflammatory and pro-oncogenic impacts by binding to integrin α_2/β_1 integrin receptors on cell surfaces, activating PI3K, Akt, and NF-κB to enhance proliferation, proinflammatory cytokines, and T cell suppression (Long et al., 2019).

WESTERN DIETARY PATTERN—SYSTEMIC PATHOPHYSIOLOGIC EFFECTS

Dysbiosis is implicated as a bridge between changing gut microbiome composition and the incipient manifestation of extraintestinal tumors. The microbial balance shifts away from commensal bacteria in the gut, creating a favorable environment for chronic inflammation as well as the suppression of immune surveillance (Zhou et al., 2020; Kovács et al., 2020). Intriguingly, it has been hypothesized that pathogenic bacteria, bacterial products, and metabolites escape into the systemic circulation via increased leakiness of tight junctions and contribute to promoting inflammatory pathways in other organs. This amounts to an organismal-level of carcinogenic circulatory signaling instigated by the diet-deregulated microbiome. Contemporary efforts in this field have examined the contribution of the gut metagenome to the development of conditions such breast, liver, and pancreatic cancers (**Figure 3**) (Chen et al., 2019; Jain et al., 2021).

Breast cancer is a multifactorial disease, aside from the rare minority of hereditary cases driven by BRCA1 and BRCA2 mutations, and familial predisposition involving genetic modifiers such as CHK2—that is initiated by a sequence of pro-carcinogenic events, including destabilized hormonal

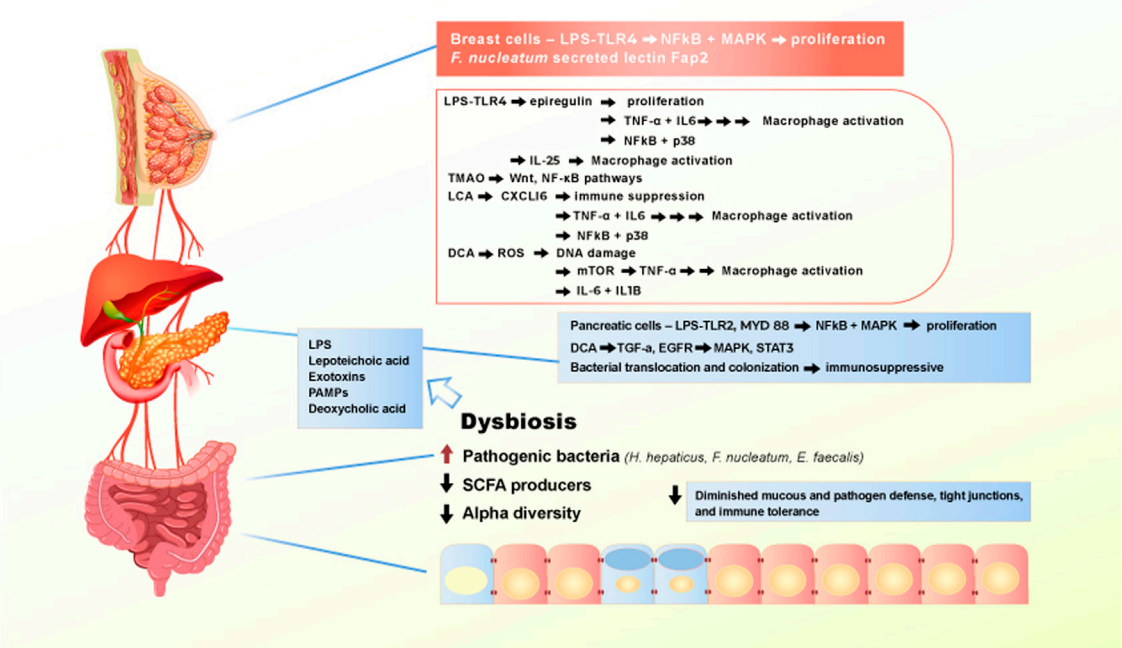

FIGURE 3 | Effect of Western diet on the gut microbiome and extraintestinal pro-carcinogenic pathways. Adherence to Western diet promote the establishment of chronic dysbiosis in the gut, leading to translocation of pathogenic bacteria and toxic bacterial metabolites entering systemic circulation and reaching extraintestinal organs. Main drivers of the pro-carcinogenic and pro-inflammatory processes are LPS, lipoteichoic acid, numerous exotoxins, PAMPs, and deoxycholic acid. When bacterial metabolites reach liver, pancreas, and breast, they bind to receptors, such as TLR4 to activate signaling pathways that drives inflammation and proliferation.

homeostasis. Growing incidence of obesity induced breast cancer, led to the acknowledgement of a gut-breast axis. Ley et al., have demonstrated that gut microbial composition differs between lean and obese people. The group has observed that Bacteroides phyla is significantly decreased in obese participants, supporting the evidence that gut microbiome mirrors the dietary and lifestyle choices (Ley et al., 2006). Moreover, Shively et al. designed a study focusing on the effect of Mediterranean versus Western diets on mammary gland microbiome of non-human primates. The group observed that upon administration of Mediterranean diet, *Lactobacillus* abundance increases in the mammary gland microbiome. Multiple studies have also shown altered gut microbiome in breast cancer patients as compared to healthy individuals (Shively et al., 2018; Iyengar et al., 2019; Klann et al., 2020; Yaghjyan et al., 2021). Nagrani et al., have demonstrated that obesity is a key risk factor in developing breast cancer, regardless of menopause manifestation and administration of hormonal therapy (Nagrani et al., 2016). The population-based study was performed between 2009 and 2013 amongst Mumbai female population, revealed the consistency of breast cancer diagnosis amongst women with higher BMI (Nagrani et al., 2016). Interestingly, Kabat et al. have conducted a 15-years prospective cohort study, focusing on the incidence of postmenopausal breast cancer and its association with obesity and metabolic syndrome (Kabat et al., 2017). Amongst 21,000 enrolled postmenopausal participants, 1,176 cases of invasive breast cancer were documented. The 15 years-follow up showed that obesity is the key risk factor of breast cancer manifestation, irrespective of metabolic dysregulation. Kabat et al. confirmed that the increase in BMI is directly proportional to the development of breast tumorigenesis (Kabat et al., 2017).

Parhi et al. showed that the increasing abundance of *F. nucleatum* positively correlates with metastatic progression of breast cancer in mice (Parhi et al., 2020). They showed that colonization of breast cancer cells is solely dependent on the *F. nucleatum* secreted lectin Fap2. Interestingly, inactivation of Fap2 significantly suppressed the tumor growth. Furthermore, metastatic progression of the cancer was facilitated by Fap2 and TIGIT binding, as this interaction results in abrogation of NK cells and tumor-infiltrating T-cells. Remarkably, breast cancer progression and speed of metastatic lesion formation was slowed down upon administration of antibiotic therapy (Parhi et al., 2020). This provides some evidence for the direct causal contribution of the gut microbiome to carcinogenesis beyond CRC, specifically to breast cancer progression.

Furthermore, Rao et al. have shown that chronic inflammation in the gut is a precursor of tumorigenesis in the mammary glands (Rao et al., 2006). They introduced *Helicobacter hepaticus* to female mice and observed the development of both mammary carcinoma and intestinal tumor. The main mechanism *H. hepaticus* utilizes to induce carcinogenesis was solely TNF-α dependent. By increasing the population of pro-inflammatory cytokines that enter the systemic circulation, *H. hepaticus* can establish the pro-inflammatory microenvironment in the breast tissues as well. This finding supports the claim that intestinal

inflammation can result in systemic inflammation, causing neoplastic formation in distant organs (Rao et al., 2006). Rutkowski et al. showed that depletion of commensal bacteria in the gut increases the activity of TLR5, resulting in the production of pro-inflammatory cytokines such as IL-6 and elevation of the γδ T cell pool. This sequential cascade spreads inflammation and can fuel tumor microenvironment in the mammary tissues, resulting in tumor formation (Rutkowski et al., 2015). Recently, Zhu et al., using shotgun metagenomic analysis of both premenopausal and postmenopausal breast cancer patients, have shown that breast cancer patients have a unique alteration of their gut microbiome in comparison to premenopausal healthy controls (Zhu et al., 2018). They demonstrated an enrichment of bacterial population involved in LPS biosynthesis, which is a stimulus for chronic intestinal inflammation (Hsu et al., 2011; Yin et al., 2016; Zhu et al., 2018). Taken together, these and other studies demonstrated that commensal bacteria are vitally important to maintaining immune homeostasis, not only locally in the gut microbiome, but also systemically in distant organs.

In the recent years, with the rising incidence of pancreatic cancer, the gut microbiome was explored as a potential driver of pancreatic malignancies. Immediate anatomical connection between pancreas and gastrointestinal system implicates a role of gut-pancreas axis in pathological conditions like type II diabetes, pancreatitis, and pancreatic ductal adenocarcinoma (PDAC). There is a unique alteration of gut microbiome between PDAC patients in comparison to healthy individuals (Pushalkar et al., 2018). Maekawa et al. have demonstrated that population of *Enterococcus faecalis*, a virulent bacterium that is more abundant in people adhering to the Western diet, is elevated in patients with pancreatic cancer, supporting the evidence of a potentially deleterious crosstalk between the gut microbiome and pancreas (Maekawa et al., 2018).

It is hypothesized that LPS translocated from the gut microbiome can cause high grade inflammation and tumorigenesis in the pancreas via the activation of MYD88 and TLR2. Interestingly, Nagathihalli et al. demonstrated that secondary bile acids, particularly deoxycholic acid (DCA) promote tumorigenesis in CRC and PDAC (Nagathihalli et al., 2014). It was observed that in pancreatic tissue, DCA modulate carcinogenic activity through TGF-α and EGFR interacting with pancreatic cells, further activating MAPK and STAT3 signaling pathways.

In mice and humans, there is a characteristic change in the microbiome signature associated with cancerous pancreas as compared to that in healthy pancreas. Pushalkar et al. have recently demonstrated that the microbiome in PDAC shifts from Bacteroidetes and Firmicutes to Proteobacteria, Actinobacteria and Fusobacteria, resulting in dysbiosis and increased bacterial translocation into the pancreatic duct (Pushalkar et al., 2018). Several studies have demonstrated that progressive carcinogenesis of PDAC is solely dependent on toll-like receptor (TLR) activation. Activation of the TLR family results in dramatic suppression of immune surveillance through increased pool of Th2-deviated $CD4^+$ T cells and polarization of tumor-associated macrophages M1, leading to the rapid progression of PDA in mice. Removal of the microbiome-derived pathogens results in positive outcomes for PDAC by causing a reduction in myeloid-derived suppressor cells and an increase in M1 macrophage differentiation (Zambirinis et al., 2015; Pushalkar et al., 2018). In addition, emerging data indicates the mycobiome may play a significant role in pancreatic oncogenesis. Aykut et al. demonstrated that certain genera of endoluminal fungi promote the development of pancreatic oncogenesis in mice, whereas the fungal ablation was shown to be tumor-protective in this mouse model of pancreatic cancer (Aykut et al., 2019).

The role of the gut-liver axis has been extensively explored in the last decade, due to the increasing evidence of strong association between metabolic disease, obesity, adherence to a Western diet, and liver cancer. Although up to now the full spectrum of gut-liver crosstalk is yet to be determined, gut microbiome seems to add a tremendous contribution in the liver homeostasis.

Gut-liver axis and bi-directional metabolite translocation is thought to be maintained through biliary duct, portal vein and systemic circulation (Schwabe and Greten, 2020; George et al., 2021). Patients with hepatocellular carcinoma (HCC) have been shown to have reduced SCFA production and increased proinflammatory bacterial species (Muñoz et al., 2019; Suriguga et al., 2020). The disruption of gut barrier in events like dysbiosis and leaky gut, result in continuous secretion of microbial associated molecular patterns (MAMPs) such as LPS into the portal vein and systemic circulation. MAMPs act as ligand to TLR4 and other members of TLR family, initiating cascade of inflammatory reactions via activation of molecular pathways like Wnt and NF-κB, promoting production of inflammatory like cytokines IL-6 and TNF- α (Dapito et al., 2012). These TLR4 type receptors are found on multiple cell types in the liver, including hepatocytes, stellate cells, and Kupffer cells, that lead to changes in proliferation, fibrosis, and immune regulation (Yu and Schwabe, 2017; Yu et al., 2010). Fox et al. observed that upon *H. Hepaticus* infection, mice had increased promotion of tumorigenesis in the liver and lower bowel. The group observed activation of NF-κB pathway, which increased T helper 1 (Th1) immune response in both liver and colon. Moreover *H. Hepaticus* infection activated Wnt/β-catenin pathways, which led to cell proliferation as well as poor clearance of damaged hepatocytes (Fox et al., 2010).

Nutrients in food products can directly interact with gut microbiome, altering its composition. An emerging biomarker for some cancers is trimethylamine N-oxide (TMAO), a secondary metabolite produced upon red meat consumption. L-carnitine and choline from meat are metabolized to trimethylamine (TMA) by gut bacteria, this is absorbed and goes to the liver via the portal vein, where it is metabolized into TMAO. There are several lines of evidence linking elevated TMAO production with carcinogenesis and cardiovascular and other NCD (Liu et al., 2018; Zmora et al., 2019; Li et al., 2021a). It has been reported that TMAO production alters the population of the mucin-degrading bacteria *Akkermansia muciniphila* in the gut lumen, resulting in disruption of the protective mucous layer that covers intestinal epithelial cells (Ijssennagger et al., 2015; Zhu

et al., 2016). TMAO was also found to be elevated in the serum of CRC patients, and the level of TMAO correlated inversely with patient prognosis, higher levels translating into less survival.

A recent population-based study on 671 patients with primary liver cancer (PLC), demonstrated that TMAO serum level was significantly elevated in patients with PLC compared to healthy controls (Liu et al., 2018). Interestingly, TMAO level was significantly decreased by reducing consumption of animal-based products (Oellgaard et al., 2017; Nowinski and Ufnal, 2018). A cohort study based in Finland showed that TMAO level positively associated with development of aggressive prostate cancer (Mondul et al., 2015). Additional epidemiological studies demonstrated the association of TMAO with liver cancer and CRC (Cho et al., 2017; Guertin et al., 2017). It is still unclear how exactly TMAO modulates carcinogenic processes. A possible mechanism that TMAO production enhances the activation of the Wnt and NF-κB signaling pathways, promoting a pro-inflammatory environment (Xu et al., 2015; Chan et al., 2019).

Bile acid homeostasis is also one of the key components of normal liver and intestine functioning. Bile acids that escape the absorption by the small intestine, travel to the colon, where they undergoes conversion into secondary bile acids. Interestingly secondary bile acid is increased in CRC and HCC patients, as well as the bacteria metabolizing the secondary bile acids (Modica et al., 2009). Large amounts of secondary bile acids can reach systemic circulation via portal vein, leading to liver inflammation (Wang et al., 1999; Zhou et al., 2020). The secondary bile acid deoxycholic acid can induce ROS mediated DNA damage and alter hepatic stellate cell phenotype resulting in IL-6 and IL-1beta production. One of the key components in bile acid homeostasis is farnesoid X receptor (FXR) (Yoshimoto et al., 2013). FXR belongs to the nuclear receptor family, that are mainly expressed in liver, kidneys, and intestine. Different types of bile acids, including taurocholic acid (TCA), deoxycholic acid (DCA) and cholic acid (CA), act as ligand for FXR, this interaction leads to the suppression of bile acid production. In cases of FXR deactivation, the bile homeostasis is disrupted resulting in continuous production of bile acids (Modica et al., 2009; Gonzalez et al., 2016).

Ma et al., has recently demonstrated that increase in natural killer T cells population inhibits the tumor progression in liver (Ma et al., 2018). It was observed that increase in Clostridium Cluster XIV abundance promoted secondary bile acid production, inhibited NKT cells, consequently leading to the progression of liver tumorigenesis. By administrating vancomycin antibiotic treatment researchers were able to deplete Gram-positive bacterial population in mice microbiome. Antibiotic treatment allowed reduced production of secondary bile acid and an increase in the translocation of CXCL16 ligand from the gut to the liver. CXCL16 is the key regulator of liver sinusoidal endothelial cells, by elevating the levels of CXCL16 researchers were able to improve the liver barrier from the gut circulated blood. This evidence supports the association between gut microbiome composition and liver homeostasis. Microbiome composition influences every organ and system in the entire body, however the majority of studies focused on specific microbes out of entire microbial populations, perhaps limiting the understanding of the microbial activity in certain diseases. Therefore, taking more systematic approach and trying to mimic entire gut microbiome signatures may unravel new avenues in cancer research.

ALCOHOL—MOLECULAR MECHANISMS OF LOCAL AND SYSTEMIC EFFECTS

Alcohol use is common worldwide across most dietary patterns. Estimates from an international WHO survey indicate that the mean lifetime prevalence of alcohol use in all countries is 80%, with a range of 3.8–97.1%. The combined average population lifetime prevalence of alcohol use disorders is 8.6%, ranging from 0.7% in Iraq to 22.7% in Australia. As of 2016, the WHO estimated that 2.3 billion people were current drinkers and 283 million people (5.1% of adults) had alcohol use disorder. Since alcohol use is so common, most studies on the effect of specific diets on the intestinal microbiome likely include both alcohol and non-alcohol users. Approximately 4% of all cancers are caused by the overconsumption of alcohol, primarily including cancers of the upper aerodigestive tract, liver, colorectum, and breast. Alcohol has a myriad number of toxic and proinflammatory effects (Rumgay et al., 2021) that are beyond the scope of this review, we will focus here on microbiome-related changes.

Mouse models have revealed profound changes in intestinal barrier function induced by chronic alcohol use. The first changes observed were increased intestinal bacterial overgrowth and altered microbiome profiles (Yan et al., 2011). This is also associated with decreased expression of intestinal bactericidal c-type lectins Reg3b and Reg3g, which help regulate luminal bacteria (Yan et al., 2011). Interestingly, treatment with prebiotics could help restore Reg3g levels, reduce bacterial overgrowth, and decrease steatohepatitis. Alcohol use for 8 weeks results in reduced intestinal barrier function and increased intestinal inflammation characterized by an increased number of inflammatory cells expressing tumor necrosis factor alpha (TNF-a) (Chen et al., 2015). This is accompanied by translocation of microbial products to the liver and increased steatohepatitis.

Studies of the effect of alcohol on the microbiome in humans have focused on subjects with moderate to heavy alcohol use, with or without concomitant liver disease (**Figure 4**). With moderate alcohol and before the development of significant liver disease, initial changes include an increase in the numbers of bacteria cultured from the small intestine, similar to what is observed in mouse models of alcohol feeding. Further studies have shown additional qualitative changes in 16S rRNA analysis of bacterial species, including a relative increase in *Proteobacteria* and a decrease in *Bacteriodaceae*, however overall alpha diversity remains stable. Increasing burden of alcohol use with concomitant liver fibrosis or cirrhosis results in further changes in the microbiome, resulting in major metabolic consequences affecting intestinal cells and systemic pathways (Fairfield and Schnabl, 2021). Analysis of stool specimens of

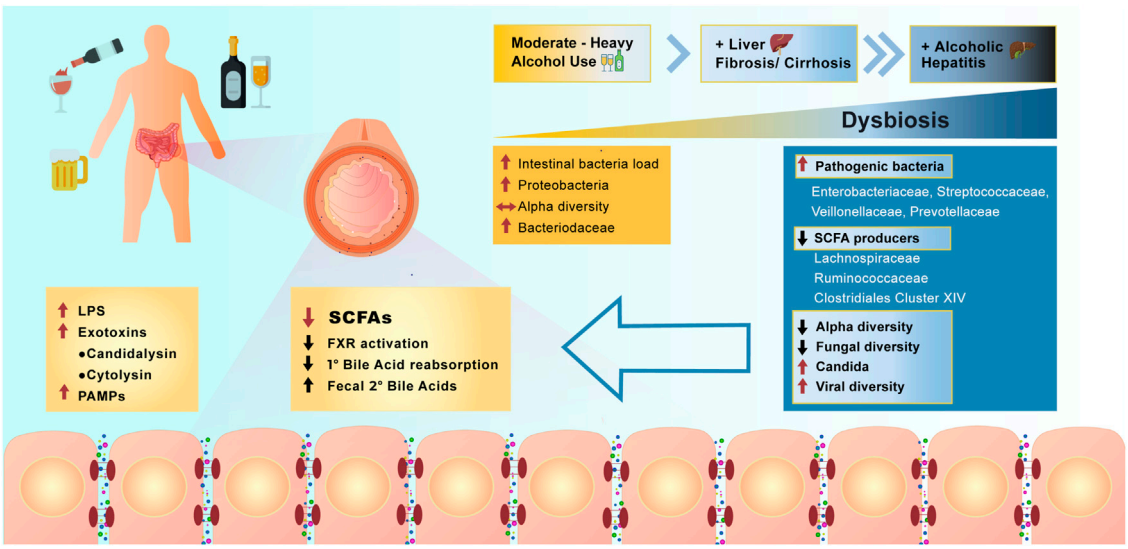

FIGURE 4 | Effect of alcohol overconsumption on the gut microbiome and intestinal homeostasis. Over consumption of alcohol positively correlates with the severity of manifested dysbiosis in the lower bowel and occurrence liver diseases. Dysbiosis resulted from alcohol overconsumption, leads to the increase in abundance of pathogenic bacteria, increase in viral diversity, production of LPS, secondary bile acids and activation of farnesoid X receptor (FXR). Alcohol overconsumption also downregulates production of SCFAs, results in decreased alpha and fungal diversity and decrease reabsorption of primary bile acids (PAMPs = pathogen associated molecular patterns).

patients with cirrhosis and alcoholic hepatitis demonstrate a decrease in SCFA producing bacteria, with an increase in potentially pathogenic bacteria, such as *Enterovaeteriaceae, Streptococcaceae, Veillonellaceae,* and *Prevontellaceae*. There is an overall decrease in alpha diversity in luminal bacteria. With the most severe form of alcoholism and liver disease, alcoholic cirrhosis and alcoholic hepatitis, further reductions in fungal diversity with an overgrowth of *Candida* species has been demonstrated (Yang et al., 2017a). In patients with alcoholic hepatitis these changes are accompanied by an increase in the viral microbiome diversity compared with subjects with alcohol use disorder and non-alcoholic controls (Jiang et al., 2020). The viral diversity is in large part related to increases in *Escherichia-*, *Enterobacteria-*, and *Enterococcus* bacteriophages and an increase in an often underappreciated non-bacterial component of the microbiome—mammalian viruses, such as *Parvoviridae* and *Herpesviridae*. In contrast, decreased viral diversity was found in patients with non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH)-cirrhosis, indicating that increased fecal viral diversity is unique to the effects of alcohol and/or alcohol-related liver disease (Lang et al., 2020).

