THE MICROBIOME IN HEPATOBILIARY AND INTESTINAL DISEASE

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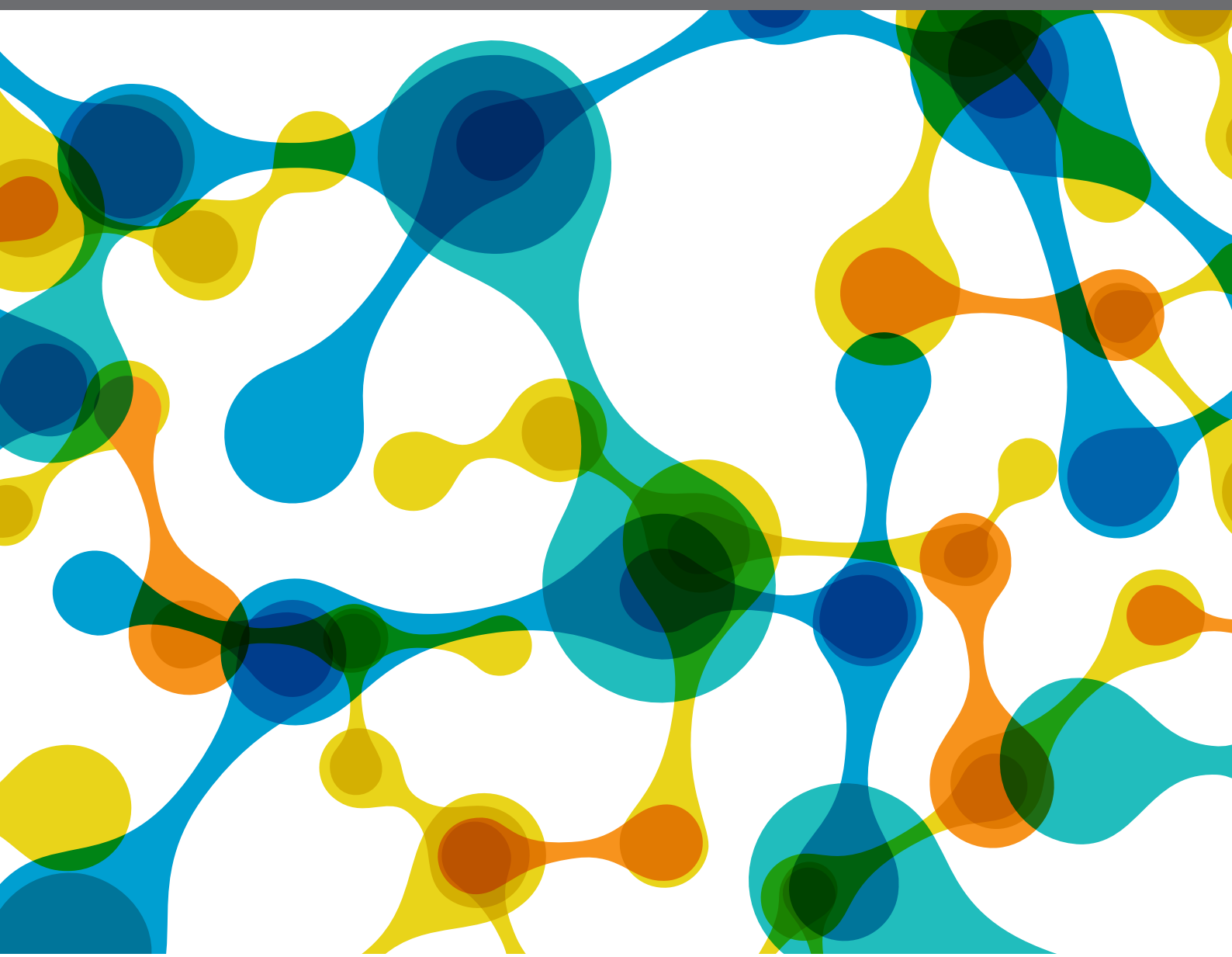

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THE MICROBIOME IN HEPATOBILIARY AND INTESTINAL DISEASE

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Table of Contents

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Editorial: The Microbiome in Hepatobiliary and Intestinal Disease

Phillipp Hartmann[1,2]*

[1]Department of Pediatrics, University of California, San Diego, San Diego, CA, United States, [2]Division of Gastroenterology, Hepatology and Nutrition, Rady Children's Hospital San Diego, San Diego, CA, United States

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Editorial on the Research Topic

The Microbiome in Hepatobiliary and Intestinal Disease

INTRODUCTION

This Editorial provides a brief overview of the changes in the intestinal microbiome with focus on the bacterial microbiome in a wide range of diseases affecting the human digestive system and then highlights the specific articles of this Research Topic.