The changes in the microbiome associated with heavy alcohol use have been correlated with worse medical outcomes in individual patients. Lang et al., have shown that patients with alcoholic cirrhosis have reduced fungal diversity and an overgrowth of *Candida* species compared with healthy individuals and non-alcohol-related cirrhosis. In the alcoholic cirrhosis subjects, this is accompanied by increased systemic fungal antigens and higher risk for death (Lang et al., 2020). These findings are duplicated in mouse models of alcoholic hepatitis with demonstration of increased circulating fungal antigens and increased liver inflammation via stimulation of C-type lectin like receptor CLEC7A on hepatic Kupffer cells resulting in cytokine IL-1b release (Yang et al., 2017b; Yang et al., 2019). Furthermore, qualitative differences in fecal viral taxa are associated with worse 90-days survival in patients with alcoholic hepatitis (Jiang et al., 2020). Currently there is much interest in whether interventions targeting the specific fungal and virome changes associated with poor prognosis can improve patient outcomes. To demonstrate the feasibility of this approach, Duan et al. showed that patients with alcoholic hepatitis have increased abundance of cytolysin-positive *Enterococcus faecalis*, which correlates with increased mortality (Duan et al., 2019). Germ free mice colonized with cytolysin-positive *E. faecalis* from patients with alcoholic hepatitis had worse liver disease in a model of alcohol induced liver disease. Mice were treated with bacteriophages that target and destroy cytolysin-positive *E. faecalis* and they showed that this could ameliorate alcohol induced liver disease in mice. Further studies targeting specific pathogenic bacteria implicated by metagenomics in human diseases are needed.

THE NEXT STEP—GENETIC EPIDEMIOLOGY AND POPULATION GENOMICS COULD REVEAL POSSIBLE LINKS BETWEEN HOST GENETICS AND MICROBIOME

In addition to diet, host genetics is an important factor that influences the gut microbiome, predisposing individuals to microbiome-modulated pathologies. A genetic bias for microbiome composition is suggested by studies of association

and heritability of certain bacterial species in related individuals. Monozygotic twins have higher correlation of microbiome signatures than dizygotic twins (Goodrich et al., 2016). Host genetic factors can influence the microbiome in mice (Benson et al., 2010). Genome-Wide Association Studies (GWAS) of the microbiome have revealed only limited links to host genetics. The detection of only a small number of associated loci is primarily due to inter-individual variability, microbiome heterogeneity, and potential overshadowing of host-genetics factors by the contributions of the diet, environment, and lifestyle to microbiome composition (Kurilshikov et al., 2021). While the link between host genetics and microbiome diversity may be weak, genetics can modulate specific microbiome-diet, microbiome-environment, and microbiome-lifestyle interactions in ways that lead to specific disease outcomes which are traceable to specific molecular interactions between host- and microbiome-derived components. Well-controlled studies, especially those utilizing homogenous populations, may be able to reveal these impacts more easily. Despite these limitations, meta-analyses of GWAS have proven effective in identifying specific molecular mechanisms connecting the microbiome to numerous diseases, including cancer. In particular, TMAO, the microbiome-generated metabolite of red meat and fat previously connected with cardiometabolic risks, was additionally linked to CRC risk by this approach (Xu et al., 2015). Aside from colon cancer, other cancers, including bladder and prostate cancer, show clear associations with the microbiome in GWAS. These signals were identified through correlating significant disease-associated genetic variants from GWAS with microbiome data. The putative causative relationships do not just go "From microbiome to cancer", as they also go the other way around: "From cancer to microbiome." Atrial fibrillation, chronic kidney disease, and prostate cancer, as predicted by host genetics, have potential causal effects on the abundance of specific gut microbiome components (Xu et al., 2015). This bidirectionality of the cancer-microbiome axis is important to take into account, because—while the idea that the microbiome can regulate, or influence disease pathogenesis is more intuitive and widely accepted—it means that a disease state can also influence the microbiome (with potential downstream feedback effects on the disease).

Given the abundance of long non-coding RNA (lncRNA) genes compared to conventional protein-coding genes in humans, it is expected that GWAS-driven interrogation of the microbiome-disease connection will uncover lncRNA contributors to this class of regulatory phenomena. Accounting for well over half of human genes, lncRNAs are now increasingly understood to be fundamental and essential to all normal cellular and developmental processes, as well as all human diseases, in which they have been examined (Hong et al., 2020). There is already a precedent: non-coding genetic variants from GWAS, including those in a lncRNA gene, have been associated with defined phyla within the microbiome (Hughes et al., 2020). Mouse microbiome models demonstrate that lncRNAs can be microbiome targets in regulatory networks, building a case for microbiome-lncRNA interactions, with functions mediated in part through the role of lncRNA in immunity (Allen and Sears, 2019) (Hong et al., 2020).

A recent study of 33 CRC patients has revealed a connection between the high abundance of *F. nucleatum* and elevated glucose metabolism: a spectrum of upregulated lncRNA in CRC cells infected with *F. nucleatum* that had increased glycolysis rate compared to uninfected CRC cells. The ENO1-IT1 lncRNA, a positive *cis*-regulator of the pro-oncogenic, pro-glycolysis ENO1 gene is the most overexpressed lncRNA correlated with glycolysis. Upon *F. nucleatum* infection, the transcription factor SP1 promotes the expression of ENO1-IT1 lncRNA through the ENO1 signaling pathway. ENO1-IT1 eventually forms a complex with KAT7 guide protein that increases the histone acetylation leading to the CRC progression (Hong et al., 2020). Chen at el., recently observed that *F. nucleatum* directly drives the overexpression of long non-coding RNA Keratin7-antisense (KRT7-AS) and Keratin7 (KRT7) in human CRC cells. *In vitro* and *in vivo* analysis showed that *F. nucleatum* activates the NF-κB signaling pathway, which upregulates KRT7-AS (a positive regulator of CRC metastasis) which in turn, serves as an activator of KRT7, stimulating cell migration (**Figure 2**) (Chen et al., 2020b).

Beyond specific case studies of human microbiome-lncRNA interactions, mouse models suggest that whole-genome lncRNA expression profiles distinguish between mouse gut microbiomes better than protein-coding gene mRNA signatures (Liang et al., 2015)– a key finding that cements the importance of lncRNAs as key components of the microbiome-disease axis and justifies further investigation of the molecular and functional underpinnings of their global relationship to the microbiome-cancer interface. The fundamental contribution of lncRNAs to cancer is, by now firmly established (Allen and Sears, 2019). Oncogenic lncRNAs have an array of roles linked to microbiome-related metabolites in cancer. *Fusobacterium* promotes the EMT transition in cancer through a long non-coding RNA gene that also serves as a microRNA host gene (Zhang et al., 2020). Nevertheless, rather than serving solely as drivers of disease progression, ncRNAs, if arising from disease-protective microbiomes and/or under disease-risk-reducing dietary conditions, may have protective roles. This possibility is consistent with the observed downregulation of several microRNAs in *F. nucleatum*-rich tumors in patients with recurrent CRC (Allen and Sears, 2019).

Concordant with this hypothesis, 30 lncRNAs have recently been highlighted to be involved in the inhibition of CRC progression by sodium butyrate (NaB) (Xiao et al., 2018). Butyrate-responsive lncRNAs have also been identified in a lung cancer model (Xi et al., 2021). RAD51-AS1, the endogenous antisense transcript that overlaps and modulates the tumor suppressor RAD51, regulates lactate in CRC (Li et al., 2021b). Although direct links with the microbiome have not yet been proven for these lncRNAs, it is reasonable to posit that, because the microbiome is tightly coupled to SCFA and lactate metabolism, so are these lncRNAs, given their direct interactions with these metabolites and pathways.

The specific molecular mechanisms that are responsible for, and mediate, the existence of multiple directional nodes joining microbiome-derived and host RNA-derived edges in the microbiome-cancer regulatory network should be characterized

in future work, so that they can be rationally targeted for therapeutics.

FUTURE DIRECTIONS

In this review we discussed recent studies that highlight role of microbiome, in particular abundance of certain pathogenic bacteria including *F. nucleatum* and *pks*$^{+}$ *E. coli*, that drive the process of carcinogenesis through induction of DNA damage, overexpression of oncogenes like Myc and triggering production of highly pro-inflammatory agents like IL-6. This review allows the reader to clearly grasp the main metabolic and molecular events that lead towards intestinal and systemic carcinogenesis upon adherence to low fiber, high fat Western dietary pattern. To compare, this review describes recent studies that uniformly demonstrate plant-based diet as a protective factor from set of metabolic conditions including obesity and from various types of cancer, including CRC. Due to the limitations of space the review is primarily focused on human studies, and extensive review of many animal studies related to microbiome and cancer are not included. The protective properties of plant-based diet are associated with the increased production of SCFAs by the commensal bacterial in the gut. The SCFAs are one of the key regulators of immune tolerance, improved gut barrier junctions, and the intestinal clearance. The depletion of SCFA producers or SCFA receptors result in adverse effects including high-grade inflammation and poor cancer prognosis. In contrast, elevating the abundance of SCFA producing bacteria in the gut microbiome through the dietary intervention, results in downregulation of inflammation and inhibition of tumor microenvironment. Although microbiome-cancer axis has been extensively studied in the past three decades, there is tremendous amount of vital information that is yet to be discovered. This review indicates that diet is the major regulator of gut microbiome and can act as a first line preventive measure from developing carcinogenic conditions. Moreover, major bacteria and metabolites that are associated with detrimental effects of Western diet on intestinal and systemic homeostasis that were discussed in this review, can serve as a potential therapeutic targets in a variety of diseases, especially cancer. Diet, as a key component of our life should not be regarded only from the nutritional point of view. Being selective, consistent, and conscious about the personal diet may prevent a one's spectrum of health conditions and improve the quality of life.

Growing numbers of studies are focusing on manipulation of gut bacterial composition to enhance the efficacy of anti-cancer therapies. Several recent studies have shown significant improvement in anti-CTL4 and anti-PD1 based therapies, via alternating gut microbiome composition towards certain commensal bacterial species, including *Bifidobacterium species* (Sivan et al., 2015; Vetizou et al., 2015). Using dietary intervention, to enhance certain commensal bacteria population, can be a supportive measure in vast array of diseases, including cancer. Personalized targeted microbial therapy is one of the most promising novel therapeutics. Currently used antibiotics are of a broad spectrum and are detrimental for both commensal and pathogenic bacteria in the gut microbiome. In situations like cancer, antibiotic established dysbiosis is a potential threat to the successful cancer therapy. Using novel bioinformatic tools and established metagenomic and metabolomic data, we can now begin to create a more personalized approach to cancer therapy and prevention. Soon we will be able to monitor and shift microbial signatures to high SCFA and anti-inflammatory metabolite producers, and target specific harmful bacteria using selective antibiotics, bacteriophages, or competitor probiotic species. Numerous randomized trials of specific gut microbiome therapeutics will be needed to expand and prove these concepts.

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

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.725821/full#supplementary-material

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REVIEW
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Microbiome-Mediated Immune Signaling in Inflammatory Bowel Disease and Colorectal Cancer: Support From Meta-omics Data

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Chronic intestinal inflammation and microbial dysbiosis are hallmarks of colorectal cancer (CRC) and inflammatory bowel diseases (IBD), such as Crohn's disease and ulcerative colitis. However, the mechanistic relationship between gut dysbiosis and disease has not yet been fully characterized. Although the "trigger" of intestinal inflammation remains unknown, a wealth of evidence supports the role of the gut microbiome as a mutualistic pseudo-organ that significantly influences intestinal homeostasis and is capable of regulating host immunity. In recent years, culture-independent methods for assessing microbial communities as a whole (termed meta-omics) have grown beyond taxonomic identification and genome characterization (metagenomics) into new fields of research that collectively expand our knowledge of microbiomes. Metatranscriptomics, metaproteomics, and metabolomics are meta-omics techniques that aim to describe and quantify the functional activity of the gut microbiome. Uncovering microbial metabolic contributions in the context of IBD and CRC using these approaches provides insight into how the metabolic microenvironment of the GI tract shapes microbial community structure and how the microbiome, in turn, influences the surrounding ecosystem. Immunological studies in germ-free and wild-type mice have described several host-microbiome interactions that may play a role in autoinflammation. Chronic colitis is a precursor to CRC, and changes in the gut microbiome may be an important link triggering the neoplastic process in chronic colitis. In this review, we describe several microbiome-mediated mechanisms of host immune signaling, such as short-chain fatty acid (SCFA) and bile acid metabolism, inflammasome activation, and cytokine regulation in the context of IBD and CRC, and discuss the supporting role for these mechanisms by meta-omics data.

Keywords: gut microbiome, inflammatory bowel diesases, colorectal cancer, metagenomics, metaproteomics, metabolomics

INTRODUCTION

The community of microorganisms that inhabit the human gastrointestinal (GI) tract and their collective genetic material are referred to as the gut microbiome. The composition and function of the gut microbiome have been widely implicated in human health and disease, particularly in GI diseases such as inflammatory bowel diseases (IBD) and colorectal cancer (CRC), where specific alteration of the gut microbiome is associated with pathology (Sobhani et al., 2011; Knox et al., 2019b; Kaplan et al., 2019). The global incidence of IBD, including component diseases Crohn's disease (CD), ulcerative colitis (UC), and IBD-unclassified (IBD-U), has risen considerably in recent decades (Molodecky et al., 2012; Kaplan et al., 2019; El-Matary and Bernstein, 2020). Increasing rates of pediatric-onset IBD in North America are of particular concern not only because disease in younger patients is more extensive and can lead to a range of developmental issues (Carroll et al., 2019) but also because of associated long-term cancer risk (Akimoto et al., 2020; El-Matary and Bernstein, 2020). Chronic intestinal inflammation is a precursor to CRC, and IBD is an established risk factor for developing both early- and late-onset CRC (Akimoto et al., 2020; Gausman et al., 2020).

Recent advances in culture-independent techniques for studying microbial communities have enabled extensive characterization of the healthy human gut microbiome (Human Microbiome Project Consortium, 2012; Méndez-García et al., 2018) and provide context for the host-microbiome interactions within the gut microenvironment. Although perturbations in the microbial community composition—dysbiosis—have been characterized in both IBD and CRC, the cause and effect relationship between inflammation and dysbiosis remains unclear. A variety of other host-mediated factors such as genetic susceptibility, epigenetics, diet, antibiotic use, and smoking status have been associated with IBD or CRC risk or both (Glória et al., 1996; Jostins et al., 2012; Kaplan et al., 2019; Lorzadeh et al., 2021), yet no exact trigger has been identified. The gut microbiome is thought to be directly implicated in the etiopathogenesis of both IBD and CRC (Sellon et al., 1998; Peloquin and Nguyen, 2013); current theories hypothesize that alterations in the normal gut microbiome, caused by some environmental exposure [for example, antibiotic use (Ungaro et al., 2014)], can trigger an inflammatory immune response that persists in the genetically susceptible host (Yang and Jobin, 2017; Knox et al., 2019b; Kaplan et al., 2019; Szamosi et al., 2020).

The intestinal epithelium is a crucial interface for host-microbiome interactions. In a healthy gut, the host's immune system must be able to recognize and tolerate commensal organisms while retaining its ability to defend against pathogens. For example, microbial taxa that are considered protective stimulate $CD4^+$ T regulatory (T_{reg}) cell proliferation and maintenance of gut immune homeostasis (Atarashi et al., 2011; Knox et al., 2019b), whereas pathogenic organisms are recognized by Toll-like receptors (TLRs) and NOD-like receptors (NLRs) on $CD4^+$ T cells, resulting in a coordinated adaptive immune response (Himmel et al., 2008). Similarly, the gut microbiota responds to host immune activation and local inflammation by altering gene expression and metabolite production (Becattini et al., 2021). Gathering a better understanding of this complex, bi-directional signaling is the basis for untargeted microbiome functional characterization.

Techniques for characterizing entire microbial communities and their physiological contributions (termed meta-omics) have now grown beyond taxonomic identification and genome mapping as studied via metataxonomics and metagenomics, respectively, into new fields of research (**Figure 1**). Metatranscriptomics, metaproteomics, and metabolomics are omics approaches that allow for further characterization of the gut microbiome in health and disease (**Figure 1**). Metagenomics describes the genetic content of a microbial community within a sample (typically stool or intestinal biopsy in the case of the GI microbiome), whereas metatranscriptomics utilizes reverse transcription to evaluate gene expression patterns from microbial messenger RNA (mRNA). Both techniques involve shotgun sequencing of nucleic acids isolated from a biological specimen and allow for prediction of downstream functional activity (**Figure 1**). Metaproteomics generally uses liquid chromatography with tandem mass spectrometry (LC-MS/MS) to isolate and quantify proteins, which are then evaluated to identify potential biomarkers of disease. Lastly, metabolomics can be used to search for a wide range of biomarkers, including amino acids, fatty acids, sugars, and vitamins. Using this approach, metabolites are detected through either nuclear magnetic resonance (NMR) or MS coupled with chromatography, the latter offering a wider range of detection and higher sensitivity. Stool is frequently used as a proxy for the luminal GI microbiome; however, the mucosal-associated microbiome—captured through endoscopic biopsy—will differ from that of the lumen and can provide site-specific evidence. Likewise, many microbiome studies focus on the bacterial component of the microbiome as a proxy for the entire community, which additionally contains eukaryotes and/or viruses. Meta-omics approaches may be used individually or in combination to study the microbiome. The application of multiple techniques to samples from the same cohort yields correlated functional profiles (Lloyd-Price et al., 2019), supporting the usefulness of microbial sequence data for downstream prediction of functional activity (**Figure 1**). Uncovering particular taxonomic and metabolic contributions in the context of IBD and CRC using these approaches gives insight into the metabolic microenvironment of the gut and the role of microorganisms within that environment. The potential of stool meta-omics techniques as non-invasive screening and detection tools is especially attractive. While this review focuses on microbiome-mediated signaling specifically, meta-omics can also be used to profile and predict disease-specific, location-specific, or longitudinal changes in microbial communities and many such studies have provided valuable insights into microbial community dynamics (Gevers et al., 2014; Hall et al., 2017; Schirmer et al., 2018).

Investigators face several challenges when using meta-omics data for microbiome research as well as for disease investigation in a clinical setting. Prior to data generation there are a number of

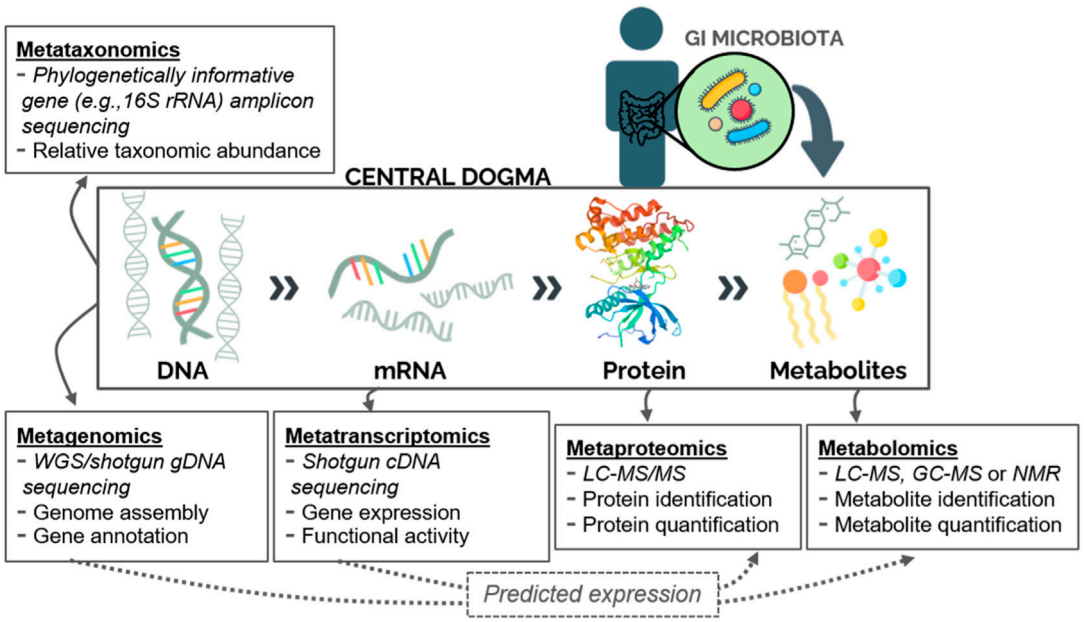

FIGURE 1 | Meta-omics techniques for studying the human gut microbiome. Microbial communities can be characterized based on their collective gene content (metataxonomics or metagenomics), gene transcripts (metatranscriptomics), protein pool (metaproteomics), or metabolite pool (metabolomics). Additionally, sequence data can be used to predict downstream expression of proteins or metabolites (dashed arrows). Abbreviations: WGS: Whole-genome sequencing; LC: Liquid chromatography; MS: Mass Spectrometry; GC: Gas chromatography; NMR: Nuclear magnetic resonance.

ways in which bias may be introduced during sample collection and storage (Alberti et al., 2014; Reck et al., 2015; Neuberger-Castillo et al., 2021). Furthermore, due to the dynamic and compositional nature of microbiomes, many of the techniques described above require large sample sizes and result in even larger datasets that must be properly assessed for quality before their interpretation (Zhang et al., 2021a). Accurate reference-based taxonomic assignment of sequences depends on the quality of database that is used and achieving statistical significance, in differential expression analysis for example, can be challenging (Zhang et al.,2021b). There is considerable interest in evaluating individual GI microbiomes within a clinical setting to aid screening and diagnosis using a personalized approach (Knox et al., 2019a). However, many of the techniques discussed above require a substantial computational effort and corresponding technical expertise, making them difficult to implement routinely in a clinical setting. Readers are referred to the following discussions regarding specific challenges associated with meta-omics techniques (Wishart 2016; Quince et al., 2017; Shakya et al., 2019; Zhang et al., 2019; Krassowski et al., 2020).

META-OMIC EXPLORATIONS OF THE GUT MICROBIOME IN IBD AND CRC REVEAL COMMON PATTERNS OF METABOLIC DYSREGULATION

The implication of the gut microbiome in CRC and IBD etiology has prompted many researchers to employ meta-omics to study microbiome function and activity (Franzosa et al., 2019; Lloyd-Price et al., 2019; Ocvirk et al., 2020). Indeed, IBD serves as a model disease for the integrative human microbiome project (iHMP), which began in 2014 and collects host and microbiome-associated data using multiple meta-omics strategies (The Integrative HMP (iHMP) Research Network Consortium, 2014; Lloyd-Price et al., 2019). Results from the iHMP and other studies reporting statistically significant changes in the microbiome between health and disease are shown in **Table 1**. Extensive characterization of CRC-associated (Wirbel et al., 2019) and IBD-associated (Lloyd-Price et al., 2019) microbiomes has demonstrated that although the dysbiotic communities differ on a taxonomic level (*i.e.,* biomarker species), there are similarities between the two diseases at the functional level (*e.g.,* depletion of butyrate-producers, bile acid dysregulation). Consequently, in this review we restrict our discussion to the functional potential and activity of disease-associated microbiomes, rather than focusing on their taxonomic composition.

Meta-omics studies have revealed that microbial dysbiosis in IBD and CRC goes beyond taxonomic imbalance. While there are discrepancies regarding the differential expression of specific proteins or metabolites between cohorts, the research collectively paints a picture of systemic dysregulation of multiple microbe-mediated compounds in disease. For example, amino acid and fatty acid metabolism are commonly dysregulated in IBD or CRC compared to healthy controls (**Table 1**), although the pattern of dysregulation is inconsistent. Other biochemical classes and pathways significantly dysregulated in a subset of studies include bile acids, vitamins B3 and B5, and sphingolipids. Interestingly, a

TABLE 1 | Meta-omic studies reporting statistically significant changes in the GI microbiome in CRC or IBD. Abbreviations: healthy controls (HC); irritable bowel syndrome (IBS); high-resolution magic angle spinning nuclear magnetic resonance (HR-MAS NMR); gas chromatography (GC); capillary electrophoresis (CE); ion cyclotron resonance Fourier transform mass spectrometry (ICR-FT/MS); time-of-flight mass spectrometry (ToFMS); liquid chromatography (LC); high performance LC (HPLC); ultra-performance LC (UPLC); medium-chain fatty acids (MCFA); long-chain fatty acids (LCFA).

Cohort type (N)	Sample type(s)	Method	Increased in disease	Decreased in disease	References
CRC					
CRC (31): Colorectal tumour biopsy vs. normal tissue	Mucosal biopsy	Metabolomics: HR-MAS NMR and GC/MS	Choline-containing compounds, taurine, scyllo-inositol, lactate, phosphocholine, phosphate, L-glycine, 2-hydroxy-3-methyl valerate, L-proline, L-phenylalanine, palmitic acid, margaric acid, oleic acid, stearic acid, uridine, 11-eicosenoic acid, propyl octadecanoate, cholesterol	Lipids, polyethylene glycol, glucose, fumarate, malate, mannose, galactose, 1-hexadecanol, arachidonic acid	Chan et al. (2009)
CRC (11) vs. HC (10)	Stool, mucosal biopsy	Metabolomics: GC/ToFMS	Uracil, uridine, proline	Fructose, linoleic acid, nicotinic acid, glucose, galactose, 3-phosphoglycerate, citric acid, inosine, creatine	Phua et al. (2014)
Meta-analysis of CRC (386) vs. tumor-free controls (392)	Stool	Metagenomics: WGS	Metabolic modules: amino acid degradation, organic acid metabolism, glycoprotein degradation, bile acid conversion	Metabolic modules: carbohydrate degradation	Wirbel et al. (2019)
CRC stage 0 (73); stage I/II (111); stage III/IV (68); multiple polypoid adenomas (MP, 67); vs. HC (251); normal with history of surgery (HS, 34)	Stool	Metagenomics: WGS, Metabolomics: CE/ToFMS	Taxonomy: sulfide-producing species – *Desulfovibrio vietnamensis, D. longreachensis, Bilophilia wadsworthia* Metabolites: deoxycholate (MP vs. HC); glychocholate, and taurocholate, branched-chain amino acids, phenylalanine, tyrosine and glycine (S0 vs. HC); serine (SIII/IV vs. HC), Pathway gene abundance: amino acid metabolism, sulfide production, phenylalanine and tyrosine biosynthesis (all CRCs vs. HC); sulfate reductase (*dsrA*), cofactor and vitamin biosynthesis, lysine biosynthesis and degradation, methane metabolism (SIII/IV vs. HC)	Taxonomy: butyrate-producing species - *Lachnospira multipara, Eubacterium eligens,* Pathway gene abundance: tryptophan biosynthesis (SIII/IV vs. HC)	Yachida et al. (2019)
Healthy Alaskan Natives (high-risk group for CRC) (32) vs. Healthy Rural Africans (low risk for CRC) (21)	Stool, urine	Metataxonomics: 16S rRNA sequencing, Metabolomics: ^{1}H-NMR spectroscopy, GC, HPLC-MS	*Enriched in high-risk population:* Actinobacteria, Verrumicrobia, Lachnospiraceae, *Bifidobacterium spp., Escherichia-Shigella spp.,* choline, formate, cholate, chenodeoxycholate, deoxycholate, conjugated bile acids, nicotinamide/niacin metabolites	*Enriched in low-risk population:* Ruminococcaeceae, Prevotellaceae, *Prevotella 9,* Ruminococcaceae, *Succinivibrio, Eubacterium coprostanoligenes*, amino acids, purines[a], pyrimidines[a], butyrate, propionate, nicotinate	Ocvirk et al. (2020)
CRC (14) vs. HC (14)	Stool	Metaproteomics: LC-MS/MS	Desulfobacterales, *Methanobacteriaceae, Sporolactobacillaceae, Bacteroides fragilis, Peptostreptococcus anaerobius,* DNA replication, recombination, and repair proteins, iron intake and transport proteins, superoxide dismutases	*Sutterellaceae, Epulopiscium, Gordonibacter,* NADH:flavin oxidoreductases/NADH oxidases, energy production and conversion proteins, amino acid transport and metabolism, coenzyme transport and metabolism, lipid transport and metabolism, translation machinery, cell wall, membrane, and envelope biogenesis, cell motility, post-translational modification, protein turnover, and chaperones, inorganic ion transport and metabolism	Long et al. (2020)
IBD					
Identical twin pairs (N = 17 pairs): discordant colonic CD*(4p);	Stool	Metabolomics: ICR-FT/MS	Bile acid metabolism[b] (glycocholate, glycochenodeoxycholate taurocholate, Trihydroxy-6β-	Arachidonic acid metabolism/ prostaglandins[a,b] (PGs; PGF2a)	Jansson et al. (2009)

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TABLE 1 | (*Continued*) Meta-omic studies reporting statistically significant changes in the GI microbiome in CRC or IBD. Abbreviations: healthy controls (HC); irritable bowel syndrome (IBS); high-resolution magic angle spinning nuclear magnetic resonance (HR-MAS NMR); gas chromatography (GC); capillary electrophoresis (CE); ion cyclotron resonance Fourier transform mass spectrometry (ICR-FT/MS); time-of-flight mass spectrometry (ToFMS); liquid chromatography (LC); high performance LC (HPLC); ultra-performance LC (UPLC); medium-chain fatty acids (MCFA); long-chain fatty acids (LCFA).

Cohort type (N)	Sample type(s)	Method	Increased in disease	Decreased in disease	References
discordant ileal CD*(2p); concordant ICD*(2p); concordant CCD*(2p) vs. HC (7 pairs), *in remission			cholanate), amino acid metabolism[b], tyrosine[b], tryptophan (ICD only), phenylalanine (ICD only), fatty acid biosynthesis (ICD only; oleic acid, stearic acid, palmitic acid, linoleic acid, arachidonic acid), Urea cycle[a,b], vitamin B6 metabolism[b]		
CD (83); UC (68); pouchitis (13) vs. HC (40)	Stool	Metabolomics: GC-MS	Styrene[c]	MCFAs, hexanoate[b], protein fermentation metabolites	De Preter *et al.* (2015)
Paediatric IBD in remission -CD (26); UC(10) vs. healthy 1st-degree relatives (54)	Stool	Metataxonomics: 16S rRNA sequencing, Metabolomics: UPLC/ToFMS	Enterobacteriaceae, cholate[b], conjugated and sulphated bile acids[b], taurine[b], tryptophan[b], adrenate[b]	Stercobilin[b], acetyl-glutamic acid[b], boldione[b], estradiol[b], androstenedione[b], azelaic acid[b]	Jacobs et al. (2016)
Paediatric IBD (newly diagnosed, treatment naive)-CD (36); UC (20); IBD-U (13) vs. endoscopic non-IBD controls (29)	Stool, blood	Metabolomics: UPLC-MS/MS	Folate and pterine biosynthesis, purine metabolism[b], amino acid metabolism, nicotinate and nicotinamide metabolism[b], urea cycle, protein biosynthesis, bile acid biosynthesis[c], sphingolipid metabolism[c], ammonia recycling[c], taurine metabolism[c], oxidation of branched-chain FAs[c], phospholipid metabolism[c], glycerolipid metabolism[c]	L-tryptophan, kynurenic acid, aspartate, threonine, asparagine, cytosine, histidine[b], taurine[b]	Kolho et al. (2017)
CD (208); UC (126); IBD-U (21); IBS (412) vs. HC (1,025)	Stool	Metagenomics: WGS	Sugar[a] degradation, succinate fermentation, Aspartate and asparagine biosynthesis, arginine biosynthesis, lysine biosynthesis[b], proline biosynthesis[c], aromatic compounds degradation, saturated FA elongation[b], specific fatty acid and lipid biosynthesis[b], thiamin salvage[c], *Bacteroides spp.,* Enterobacteriaceae[b], Bacteroidaceae	Pyruvate and mixed acid fermentation[b], general amino acid biosynthesis (see exceptions in previous column), tryptophan degradation[b], valine degradation[b], coenzyme A biosynthesis[b], coenzyme B biosynthesis, specific fatty acid and lipid biosynthesis[b], nucleotides and nucleosides biosynthesis, phosphopantothenate biosynthesis[b], *Faecalibacterium prausnitzii*[b], *Bifidobacterium longum*[b], *Roseburia hominis,* Actinobacteria, Rikenellaceae, Akkermansiaceae, Firmicutes	Vich Vila et al. (2018)
Pediatric IBD (treatment naïve): CD (25); UC (22) vs. non-IBD (24)	Mucosal-luminal interface (MLI) biopsy	Metaproteomics: MS	DNA replication, recombination, and repair proteins, defence mechanism proteins (CRISPR/Cas), cell wall, membrane and envelope biogenesis proteins, amino acid transport and metabolism, mobilome, cysteine degradation, Proteobacteria, Verrucomicrobia, Ascomycota, Spirochetes, *F. prausnitzii* strain L2-6	Cysteine biosynthesis, *Bacteroides*	Zhang et al. (2019)
CD (68); UC (53) vs. non-IBD (34)	Stool	Metagenomics: WGS, Metabolomics: LC-MS	Sphingolipids, carboximic acids[a], bile acids (cholate, chenodeoxycholate)[a], organonitrogen compounds, cholesteryl esters, phenylacetamides[b], phosphatidylcholines, α-amino acids, lactate	LCFAs, butyrate[d], propionate[d], secondary bile acids (lithocholate, deoxycholate) [d], flavonoids, indoles[a,b], cinnamic acids[a], triacylglycerols, tetrapyrroles[a,b], triterpenoids, alkyl-phenylketones, brassinolides[a,b], ergosterols[a], quinolines[a,b], vitamin D[a], stigmastanes[a], lactones, β-diketones[b], cholesterols[a], phenylbenzodioxanes, pantothenate (vitamin B5)	Franzosa et al. (2019)

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TABLE 1 | (*Continued*) Meta-omic studies reporting statistically significant changes in the GI microbiome in CRC or IBD. Abbreviations: healthy controls (HC); irritable bowel syndrome (IBS); high-resolution magic angle spinning nuclear magnetic resonance (HR-MAS NMR); gas chromatography (GC); capillary electrophoresis (CE); ion cyclotron resonance Fourier transform mass spectrometry (ICR-FT/MS); time-of-flight mass spectrometry (ToFMS); liquid chromatography (LC); high performance LC (HPLC); ultra-performance LC (UPLC); medium-chain fatty acids (MCFA); long-chain fatty acids (LCFA).

Cohort type (N)	Sample type(s)	Method	Increased in disease	Decreased in disease	References
CD (67); UC (38) vs. non-IBD (27)	Stool, colon biopsy, blood	Metagenomics: WGS, Metatranscriptomics, Metabolomics: LC-MS	Cholate[b], chenodeoxycholate[b], taurochenodeoxycholate[b], C8 carnitine[b], anti-ompc[b], calprotectin[c], adrenate, arachidonate, putrescine, taurine, *Escherichia coli, Klebsiella pneumoniae, Roseburia gnavus*[b]	Deoxycholate, lithocholate, propionate, C16:0 LPE, adipate, C20:4 carnitine, 3'-O-methyladenosine, suberate, nicotinate (B3), pantothenate (B5), *F. prausnitzii*, *Alistipes finegoldii*, *Alistipes shahii*, *Alistipes putredinis, Subdoligranulum unclassified*	Lloyd-Price *et al.* (2019)
Treatment-naive UC (18) vs. HC (14)	Mucosal biopsy	Metabolomics: GC-ToFMS, UPLC-MS	Lysophospholipids[c], acyl carnitines[c], arachidonate[c], asparagine[c], citrulline[c], dimethylarginine[c], glutamyl-L-amino acids[c], glutamate[c], kynurenine[c], L-valine[c], L-isoleucine[c], nicotinamide[c]	beta-alanine[c], creatine[c], eicosapentaenoate[c], fructose[c], glutaryl-carnitine[c], glycerol-3-phosphate[c], guanosine[c], leucylglycine[c], linoleate[c], L-glutamine[c], methylmalonyl carnitine[c]	Diab et al. (2019)

[a]*Includes derivatives of the molecule class.*
[b]*Significantly different in CD only.*
[c]*Significantly different in UC only.*
[d]*Difference not statistically significant in this cohort.*

similar pattern of metabolic dysregulation was apparent in the metabolome of a healthy CRC high-risk population (based on heritage and diet) compared to a healthy low-risk population (Ocvirk et al., 2020) (**Table 1**), suggesting that dysbiotic microbiomes are present before disease manifestation and contribute to CRC development. These shared patterns of dysregulation could indicate a shared etiology between IBD and CRC. The remainder of this review will summarize the biochemical significance of commonly dysregulated metabolites and pathways in the context of chronic inflammation and host immunity from *in vitro* and *in vivo* experimental research.

MICROBIOME-MEDIATED MECHANISMS AND IMPACT ON HOMEOSTASIS

Amino Acid Metabolism and Polyamines

Many amino acids have an essential role in host immune signaling, and some are precursors for tumour-promoting compounds such as polyamines, which are capable of modulating systemic and mucosal adaptive immunity (Rooks and Garrett, 2016; Thomas et al., 2019). Genes involved in amino acid degradation were found to be enriched in a meta-analysis of CRC metagenomic profiles (Wirbel et al., 2019), and differential abundances of specific amino acids and related pathways are prevalent in IBD and CRC meta-omics studies (**Table 1**).

Both host and microbial cells utilize the essential amino acid tryptophan. Catabolism of tryptophan by macrophages leads to suppressed T cell proliferation *in vitro* (Munn et al., 1999). Tryptophan degradation has also been shown to modulate the differentiation of T cell subsets, affecting mucosal immunity and epithelial barrier integrity (Shapiro et al., 2014). Microbial metabolism of tryptophan can result in the production of kynurenines and indole-3-aldehyde, metabolites with immunomodulatory and anti-inflammatory effects including aryl hydrocarbon receptor activation promoting T_{reg} cell development and local IL-22 production (Zelante et al., 2013). Both tryptophan and kynurenic acid were found to be depleted in newly diagnosed, treatment-naive pediatric IBD patients (Kolho et al., 2017), whereas patients with remissive IBD displayed increased levels of tryptophan in stool compared to healthy controls (Jansson et al., 2009; Jacobs et al., 2016), supporting an immunoprotective role. Decreased tryptophan degradation in CD patients has also been predicted from deep sequencing of fecal metagenomes (Vich Vila et al., 2018). In contrast, kynurenine levels were elevated in mucosal biopsy samples of treatment-naive UC patients compared to healthy controls (Diab et al., 2019), as well as in stool from late-stage CRC patients (Yachida et al., 2019). Whether these discrepancies result from differing sample types or the contradictory effects of bioactive kynurenines (Rossi et al., 2019) is not yet understood.

Polyamines putrescine, spermidine, and spermine are derived from the amino acids arginine and ornithine in bacterial and mammalian cells (Gerner and Meyskens, 2004; Pegg, 2016; Rooks and Garrett, 2016). Other polyamines, such as trimethylamine (TMA), are synthesized by bacteria from the quaternary ammonium compounds choline and carnitine (Gerner and Meyskens, 2004; Thomas et al., 2019). Polyamines are essential metabolites for both the host and the microbiota. They are present at high levels in the GI tract, where they enable rapid turnover of intestinal epithelial cells and regulate specific

eukaryotic gene expression among many other functions. For many decades now, polyamines have been implicated in tumorigenesis, including CRC (Gerner and Meyskens, 2004; Peng et al., 2021), due to their essential role in cell proliferation. Bacterial TMA production has also been linked to the consumption of red meat (a source of carnitine) and cardiovascular disease (Koeth et al., 2014).

In meta-omic CRC studies, levels of choline and choline-containing compounds (precursors of TMA) were found to be elevated in colorectal tumour biopsies relative to normal tissue from the same patients (Chan et al., 2009) and in stool from a high-risk group for CRC compared to a low-risk group (Ocvirk et al., 2020). Yachida et al. (2019) reported differentially abundant acetylated and diacetylated derivates of spermine, which have been previously proposed as tumour markers (Kawakita and Hiramatsu, 2006), at different stages of CRC. Of note, the polyamine putrescine and certain carnitine derivatives were reported to be differentially abundant in IBD compared to healthy controls (Diab et al., 2019; Lloyd-Price et al., 2019), supporting a role for polyamine metabolism in the etiology of IBD as well as CRC. Carnitine additionally serves as an organic compatible solute (osmoprotectant) for bacterial cells under osmotic stress (Meadows and Wargo, 2015). Hyperosmolarity is correlated to inflammation and serves as the primary inflammatory mechanism for DSS and other chemically-induced colitis models (Schwartz et al., 2009).

Levy et al. (2015) described altered amino acid and polyamine metabolism in the gut microbiome of NLRP6 inflammasome-deficient mice. Taurine—a bile acid conjugate—was depleted and shown to induce NLRP6-dependent IL-18 secretion by triggering intestinal inflammasome activation *in vitro*. The authors propose taurine as a microbiota-dependent positive inflammasome/IL-18 modulator and the polyamine spermine as an inflammasome/IL-18 suppressor. Epithelial IL-18 secretion leads to downstream antimicrobial peptide production, which in turn affects the intestinal microenvironment. The addition of taurine, histamine, or spermine to drinking water induced compositional changes in the gut microbiome of wild-type mice, but not in inflammasome-deficient mice nor anaerobic cultures, suggesting that the capacity of these molecules to alter the gut microbial balance depends on host signaling via NLRP6. When dysbiotic microbiota from inflammasome-deficient mice was transferred into a wild-type host, the authors observed dominance of the dysbiotic microbiota, leading to inflammasome suppression and reduced levels of colonic IL-18. These results highlight the complex interplay of host and microbial metabolism and the delicate inflammatory balance in the gut microenvironment. Patterns of dysregulation regarding amino acid metabolism in IBD (particularly in CD), are inconsistent with those observed in Irritable Bowel Syndrome (IBS) patients (Vich Vila et al., 2018), suggesting a disease-specific mechanism.

Bile Acids

In healthy states, primary bile acids (BAs), including cholate and chenodeoxycholate, are synthesized from cholesterol in the liver and conjugated with glycine or taurine before excretion. The majority of primary BAs are reabsorbed in the ileum; however, a small proportion (approximately 5%) enter the colon where they are deconjugated and converted to secondary BAs, including deoxycholic acid and lithocholic acid, respectively, by the microbiota (Peng et al., 2021). Dominant members of the healthy stool microbiome (Human Microbiome Project Consortium, 2012), namely Firmicutes (including *Clostridium* spp., *Ruminococcus gnavus* and *Faecalibacterium prausnitzii*) and Bacteroidetes, are potent BA deconjugators and secondary BA transformation is attributed to metabolic activity by the genera *Bacteroides, Clostridium, Eubacterium, Lactobacillus*, and *Escherichia* (Duboc et al., 2013; Jia et al., 2018). In this regard, metabolism by the microbiome determines the composition of the BA pool in the gut. Bile acids in the intestine act primarily as detergents, facilitating the absorption of lipids and fat-soluble compounds. However, they can also act like hormones, regulating nutrient metabolism by activating specific nuclear and G protein-coupled receptors. They have also been shown to act as antimicrobial agents in the gut through their detergent activity (Shapiro et al., 2014; Ridlon et al., 2016). Ridlon et al. (2014, 2016) present in-depth reviews of bile acid metabolism and the gut microbiome.

Bile acids have been implicated in tumorigenesis, and particularly in CRC, through their capacity to induce oxidative stress and DNA damage at high concentrations (Bernstein et al., 2009; Ajouz et al., 2014; Jia et al., 2018). Increased levels of secondary BAs, specifically hydrophobic deoxycholic acid, have been correlated with the presence of colorectal adenomas (precursor lesions for CRC) and are capable of promoting intestinal tumorigenesis in experimental mouse models (Bernstein et al., 2009; Yachida et al., 2019; Peng et al., 2021). *Bilophilia wadsworthia*, whose growth is stimulated by the conjugated primary BA taurocholate, was enriched in patients with multiple polypoid adenomas and significantly correlated with the concentration of deoxycholic acid in stool (Yachida et al., 2019). Dietary fat intake positively influences secondary BA production, and Western-style diets with high fat intake have been implicated in the etiology of CRC (Bernstein et al., 2009; Ajouz et al., 2014). Metagenomic meta-analysis of the gut microbiome in CRC has indicated that genes for secondary BA conversion are consistently enriched in disease (Wirbel et al., 2019), and deoxycholic acid, along with cholate and chenodeoxycholate, were found to be enriched in a metabolomic analysis of a CRC high-risk cohort (Ocvirk et al., 2020). Another metabolomic analysis reported significantly elevated levels of glycine and taurine conjugates of cholate in early-stage CRC (Yachida et al., 2019). Metaproteomic analysis of CRC by Long et al. (2020) revealed elevated abundance of genera involved in BA metabolism and enrichment of DNA repair proteins and superoxide dismutases, indicating oxidative stress response (Jia et al., 2018; Long et al., 2020) (**Table 1**).