GUT MICROBIOME CHANGES IN DISEASES AFFECTING THE HUMAN DIGESTIVE SYSTEM

Essentially all diseases affecting the human digestive system are associated with significant increases and decreases of sub-populations of the gut microbiome compared with controls subjects; recurrent changes of the intestinal bacterial microbiome observed across 30 different conditions of the human digestive system are summarized in **Figure 1**. Briefly, *Streptococcus* (Llopis et al., 2016; Maccioni et al., 2020; Gao et al., 2021), *Actinomyces* (Ciocan et al., 2018; Maccioni et al., 2020; Gao et al., 2021), and *Rothia* (Ciocan et al., 2018; Maccioni et al., 2020) are increased, whereas *Faecalibacterium* (*prausnitzii*) (Gao et al., 2020a; Gao et al., 2020b; Maccioni et al., 2020) and *Bacteroides* (Puri et al., 2018; Gao et al., 2020a; Maccioni et al., 2020; Gao et al., 2021) are decreased in abundance in alcohol-associated liver disease (ALD). *Faecalibacterium* (*prausnitzii*) (Wong et al., 2013; Da Silva et al., 2018; Oh et al., 2020a) is also detected at diminished concentrations in non-alcoholic fatty liver disease (NAFLD) similar to *Coprococcus* (Zhu et al., 2013; Wang et al., 2016; Da Silva et al., 2018), whereas *Escherichia (coli)* (Zhu et al., 2013; Jiang et al., 2015; Oh et al., 2020a) and *Lactobacillus* (Raman et al., 2013; Jiang et al., 2015; Da Silva et al., 2018) are increased in NAFLD. Liver cirrhosis is associated with elevated intestinal levels of *Enterococcus (faecalis)* (Zhao et al., 2004; Chen et al., 2011; Bajaj et al., 2012), *Prevotella* (Qin et al., 2014; Chen et al., 2016; Shao et al., 2018; Ponziani et al., 2019; Zeng et al., 2020), *Clostridium* (Zhao et al., 2004; Chen et al., 2011; Bajaj et al., 2012; Qin et al., 2014; Heidrich et al., 2018; Shao et al., 2018), *Veillonella* (Qin et al., 2014; Chen et al., 2016; Shao et al., 2018; Oh et al., 2020a; Zeng et al., 2020), *Lactobacillus* (Qin et al., 2014; Heidrich et al., 2018; Shao et al., 2018; Ponziani et al., 2019; Zeng et al., 2020), *Atopobium* (Chen et al., 2016; Ponziani et al., 2019; Zeng et al., 2020), and *Streptococcus* (Qin et al., 2014; Shao et al., 2018; Ponziani et al., 2019; Oh et al., 2020a), and with reduced levels of *Dorea* (Bajaj et al., 2012; Oh et al., 2020a), *Alistipes* (Qin et al., 2014; Shao et al., 2018; Oh et al., 2020a), and *Subdoligranulum* (Bajaj et al., 2012; Qin et al., 2014; Shao et al., 2018). Gut microbiome changes in hepatocellular carcinoma (HCC) are similar to the

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***Correspondence:**
Phillipp Hartmann
phhartmann@ucsd.edu

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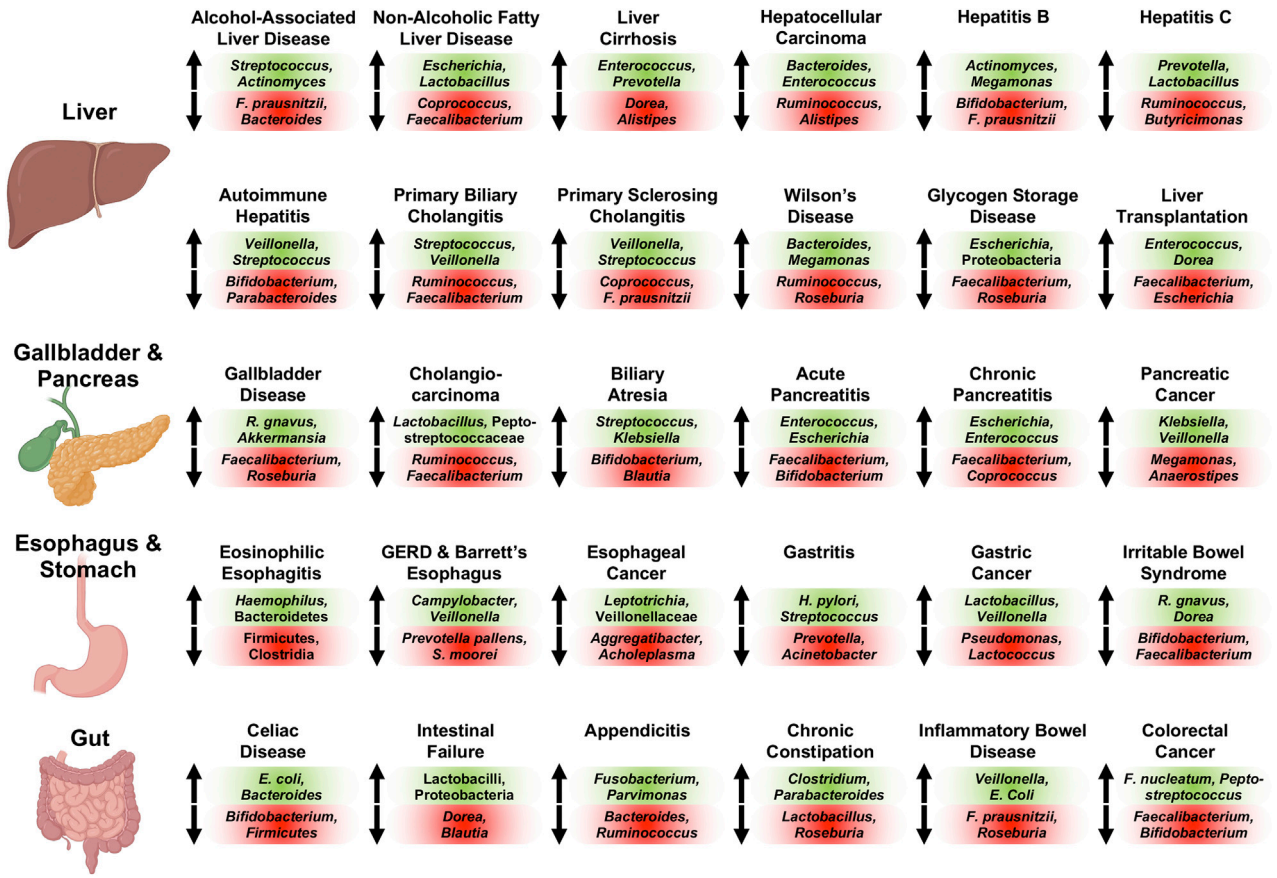