Despite their link to carcinogenesis, non-sulphated secondary BAs have been shown to exert anti-inflammatory effects *in vitro* (Duboc et al., 2013). Signaling via the main BA receptors, G protein-coupled receptor (GPCR) TGR5 and nuclear receptor farnesoid X receptor (FXR), leads to downstream inhibition of NF-κB mediated pro-inflammatory innate immune response

(Shapiro et al., 2014). FXR activation has been shown to protect mice from induced colitis via the downregulation of pro-inflammatory cytokines (Gadaleta et al., 2011). It has also been demonstrated to play a role in maintaining intestinal epithelium integrity by preventing bacterial overgrowth (Inagaki et al., 2006). Conversely, inactivation of FXR and subsequent BA dysregulation has been associated with increased colon cell proliferation and tumorigenesis in mice fed a Western-style diet (Dermadi et al., 2017). Individual BAs have variable ability to activate FXR and TGR5; unconjugated BAs are considered to have a greater FXR activation potential than their conjugated counterparts, and secondary BAs (including conjugated forms) are potent activators of TGR5 (Jia et al., 2018). Meta-omics of IBD indicates that primary conjugated and unconjugated BAs are enriched, and secondary BAs are depleted in disease (Jansson et al., 2009; Jacobs et al., 2016; Franzosa et al., 2019; Lloyd-Price et al., 2019). This pattern has likewise been observed in IBD serum metabolomics (Duboc et al., 2013), indicating systemic disruption. These results support a disease model in which gut dysbiosis alters the BA pool in favour of primary BAs. The subsequent reduction in secondary BAs drives host immune signaling toward an inflammatory phenotype.

Fatty Acids

Fatty acids, especially short-chain fatty acids (SCFAs), have received a great deal of attention in GI health. Colonocytes use SCFAs derived from gut microbial fermentation of dietary polysaccharides as an important source of energy (Shapiro et al., 2014; Rooks and Garrett, 2016; Hanus et al., 2021). These microbial metabolites can also contribute to host immunostasis through GPCR signaling and histone deacetylase (HDAC) inhibition, promoting an anti-inflammatory phenotype in the gut and strengthening the intestinal barrier (Shapiro et al., 2014; Rooks and Garrett, 2016). In particular, the SCFA butyrate and butyrate-producing bacteria, such as *Faecalibacterium prausnitzii* and *Roseburia hominis*, are considered to be beneficial, and their depletion is characteristic of IBD dysbiosis (Machiels et al., 2014; Patterson et al., 2017; Forbes et al., 2018; Alameddine et al., 2019). Butyrate is thought to contribute to intestinal health through a variety of mechanisms, including promotion of T_{reg} differentiation and macrophage polarization, inhibition of LPS-induced pro-inflammatory cytokine production, promotion of apoptosis in colonocytes, and strengthening intestinal epithelial cell (IEC) barrier function (Alameddine et al., 2019; Knox et al., 2019b; Silva et al., 2020; Hanus et al., 2021).

The protective role of SCFAs in CRC has also been suggested via their ability to epigenetically modulate tumour suppressor gene translation through HDAC inhibition and activation of GPCR signaling pathways resulting in colon adenoma and carcinoma cell apoptosis (Ou et al., 2013; Hanus et al., 2021; Peng et al., 2021). Although most of the meta-omic explorations of CRC discussed in this review did not report statistically significant differences in SCFAs between disease and controls, butyrate-producing species such as *Lachnospira multipara* and *Eubacterium eligens* were significantly ($p < 0.005$) depleted in CRC for one study, which also reported significant elevation of phenylpropionate in late-stage CRC, specifically (Yachida et al., 2019). Butyrate was found to be significantly less abundant in the high-risk CRC population described (Ocvirk et al., 2020) compared to the low-risk population. Similar results have been reported in another CRC-risk cohort study (Ou et al., 2013). The observation that butyrate and butyrate-producing species are depleted in both IBD and CRC suggests a metabolic link between diseases and possible mechanism for increased risk of CRC development among IBD patients via epigenetics.

The gut microbiota has also been implicated in the metabolism of a wide range of FAs, such as the polyunsaturated FAs arachidonic acid and linoleic acid, since conjugation and transformation of these metabolites was found to be dependent on the gut microbiota (Kishino et al., 2013). Linoleic and arachidonic acid are essential for the production of prostaglandins (PGs) and other eicosanoids, molecules that contribute to immune signaling and inflammation via cytokine production (Jansson et al., 2009). Increased production of PGs drives chronic intestinal inflammation and has been identified in the intestinal mucosa of IBD patients (Raab et al., 1995). However, there also exists a role for PGs in the maintenance and repair of intestinal epithelium (Wang et al., 2005). In CRC, arachidonic and linoleic acid were decreased in some cohorts (Chan et al., 2009; Phua et al., 2014), while others reported decreases in global lipid metabolism (Long et al., 2020).

Elevated levels of arachidonic acid were observed in IBD cohorts (Jansson et al., 2009; Diab et al., 2019; Lloyd-Price et al., 2019). A decrease in arachidonic acid metabolism and subsequent PG production, as observed in Jansson et al. (2009), may explain the accumulation of this FA in the IBD environment. Overall, the meta-omics data indicates broad disruptions in FA metabolism in disease, especially in IBD, where depletion of short-, medium-, and long-chain FAs has been consistently reported. Further experimental characterization of the complex interactions of FAs and their metabolites with host immune regulation in the context of gut inflammation may help to elucidate the precise role of FA metabolism in pathogenesis.

Vitamins B3 & B5

B vitamins are key intermediates in essential cofactor metabolism and are indispensable for cellular life. These essential micronutrients are obtained from dietary sources, bacterial sources, or both. A deficiency of these vitamins from increased cellular demand or absorption defect leads to various physiological disruptions. Many, but not all, members of the gut microbiota are prototrophic for B vitamin synthesis (Peterson et al., 2020). It has been suggested that auxotrophic human gut bacteria rely on B vitamin sharing within the GI microenvironment for survival, as humanized gnotobiotic mice supported B vitamin auxotroph survival for at least 4 weeks regardless of dietary vitamin intake (Sharma et al., 2019).

Vitamin B3, also known as niacin or nicotinic acid, is a precursor of nicotinamide adenine dinucleotide (NAD), an essential cofactor involved in cellular redox reactions. Bacteria synthesize niacin from aspartic acid or tryptophan via the kynurenine pathway, ultimately resulting in NAD production.

Human cells can also synthesize NAD *via* niacin-independent salvage pathways. In addition to its role in essential redox reactions, NAD plays a role in epigenetic enzyme regulation and genomic stability, as well as reactive oxygen species inhibition (Peterson et al., 2020). Administration of niacin disrupts NF-κB signaling, leading to suppression of inflammatory cytokines; its effects have been investigated in the context of several human diseases, including atherosclerosis, fatty liver disease, and Parkinson's disease (Su et al., 2015; Peterson et al., 2020). Niacin is also known to affect fatty acid synthesis via NAD, and niacin or tryptophan deficiency can result in Pellagra, a disease whose symptoms include skin inflammation, diarrhea, and dementia. In mice, a diet supplemented with niacin and tryptophan was associated with increased expression of intestinal antimicrobial peptides and administration of this diet to colitis-susceptible mutant mice shifted the composition of the gut microbiota toward that of wild-type mice (Hashimoto et al., 2012). The receptor for niacin, GPR109A, is present on monocytes and macrophages and, notably, is also a receptor for the SCFA butyrate. Activation of GPR109A has been shown to suppress colonic inflammation and carcinogenesis via targeted T cell differentiation in mice and was deemed essential for butyrate-mediated IL-18 expression in the colonic epithelium (Singh et al., 2014).

Regarding meta-omics, niacin was reported to be decreased in CRC compared to healthy controls (Phua et al., 2014). The low-risk CRC cohort revealed elevated levels of niacin compared to the high-risk group (Ocvirk et al., 2020). In the same study, nicotinamide, a vitamer of niacin with anti-inflammatory properties (Peterson et al., 2020), and other niacin metabolites were enriched in the high-CRC-risk population (Ocvirk et al., 2020). Nicotinamide is suggested to play a role in cancer chemoprevention via enhanced DNA repair and suppression of pro-inflammatory mediators (Nikas et al., 2020). Loss of niacin-producing organisms in the gut could thus decrease the bioavailability of niacin to IECs, promoting inflammation and carcinogenesis. In IBD, vitamin B3 and associated metabolites appear to be differentially expressed in disease (**Table 1**). However, the pattern of dysregulation is inconsistent between cohorts (Kolho et al., 2017; Diab et al., 2019; Lloyd-Price et al., 2019). Differences in the type of biological sample(s) used, as well as cohort design, likely contribute to these inconsistencies. As already discussed, the stool and mucosal GI metabolic profiles can provide distinct contextual information (e.g., increased metabolite utilization or reabsorption in the GI tract mucosa may result in decreased stool concentrations or vice versa). The interpretation of these results is additionally challenging since niacin/NAD metabolism affects many cellular processes.

Vitamin B5, pantothenic acid, is essential for coenzyme A (CoA) synthesis and is abundant in a large variety of foods, so deficiency is rare (Peterson et al., 2020). The sodium-dependent multivitamin transporter (SMVT) facilitates the uptake of vitamins B5 and B7. It has been demonstrated to play a role in gut permeability in mice (Sabui et al., 2018), implicating one or both of these vitamins in gut homeostasis. While there is a lack of evidence for the pantothenic acid disruption in CRC meta-omics, three relatively large-scale IBD studies reported significantly depleted vitamin B5 in disease compared to healthy controls (Vich Vila et al., 2018; Franzosa et al., 2019; Lloyd-Price et al., 2019) (**Table 1**). Depletion of pantothenic acid in the gut may be a symptom of IBD-related dysbiosis and the loss of B5-producing organisms; however, further investigation into the role of vitamin B5 in IBD is needed to understand the cause and effects of this perturbation.

Sphingolipids

Sphingolipids such as sphingomyelin (SM) and glycosphingolipids (GSLs) are essential structural components of IEC membranes. In addition to a direct role in maintaining epithelial barrier integrity, sphingolipids and related metabolites have also been demonstrated to participate in immune signaling and modulation of inflammation (An et al., 2014; Abdel Hadi et al., 2016). In a mouse model, early-life exposure to microbial sphingolipids was shown to reduce invariant natural killer T (iNKT) cell proliferation and protect the adult host from iNKT cell-mediated colitis (An et al., 2014). Sphingolipid metabolism is complex and is reviewed in detail elsewhere (Abdel Hadi et al., 2016).

Sphingosine and ceramide are sphingolipids that, when accumulated on the surface of IECs, increase epithelial permeability and disrupt normal barrier function; ceramide was additionally found to induce either pro- or anti-inflammatory effects depending on its enzymatic origin (Abdel Hadi et al., 2016). The production of PGs from arachidonic acid, as previously discussed, can also be modulated by sphingolipid composition in cellular membranes (Nakamura and Murayama, 2014). Meta-omics of IBD reveals enrichment of sphingolipids (specifically, ceramide and sphingomyelin) and sphingolipid metabolism in disease (Kolho et al., 2017; Franzosa et al., 2019) (**Table 1**), supporting their role as pro-inflammatory mediators. No significant differences in sphingolipid metabolism were reported for CRC (**Table 1**). However, disruptions in arachidonic acid metabolism and PG production (Chan et al., 2009) may indicate undetected upstream changes in sphingolipid composition. Experimental evidence has tied sphingolipid receptor expression to tumour suppression in CRC (Petti et al., 2020). Due to their role in intestinal epithelial barrier maintenance and their varied capability for immune signaling, further characterization of sphingolipids in intestinal disease is warranted. Notably, further experimentation with early-life exposure to microbial sphingolipids and the resulting effects on host immune development could have implications related to the hygiene hypothesis (see below) and IBD development.

SUMMARY

Technological advancements in recent decades have enabled extensive characterization of the human gut microbiome via meta-omics techniques. IBD, in particular, is commonly modelled in microbiome research, and the association with CRC is thought to indicate some degree of shared etiology between the two diseases. Genetic, environmental, and

immunologic factors are thought to contribute to disease development; however, the mechanisms by which they do so are complex and not fully understood (Sobhani et al., 2011; Carroll et al., 2019). The intestinal microbiota have been implicated in the pathogenesis of both IBD and CRC. In the absence of a directly causative agent, the microbial community within the gut is being investigated as a mutualistic pseudo-organ capable of influencing homeostasis in its host.

It has been suggested that increased urbanization and hygienic behaviours (*e.g.,* household use of disinfectants) results in reduced colonization by commensal organisms in early life and that this lack of exposure directly affects immune system development, resulting in a predisposition to aberrant immune responses—*i.e.,* IBD—later in life (Weinstock and Elliott, 2009). Much support for this "hygiene hypothesis" comes from experimental studies regarding helminth and parasite colonization; however, these organisms are not typically captured in microbiome studies. Given the impact of the early-life environment on individual microbiome development and subsequent health outcomes (Nielsen et al., 2020; Boutin et al., 2021), the role of commensal bacteria in immune development and sensitization should not be ignored. Meta-omic cohort studies can provide insights into the mechanisms of aberrant immune response in disease and identify key metabolites whose role in immune development warrants further study.

An advantage of functional meta-omics is the potential to discover molecular biomarkers of disease that can aid in non-invasive screening and diagnosis, or in the case of IBD, distinguishing component diseases from one another. Meta-omic characterization of IBD and CRC in human cohorts has revealed similar patterns of metabolic dysregulation that implicate a disruption in host-microbiota cross-talk leading to aberrant immune response and inflammation. Dysregulated metabolism of amino acids, polyamines, bile acids, fatty acids, and B vitamins has been reported in both IBD and CRC cohorts, supporting a degree of shared microbiome-mediated etiology between the diseases. Several of these metabolites directly or indirectly affect NF-κB activation, a key transcription factor within the intestinal tumour microenvironment (Schwitalla et al., 2013). Protective microbially-mediated compounds such as tryptophan and butyrate have been shown to impact T_{reg} cell proliferation, and their relative decrease in disease not only reflects the loss of beneficial organisms, but also potentially drives autoinflammation through imbalanced T cell differentiation (Dominguez-Villar and Hafler, 2018). Although IBD are risk factors for CRC, not all IBD patients develop malignancies. Changes in the GI microbiome have been associated with cancer development in animal studies; however, the precise role of the microbiome in mediating carcinogenesis has yet to be discovered.

The human genetic landscape is one of many health determinants that can influence the development of individual gut microbiomes (Goodrich et al., 2014). Alternatively, studies in mice have revealed that dysbiotic fecal microbiota from a genetically susceptible (*e.g.,* Nod2 or Asc deficient) host can be dominantly transferred to healthy, wild-type recipients and is sufficient to increase sensitivity to DSS-induced colitis and tumorigenesis (Couturier-Maillard et al., 2013; Levy et al., 2015), indicating that dysbiotic communities have the capacity to increase disease risk in wild-type recipients *via* molecular signaling (*e.g.,* cytokine modulation) under these experimental conditions. These data support a model whereby inflammation and disease susceptibility depend on critical communication between the microbiome and the immune system of the host. Dysbiosis may be driven by a multitude of factors, including genetic susceptibility, diet, antibiotic usage, smoking status and other environmental exposures, which could have individual as well as cumulative effects on the gut microenvironment, including epithelial barrier integrity and inflammation. Despite these diverse influences, there appears to be some degree of shared microbially-mediated metabolic dysregulation in IBD and CRC, supporting a common etiology related to the GI microbiome. The discovery of significant metabolic differences between the microbiomes of healthy individuals in either high- or low-risk CRC cohorts suggests a model of dysbiotic metabolism in asymptomatic individuals leading to enhanced risk of immune imbalance and symptomatic disease.

Due to a high degree of variability in meta-omics data, systematic meta-analyses can be particularly useful in revealing disease-specific microbiome signatures across cohorts (Wirbel et al., 2019). Future research directions include longitudinal metabolic characterization of the IBD microbiome, including quantification of specific metabolites discussed here, toward development of CRC in order to identify why some IBD patients develop CRC and others do not. The role of microbial metabolites in infant immune system development may also provide valuable insights into immune modulation and disease susceptibility. Additionally, data produced from downstream meta-omics techniques (*i.e.,* metaproteomics and metabolomics) can be used to confirm or contradict predicted functional activity from the plethora of available metataxonomics, metagenomics, or metatranscriptomics research. Even if consistent changes in gut meta-omics are found that are predictive of persons with IBD who develop CRC there remains the issue of determining cause and effect. Future studies will benefit from large cohort sizes, detailed metadata, considerate sample collection and storage techniques, and robust statistical approaches in order to address the many challenges associated with meta-omics research. However, there are studies that show that colon neoplasia is most likely to develop in the setting of active inflammation and is unlikely to develop in the absence of inflammation (Rubin et al., 2013; Shaffer et al., 2021; Yvellez et al., 2021). The association of meta-omics changes with other non-IBD chronic immune mediated inflammatory diseases suggests that the intestinal microbial milieu may drive systemic inflammation (Forbes et al., 2018). An evolving paradigm of gut microbial changes driving inflammation and the knowledge that gut inflammation can drive neoplasia development, makes it realistic to consider that microbial changes may underlie the ultimate development of neoplasia in persons with IBD and identifying these changes early on can be a mechanism to interrupt the inflammation-neoplasia paradigm

in IBD. As the field continues to expand, overcoming barriers to clinical implementation of meta-omics will pave the way for personalized approaches to diagnosis and screening.

AUTHOR CONTRIBUTIONS

MP participated in developing the idea for the article, drafting the article, and participated in the final editing. JF participated in developing the idea for the article and participated in the final editing. NK participated in developing the idea for the article and participated in the final editing. CB participated in developing the idea for the article and participated in the final editing. GV participated in developing the idea for the article and participated in the final editing.

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REVIEW
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Gut Microbes and Hepatic Encephalopathy: From the Old Concepts to New Perspectives

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Hepatic encephalopathy (HE) is a severe complication of advanced liver disease and acute liver failure. The clinical spectrum ranges from minor cognitive dysfunctions to lethargy, depressed consciousness, and coma and significantly impact the quality of life, morbidity, and mortality of the patients. It is commonly accepted that the gut milieu is essential for the development of HE; however, despite intensive research efforts, the pathogenesis of HE is still not fully elucidated. As our knowledge of gut microbiota moves from the pioneering era of culture-dependent studies, the connection between microbes, inflammation, and metabolic pathways in the pathogenesis of HE is becoming increasingly clear, providing exciting therapeutic perspectives. This review will critically examine the latest research findings on the role of gut microbes in the pathophysiological pathways underlying HE. Moreover, currently available therapeutic options and novel treatment strategies are discussed.

Keywords: hepatic encephalopathy, gut microbes, gut-liver-brain axis, antibiotics, probiotics, fecal microbiota transplantation

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INTRODUCTION

Hepatic encephalopathy (HE) encompasses a broad spectrum of neurological or psychiatric abnormalities ranging from minor cognitive dysfunction to lethargy, depressed consciousness, and coma occurring in patients with liver insufficiency or portosystemic shunting (Vilstrup et al., 2014). According to the severity of the clinical presentation, HE has traditionally been classified into overt (OHE) and minimal HE (MHE). Clinically manifest neurological-psychiatric abnormalities characterise OHE, while neuropsychological or electrophysiological alterations without clinically detectable abnormalities are typical of MHE (Ferenci et al., 2002). HE is likely the most frequent complication of cirrhosis, with a prevalence ranging from 16 to 21% for OHE in decompensated cirrhosis to 20–80% for MHE in compensated cirrhosis (D'Amico et al., 1986; Allampati et al., 2016). The onset of HE is associated with a high risk of recurrence, hospital admission rate, and poor survival and impacts the quality of life of patients and their caregivers (Cordoba et al., 2014).

Despite intensive research efforts, the pathogenesis of HE is still not fully elucidated. Thus, effective therapies for treating and preventing HE are still lacking, hence the urgent need to update our knowledge, moving from the old concepts to the newest perspectives.

From the Ammonia Hypothesis to the Gut-Liver-Brain Axis

It is commonly accepted that neurological impairment and cognitive decline provoked by liver dysfunction result from blood-derived factors influencing the permeability and altering the integrity of the blood-brain barrier. Since the description of the "meat intoxication syndrome" in portocaval-shunted dogs, at the end of the 19th century, ammonia has been considered the critical metabolic factor underlying HE's development (Amodio, 2015).

Ammonia primarily derives from the gut as an end product of protein digestion, amino acid deamination, and bacterial urease activity. Furthermore, multiple organs, such as the brain, muscle, and kidney, contribute to ammonia production by deaminating glutamine via glutaminase. In physiological condition, the liver efficiently extracts (85%) the ammonia from portal blood and, through the urea cycle, convert it into urea, then excreted in the kidneys (75%) and the intestine (25%). Only 15% of the ammonia pool enters the systemic circulation (Rose et al., 2020). Defects in hepatic function, portal blood flow, and urea cycle enzymes or intermediates can result in hyperammonemia, as can excessive ammonia production in the gastrointestinal tract. The ammonia passes freely into the brain, where astrocytes remove it producing glutamine via glutamine synthetase. Glutamine induces astrocyte hypertonia resulting in astrocyte swelling, compromised neuronal communication, impaired function, and brain edema. However, ammonia concentration and HE severity are poorly correlated, thus demonstrating that it is only a piece of the puzzle underpinning the pathogenesis of HE (Shawcross et al., 2011). In addition to the direct role of ammonia, systemic inflammation/oxidative stress and increased blood bile acids impact the blood-brain barrier permeability, allowing an increased influx of molecules physiologically unable to cross it (Atluri et al., 2011). Consequently, the alterations of metabolites in cerebrospinal fluid and changes in neurotransmission such as increased GABAergic tone significantly modulate the onset of neurological decline (Keiding et al., 2006; Riordan and Williams, 2010). More recently, neurosteroids, endogenous benzodiazepines, and manganese have emerged as synergistic factors in the onset of HE (Rose et al., 2020). Furthermore, like a vicious circle, hyperammonaemia "*per se*" can induce neutrophil dysfunction and reactive oxygen species release, contributing to systemic oxidative stress and inflammation, exacerbating its harmful effects in the brain (Shawcross et al., 2004).

The gut-liver-brain axis refers to the bidirectional relationship between the gut and its microbiota, the liver, and the brain, resulting from integrating signals generated by dietary, genetic, and environmental factors (Mancini et al., 2018). In patients with liver cirrhosis, defective small intestinal motility, reduced gastric acid secretion, and weaker antimicrobial defense of intestinal mucosa determine small intestinal bacterial overgrowth (SIBO). The concomitant decrease in bile acids (BAs) synthesis due to liver failure synergistically act with SIBO to determine pathological changes in the intestinal microbiota composition, mainly characterised by a massive reduction in microbial diversity, a decline in autochthonous non-pathogenic bacteria (*Bacteroidetes*, *Ruminococcus*, *Roseburia*, *Veillonellaceae*, and *Lachnospiraceae*) and an overgrowth of potentially pathogenic species (*Fusobacteria*, *Proteobacteria*, *Enterococcaceae*, and *Streptococcaceae*) (Fukui, 2015; Acharya and Bajaj, 2017).

The paucity of bacteria involved in producing short-chain fatty acids (SCFAs) and converting primary into secondary BAs contribute to worsening gut dysbiosis and disrupting intestinal barrier integrity. Indeed, SCFAs (mainly butyrate, acetate, and propionate), produced in the colon by bacterial fermentation of dietary fibers and resistant starch, exert several beneficial functions: preserving intestinal barrier integrity, nourishing colonocytes promoting, mucus production, and reducing of colonic inflammation (Nava and Stappenbeck, 2011; Rowland et al., 2018). Furthermore, the lower abundance of 7α-dehydroxylating bacteria in the colon (*Lachonospiraceae*, *Ruminococcaceae*, and *Blautia*) due to a reduction in primary BAs determines a change in secondary-to-primary BAs ratio that can favor the overgrowth of pathogenic taxa (Kakiyama et al., 2013; Ridlon et al., 2013).

The impaired intestinal barrier integrity enhances bacterial translocation and the release of bacterial endotoxins in circulation, such as lipopolysaccharides, flagellin, peptidoglycan, and microbial nucleic acids, perpetuating liver damage and contributing to systemic inflammation responsible for blood-brain barrier dysfunction and neuroinflammation (Shawcross et al., 2004; Shawcross et al., 2007; Shawcross et al., 2011; Dhiman, 2012).

Dysregulation of blood-brain barrier permeability may also lead to a dramatic increase of certain BAs such as lithocholic, taurocholic, and glycocholic acid in the cerebrospinal fluid of patients with HE or brain tissue of rodent model of HE (Tripodi et al., 2012; McMillin et al., 2016; Weiss et al., 2016).

Although a precise role for BAs in the pathogenesis of HE has not yet been completely defined, they are likely involved in aberrant neuronal signalling and the promotion of neuroinflammation through microglia activation (DeMorrow, 2019).

Overall, HE can be considered a typical gut-liver-brain axis disease model (**Figure 1**).

GUT MICROBIOTA IN LIVER CIRRHOSIS

Culture-Based Studies on Gut Microbiome in Human Cirrhosis

First attempts to characterise gut microbiota composition employed culture-based technologies and analysed microbial changes after HE therapy (**Table 1**). Riggio et al. observed a significant growth, defined as more than 2 log increases of non-urease producing *Lactobacilli* spp. after both lactulose and lactitol treatment and a reduction in proteolytic bacteria (*Enterobacteria* and *Enterococci*) after lactitol alone (Riggio et al., 1990). Lactitol administration for 4 weeks was also associated with an increased occupation ratio (number of specific bacteria/total number of bacteria detected) of anaerobic *Bifidobacterium* and a rise in *Lactobacilli* total count. Furthermore, a reduction in *Clostridium*

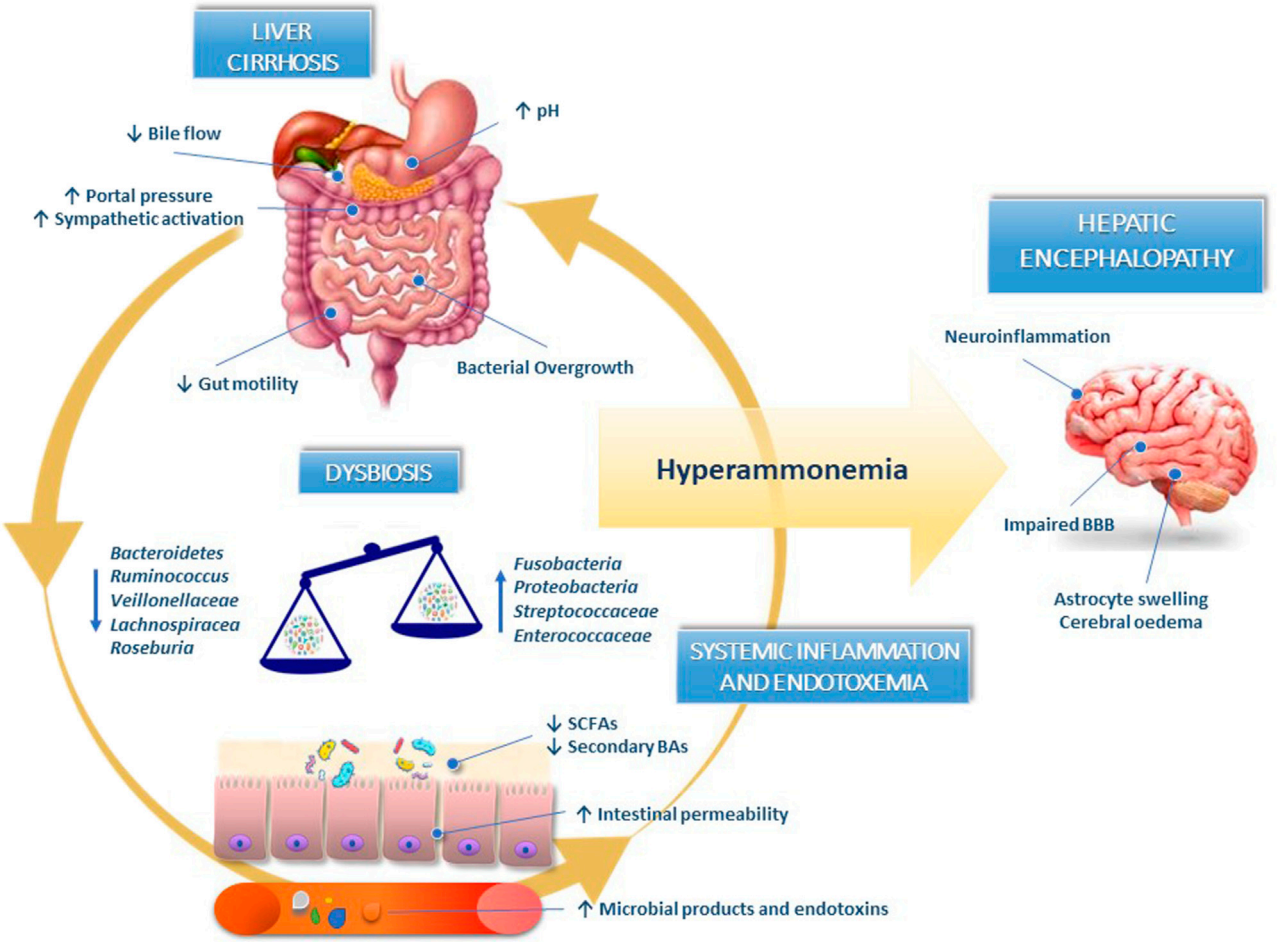

FIGURE 1 | Gut-liver-brain axis in the pathogenesis of hepatic encephalopathy. In liver cirrhosis, the decrease in bile acids synthesis, defective small intestinal motility and reduced gastric acid secretion induce small intestinal bacterial overgrowth and dysbiosis. The reduced abundance of bacteria synthesising short-chain fatty acids and converting primary into secondary bile acids contribute to worsening gut dysbiosis and disrupting intestinal barrier integrity. Pathological bacterial translocation and release of bacterial endotoxins in circulation perpetuate liver damage and contribute to systemic inflammation responsible for blood-brain barrier dysfunction and neuroinflammation. BAs, bile acids; BBB, blood-brain barrier; SCFAs, short-chain fatty acids.

TABLE 1 | Culture-based studies on gut microbiome in human cirrhosis.

Author	Population	Sample	Methods	Results
Riggio et al. (1990)	Cirrhotic patients	Stool	Culture	↑ *Lactobacilli* spp. after both lactulose and lactitol therapy ↓ *Enterobacteria* and *Enterococci* after lactitol
Tarao et al. (1995)	Cirrhosis with HE	Stool	Culture	↑ Occupation ratio of *Bifidobacterium* and ↓ *Clostridium* and *Bacteroides* after lactitol treatment ↓ Serum ammonia and improvement of mental status and asterixis
Liu et al. (2004)	Cirrhosis with HE	Stool	Culture	↑ *Lactobacillus* spp. and ↓ Escherichia *coli* and *Staphylococcus* spp. ↓ Serum ammonia and reversal of MHE in 50% of patients

and *Bacteroides*, considered to be ammonia-producing bacteria, was observed. These changes in gut microbiota paralleled the decrease in venous ammonia level and improvement of mental status and asterixis (Tarao et al., 1995). Last, in a randomised, placebo-controlled trial involving 55 patients with MHE, Liu et al. demonstrated that a symbiotic treatment with probiotics and fermentable fiber effectively increased the fecal content of *Lactobacillus* spp. at the expense of the overgrowth of pathogenic bacteria, such as *Escherichia coli* and *Staphylococcus* spp. Symbiotic treatment was further associated with reduction of serum ammonia and reversal of MHE in 50% of the patients compared to the placebo group (Liu et al., 2004). Although the data derived from these pivotal studies were the cornerstones of the "microbial revolution" in the pathogenesis of

TABLE 2 | Culture-independent studies on gut microbiome in human cirrhosis with or without HE.

Author	Population	Sample	Methods	Results
Chen et al. (2011)	Cirrhosis vs healthy control	Stool	16S sequencing	*Proteobacteria* and *Fusobacteria* phyla and *Streptococcaceae*, *Veillonellaceae* and *Enterobacteriaceae* families higher in cirrhotic patients than in controls *Bacteroidetes* phylum and *Lachnospiraceae* family lower in cirrhotic patients than in controls
Bajaj et al. (2014b)	Cirrhosis vs healthy controls	Stool	16S sequencing, MTPS	↓ Autochthonous taxa (*Lachnospiraceae*, *Ruminococcaceae*, and Clostridiales XIV), non-autochthonous taxa (*Enterobacteriaceae* and *Bacteroideaceae*) ratio
Bajaj et al. (2012b)	OHE/non-OHE/ control	Stool	16S sequencing, MTPS	↑*Enterobacteriaceae*, *Alcaligeneceae*, and *Fusobacteriaceae* and ↓ *Ruminococcaceae* and *Lachnospiraceae* in cirrhotic group versus controls ↑ *Enterobacteriaceae*, *Alcaligenaceae*, *Lactobacilaceae*, and *Streptococcaceae* in OHE versus controls ↑ *Veillonellaceae* in OHE versus no OHE *Alcaligeneceae* and *Porphyromonadaceae* associated with poor cognition
Bajaj et al. (2012a)	OHE/no-OHE/ control	Stool Sigmoid mucosa	16S sequencing	↑*Dorea*, *Subdoligranulum*, Incertae Sedis XIV, *Blautia*, *Roseburia*, *Faecalibacterium* and ↓ *Enterococcus*, *Burkholderia*, *Proteus* in cirrhosis ↑ *Enterococcus*, *Veillonella*, *Megasphaera*, and *Burkholderia* and ↓ *Roseburia* in OHE mucosal microbiome
Zhang et al. (2013)	MHE/no MHE/ control	Stool	16S sequencing	↑ *Veillonellaceae* and *Streptococcaceae* in cirrhotics *Streptococcus salivarius* was higher in MHE *Veillonella parvula* and *Streptococcus salivarius* were correlated with cognitive function and ammonia level
Bajaj et al. (2015a)	Previous HE/non-HE/control	Saliva	16S sequencing	↑*Enterobacteriaceae*, *Enterococcacea* and ↓ in autochthonous microbiota and *Erysipelothricaceae* in previous HE, compared to non-HE patients and controls
Ahluwalia et al. (2016)	Previous HE/non-HE/control	Stool	16S sequencing	*Streptococcaceae*, *Enterobacteriaceae*, *Lactobacillaceae* and *Peptostreptococcaceae*, were positively linked with hyperammonemia-associated astrocytic changes *Porphyromonadaceae*, were correlated with neuronal integrity and oedema
Iebba et al. (2018)	Cirrhosis vs control	Stool	16S sequencing and NMR metabolism	*Stenotrophomonas pavanii*, *Methylobacterium* as well as metabolites (methanol, threonine), enhanced the risk of HE
Sung et al. (2019)	AHE vs. cirrhosis/ control	Stool	16S sequencing	↑ *Firmicute*, *Proteobacteria* and *Actinobacteria* during AHE *Alistipes*, *Bacteroides*, *Phascolarctobacterium* were associated with HE recurrence *Clostridium-XI*, *Bacteroides*, *Bacteroides*, *Lactobacillus*, *Clostridium sedis* were associated with overall survival at 1-year follow-up
Bloom et al. (2021)	Previous OHE/ no-OHE	Stool	Shotgun sequencing and LC-MS/MS	*Anaeromassilibacillus species*, *Anaerostipes caccae*, *Bacteroideseggerthii*, *Clostridium species*, *Faecalicatena contorta*, *Holdemaniafiliformis*, *Neglecta timonensis*, and *Ruminococcus* species were linked to a history of OHE Lower concentrations of 6 faecal SCFAs in patients with a history of OHE

OHE, overt hepatic encephalopathy; MHE, minimal hepatic encephalopathy; AHE, acute hepatic encephalopathy; NMR, nuclear magnetic resonance; LC-MS/MS, liquid chromatography tandem mass spectrometry.

chronic liver diseases and HE, culture-based methodologies used to characterise the microbial communities hamper the results. Indeed, most bacterial species inhabiting the gut can either not be cultured or reliably distinguished. Further, these techniques are only qualitative or, at best, semi-quantitative.

Culture-Independent Studies on Gut Microbiome in Human Cirrhosis

The introduction of culture-independent techniques has revolutionised the field of intestinal microbiology (**Table 2**). Through the sequencing of the bacterial 16S ribosomal RNA (16S rRNA) gene containing variable regions useful for phylogenetic identification, more recent studies better defined the taxonomic profile of gut microbiota in patients affected by chronic liver diseases in respect to healthy individuals (Fraher et al., 2012; Qin et al., 2014).

Chen et al. first characterised fecal microbial communities in patients with liver cirrhosis using pyrosequencing of the 16S rRNA V3 region (Chen et al., 2011). Compared to healthy individuals, cirrhotic patients had lower microbial diversity, as estimated by the Shannon diversity index, and changes in the intestinal microbial community composition both in terms of phyla (with a marked decrease in the relative abundance of *Bacteroidetes* and enrichment in *Proteobacteria* and *Fusobacteria*) and families (enrichment in *Enterobacteriaceae*, *Pasteurellaceae*, *Streptococcaceae*, *Veillonellaceae* and depletion in *Lachnospiraceae*). Interestingly, *Streptococcaceae* showed a positive correlation trend, whereas *Lachnospiraceae* negatively correlated with the severity of cirrhosis assessed by the Child-Pugh score. The enrichment of *Streptococcus*, *Veillonella*, and *Enterobacteriaceae* in fecal microbiota might result from a relocation of small intestinal bacteria. On the other hand, a decline in species involved in SCFAs metabolism, such as *Lachnospiraceae*, could lead to a higher colonic pH and ammonia production (Justesen et al., 1984; Vince et al., 1990).

In a more extensive study involving 244 patients covering the spectrum from healthy controls to decompensated cirrhosis, the

authors found a progressive decrease in the ratio between potentially beneficial autochthonous taxa (*Lachnospiraceae*, *Ruminococcaceae*, and Clostridiales XIV) and harmful non-autochthonous taxa (*Enterobacteriaceae* and *Bacteroideaceae*), so-called cirrhosis dysbiosis ratio, paralleling the worsening of liver disease and higher endotoxemia (Bajaj et al., 2014). Thus, the imbalance of intestinal microbiota composition negatively affects the natural history of liver disease leading to hepatic and extra-hepatic complications.

Gut Microbiota and Hepatic Encephalopathy

Compared with cirrhotic patients without cognitive dysfunction, patients with both MHE and OHE had specific alterations of gut microbiota profile. Bajaj et al. firstly demonstrated that the differences in stool microbiome composition between healthy controls and cirrhotic were more pronounced analysing the results according to HE. In detail, the abundance of *Lachnospiraceae* and *Ruminococceae* was significantly higher in the control group, whereas *Enterobacteriaceae*, *Fusobacteriaceae*, *Alcaligenaceae*, *Lactobacillaceae*, and *Leuconostocaceae* were significantly lower in the controls compared with cirrhotic patients, irrespective of HE. However, the HE group differed from controls on several additional bacterial families compared with cirrhotics without HE with a significantly higher concentration of *Enterobacteriaceae*, *Alcaligenaceae*, *Lactobacilaceae*, and *Streptococcaceae*. Moreover, altered flora (higher *Veillonellaceae*), poor cognition, endotoxemia, and inflammation (IL-6, TNF-α, IL-2, and IL-13) were observed in HE compared with cirrhotics without HE (Bajaj et al., 2012b). More strikingly, the authors found that specific bacterial families (*Alcaligeneceae*, *Porphyromonadaceae*, *Enterobacteriaceae*) were strongly associated with both cognition and inflammation in HE. *Alcaligeneceae*, in particular, can produce ammonia by degradation of urea, likely explaining the correlation with cognitive impairment (Bajaj et al., 2012b). Later, the same authors analysed both the stool and colonic mucosal microbiome of 60 cirrhotic patients. The sigmoid mucosal microbiome considerably differed from the corresponding stool samples, and these differences persisted in studying the group according to the presence of HE. In detail, members of genera *Enterococcus*, *Megasphaera*, and *Burkholderia* were overrepresented in HE and linked to poor cognition and inflammation, whereas *Roseburia* prevailed in the group without HE. The alteration of bile acid metabolism and the decrease of antibacterial peptides or mucins in the colon, typically occurring in the advanced stages of liver diseases, could lead to a selection of potentially pathogenic bacteria adhering to and growing in the colonic mucosa. Thus, several essential processes in the pathogenesis of HE probably occur at the mucosal surface rather than lumens, such as translocation and interaction between microbiota and the immune system (Bajaj et al., 2012a).

Zhang et al. found that the stool concentration of the gut urease-containing bacteria *Streptococcus salivarius* was significantly higher in cirrhotic patients with MHE than in those without HE. Furthermore, the change in the amount of these bacteria positively correlated with ammonia accumulation (Zhang et al., 2013). The difference in the bacterial families associated with HE reported by the authors can be explained by the high interindividual variations in gut microbiota across populations, or other unknown factors could (Yatsunenko et al., 2012).

Interestingly, dysbiosis, resulting from decreased autochthonous or commensal taxa, has also been found in the saliva of patients with cirrhosis compared to controls. Qin et al. reported that most of the enteral consortia detectable in cirrhotic (mainly *Streptococcus* spp. and *Veillonella* spp.) belong to the oropharyngeal inhabitants, suggesting an invasion of the gut by oral bacterial species (Qin et al., 2014). Salivary microbiome analysis showed an increase in pathogenic *Enterobacteriaceae* and a reduction in autochthonous microbiota *Erysipelothricaceae* in HE, compared to non-HE patients and controls, thus indicating a global mucosal-immune interface alteration (Bajaj et al., 2015a).

Gut dysbiosis can also directly impact brain homeostasis, with neuronal and astrocytic dysfunction, particularly in HE. Ahluwalia et al., using multi-modal magnetic resonance imaging (MRI), correlated specific microbial families with neuroradiological findings. Hyperammonemia-associated astrocytic changes (i.e., increased glutamate/glutamine ratio and reduced myo-inositol and choline) at the magnetic resonance spectroscopy (MRS) positively correlated with families *Streptococcaceae*, *Enterobacteriaceae*, *Lactobacillaceae*, and *Peptostreptococcaceae*, while negatively correlated with *Lachospiraceae*, *Ruminococcaeae*, and Clostridiales XIV. On the other hand, *Porphyromonadaceae* were only associated with neuronal changes on diffusion tensor imaging, used to assess neuronal integrity and edema, without linkages with ammonia (Ahluwalia et al., 2016).

Despite the impressive results, a more comprehensive microbiota analysis should combine metagenomics with other "omics" approaches, particularly metatranscriptomics and metabolomics. Metatranscriptomics allows understanding gene expression and protein activity, whereas metabolomics represents the comprehensive analysis of metabolites released of the entire micro¬bial community. Iebba et al. made one of the first attempts to integrate these different approaches. Through the combination of 16s DNA sequencing, nuclear magnetic resonance (NMR) metabolomics, and network analysis, they observed that the translocation of certain species (*Stenotrophomonas pavanii*, *Methylobacterium extorquens*) into the peripheral blood system, as well as metabolites (methanol, threonine), enhanced the risk of HE (Iebba et al., 2018).

Identifying specific gut microbiota provides new strategies for clinical diagnosis, treatment, and eventually weighing the prognosis of HE. In this regard, in hospitalised patients with cirrhosis, dysbiosis on admission (mainly changes in Proteobacteria constituents) was associated with increased risk of extra-hepatic organ failure, acute liver failure, and death, independent of clinical factors (Bajaj et al., 2019b).

Stool and salivary unique microbiome patterns predicted readmission and mortality at 90 days in cirrhotic patients, respectively (Bajaj et al., 2015b).

Sung et al. profiled dynamic changes in gut microbiomes of cirrhotic patients with overt HE at the acute episode before

treatment, 48–72 h after active treatment, and the inactive stage (2–3 months after the episode) by comparing them with healthy individuals and patients with compensated cirrhosis. During acute hepatic encephalopathy (AHE), gut microbiome diversity and relative abundance of *Bacteroidetes* phylum diminished, whereas *Firmicutes, Proteobacteria*, and *Actinobacteria* increased. Moreover, the relative abundance of three species (*Alistipes*, *Bacteroides*, *Phascolarctobacterium*) and five operational taxonomic units (OTUs) (Clostridium-XI, *Bacteroides*, *Bacteroides*, *Lactobacillus*, Clostridium sedis) found during AHE were respectively associated with HE recurrence and overall survival during the subsequent 1-year follow-up (Sung et al., 2019).

Finally, in a prospective study involving 49 cirrhotic patients, Bloom et al. found eight species significantly less abundant in those patients with a history of OHE (*Anaeromassilibacillus species*, *Anaerostipes caccae*, *Bacteroideseggerthii*, *Clostridium species*, *Faecalicatena contorta*, *Holdemaniafiliformis*, *Neglecta timonensis*, and *Ruminococcus species*). However, none of the species was able to predict the future event of OHE. Moreover, they found an inverse correlation between bacterial species producing SCFAs and cirrhosis severity and lower concentrations of six specifical fecal SCFAs (acetate, propionate, butyrate, isobutyrate, valerate, and succinate) in patients with a history of OHE, thus supporting a crucial role of these metabolites in HE pathogenesis (Bloom et al., 2021).

THERAPY

Given the fundamental role of gut microbiota alteration in HE development, it is not surprising that most therapeutic strategies recommended by current guidelines primarily target gut microbiota or their bioproducts.