FIGURE 1 | Representative associated intestinal bacterial microbiome changes across diseases of the human digestive system with focus on genera. Changes described here were observed in various publications for the respective conditions as detailed in the manuscript. Bacterial populations on green background are increased in abundance and populations on red background are decreased in abundance for the respective disease. Of note, *Veillonella* and Veillonellaceae, *Streptococcus*, *Lactobacillus* and Lactobacilli, *Escherichia (coli)*, *Enterococcus (faecalis* and *faecium*), and *Klebsiella* are oftentimes found at increased concentrations, whereas *Faecalibacterium (prausnitzii)*, *Ruminococcus* and Ruminococcaceae (except *Ruminococcus gnavus*), *Bifidobacterium*, *Roseburia*, and *Coprococcus* are frequently decreased in abundance in various digestive diseases. Created with a license from Biorender.com. *E. coli, Escherichia coli*; *F. nucleatum, Fusobacterium nucleatum; F. prausnitzii, Faecalibacterium prausnitzii*; GERD, gastroesophageal reflux disease; *R. gnavus, Ruminococcus gnavus; S. moorei, Solobacterium moorei.*

ones identified in liver cirrhosis, indicating their relationship also on a microbial level. HCC is frequently linked to high intestinal amounts of *Bacteroides* (Ponziani et al., 2019; Huang et al., 2020; Zeng et al., 2020), *Enterococcus* (Ni et al., 2019; Ponziani et al., 2019; Xin et al., 2019), *Veillonella* (Ni et al., 2019; Zeng et al., 2020), and *Atopobium* (Ni et al., 2019; Zeng et al., 2020), and low amounts of *Ruminococcus* (Ren et al., 2019; Zeng et al., 2020), *Alistipes* (Ren et al., 2019; Huang et al., 2020), and *Bifidobacterium* (Ponziani et al., 2019; Xin et al., 2019). Hepatitis B is associated with increased *Actinomyces* (Wang et al., 2017; Yao et al., 2021), *Megamonas* (Wang et al., 2017; Joo et al., 2021), *Enterococcus* (*faecalis*) (Lu et al., 2011; Yao et al., 2021), *Veillonella* (Yang et al., 2020; Zeng et al., 2020; Yao et al., 2021), *Streptococcus* (Yang et al., 2020; Yao et al., 2021), and *Atopobium* (Zeng et al., 2020; Yao et al., 2021), and decreased *Bifidobacterium* spp. (Lu et al., 2011; Xu et al., 2012), *Faecalibacterium* (*prausnitzii*) (Lu et al., 2011; Yang et al., 2020), *Parabacteroides* (Wang et al., 2017; Yao et al., 2021), and *Ruminococcus* (Wang et al., 2017; Yao et al., 2021) in abundance, whereas patients with hepatitis C have been found enriched in *Prevotella* (Aly et al., 2016; Sultan et al., 2021a), *Lactobacillus* (Heidrich et al., 2018; Inoue et al., 2018), *Streptococcus* (Heidrich et al., 2018; Inoue et al., 2018), and *Veillonella* (Aly et al., 2016; Heidrich et al., 2018), and deplete of *Ruminococcus* (Aly et al., 2016; Mohieldeen et al., 2021) and *Butyricimonas* (Aly et al., 2016; Heidrich et al., 2018) compared with control subjects.