Lactulose

Lactulose, a synthetic non-absorbable disaccharide, is part of the therapeutic armamentarium to treat HE since its first trials in the 1960s (Elkington et al., 1969). Behind the cathartic effect that reduces the contact time between luminal contents and the intestinal mucosa, lactulose lowers colonic pH creating a hostile environment for urease-producing gut flora and stimulating growth-acid-resistant, non-urease producing species. Furthermore, it reduces the absorption of ammonia by non-ionic diffusion. In 2014, the European and American Associations for the Study of the Liver (EASL/AASLD) published a joint practice guideline in which they recommended lactulose as the treatment of choice for OHE and secondary prevention after an index event (Vilstrup et al., 2014).

Despite its effect on ammonia production and improvement of HE symptoms, evidence linking the impact of lactulose on species richness in the gut microbiota remains conflicting. In earlier studies using stool culture, lactulose administration altered the relative abundance of certain gut bacteria, especially acidophilic, urease-deficient bacteria, such as *Lactobacilli* and *Bifidobacteria* (Vince et al., 1974; Merli et al., 1992). More recent studies based on culture-independent methods failed to demonstrate significant differences in composition or diversity of gut microbiota associated with lactulose administration or withdrawal (Bajaj et al., 2012b). Interestingly, patients who responded to lactulose treatment had a favorable modification of bacterial taxa. A recent randomised controlled trial conducted in patients with HE found significant differences between lactulose responders and non-responders in *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* (Wang et al., 2019).

The apparent disconnection between reduction in blood ammonia and microbial changes, found in some studies, could be related to microbial changes below the detectable threshold or the relatively low sample size of the studies.

Antibiotics

Antibiotics are presumed to exert therapeutic effects by decreasing colonic populations of urease-producing bacteria and, in combination with lactulose, are historically a mainstay of HE treatment. Over time, the prescribing trends evolved from chlortetracycline in the 1950s to neomycin and others now, with antibiotics generally falling out of favor because of severe side effects.

Rifaximin is a common antibiotic with broad-spectrum activity against aerobic and anaerobic Gram-positive and Gram-negative bacteria. The administration of rifaximin in patients with HE improves hyperammonaemia, endotoxemia, cognitive dysfunction and stimulates the immune system (Takaya et al., 2018; Mangas-Losada et al., 2019). According to current clinical practice guidelines, rifaximin is recommended as add-on therapy to prevent OHE recurrence, although it is also indicated in combination with lactulose in patients with overt HE.

More recent studies demonstrated that rifaximin impacts the function or activities of the gut microbiota by increasing serum levels of long-chain fatty acids and carbohydrate metabolism intermediates in patients with minimal HE and favorably affect serum proinflammatory cytokine. Furthermore, rifaximin in patients with HE has been associated with reduced gut ammonia-production via the action of glutamine and changes in the metabolism of bacteria-produced agents, such as lipopolysaccharide and secondary bile acid (deoxycholic acid) that contribute to maintaining normal gut microbiota levels (Bajaj, 2016; DuPont, 2016; Kang et al., 2016).

Regarding the effects on the gut microbiota composition, rifaximin is associated with a modest decrease in *Veillonellaceae* and an increase in *Eubacteriaceae*. Furthermore, rifaximin diminished the diversity and abundance of ammonia-producing bacteria such as *Clostridium* and *Streptococcus*, a risk factor for HE (Bajaj et al., 2013; Zuo et al., 2017). Nevertheless, although the favorable modulation of the microbiome by rifaximin in patients with HE was effective, there was no significant change in the overall relative abundance of bacteria (Kawaguchi et al., 2019). A newer agent currently used in clinical trials for the treatment of HE is nitazoxanide, a broad-spectrum antibiotic and antiparasitic agent with activity against gut anaerobes. However, studies on the effect of microbiota composition are still lacking (Glal et al., 2021).

Probiotics

The World Health Organization defines probiotics as "live microorganisms that confer a health benefit on the host" (Hotel and Cordoba, 2001). Probiotics, with their pleiotropic effects, may be helpful to treat HE for their ability to suppress bacterial urease activity, lower ammonia absorption through pH reduction, modulate the immune response, and reduce intestinal permeability and uptake other toxins (indoles, oxindoles, phenols, and mercaptans). Furthermore, probiotics enhance the hepatic clearance of ammonia and other toxins by lowering gut-derived inflammatory signalling and oxidative stress in the liver (Solga, 2003). The most utilised probiotics include strains of lactic acid-producing bacilli (i.e., *Lactobacillus* and *Bifidobacterium*), non-pathogenic strains of *E. coli* (i.e., *E. coli Nissle* 1917), *Streptococcus salivarius*, a non-pathogenic strain of yeast (i.e., *Saccharomyces boulardii*), and a mixture of strains like VSL#3, which consists of eight different probiotic strains: *Streptococcus salivarius subp. thermophilus*, *Bifidobacterium breve*, *B. longum*, *B. infantis*, *Lactobacillus acidophilus*, *L. plantarum*, *L. paracasei*, and *L. bulgaricus*.

Several studies addressed the effect of probiotics, alone or in combination with standard therapy, in treating both MHE and OHE, with conflicting results. In MHE patients receiving probiotic Lactobacillus GG (LGG), Bajaj et al. described a significant improvement of dysbiosis characterised by the reduction of relative abundance of *Enterobacteriaceae* and increase in beneficial autochthonous taxa of *Lachnospiraceae* and Clostridiales Incertae Sedis XIV, (Bajaj et al., 2014a).

Treatment based on probiotics containing *C. butyricum* and *B. infantis* enriched Clostridium cluster I and *Bifidobacterium* abundance and decreased *Enterococci* and *Enterobacteriaceae* in MHE patients with HBV cirrhosis. Additionally, probiotic treatment was also associated with reducing venous ammonia and improved cognition (Xia et al., 2018).

The mixture of probiotics VSL#3 was found non-inferior to the standard therapy with lactulose in improving MHE and effectively preventing HE in patients with cirrhosis. (Lunia et al., 2014; Pratap Mouli et al., 2015). Moreover, a daily intake over 6 months significantly reduced the risk of hospitalisation for OHE. (Dhiman et al., 2014). On the other hand, Marlicz et al. did not find differences in incidence and grade of HE, assessed with critical flicker frequency, during probiotic supplementation (Marlicz et al., 2016). In the past years, multiple clinical trials and case reports have demonstrated the efficacy of symbiotics in HE treatment. The association of probiotics and fermentable fiber significantly increased the faecal content of non-urease-producing *Lactobacillus* species and was associated with a reduction in blood ammonia levels and reversal of MHE in 50% of patients (Liu et al., 2004). Furthermore, a 60 days treatment with a combination of *Bifidobacterium* and fructooligosaccharides was associated with a significant improvement of psychometric tests and blood ammonia levels when compared with the lactulose group in patients with HE (Malaguarnera et al., 2010).

Unfortunately, data from clinical trials on the use of probiotics to treat HE are difficult to compare because of differences in probiotic strains and delivery methods used, heterogeneity in study design and addressed endpoints (improved quality of life, progression from MHE to OHE, ammonia and endotoxemia reduction, gut microbiota modulation) (Khoruts et al., 2020). Finally, the relatively small number of colony-forming units in most commercial probiotic formulations hamper the optimistic conclusion that probiotics are sufficient to overtake the resident microbial community structure of cirrhosis and HE (Woodhouse et al., 2018).

A systematic review of 21 intervention trials including 1,420 participants indicated that probiotic supplementation vs placebo or no treatment reduced HE adverse events (including OHE development) and improved quality of life by lowering plasma ammonia concentration (Dalal et al., 2017). A more recent meta-analysis, including 14 randomised controlled trials and 1,132 patients, concluded that probiotic treatment effectively decreases serum ammonia and endotoxin levels, improves MHE, and prevents overt HE development in patients with liver cirrhosis. In addition, probiotics are as helpful as lactulose for MHE patients (Cao et al., 2018).

More recently, a meta-analysis including 25 trials and 1,563 participants found that probiotics effectively reversed minimal HE and prevented episodes of overt HE compared with placebo or no treatment; however, the evidence was low to moderate quality (Dhiman et al., 2020). Thus, drawing a definite conclusion on the efficacy of probiotics in HE still represents a tricky challenge.

Fecal Microbiota Transplantation

Fecal microbiota transplantation (FMT) refers to the transfer of stool from "healthy" donors to patients with disordered gut microbes, with the purpose to restore eubiosis (Vindigni and Surawicz, 2017).

In patients with HE, FMT may reduce ammonia synthesis by shifting the gut microbiota composition to bacterial taxa low in urease, diminishing ammonia uptake by re-establishing intestinal barrier integrity, and increasing ammonia clearance by improving liver function. Earlier studies on animal models correlated FMT with lower ammonia production in the gut, reduced risk of encephalopathy, and protective effect against carbon tetrachloride-induced acute hepatic dysfunction (Shen et al., 2015; Wang et al., 2017). Interestingly, if the donor was a patient with HE, FMT results in neuroinflammation and microbial ecological disorders (Liu et al., 2020).

In a paradigmatic case report, Kao et al. first demonstrated that serial FMT in a patient with mild HE improved the cognitive function, assessed with Stroop test and inhibitory control test (Kao et al., 2016).

Later, in a randomised controlled trial, Bajaj et al. demonstrated that FMT from a rationally selected donor (i.e., high *Lachnospiraceae* or *Ruminococcaceae*) in 10 cirrhotic patients suffering from recurrent OHE reduced hospitalisations, improved cognition, and dysbiosis compared to standard of care (Bajaj et al., 2017). Similar results were obtained in a phase 1 trial using FMT via oral capsules in recurrent OHE. Post-FMT, cognitive performance improved, and duodenal mucosal diversity increased with higher *Ruminococcaceae*, *Bifidobacteriaceae*, and lower *Streptococcaceae* and *Veillonellaceae* (Bajaj et al., 2019a).

Recently, a systematic meta-analysis comprising two randomised clinical trials, three case reports, and three rodent studies highlighted the association between FMT and improved neurocognitive tests, lower hospital readmission rate, and a reduction in serious adverse events (Madsen et al., 2021). Despite the potential benefits, the risk of infections, likely due to lack of donor screening, burdens FMT and limits its use in the context of clinical trials.

CONCLUSION

Moving from the pioneering era of culture-dependent studies, the connection between microbes, inflammation, and metabolic pathways in the pathogenesis of HE is becoming increasingly clear. PCR-based deep-sequencing technologies and metagenomic approaches are potent methods for studying microbiota and have provided high phylogenetic resolution of microbial communities inhabiting the gastrointestinal tract and their connection with the disease. However, they have substantial limitations. First, they unselectively detect microbes regardless of their viability, and different depths of sequencing lead to varying levels of selectivity. Furthermore, the results are based on the relative read abundances of microbial species in a given sample and thus do not provide exhaustive information on the function and dynamics of human-associated microbial ecosystems.

The recent evidence that viruses and fungi are active components of the gastrointestinal microbial ecosystem suggests that we are now starting to gain insight into the complexity of this organ. *In-vitro* models that resemble the human microbial environment, new methods to isolate and culture of previously unculturable bacteria, and emerging approaches to the study of the virome and mycome are now available. They will probably fill the gaps in our understanding of the microbiome's role in maintaining health and developing diseases. The next challenge is to apply this understanding to develop new therapeutic strategies that target the microbial ecosystem based on the patient's microbiome fingerprint.

AUTHOR CONTRIBUTIONS

AR and CS substantially contributed to the conception and design of the article. AR, CS, DC, PC, and ON interpreted the relevant literature. AR, CS, and DC drafted the article. AR and GN critically revised the article. All authors approved the final version of the paper.

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REVIEW
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Molecular Immune Mechanism of Intestinal Microbiota and Their Metabolites in the Occurrence and Development of Liver Cancer

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Intestinal microorganisms are closely associated with immunity, metabolism, and inflammation, and play an important role in health and diseases such as inflammatory bowel disease, diabetes, cardiovascular disease, Parkinson's disease, and cancer. Liver cancer is one of the most fatal cancers in humans. Most of liver cancers are slowly transformed from viral hepatitis, alcoholic liver disease, and non-alcoholic fatty liver disease. However, the relationship between intestinal microbiota and their metabolites, including short-chain fatty acids, bile acids, indoles, and ethanol, and liver cancer remains unclear. Here, we summarize the molecular immune mechanism of intestinal microbiota and their metabolites in the occurrence and development of liver cancer and reveal the important role of the microbiota-gut-liver axis in liver cancer. In addition, we describe how the intestinal flora can be balanced by antibiotics, probiotics, postbiotics, and fecal bacteria transplantation to improve the treatment of liver cancer. This review describes the immunomolecular mechanism of intestinal microbiota and their metabolites in the occurrence and development of hepatic cancer and provides theoretical evidence support for future clinical practice.

Keywords: intestinal flora, metabolites, liver cancer, occurrence and development, immune mechanism, therapeutics

INTRODUCTION

Liver disease is a critical and common disease, which represents a main health burden worldwide, with increasing morbidity and mortality (Acharya and Balaji., 2021). Globally, liver cirrhosis and liver cancer are the 11th and 16th most common causes of death, respectively (Asrani et al., 2019). Chronic liver disease (CLD) is caused by viral hepatitis, nonalcoholic fatty liver disease (NAFLD) and alcoholic liver disease (ALD), and can result in hepatocellular carcinoma (HCC) (Avila et al., 2020; Schwabe et al., 2020; Schwabe and Greten, 2020). Therefore, an active treatment for CLD is urgently required, especially liver cancer. However, there are currently no effective treatment measures for HCC because of its complex pathophysiological mechanism.

Recent studies have illustrated the association between intestinal microbiota and carcinogenesis, which is significant for the pathogenesis of liver cancer (McQuade et al., 2019; Kanmani et al., 2020; Schwabe and Greten, 2020). Increasing evidence has demonstrated the changes in gut microbiota

composition and function play a critical role in liver health from pre-cirrhotic stages to cirrhosis, decompensation, and the requirement of liver transplantation (Schwabe et al., 2020). The microbiota-gut-liver axis is the interaction between the liver, gut, and intestinal microbiota (Tripathi et al., 2018a). Intestinal-derived metabolites, cellular components, hormones, and other substances are transported into the liver through the portal circulation and interact with immune cells, causing inflammatory reactions and inducing the progression of various hepatic diseases. The liver is directly related to the gastrointestinal tract through the portal venous circulation and biliary system, so it is often exposed to bacterial products produced by intestinal microorganisms. The pathogenesis of HCC is linked to negative alterations to the gut microbiota, and the liver is connected to the intestine directly through the hepatic portal circulation (Gupta et al., 2019). However, the molecular mechanism underlying the role of the gut microbiota in hepatocarcinogenesis remains unclear.

Numerous studies have demonstrated that the association between the gut microbiota and the immune system may be a critical cause of hepatocarcinogenesis. The microbe-gut-liver axis plays an important role in liver cancer (Albillos et al., 2020). Various factors, such as drinking alcohol, high fat-diet, and bacterial infection can perturb the intestinal flora and destroy the intestinal barrier. This disruption results in the release of intestinal toxins and metabolites that target the liver and trigger the liver immune response *via* Kupffer cells (KCs), macrophage and neutrophil activation, and cell surface receptors combined with bacterial pathogen-associated molecular patterns (PAMPs); together, these processes activate a series of reactions that can lead to liver damage (Golonka and Vijay-Kumar., 2021). The liver may eventually develop hepatitis, cirrhosis, fibrosis, and even cancer. Intestinal bacteria and their metabolites also promote the occurrence and development of liver cancer through their receptors or inflammatory signal passageway. For example, intestinal bacteria ferment butyric acid, which is produced by non-absorbable fiber inulin, and can promote liver cancer (Wisniewski et al., 2019). Moreover, under the function of intestinal bacteria, primary bile acids can generate secondary bile acids, which will promote the occurrence and development of liver cancer (JM et al., 2014). Thus, intestinal bacteria and their metabolites are essential in the development of liver cancer and have attracted wide attention.

The gut microbiota affects immune responses, and studies have revealed a strong association between the gut microbiota and the response to immune checkpoint blockade. Therefore, methods of regulating the microbiome are being developed for various cancers. These methods include the use of fecal microbiota transplant, probiotic administration, and dietary intervention, all of which are being used experimentally for the treatment of liver cancer (McQuade et al., 2019).

Here, we summarize the molecular immune mechanism of gut microbiota in the occurrence of liver cancer and reveal the important role of the microbiota-gut-liver axis. In addition, we describe how the intestinal flora can be balanced by antibiotics, prebiotics, postbiotics, and fecal bacteria transplantation to improve the treatment of liver cancer. In conclusion, we summarize the immunomolecular mechanism of gut microorganisms and their metabolites in the occurrence of liver cancer, and provide theoretical evidence and support for future clinical practice.

LIVER DISEASES AND THE GUT MICROBIOME

The gut and liver are interconnected and interact with each other. Anatomically, the gut microbiota and their metabolites, enterogenous hormones, and nutrients help to maintain liver function and metabolism, while the liver absorbs these products from the gut and secrets bile acids to the gut (Delacroix et al., 1982). Disruptions of this interaction may lead to the development of liver diseases such as liver inflammation, ALD, NAFLD, non-alcoholic steatohepatitis (NASH), fibrosis, and cirrhosis (Seki et al., 2007; Cassard and Ciocan., 2018).

ALD

In addition to the direct toxicity of alcohol on hepatocytes cells, the pathogenesis of ALD is also related to gut microbiota disorder, loss of intestinal barrier function and activation of Toll-like receptors (TLRs) on hepatic immune cells (Albillos et al., 2020). Alcohol intake influences the gut microbiome, which occurs long before fibrosis develops (Bull-Otterson et al., 2013). In chronic alcoholism patients with jejunal inhalation, the intestinal overproduction of aerobic and anaerobic microorganisms has been widely recognized. In mice fed alcohol or in individuals with chronic ethanol abuse, metagenomic analysis of the intestinal microbiome has shown that bacterial diversity decreased and phylogeny transited to higher protein bacterial abundance (Chen et al., 2011; Yan et al., 2011; Mutlu et al., 2012). Interestingly, a specific microbial pattern, including a large number of *Bifidobacteria* and *Streptococci*, has recently found in the intestines of patients with severe alcoholic hepatitis (Llopis et al., 2016).

NAFLD

NAFLD can be broadly divided into non-progressive phenotype liver diseases, NAFL and NASH. NAFLD, closely related to obesity, has common mechanisms with type 2 diabetes mellitus, insulin resistance, and risk factors of cardiovascular disease. Previous studies have shown that a high-fat diet can change the microbiota, thereby damaging the intestinal barrier (Mouries et al., 2019), promoting the portal influx of bacterial products, aggravating non-liver inflammation, and generating metabolic abnormalities. Although a causal link between NAFLD and gut microbiota remains unclear, numerous studies have emphasized the effect of the gut microbiome in the pathogenesis of NAFLD. Indeed, preclinical studies have revealed that the prevalence of intestinal bacterial overgrowth (Kapil et al., 2016; Miele et al., 2009) and the changes in microbiota composition in NAFLD patients (Boursier et al., 2016) were higher than those in healthy controls. Using shotgun metagenomics sequencing, researchers have found a correlation between the microbiological characteristics of

patients with NAFLD and advanced fibrosis, which is characterized by the increased abundance of *Vulvobacterin* and *Escherichia coli* (Ren et al., 2019). Although NAFLD and ALD are the basic mechanisms of intestinal barrier dysfunction, there are subtle differences in their intestinal microbial composition, intestinal permeability, bile acids, and ethanol and choline metabolites, which need to be further studied.

Viral Hepatitis

Viral hepatitis, mainly including hepatitis A virus (HBV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV), and hepatitis E virus (HEV), is an infectious disease that seriously harms human health. Several studies have shown that gut dysbiosis is associated with viral hepatitis, chiefly HCV and HBV infection. HCV infection is a prime cause of cirrhosis, HCC, liver failure, and death (Craxì et al., 2008). Liver failure and disease progression in patients with HBV infection has been found to be linked to gut flora dysbiosis. HBV infection delays the development of the gut microbiota, and alters the dynamic changes in gut microbiota. One study found that the gut bacterial distribution in HBV infected mice was dominated by six phyla, namely, Bacteroidetes, Firmicutes, Verrucomicrobia, Proteobacteria, Actinobacteria, and Spirochaetes (Wang et al., 2019). Similarly, dysbiosis of intestinal flora can affect the progression of viral hepatitis.

Cholestatic Hepatitis

Cholestatic hepatitis is a type of bile duct excretion dysfunction with multiple causal factors. In patients with cholestatic hepatitis, bile cannot be actively discharged through the bile duct to the intestine and instead refluxes into the blood. One study identified seven genera, with different abundances between the intrahepatic cholestatic of pregnancy and healthy individuals. The seven genera included Escherichia/Shigella, Parabacteroides, Flavonifractor, Atopobium, Turicibacter, *Lactobacillus*, and Megamonas. In addition, the family Lactobacillaceae and the phylum Proteobacteria were also different between the two groups (Molinaro et al., 2018). Another study suggested that intestinal barrier function can be affected by cholestasis in the intestinal lumen (Abu Faddan et al., 2017). Thus, breaking the balance between the bile acids and gut microbiota can lead to inflammation.

Cirrhosis

Liver cirrhosis is accompanied by severe intestinal barrier impairment. The damage of the intestinal barrier in patients with decompensated cirrhosis is due to the damage of all levels of intestinal barrier defense, and is related to liver dysfunction, reduction of bile flow, and immune function impairment. Intestinal barrier dysfunction and the gut microbiome directly participate in the pathogenesis of compensated cirrhosis, and both are related to the incidence and severity of complications of decompensated cirrhosis, namely bacterial infection and encephalopathy. For decades, human and experimental liver cirrhosis models have highlighted changes in intestinal microbiota and bacterial overgrowth (Shah et al., 2017).

Currently, metagenomic techniques have been used to characterize the fecal microbiome in cirrhosis as one of reduced diversity, increased relative overgrowth of potentially pathogenic bacterium, such as Enterobacteriaceae, Staphylococcaceae and Enterococcaceae, and decreased relative abundance of the potentially beneficial bacterium Lachnospiraceae and Ruminococcaceae (Bajaj et al., 2013; Chen et al., 2011; Qin et al., 2014). The change in microbial composition in cirrhosis is the result of microbiome management: the decrease in intestinal movement and transport time occurs mainly at the ascites stage (Pérez Paramo et al., 2000; Gunnarsdottir et al., 2003; Yan et al., 2011); bile acid abnormalities, including a decrease in primary bile acids (PBAs) level and an increase in intestinal secondary bile acids (SBAs) level (Lorenzo-Zuniga et al., 2003; Kakiyama et al., 2013, 2014); and impaired intestinal immune function. Experimental cirrhotic ascites is related to impaired Paneth cell alpha-defensins and damage to dendritic cells (DCs) (Teltschik et al., 2012), both of which are particularly severe in rats with ascites and pathological bacterial translocation. Another factor leading to microbiota changes is hypochloremia during cirrhosis (Llorente et al., 2017). Remarkably the abnormal pattern of pathogenic bacteria is an independent factor in liver cirrhosis (Chen et al., 2011; Kakiyama et al., 2013).