The various autoimmune liver diseases exhibit similar gut microbiome alterations: Stool samples of patients with autoimmune hepatitis are characterized by large quantities of *Veillonella* (Elsherbiny et al., 2020; Liwinski et al., 2020; Lou et al., 2020; Wei et al., 2020), *Streptococcus* (Elsherbiny et al., 2020; Liwinski et al., 2020; Wei et al., 2020), *Haemophilus* (Elsherbiny et al., 2020; Lou et al., 2020), and *Klebsiella* (Lou et al., 2020; Wei et al., 2020), and small quantities of *Bifidobacterium* (Lin et al., 2015; Liwinski et al., 2020), *Parabacteroides* (Elsherbiny et al.,

2020; Lou et al., 2020; Wei et al., 2020), and Ruminococcaceae (Lou et al., 2020; Wei et al., 2020). Primary biliary cholangitis is associated with an elevated abundance of *Streptococcus* (Lv et al., 2016; Tang et al., 2018; Furukawa et al., 2020), *Veillonella* (Lv et al., 2016; Abe et al., 2018; Tang et al., 2018), *Klebsiella* (Lv et al., 2016; Tang et al., 2018), *Haemophilus* (Lv et al., 2016; Tang et al., 2018), and *Lactobacillus* (Tang et al., 2018; Furukawa et al., 2020), and diminished *Ruminococcus*/Ruminococcaceae (Lv et al., 2016; Furukawa et al., 2020) and *Faecalibacterium* (Tang et al., 2018; Furukawa et al., 2020), while primary sclerosing cholangitis is linked to enlarged proportions of *Veillonella* (Bajer et al., 2017; Kummen et al., 2017; Rühlemann et al., 2019; Cortez et al., 2020; Lapidot et al., 2021), *Streptococcus* (Sabino et al., 2016; Bajer et al., 2017; Rühlemann et al., 2019; Lapidot et al., 2021), and *Enterococcus* (Sabino et al., 2016; Bajer et al., 2017), as well as depressed proportions of *Coprococcus* (Bajer et al., 2017; Kummen et al., 2017; Rühlemann et al., 2019; Kummen et al., 2021), *Faecalibacterium prausnitzii* (Bajer et al., 2017; Lapidot et al., 2021), and Lachnospiraceae (Bajer et al., 2017; Kummen et al., 2017; Kummen et al., 2021; Lapidot et al., 2021). Enriched gut microbiota in Wilson's Disease are *Bacteroides* (Geng et al., 2018; Cai et al., 2020) and *Megamonas* (Geng et al., 2018; Cai et al., 2020), whereas *Ruminococcus* (Cai et al., 2020) and *Roseburia* (Geng et al., 2018) are reduced. Glycogen storage disease shows overrepresented intestinal *Escherichia* (Colonetti et al., 2019; Ceccarani et al., 2020) and Proteobacteria (Colonetti et al., 2019; Ceccarani et al., 2020), and underrepresented *Faecalibacterium* (Colonetti et al., 2019; Ceccarani et al., 2020) and *Roseburia* (Colonetti et al., 2019; Ceccarani et al., 2020). Liver transplantation results in expansion of *Enterococcus* spp. (Wu et al., 2012; Annavajhala et al., 2019; Song et al., 2021a), *Dorea* (Bajaj et al., 2018; Annavajhala et al., 2019), *Blautia* (Sun et al., 2017; Bajaj et al., 2018; Song et al., 2021a), and *Streptococcus* (Bajaj et al., 2018; Annavajhala et al., 2019), and reduction of *Faecalibacterium (prausnitzii)* (Wu et al., 2012; Annavajhala et al., 2019; Lu et al., 2019; Song et al., 2021a), *Escherichia* (Bajaj et al., 2017; Sun et al., 2017; Bajaj et al., 2018), *Shigella* (Bajaj et al., 2017; Sun et al., 2017; Bajaj et al., 2018), and *Bifidobacterium* (Wu et al., 2012; Bajaj et al., 2018).

Gallbladder disease is linked to enriched *Ruminococcus gnavus* (Wang et al., 2020a; Zhang et al., 2021a) and *Akkermansia* (Liu et al., 2015; Zhang et al., 2021a), and depleted *Faecalibacterium* (Wu et al., 2013; Wang et al., 2020a), *Roseburia* (Wu et al., 2013; Keren et al., 2015; Zhang et al., 2021a), and *Prevotella 9* (Wang et al., 2020a; Zhang et al., 2021a). Cholangiocarcinoma is associated with enlarged fecal proportions of *Lactobacillus* (Jia et al., 2020; Zhang et al., 2021a) and Peptostreptococcaceae (Jia et al., 2020; Zhang et al., 2021a), and smaller proportions of *Ruminococcus* (Jia et al., 2020; Zhang et al., 2021a) and *Faecalibacterium* (Zhang et al., 2021a). The intestinal contributions of *Streptococcus* (Wang et al., 2020b; Song et al., 2021b) and *Klebsiella* (Wang et al., 2020b; Song et al., 2021b) are increased in biliary atresia, and those of *Bifidobacterium* (Wang et al., 2020b; Song et al., 2021b), *Blautia* (Wang et al., 2020b; Song et al., 2021b), and *Faecalibacterium* (Wang et al., 2020b; Song et al., 2021b) are decreased. The microbial changes occurring in acute and chronic pancreatitis compared with controls are similar: Acute pancreatitis is characterized by overrepresentation of *Enterococcus* (*faecalis*) (Zhu et al., 2019; Yu et al., 2020), *Escherichia* (*coli*) (Zhu et al., 2019; Yu et al., 2020), and Enterobacteriaceae (Tan et al., 2015; Zhu et al., 2019), and underrepresentation of *Faecalibacterium* (Zhu et al., 2019; Yu et al., 2020), *Bifidobacterium* (Zhu et al., 2019; Yu et al., 2020), and *Blautia* (Zhu et al., 2019; Yu et al., 2020), whereas chronic pancreatitis exhibits elevated fecal amounts of *Escherichia* (*coli*) (Savitskaia et al., 2002; Zhou et al., 2020a; Frost et al., 2020) and *Enterococcus* (*faecalis* and *faecium*) (Savitskaia et al., 2002; Frost et al., 2020), and lower amounts of *Faecalibacterium (prausnitzii)* (Jandhyala et al., 2017; Zhou et al., 2020a; Wang et al., 2020c; Frost et al., 2020), *Coprococcus* (Zhou et al., 2020a; Frost et al., 2020), *Subdoligranulum* (Zhou et al., 2020a; Wang et al., 2020c), and *Collinsella* (Zhou et al., 2020a; Wang et al., 2020c). Pancreatic cancer is associated with an expansion of *Klebsiella* (Ren et al., 2017; Pushalkar et al., 2018; Matsukawa et al., 2021), *Veillonella*/Veillonellaceae (Ren et al., 2017; Pushalkar et al., 2018; Half et al., 2019), *Parabacteroides* (Pushalkar et al., 2018; Matsukawa et al., 2021), and *Lactobacillus* (Ren et al., 2017; Matsukawa et al., 2021), as well as reduced *Megamonas* (Ren et al., 2017; Pushalkar et al., 2018), *Anaerostipes* (Ren et al., 2017; Pushalkar et al., 2018; Half et al., 2019), *Dorea* (Ren et al., 2017; Pushalkar et al., 2018), and *Firmicutes* (Ren et al., 2017; Matsukawa et al., 2021).

Eosinophilic esophagitis is linked to high abundances of *Haemophilus* (Harris et al., 2015; Hiremath et al., 2019) and Bacteroidetes (Benitez et al., 2015; Kashyap et al., 2019; Laserna-Mendieta et al., 2021), and low abundances of Firmicutes (Benitez et al., 2015; Kashyap et al., 2019) and Clostridia (Kashyap et al., 2019). Elevated concentrations of *Campylobacter* (*concisus*) (Macfarlane et al., 2007; Blackett et al., 2013; Deshpande et al., 2018; Snider et al., 2019) and *Veillonella* (Liu et al., 2013; Deshpande et al., 2018; Snider et al., 2018), and small quantities of *Prevotella pallens* (Snider et al., 2019; Kawar et al., 2021) and *Solobacterium moorei* (Zhou et al., 2020b; Kawar et al., 2021) can be detected in gastroesophageal reflux disease (GERD) and Barrett's Esophagus. Esophageal adeno- and squamous cell carcinoma are associated with increased amounts of *Leptotrichia* (Lopetuso et al., 2020; Zhao et al., 2020), Veillonellaceae (Li et al., 2020a; Lopetuso et al., 2020; Zhao et al., 2020), and *Bifidobacterium* (Zhou et al., 2020b; Lopetuso et al., 2020; Zhao et al., 2020), and decreased amounts of *Aggregatibacter* (Chen et al., 2015; Zhao et al., 2020) and *Acholeplasma* (Chen et al., 2015; Zhao et al., 2020). Gastritis is characterized by enriched *Helicobacter pylori* (Parsons et al., 2017; Yang et al., 2019; Ndegwa et al., 2020) and *Streptococcus* (Li et al., 2009; Gao et al., 2018; Cui et al., 2019), and depleted *Prevotella* (Parsons et al., 2017; Cui et al., 2019; Ndegwa et al., 2020) and *Acinetobacter* (Parsons et al., 2017; Cui et al., 2019; Ndegwa et al., 2020). In gastric cancer, *Lactobacillus* (Qi et al., 2019; Wang et al., 2020d; Gantuya et al., 2020) and *Veillonella* (Castaño-Rodríguez et al., 2017; Qi et al., 2019; Wang et al., 2020d) are increased, whereas *Pseudomonas* (Wang et al., 2020d; Gantuya et al., 2020) and *Lactococcus* (Chen et al., 2019; Gunathilake et al., 2019; Wang et al., 2020d) are decreased. Irritable bowel syndrome is linked to overrepresented *Ruminococcus gnavus* (Rajilić–Stojanović et al., 2011; Rangel

et al., 2015) and *Dorea* (*formicigenerans*) (Rajilić–Stojanović et al., 2011; Rangel et al., 2015; Maharshak et al., 2018), and underrepresented *Bifidobacterium* (*catenulatum*) (Malinen et al., 2005; Kerckhoffs et al., 2009; Rajilić–Stojanović et al., 2011) and *Faecalibacterium* (*prausnitzii*) (Carroll et al., 2012; Rangel et al., 2015; Maharshak et al., 2018).