ROLE OF THE MICROBE-GUT-LIVER AXIS IN THE OCCURRENCE AND DEVELOPMENT OF LIVER CANCER

Recently, increasing evidence has shown that the destruction of the gut-liver axis results in the progression of most CLDs, including cirrhosis and liver cancer (Kanmani et al., 2020). Dysbiosis and leaky gut are extraordinary characteristics of liver cirrhosis, which result in an increase in intestinal bacterial infections such as spontaneous bacterial peritonitis, which are considered to be responsible for the development of liver cancer in patients with cirrhosis. Moreover, striking features are observed at all phases of CLD, accelerating the gradual progression from fibrosis to cirrhosis and HCC (Tripathi et al., 2018b; Yu and Schwable., 2017). Moreover, intestinal leakage and intestinal disorders are linked; intestine leakages make bacterial metabolites and microbe-associated molecular patterns (MAMPs) easier to transpose and reach the liver, while intestinal disorders can lead to the formation of more permeable intestinal barriers (Schwabe and Greten, 2020).

Gut Leakage

The cirrhotic liver serves as the initial site for detoxification of microbial products from portal blood. "Leaky gut" refers to a situation of increased gut permeability for microbiota and their metabolites, which often occurs in hepatic cirrhosis and represents an important pathogenetic factor for major complications. Prolonged gut transit induces intestinal bacteria overgrowth, a pathological condition in which colon bacteria translocate into the small intestine. Furthermore, intestinal flora disorders have been detected in patients with cirrhosis of the liver, characterized by excessive growth of potentially pathogenic bacteria and a decrease in non-pathogenic native bacteria

(Henao-Mejia et al., 2012). Using high-molecular weight polyethylene glycol or FITC-dextran methods, intestinal leakage can be examined in patients or animal models of ethanol-associated and biliary liver disease (Parlesak et al., 2000; Yan et al., 2011; Fouts et al., 2012).

The mechanism underlying leaky gut is multifactorial and has been studied previously (Pradere et al., 2010; Yu and Schwabe, 2017). LPS, a component of the cell wall of Gram-negative bacteria that induces an inflammatory reaction *via* TLR4, is the most commonly used marker for inflammatory bacterial translocations. The primary levels of LPS gradually increase throughout the course of CLD, with the highest levels observed in patients with stage C cirrhosis of Child Pugh (Lin et al., 1995). Similarly, bacterial DNA levels of TLR9 agonists were elevated in CLD (Bellot et al., 2010). These results indicate that livers with chronic lesions are likely to be exposed to a wide range of TLR ligands as well as other bacterial metabolites. Moreover, an increase in ethanol metabolism in the gut due to excessive alcohol consumption, promotes intestinal dysfunction and excessive bacterial growth, resulting in intestinal leakage. This results in liver damage, which facilitates the mitosis of liver cells and inhibits apoptosis, ultimately leading to the development of HCC (Méndez-Sánchez et al., 2020).

Dysbiosis

When stressed by various disease processes, the human gut microbiome experiences dysbiosis, which promotes the progression of liver fibrosis/cirrhosis by increasing the inflammatory reaction and progressing toward fibrosis/cirrhosis (Albillos et al., 2020). For instance, alcohol consumption induces direct hepatoxicity by microbiota dysbiosis, which results from bacterial proliferation in the small/large intestine or direct microbial toxicity, and by immediate local lesion of the intestinal barrier, leading to increased bacterial translocation and inflammation (Mutlu et al., 2012; Leclercq et al., 2014; Meroni et al., 2019).

Recently, studies have shown the occurrence of dysbiosis at various stages of CLD. The most relevant studies reveal gut dysbiosis in cirrhosis (Qin et al., 2014; Pasolli et al., 2016; Ponziani et al., 2019; Ren et al., 2019), mainly during the progression stage of liver cancer and those in patients with liver cancer (Grąt et al., 2016; Ponziani et al., 2019; Ren et al., 2019) Clinical studies have demonstrated that the presence of liver cirrhosis can be accurately predicted by the change in the intestinal microflora (Pasolli et al., 2016), and emphasized that end-stage CLD is the most associated-dysbiosis disease. Dysbiosis alters various processes that influence the development of CLD and the subsequent progression of liver cancer, including inflammation, lesions, fibration and regeneration. Although the most significant changes in the gut microbiota have been observed between healthy controls and patients with liver cirrhosis (Qin et al., 2014; Pasolli et al., 2016), there is growing evidence of differences between patients with liver cirrhosis and those with HCC (Grat et al., 2016; Ponziani et al., 2019; Ren et al., 2019). Indeed, in patients with HCC and cirrhosis, excessive growth of *E. coli* in the gut was first reported in 2016 (Grat et al., 2016).

The microbiome study showed a decrease in fecal bacterial diversity from healthy controls to patients with liver cirrhosis, but an increase from patients with cirrhosis to those with early liver cancer with cirrhosis. Compared to NASH-induced cirrhosis without HCC, the abundance of Ruminococcaceae and Bacteroides increased, while the abundance of Akkermansia and Bifidobacterium decreased in patients with HCC (Ponziani et al., 2019). Moreover, researchers observed that in patients with HBV-related HCC, the richness in fecal microbiota was remarkably greater than in healthy groups and those with non-HBV non-HCV (NBNC)-related HCC. Furthermore, the feces of patients with NBNC-related HCC harbored more potential pro-inflammatory bacteria including Escherichia-Shigella and Enterococcus, and a decreased abundance of Faeca libacterium, Ruminococcus, and Ruminoclostridium, contributing to the reduction of anti-inflammatory short-chain fatty acids (SCFAs) (Liu et al., 2019). (**Figure 1**)

Variations in the microbial profile of liver cancer have been observed in previous studies, which may be due to differences in etiology, geographic area, and nutrient intake. In addition, the differences between patients with cirrhosis and HCC and those with cirrhosis of the liver are less marked than those observed between healthy patients and those with liver cirrhosis. Therefore, not only is the microbiome-based diagnostic test more potent than the liver cirrhosis screening test for liver cancer, it is also possible that microorganisms that have a functional impact on the development of liver cancer are primarily affected by cirrhotic alterations rather than specific HCC alterations.

ROLE OF GUT MICROBIOTA AND THEIR METABOLITES ON THE IMMUNE MECHANISMS OF THE PROGRESSION OF LIVER CANCER

LPS and TLRs

Intestinal microorganisms can affect the health of their hosts by producing cellular components such as LPS, peptidoglycans, lipoteichoic acid, flagellin, and DNA. The cellular components are transmitted from the portal circulation to the liver, where they actively interact with immune cells, causing inflammation and the progression of various liver diseases. Among the bacterial components, LPS is a key inflammatory molecule, which is increasingly translocated to the liver during intestinal dysbiosis (Kanmani et al., 2020). LPS can induce a systemic proinflammatory and fibrotic condition, where the transduction of insulin signals is disrupted, leading to an increase in the net lipidization of adipose tissue and the transport of free fatty acids from adipose tissue to the liver (Kang et al., 2017). Once excessive lipids are exposed to cellular stress in the liver, they are ultimately expanded through the proinflammatory environment of the liver and system. Subsequently, an acute reaction and liver fibrosis develop, which ultimately progress to cirrhosis of the liver (Kumar et al., 2017), and even liver cancer.

PAMPs and metabolites are derived from the actions of the intestinal microbiome on exogenous (food and environmental

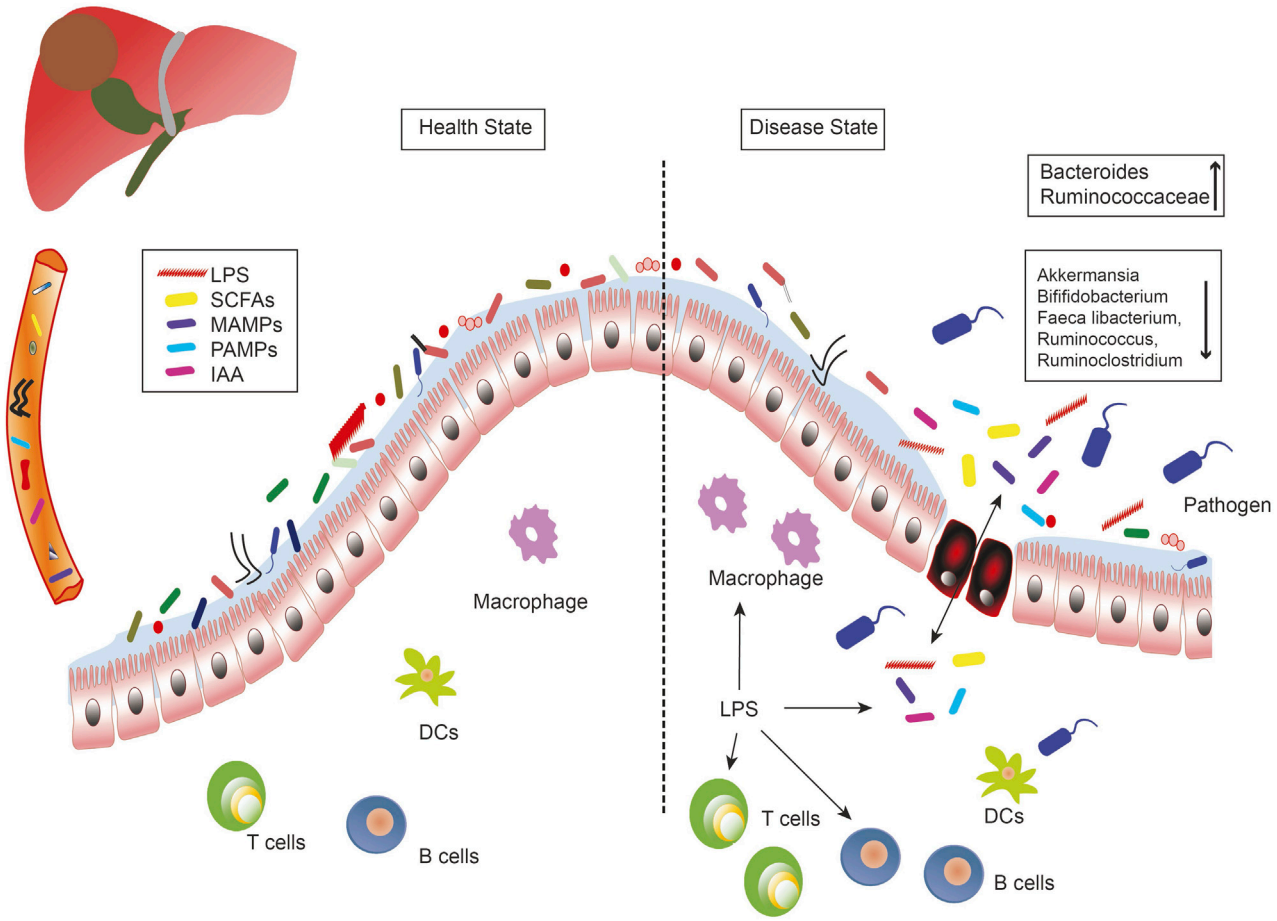

FIGURE 1 | The role of the microbe-gut-liver axis in the occurrence and development of liver cancer. Intestinal microorganisms, metabolites (SCFAs, IAA), and bacterial components (LPS, MAMPs, PAMPs) are transported to the liver through the portal vein, where they interact with immune cells and generate inflammatory responses to induce liver diseases. Intestinal leakage and dysregulation of flora are the important characteristics of the liver disease. DCs, dendritic cells; IAA, indole acetic acid; LPS, lipopolysaccharides; MAMPs, microbe associated molecular patterns; PAMPs, pathogen associated molecular patterns; SCFAs, short-chain fatty acids.

exposure) and endogenous substrates (amino acids and bile acids), which can reach the liver through the portal route and promote inflammatory reactions. In 1989, Charles Janeway first proposed that pattern recognition receptors (PRRs) recognize PAMPs to activate innate immunity and adaptive immunity (Janeway, 1989). TLRs, type-I transmembrane proteins, were the first identified PRRs to be capable of sensing pathogen infection. TLRs are the main receptors in cirrhosis for the identification of gut bacteria and represent an essential component of the congenital immune system, triggering the cascade of signals associated with the progression of cirrhosis. Among the ten human TLRs, TLR2, TLR4, TLR5, and TLR9 identify bacterial infection. TLR4 is the most widely studied PRR. Through LPS-binding protein, CD14 and myeloid differentiation protein 2 (MDP2), TLR4 can recognize and bind to LPS to produce response (Wen et al., 2017). Preclinical studies have confirmed that the contribution of PAMPs to NAFLD liver damage by a reduction in liver degeneration, inflammation, and fibrosis in mice with TLR4- or TLR9 deficiencies with a high-fat or low-choline diet (Rivera et al., 2007; Saberi et al., 2009; Miura et al., 2010). In addition, the absence of inflammatory microorganisms associated with changes in intestinal flora in mice led to steatosis and inflammation of the liver through the endovascular flow of TLR4 and TLR9 agonists, ultimately resulting in increased expression of TNF-αand inflammation in the liver, which is particularly severe in mice with liver steatosis (Henao-Mejia et al., 2012). Unlike TLR4 identifying Gram-negative bacteria, TLR2 primarily identifies Gram-positive bacteria. Lipoteichoic acid is a bacterial component of the intestine which, as a ligand of TLR2, contributes to the development of HCC in obese mice by increasing tumor-promoting senescence-associated secretory phenotype (SASP) of hepatic stellate cells (HSCs) and COX2 expression. COX2-induced prostaglandin E2 (PGE2) neutralizes anti-tumor immunity and facilitates the progression of HCC. TLR5 and TLR9 identify Gram-positive and Gram-negative bacterial flagellin and CpG DNA, respectively.

Immune Cells in Liver Cancer

Monocytes/Macrophages

Under the stimulation of endotoxin, monocytes from circulating sources can infiltrate the liver and differentiate into macrophages, which promote inflammatory mediators and oxygen free radicals to induce liver dysfunction and even liver failure. Monocyte-derived macrophage transformations are key events in hepatic inflammation (Zhao et al., 2018). In the liver, KCs and bone marrow-derived macrophages identify PAMPs from enterohepatic circulation *via* TLR4. TLR4 upregulation facilitates binding with its ligand, myeloid differentiation primary reaction 88, and leads to activation of mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK), and subsequent activation of the NF-κβ pathway. This in sequence induces the release of TNF-α, IFN-γ, prostaglandin-2, IL-1α, IL-1β, IL-6, reactive oxygen species (ROS), and nitric oxide, to maintain the liver inflammatory reaction. NF-κB also activates anti-apoptotic genes with important carcinogenic effects. An increase in TNF-α production has been shown to decrease tight junction (TJ) proteins and leads to intestinal barrier damage (Méndez-Sánchez et al., 2020). In addition, the activation of the TLR4 signaling pathway and intestinal bacteria accelerates the progression of liver cancer through mediating increased cell proliferation, expression of hepatomitogen epiregulin, and inhibition of apoptosis (Dapito et al., 2012).

KCs are resident macrophages in the liver and are essential to maintain immune tolerance by phagocytosing toxins from bacteria and xenobiotics. In the case of KC depletion, circulating monocytes can adopt the transcriptional profile of KCs. In the KC niche, interactions with HSCs, hepatocytes, and endothelial cells can also trigger monocyte recruitment. A cascade of interactive signals from Notch, liver X receptor-α, and transforming growth factor-β1 (TGF-β1) recruits monocytes to fill the population with liver macrophages into KCs. Similar to cyclic monocytes/macrophages, KCs can differentiate into M1- and M2-type cells in the chronic inflammatory process of the liver, but these subtypes serve as a double-edged sword in the context liver health. For NAFLD and NASH, M2-polarized macrophages play a protective role in resisting apoptosis and avoiding lipid accumulation, while a predominance of M1 macrophages aggravates these liver diseases. In contrast, *in vitro* studies of mature HCC cell lines and clinical HCC specimens (such as blood and liver tissue) have shown that a predominant M2 phenotype in liver cancer promotes tumor growth, cell migration, metastasis dependent on epithelial-mesenchymal transformation, drug resistance, and facilitates an immunosuppressive tumor microenvironment. In addition, intestinal flora disorder induces the production of IL-25 and promotes the progression of liver cancer by activating macrophage substitution and CXCL-10 secretion in the tumor microenvironment (Li et al., 2019).

Neutrophils

The neutrophil/lymphocyte ratio has been widely defined as a biological marker for diagnosis, prognosis, overall survival, preoperative, postoperative, and relapse of HCC. The spectrum of cytokines surrounding the tumor microenvironment can determine whether neutrophils differentiate as N1 or N2 immunosuppressant anti-tumor subtypes. In the early-stage of liver cancer, liver cancer-associated neutrophils (LCANs) are located in the periphery and have a cytotoxic N1 phenotype for tumor cells. Neutrophils differentiate into N2 phenotypes at an advanced stage of liver cancer, due to a shift in cytokine production in favor of CCL2 and CCL17, as well as an up-regulation of TGF-β. Thus, neutrophil and TGF-β blockage can slow the growth of liver cancer, and, in particular, inhibition of TGF-β can transform the LCAN population from an N2 to N1 phenotype. The intestinal microflora has no direct effect on the pathology of LCAN in HCC. However, the diversity and composition of intestinal microorganisms can affect the circulation and the level of liver neutrophils. Thus, the greater the diversity of intestinal flora, the lower the proportion of neutrophils/lymphocytes in the blood (Golonka and Vijay-Kumar., 2021).

Lymphocytes

When viable bacteria cross the barrier from the intestinal lumen, bacteria are commonly translocated from the gut to the hepatic or circulatory system. KCs activate the congenital activate immune response by releasing cytokines and producing ROS. Released cytokines stir up immune cells such as neutrophils and monocytes in the liver to control invasive microorganisms, but they also promote liver damage. The supplement of lymphocytes and neutrophils and the activation of HSCs occur, which ultimately leads to collagen production and fibrosis. Finally, constant natural killer cells (iNKT), usually present in the intestine, can also migrate into the liver during bacterial translocations caused by chronic alcohol abuse, and have been found to contribute to the apoptosis of liver cells (Bruellman and Llorente., 2021). In addition, the reduction of the intestinal microflora through antibiotic treatment inhibits the development of HCC in mice by increasing the accumulation of NKT cells in the liver and $CD4^+$ or $CD8^+$ memory T cells (Ma et al., 2018). Higher levels of PBAs, CXCL16, and CXCR6 can alter the accumulation of NKT cells in the liver cancer mice.

DCs

DCs in the gut can carry bacteria to mesenteric lymph nodes, causing a more localized immune response. Prolonged inflammation can impair the functioning of the lymphatic system and further damage the immune system of the liver. One of the main mechanisms of protection of the liver for the host is the secretion of IgA in the bile attached to bacteria. Disturbances in the host can cause the liver's immune system to further regulate the microflora to protect against damage to the intestinal barrier. More recently, DCs have received increasing attention in the cellular treatment of tumors (Montico et al., 2017; Saxena and Bhardwaj., 2017). Immature dendritic cells (imDCs) have the ability to phagocytize and process the antigen, but their ability to present antigen is low. In both *in vivo* and *in vitro* studies, various methods have been used to induce imDCs maturation to increase their antitumor activity (Crottes et al., 2016). Researchers have used different antigenic formulas to modify and charge DCs before injecting them into animal

models or patients, resulting in anticancer effects (Funda et al., 2018). (**Figure 3**).

Gut Microbiota Metabolites and Liver Cancer

PBAs and SBAs

In the liver, cholesterol is metabolized into PBAs, which are stored in and released from the gallbladder into the small intestine, and they can be used to dissolve lipids and fat-soluble dietary vitamins (JM et al., 2014). Considerable amounts of PBAs are reabsorbed from the ileum into the liver; a small proportion (~3%) are easily deconjugated, which enters the large intestine and metabolized into SBAs by gut bacteria (Jia et al., 2018). SBAs are increasingly considered as cancer-promoting metabolites. The intestinal microflora has been shown to use bile acids as messengers to impair immune functions and influence anti-cancer effects (Wahlstrom et al., 2016; Ma et al., 2018). Studies have suggested that Gram-positive bacteria will accumulate in mice fed a high-fat diet with enhanced bile acid processing capacity (Yoshimoto et al., 2013). Thus, when the antibiotic vancomycin inhibits endogenous production of deoxycholic acid (DCA), a high-lipid diet leads to an increase in the levels of DCA produced by bacteria, together with dimethylbenzanthracene and high-fat diet, which contributes to the development of liver cancer (Yoshimoto et al., 2013).

The liver is sensitive to the metabolites of intestinal bacteria and changes in the gut microflora affect the functioning of the liver's immune cells. The intestinal microorganisms participate in the physiological activity of the host by acting on the pool of bile acids, and thus regulate hormonal secretion and immunization by the metabolites produced. Ma et al. reported that reducing the abundance of intestinal Clostridial bacteria can increase PBAs levels and inhibit liver tumors. Moreover, DCA can increase TLR2 expression in HSCs together with increased TLR2 agonist lipoteichoic acid, which results in the tumor-promoting SASP (Loo et al., 2017).

The onset and development of primary liver cancer are modulated by SBAs through several different mechanisms, including DNA injury, inflammation-related tumorigenesis, and hepatotoxicity (Yoshimoto et al., 2013; Bourzac, 2014), which favor an immunosuppressive tumor microenvironment by reducing the accumulation of natural killer T (NKT) cells in the liver (Hartmann and Kronenberg., 2018; Ma et al., 2018; Mossanen et al., 2019) Another mechanism by which intestinal microorganism-induced changes in the metabolism of bile acids control the growth of liver cancer is *via* the regulation of CXC chemokine ligand 16 (CXCL16) expression in the liver and the recruitment of NKT cells by CXCL16 (Ma et al., 2018). PBAs have been shown to play a key role in the up-regulation of CXC16 in endothelial cells in the liver sinus, which in turn contributes to the recruitment of NKT cells. NKT cells subsequently kill tumor cells in a CD1-dependent manner. In another study, antibiotics inhibited the development of liver cancer induced by high cholesterol and a fat NASH diet, along with a significant reduction in SBAs, which activate the mammalian target of rapamycin (mTOR) pathway in liver cells (Yamada et al., 2018).