Celiac disease is associated with enlarged proportions of *Escherichia coli* (Nadal et al., 2007; Collado et al., 2009; Schippa et al., 2010), *Bacteroides* (*fragilis* and *vulgatus*) (Nadal et al., 2007; Collado et al., 2009; De Palma et al., 2010; Schippa et al., 2010; Sánchez et al., 2012), and *Staphylococcus* (Collado et al., 2009; Sánchez et al., 2013), and contracted contributions of *Bifidobacterium* (Sanz et al., 2007; Collado et al., 2009; De Palma et al., 2010) and Firmicutes (Sánchez et al., 2013; Iaffaldano et al., 2018). The gut microbiome of patients with intestinal failure is enriched in *Lactobacillus*/Lactobacilli (Joly et al., 2010; Korpela et al., 2017) and Proteobacteria (Davidovics et al., 2016; Korpela et al., 2017), and diminished in *Dorea* (Huang et al., 2017; Piper et al., 2017) and *Blautia* (Huang et al., 2017; Piper et al., 2017). Acute appendicitis is characterized by elevated levels of *Fusobacterium* (Swidsinski et al., 2011; Guinane et al., 2013; Jackson et al., 2014; Zhong et al., 2014; Rogers et al., 2016), *Parvimonas* (Guinane et al., 2013; Jackson et al., 2014; Zhong et al., 2014; Rogers et al., 2016), *Campylobacter jejuni* (Campbell et al., 2006; Oh et al., 2020b), and *Gemella* (Guinane et al., 2013; Zhong et al., 2014), and reduced levels of *Bacteroides* (Swidsinski et al., 2011; Samuelsson et al., 2013; Zhong et al., 2014; Rogers et al., 2016), *Ruminococcus* (Samuelsson et al., 2013; Munakata et al., 2021), and *Faecalibacterium* (*prausnitzii*) (Swidsinski et al., 2011; Samuelsson et al., 2013). The gut microbiome signature of chronic constipation consists of large proportions of *Clostridium* (Zoppi et al., 1998; Zhu et al., 2014) and *Parabacteroides* (de Meij et al., 2016; Li et al., 2020b), and depressed amounts of *Lactobacillus* (Khalif et al., 2005; Moraes et al., 2016; Jomehzadeh et al., 2020) and *Roseburia* (Mancabelli et al., 2017; Li et al., 2020b). Stool analysis of patients with inflammatory bowel disease (IBD) frequently demonstrates overrepresentation of *Veillonella* (Gevers et al., 2014; Mottawea et al., 2016; Santoru et al., 2017; Schirmer et al., 2018) and *Escherichia coli* (Schwiertz et al., 2010; Sha et al., 2013; Gevers et al., 2014; Santoru et al., 2017), and underrepresentation of *Faecalibacterium* (*prausnitzii*) (Schwiertz et al., 2010; Joossens et al., 2011; Morgan et al., 2012; Kumari et al., 2013; Gevers et al., 2014; Machiels et al., 2014; Schirmer et al., 2018), and *Roseburia* (Morgan et al., 2012; Kumari et al., 2013; Rajilić-Stojanović et al., 2013; Gevers et al., 2014; Machiels et al., 2014). *Fusobacterium* (*nucleatum*) (Kostic et al., 2012; Ahn et al., 2013; Warren et al., 2013; Zeller et al., 2014; Gao et al., 2015; Mira-Pascual et al., 2015; Yu et al., 2017; Dai et al., 2018; Guo et al., 2018) is commonly enriched in colorectal cancer along with *Peptostreptococcus* (Wang et al., 2012; Ahn et al., 2013; Zeller et al., 2014; Gao et al., 2015; Yu et al., 2017), whereas *Faecalibacterium* (*prausnitzii*) (Balamurugan et al., 2008; Kostic et al., 2012; Guo et al., 2018) and *Bifidobacterium* (Mira-Pascual et al., 2015; Dai et al., 2018; Guo et al., 2018) are depressed in number.

Fungi, viruses, and other non-bacterial populations are also detected at aberrant proportions in disorders of the digestive system, e.g., the fungus *Candida albicans* is increased in ALD (Lang et al., 2020a; Hartmann et al., 2021), NAFLD (Demir et al., 2021), gastric cancer (Zhong et al., 2021), IBD (Sokol et al., 2017), and colorectal cancer. (Starý et al., 2020) Viruses have also been correlated with disease activity in alcoholic hepatitis (Jiang et al., 2020) and NAFLD (Lang et al., 2020b) among others. Archaea have been investigated as well, *Methanosphaera stadtmaniae* (Blais Lecours et al., 2014) has been found to be more abundant and *Methanobrevibacter smithii* (Ghavami et al., 2018) has been found to be depleted in the gut microbiome of patients with IBD.

DISEASE ASSOCIATION INDEX

When evaluating the microbiome findings of these 30 disorders of the digestive system above, striking observations can be made: *Faecalibacterium* (*prausnitzii*) is associated with 16 of these conditions, and this bacterium is decreased in all of these 16 conditions. In contrast, the fecal abundance of *Veillonella* and Veillonellaceae is increased in 13 out of 13 diseases and that of *Streptococcus* is increased in 10 out of 10 conditions in which a robust association has been demonstrated. To evaluate how likely a microbial population is increased or decreased across diseases that it is associated with, a *Disease Association Index* (*DAI*) can be calculated by dividing the increased-decreased net value (= the number of diseases in which the microbial population is increased minus the number of diseases in which the microbial population is decreased) by the total number of conditions that the population has been associated with. E.g. the DAI for *Faecalibacterium* (*prausnitzii*) among the digestive diseases discussed above is −1 (= (0–16)/16); the DAI for *Veillonella* and Veillonellaceae as well as for *Streptococcus* is +1 (= (13–0)/13) and +1 (= (10–0)/10), respectively. The DAI ranges from −1 to +1; the higher the value the more likely the microbial population to be increased in the evaluated diseases, and the lower the more likely that the population is decreased in the analyzed conditions. The closer the DAI is to 0, the more ambivalent is the microbial population. A DAI of +0.6 or higher, and a DAI of −0.6 or lower indicates that the abundance of a microbe can be considered highly positively or negatively correlated with disease, respectively. *Ruminococcus* has a DAI of −0.6 (2 increases/8 decreases), indicating that it is predominantly decreased in digestive diseases (of note, the species *Ruminococcus gnavus* is responsible for both increased abundances of *Ruminococcus* in these diseases, see above). Additional notable DAIs: *Bifidobacterium* −0.8 (1 increase/9 decreases), *Lactobacillus*/Lactobacilli +0.78 (8/1), *Escherichia* (*coli*) +0.71 (6/1), *Enterococcus* (*faecalis/faecium*) +1 (7/0), *Dorea* −0.2 (2/3), *Prevotella* −0.2 (2/3), *Bacteroides* (*fragilis/vulgatus*) +0.2 (3/2), *Roseburia* −1 (0/5), and *Klebsiella* +1 (4/0).

Targeted repletion trials for bacterial populations that are predominantly decreased (such as repletion of *Faecalibacterium* (*prausnitzii*) in a murine colitis model), (Martín et al., 2014) or targeted elimination trials for populations that are predominantly increased (such as

elimination of cytolytic *Enterococcus faecalis* via targeted bacteriophages in a murine model of alcohol-induced liver disease) (Duan et al., 2019) could be attempted in the future.

ARTICLES IN THIS RESEARCH TOPIC

The articles in this Research Topic are very diverse and wide-ranging. Zheng et al. describe microbiome and metabolite differences in various autoimmune liver diseases (Zheng et al.). Warner et al. characterize the role of human beta defensin-2 in alcohol-induced liver injury in mice (Warner et al.). Chen et al. discuss the role of the microbiota in the pathogenesis of chemical-induced acute liver injury models in rodents and the protective use of probiotics herein (Chen et al.). Zhang et al. demonstrate that hepatic branch vagotomy results in decreased dysbiosis but increased hepatic steatosis and continued neuro-inflammation in murine cirrhosis secondary to carbon tetrachloride injections (Zhang et al.). Song et al. analyzed changes of the gut microbiome in patients with biliary atresia after liver transplantation (Song et al.). Chen et al. report microbiome and metabolite shifts in a mouse model of gallstone disease (Chen et al.). Rao et al. discuss microbiome changes in cholangiocarcinoma and related precancerous conditions (Rao et al.). Jihan et al. identified specific microbiome signatures in cancers affecting the esophagus, stomach, colon, and rectum (Wang et al.). Busing et al. review various changes in the microbiota and metabolism in eosinophilic esophagitis (Busing et al.). Wang et al. associate intratumor microbiome signatures with subtype, tumor stage, and survival in patients with esophageal carcinoma (Wang et al.). Hu et al. discuss alterations seen in the gut microbiota in food allergies and other allergic conditions (Hu et al.). Chu et al. use a variety of mouse models to induce gastritis and analyze the associated modulations of the intestinal microflora (Chu et al.). Sultan et al. discuss metabolite alterations associated with intestinal dysbiosis in IBD (Sultan et al.). Houshyar et al. review what is known about the role of fungi and archaea in IBD (Houshyar et al.). Zhao et al. evaluate the role of gut bacteria in a rat model of intra-abdominal hypertension (Zhao et al.). Montanari et al. detail the relationship of an pro-inflammatory state and gut dysbiosis, and the effects of diet and medications on the gut microbiota observed in disorders of inborn errors of metabolism (Montanari et al.). Lastly, Li et al. introduce *Amadis*, a comprehensive, manually curated database that documents experimentally supported microbiota-disease associations (Li et al.).

AUTHOR CONTRIBUTIONS

PH was responsible for the preparation of the manuscript.

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REVIEW
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Gut Microbiota and Chemical-Induced Acute Liver Injury

Tao Chen[1,2], *Rui Li*[2] *and Peng Chen*[2*]

[1] Department of Physiology, School of Basic Medical Sciences, Gannan Medical University, Ganzhou, China, [2] Department of Pathophysiology, Guangdong Provincial Key Laboratory of Proteomics, School of Basic Medical Sciences, Southern Medical University, Guangzhou, China

Background: Drug overdose or chemical exposures are the main causes of acute liver injury (ALI). Severe liver injury can develop into liver failure that is an important cause of liver-related mortality in intensive care units in most countries. Pharmacological studies have utilized a variety of comprehensive chemical induction models that recapitulate the natural pathogenesis of acute liver injury. Their mechanism is always based on redox imbalance-induced direct hepatotoxicity and massive hepatocyte cell death, which can trigger immune cell activation and recruitment to the liver. However, the pathogenesis of these models has not been fully stated. Many studies showed that gut microbiota plays a crucial role in chemical-induced liver injury. Hepatotoxicity is likely induced by imbalanced microbiota homeostasis, gut mucosal barrier damage, systemic immune activation, microbial-associated molecular patterns, and bacterial metabolites. Meanwhile, many preclinical studies have shown that supplementation with probiotics can improve chemical-induced liver injury. In this review, we highlight the pathogenesis of gut microorganisms in chemical-induced acute liver injury animal models and explore the protective mechanism of exogenous microbial supplements on acute liver injury.