The liver influences the Th17 and Treg balance of the intrinsic layer *via* a derivative of lithocholic acid (LCA), a bile acid metabolite. 3-OxoLCA can combine with retinoic acid-associated orphan receptors γT (ROR γT) to prevent the proliferation of Th17 cells, while isoalloLCA produces mitochondrial ROS, leading to the up-regulation of forkhead box protein 3 (FOXP3), a regulator of Treg differentiation. In mice fed a Lieber DeCarli ethanol diet, the farnesoid X receptor (FXR), a regulator of bile acid and lipid metabolism, was lowered, resulting in an increase in bile acid levels produced and secreted by the liver. Degeneration of liver fat and ALD are likely to occur in mice depleted by FXR, as well as in chronic feeding models with excess ethanol. Experimental treatment of DK-naive T cells isolated from mice defected by FXR with isoalloLCA and 3-oxolca did not alter the expression of FOXP3 in relation to the control group. Moreover, in cells treated with 3-oxoLCA, FXR did not help inhibit Th17 cells (Bruellman and Llorente., 2021).

SCFAs

SCFAs represent an important category of bacterial metabolites, and are considered to be the richest microbiome-derived metabolites in the gut (Zhang Z. et al., 2019). Intestinal flora produce various gastrointestinal enzymes, including propionate and acetate coenzyme A transferase, and butyrate kinase, all of which transfer complex or undigested carbohydrates of diets into host absorbable SCFAs, mainly acetic acid, propionic acid, and butyrate (El Kaoutari et al., 2013; Wisniewski et al., 2019). For example, Bacteroidetes produce acetate and propionate, which can be delivered directly through the portal vein to peripheral tissues, including the liver and adipose tissue, for lipogenesis and gluconeogenesis (Koh et al., 2016; Wilson et al., 2017). In addition, SCFAs have a strong ability to inhibit intestinal inflammation and prevent pathogen invasion, as well as to maintain barrier integrity, primarily by activating GPCRs or inducing their inhibitory effects on HDACs to further influence gene expression (Zhang Z. et al., 2019). Although SCFAs are generally considered health-promoting factors, particularly for colonic epithelial cells, one study showed that high dietary consumption of inulin, a non-absorbable fiber that is converted to butyrate, promoted the development of HCC in mice with dysbacteriosis (Singh et al., 2018). Evidence has shown that an imbalance between T helper 17 cells (Th17s) and regulatory T cells (Tregs) is associated with aberrant immune responses. Recent advances in culture-independent techniques for the detection and identification of intestinal commensal bacteria enabled the discovery that Th17 and Treg differentiation are regulated by SCFAs, particularly butyrate, produced by the gut microbiota. This finding provided a mechanistic link between dysbiosis, defined as changes in the composition of the gut microbiota, and various inflammatory diseases. On this basis, research suggested that dysbiosis with reduced production of SCFAs leading to Th17/Treg imbalance, is involved in the etiology of liver cancer. Thus, the inhibition of fermentation by drugs or the depletion of fermentation bacteria can significantly reduce SCFAs and prevent HCC (Singh et al., 2018). In contrast, another study showed that propionate improves the cytotoxic effect of cisplatin on liver cancer by modulating the G-protein coupled receptor 41 (GPR41) signaling pathway (Kobayashi et al., 2018).

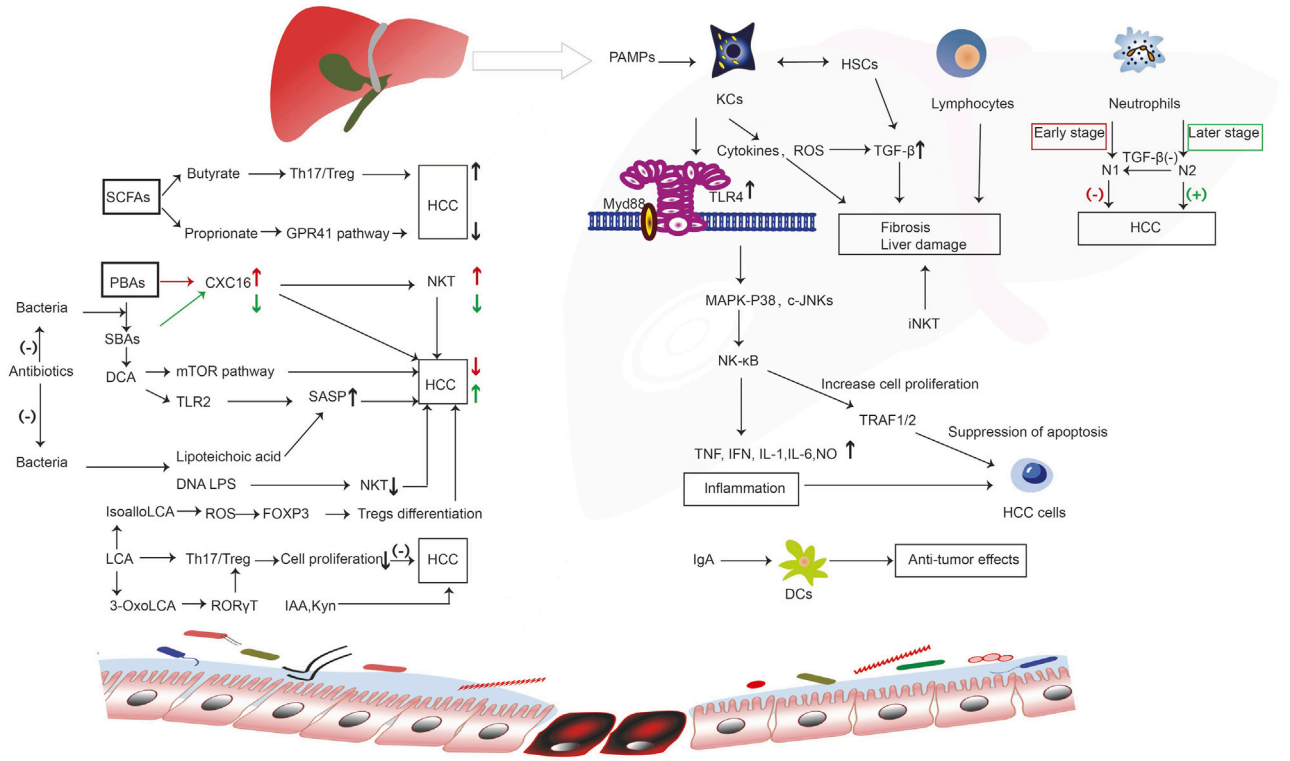

FIGURE 2 | The immune molecular mechanism of intestinal microorganisms and their metabolites in the occurrence and development of liver cancer. Bacterial components such as LPS and SCFA butyrate bind to receptors to regulate the activity of immune cells. Liver-derived PBAs enter the intestinal tract and generate SBAs under the action of bacteria, which promotes the occurrence of liver cancer through a series of immune reactions. However, SCFA proprionate binds to GPR41 receptor, and the microbial metabolism produces IAA and Kyn to inhibit the occurrence of liver cancer through a series of reactions. Enterogenous microorganism and metabolic products reach the liver, activating liver immune cells, including KCs, lymphocytes, and neutrophils; these cell surface receptors and PAMPs undergo ligand binding, triggering a series of immune cascade, induce inflammation, promote or inhibit liver cell hyperplasia of fibers, liver cell proliferation, differentiation, and apoptosis, thus playing a role in promoting or inhibiting liver cancer; Intestinal DCs can interact with IgA that is released by the liver to impart an anti-cancer effect. However, SCFAs and BAs (PBAs, SBAs, DCA, LCA) produced by intestinal microorganisms can bind to corresponding receptors, trigger a series of immune responses, regulate CXC16 levels, SASP, NKT, Treg differentiation, and Th17/Treg, and promote or inhibit liver cancer. CXC16, CXC chemokine ligand 16; c-JNKs, c-Jun N-terminal kinases; DCs, dendritic cells; DCA, deoxycholic acid; FOXP3, forkhead box protein 3; GPR41, G-protein coupled receptor 41; HCC, hepatocellular carcinoma; HSCs, hepatic stellate cells; iNKT, invariant natural killer; IAA, indole-3-acetic acid; IFN, interferon; IL-1, interleukin-1; IL-6, interleukin-6; KCs, kupffer cells; Kyn, kynurenine; LCA, lithocholic acid; LTA, lipoteichoic acid; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; NF-kB, nuclear factor-kB; N1, neutrophils 1; N2, neutrophils 2; NKT, natural killer; NO, nitric oxide; PBAs, primary bile acids; RORγT, retinoic acid-associated orphan receptors γT ; ROS, reactive oxygen species; SCFAs, short-chain fatty acids; SASP, senescence-associated secretory phenotype; SBAs, secondary bile acids; TLR, toll-like receptor; TGF-β1, transforming growth factor-β1; Th17/Treg, T helper 17/ regulatory T; TRAF1/2, tumor necrosis receptor associated factors 1/2.

Other Gut Metabolites

Indol-3-acetic acid (IAA), an intestinal-derived metabolite, reduces high-fat diet-induced hepatotoxicity by improving liver lipogenesis, lipid metabolism, and inflammatory and oxidative stress in mice (Ji et al., 2019). Moreover, in SK-HEP-1-implanted nude mice, IAA can induce tumor regression, and may represent a novel cancer therapeutic agent (Park et al., 2009). Another study showed the IAA confers protection against HCC by affecting antioxidant gene expression and DNA fragmentation (Mourão et al., 2009). As an endogenous ligand of the human activation of the aryl hydrocarbon receptor, the tryptophan (Trp) catabolite kynurenine (Kyn) is a liver-derived Trp-degrading enzyme that may be associated with HCC (Opitz et al., 2011). The inability of D-Trp-6-luteinizing hormone releasing hormone cannot inhibit the development of HCC (Guéchot et al., 1989). The transcription of enzymes implicated in L-Trp metabolism causes DNA injury during the early stages of tumorigenesis (Tummala et al., 2014). (**Figure 2**).

In short, gut microbiota and their metabolites reach the liver and activate liver immune cells, including KCs, lymphocytes, and neutrophils, inhibiting or promoting the occurrence and development of liver cancer through various immune response mechanisms. Intestinal DCs interact with IgA released by the liver, thereby producing anti-cancer effects. Intestine microbe metabolites (e.g., PBAs, SBAs, DCA, LCA, SCFAs) regulate the development and progression of liver cancer by stimulating or inhibiting immune reactions and inflammation in the liver.

In addition, we also focused on the relationship between the changes of liver cancer biomarkers and intestinal microflora and metabolites. As it is well known, serum alpha-fetoprotein (α-AFP), structure-specific recognition protein 1 and lamin B1 are important biomarkers for the diagnosis of liver cancer, as well

as important indicators for the evaluation of therapeutic effect and prognosis (Feng et al., 2021). Studies have shown that these biomarkers are involved in the immune and inflammatory responses in the development and progression of liver cancer, among which, the level of α-AFP is associated with the diversity of intestinal microorganisms (Zhang et al., 2019a). Elshaer et al. reported that in thioacetame-induced liver cirrhosis rats, the TLR4-CXCL9 pathway was activated and the serum α-AFP level increased, which could be prevented by administration of *Lactobacillus* Plantarum (Elshaer et al., 2019). Another study reported that the levels of serum zonulin, a marker of intestinal permeability, increased significantly in patients with liver cirrhosis and HCC. The combination of zonulin and AFP exhibited a significantly larger receiver operating characteristic curve compared to zonulin or AFP alone, suggesting that their combination confers significant benefit to diagnostic accuracy in differentiating liver cirrhosis from HCC (Wang et al., 2019). However, to the best of our knowledge, there are limited studies on the effects of intestinal bacteria and metabolites on liver cancer biomarkers. In future, it will be possible to pay attention to their relationship, or even combine their detection, to provide a basis for the diagnosis, treatment, and prognosis of liver cancer.

POTENTIATING THE ANTICANCER EFFECTS OF THERAPEUTIC DRUGS BY REGULATING THE GUT MICROBIOTA

Changes in intestinal flora are associated with resistance to chemotherapy drugs. Using antibiotics, post-biotics, probiotics, fecal microflora transplant (FMT), or nanotechnologies, to regulate microflora may enhance the anti-cancer effects of chemotherapy agents (Compare et al., 2017; Cheng et al., 2020).

Antibiotics

Studies have shown that antibiotic treatment at advanced stages of HCC in mice may be effective in reducing cancer development (Meroni et al., 2019). For example, rifaximine is currently being tested on the microflora in various clinical trials of CLD as the safest non-absorbable antibiotic (Wiest et al., 2017). Rifaximine is a broad-spectrum compound that reduces endotoxin and anti-inflammatory effects independent of its bactericidal effects. The combination of rifaximine and simvastatin for the treatment of key mechanisms of liver cirrhosis progression, namely liver and intestinal and systemic inflammatory reactions, is currently undergoing a clinical phase 3, multicenter, double-blind, placebo-controlled trial to prevent ACLF in patients with decompensated cirrhosis. Recent studies have shown that the levels of some SBAs in the liver decreased after antibiotics depleted commensal bacteria from the gut microbiome in mice. This relieved inhibition of CXCL16, a potent recruiter of NKT cells, and also increased the levels of certain PBAs known to induce CXCL16, resulting in the accumulation of hepatic NKT cells and a reduction in liver tumors.

Postbiotics

Recent data indicate that the potential mechanisms for controlling micro-organism-based intestinal homeostasis depend on their metabolites, also known as postbiotics (Tsilingiri and Resscigno., 2013). Postbiotics are advanced microbiology tools that can be used to maintain long-term health benefits. Their compounds are variable and depend on the strain and their metabolic state and include SCFAs, SBAs, proteins, enzymes, peptides, bacteriocins, polysaccharides, vitamins and organic acids (Compare et al., 2017), all of which have been described as having an immuno-regulatory and protective effect in the intestinal barrier. Postbiotics can strengthen the structure of the close bonding of the epithelium by increasing the expression of TJs proteins and intestinal mucin, which can promote the restoration of intestinal barrier function (Tsilingiri et al., 2012). In particular, postbiotic agents can act on immune cells and protect intestinal tissues from immunopathological damage by increasing the secretion of anti-inflammatory cytokines such as IL-10 (Mileti et al., 2009; Compare et al., 2017).

Postbiotic agents also manifest as factors inducing the elasticity of the microflora. They may act as inhibitors of pathogenic bacteria or possibly as signal quorum molecules, regulating the density of bacterial cells and supporting the formation of biological membranes of microbial composition (Fanning et al., 2012). Studies have shown that postbiotic agents have anti-proliferation, anti-inflammatory, and anti-cancer properties, and are able to regulate the effectiveness of cancer treatments and reduce the side effects of traditional treatments on patients with cancer (Rad et al., 2021).

Probiotics

Numerous clinical trials have been conducted to study the effects of prebiotics/probiotics on cancer. Some trials showed improved clinical outcomes in patients using probiotics (Mego et al., 2015; Theodoropoulos et al., 2016; Flesch et al., 2017; Tian et al., 2019), while others were unable to verify the significant effects of receiving probiotics. A prospective clinical study in patients with colorectal cancer showed that *Lactobacillus acidophilus* NCFM and *Bifidobacterium lactis* Bl-04 increased the levels of *butyrate-producing bacteria,* including *Faecalibacterium and Clostridiales* spp., but decreased the level of bacteria associated with colorectal cancer, such as *Fusobacterium* and *Peptostreptococcus* (Hibberd et al., 2017). In addition to altering the features of microorganisms, probiotics have also been reported to suppress the development of cancer in animal models. Zhang *et al.* established a model of liver cancer in rats using diethylnitroamine to reveal that oral VSL#3 probiotic mixture reduced intestinal inflammatory reactions, maintained the integrity of the intestinal mucosa, and inhibited tumor growth (Zhang et al., 2012). A subsequent study showed that the probiotic mixture Prohep decreased the number of Th17 cells in the tumor and thus inhibited the development of liver cancer in a mouse model grafted under the skin (Li et al., 2016). Clinical trials evaluating the therapeutic potential of VSL#3 in patients with liver cirrhosis (Dhiman et al., 2014) or NAFLD (Anderson et al., 2004) have shown that probiotics alleviate the severity of diseases closely related to the progression of liver cancer (Michelotti et al., 2013).

However, some clinical trials have found no evidence of the clinical benefits of probiotics in the treatment of cancer (McNaught

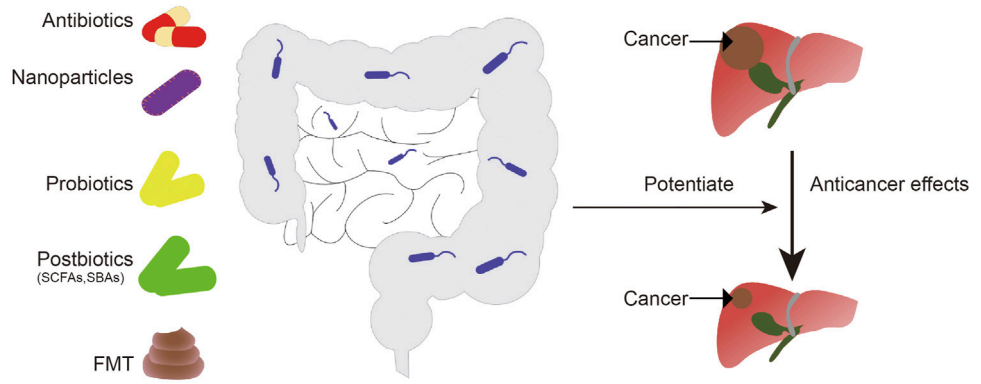

FIGURE 3 | Intestinal microorganisms can be regulated by antibiotics, probiotics, postbiotics, fecal bacteria transplantation, and nanoparticles to enhance the anti-liver cancer effect of chemotherapy drugs. FMT, fecal microbiota transplantation; SCFAs, short-chain fatty acids; SBAs, secondary bile acids.

et al., 2002; Anderson et al., 2004). Postoperative treatment of patients with head and neck cancer with mixed strains of *Lactobacillus* and Bifidobacterium did not improve clinical outcomes. Moreover, patients treated with probiotics and those treated with placebo were shown to have similar postoperative infection rates, inflammatory markers, and levels of diaminoxydase, which are indicators of intestinal permeability (Lages et al., 2018). The conflicting results of clinical trials can be explained by differences in the microbiome and host genome between individuals. Colonization and function of probiotics are affected by native microflora, host gene expression features, and other exogenous factors (Morgan et al., 2012; Lages et al., 2018). Evaluating the clinical benefits of probiotics in patients with cancer therefore remains a challenge. Moreover, most probiotic clinical trials have limitations such as reduced sample size, short duration of treatment, and lack of follow-up of long-term effects of probiotics on patients. Thus, well-designed studies are essential to evaluate the probiotic treatment of cancer patients with cancer.

FMT

FMT is defined as the digestive transmission of intestinal microflora from healthy donors to unhealthy recipients, with the objective of restoring intestinal homeostasis or establishing a new balance to eliminate or improve disease (van Nood et al., 2013). FMT has been recognized by official guidelines as the standard treatment for recurrent *clostridium difficile* infection (CDI), with a cure rate close to 90% (van Nood et al., 2013; Chen et al., 2019). A previous clinical showed that patients with liver cirrhosis and recurrent liver encephalopathy were well tolerated and safe in the long term by oral administration in capsules after pre-treatment with antibiotics (Bajaj et al., 2018; Bajaj et al., 2019a; Bajaj et al., 2019b). Moreover, FMT has been shown to restore antibiotic-related microbiological biodiversity and reduce function destruction, resulting in continuous improvement in cognitive function parameters and reduced rates of liver encephalopathy re-emergence and liver-related hospitalization (Bajaj et al., 2018; Bajaj et al., 2019a; Bajaj et al., 2019b). FMT has also been shown to reverse early portal hypertension, intra-hepatic endothelial dysfunction, and insulin resistance in rats on a high-fat, fructose diet (Garcia-Lezana et al., 2018). Generally, to make FMT more viable in the treatment of cancer, the choice of the ideal donor remains a crucial question, as preliminary evidence indicates that the donor's intestinal microbiome is a determining factor in the response rate to the patient in cancerous mice (Routy et al., 2018). However, until we can identify a microflora that supports cancer immunotherapy, therapists should make use of the balanced fecal microflora of healthy donors rather than the patient's disturbed microflora. There is no consensus on which species or combinations of bacteria are the best option to enhance immune effects, and further research is needed in this area.

Nanoparticles

Ongoing clinical trials have shown that nanotechnology can be used to target cancer-related bacteria or to release anticancer agents in a controlled manner, with fewer side reactions (Angsantikul et al., 2018; Song et al., 2019). Given the impact of nanotechnology on cancer prevention and treatment, the assessment of toxicity, side effects, and downstream mechanisms mediated by nanoparticles should be considered. In addition, the interactions between NPs and the immune system may have effects on the gut microbiota. For example, exosome-like NP (ELNs) RNAs regulate gut microbiota to enhance the function of the gut barrier (Teng et al., 2018), and phage-guided irinotecan-loaded dextran NPs promote release of bacterial derived butyrate, which may improve the therapeutic strategy of tumor (Kannen et al., 2019). By combining Fe@Fe3O4 NPs with ginsenoside Rg3 (NpRg3), a new nano-drug is currently being developed, with good efficacy for liver cancer (Ren et al., 2020). (**Figure 3**).

CONCLUSION AND PERSPECTIVES

In conclusion, this review summarizes the molecular immune mechanisms of gut microbiota in the occurrence and development of liver cancer, revealing the important role of the microbiota-gut-liver axis on liver cancer. In addition, we describe how to balance the intestinal flora by regulating diet,

antibiotics, prebiotics, postbiotics, and fecal bacteria transplantation to improve the treatment of liver cancer.

However, this paper still has some limitations. In this review, there are more studies on the cell and animal models, but fewer clinical studies, especially on the relationship between microbial metabolites and liver cancer, which need to be further strengthened. In terms of the molecular immune mechanism, there are still many problems that need to be resolved in the study of microbes and metabolites and liver cancer; for example, whether SCFAs play a role in the occurrence and development of liver cancer and whether there are differences between different types and different individuals, particularly individual differences in microbes and whether the results are consistent in animals and humans. Second, further investigations on the regulation of intestinal microbiota to enhance the efficacy of tumor chemotherapy and preclinical and clinical studies on HCC are warranted. In conclusion, intestinal microbes and their metabolites are closely related to the occurrence and development of liver cancer. Regulating intestinal microorganisms represents a promising strategy to enhance the efficacy of immunotherapy in liver cancer and reduce adverse reactions.

AUTHOR CONTRIBUTIONS

JZ and ZL initiated and made the outline of the manuscript. CB, GX, and CL drafted the manuscript, with contributions from all other authors.

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