Keywords: chemical, gut-liver axis, gut microbiota, intestine, acute liver injury

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Xiaomei Fan, Jinan University, China
Geetha Samak, DVS College of Arts and Science, India

***Correspondence:**
Peng Chen
perchen@smu.edu.cn

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INTRODUCTION

Acute liver injury (ALI) is a common disease that seriously threatens the life and health of the patients. The main pathological manifestation is a sharp decline in liver function caused by the necrosis of a large number of hepatocytes (Lee, 2004; Bernal et al., 2010; Bunchorntavakul and Reddy, 2018). ALI caused by multiple etiologies has become a crucial public-health issue at both regional and global scales. The most common causes of ALI in developed countries are acetaminophen (APAP) induced hepatotoxicity, drug induced liver damage, autoimmunity, and viral hepatitis B, which accounts for about 70% of the cases; on the other hand, the most common causes in developing countries are viral hepatitis A, B, and E (Stravitz and Lee, 2019). Currently, liver transplantation (LT) is the only clinically essential therapy for the treatment of acute liver failure (ALF). However, the rapid progression of ALI, the scarcity of liver sources, and the high medical costs limit its application. Therefore, it is necessary to find other effective interventions for severe acute liver injury patients. Recently, there is increasing evidence points to the intimate connection between gut microbiota and liver injury. However, compared with ALI, these studies mostly focused on chronic liver diseases,

such as non-alcoholic fatty liver disease (NAFLD), autoimmune hepatitis, liver cirrhosis, and hepatocellular carcinoma (Compare et al., 2012; Lee and Suk, 2020; Wei et al., 2020). In recent years, an increasing number of preventive or adjunct therapeutic measures for ALI have been proposed based on the regulation of gut microbiota (Kim et al., 2018; Jiang et al., 2021). The gut contains around 1,000 different species of bacteria, which play a crucial role in the survival of organisms. The crosstalk between gut and liver is increasingly recognized. Gut microbiota has been defined as a significant micro-ecosystem, which is symbiotic with the organism and participates in a variety of physiological and pathological processes (Sharma and Gilbert, 2018).

In this review, we focused on the current knowledge of the gut microbiota's contribution and the protective mechanism of exogenous microbial supplements on chemically induced ALI.

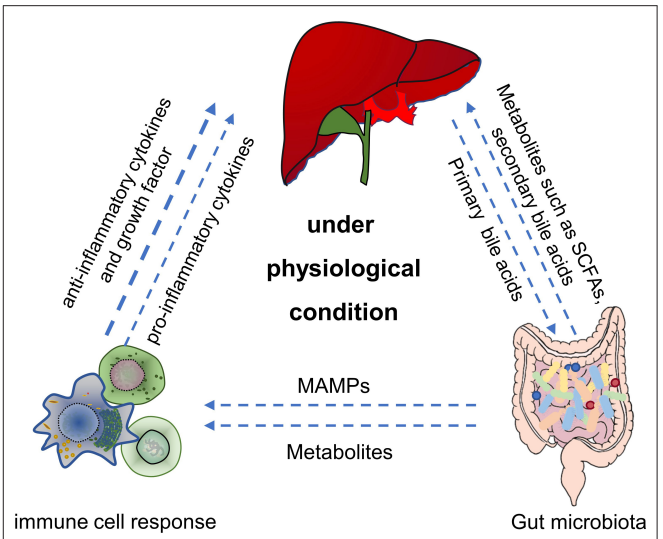

FIGURE 1 | The main role of gut microbiota in gut-liver-immune axis under physiological condition. First, by utilizing the nutrients and metabolic substrates (such as primary bile acids from liver), gut microbiota can produce various bioactive metabolites (such as acetate, propionate, butyrate, secondary bile acids, and amino acids) which were absorbed through the portal vein into the liver, to regulate hepatic function. Second, gut microbiota can also directly or indirectly establish and balance the hepatic immune response through metabolites, secondary bile acids and MAMPs. SCFAs, short chain fatty acids. MAMPs, microbial-related molecular patterns. Figure was partly adapted from Smart Servier Medical Art (https://smart.servier.com/), which is licensed under a Creative Common Attribution 3.0 Generic License.

ROLE OF THE MICROBIOTA IN THE IMMUNE HOMEOSTASIS OF THE GUT, LIVER AND LIVER DISEASE

Immune Homeostasis of the Gut and Liver

The immune system plays a critical role in maintaining the symbiotic relationship between the microbiome and the host. Intestinal mucosa-associated lymphoid tissue contains a variety of immune cells, such as antigen-presenting cells (APCs), innate lymphocytes, T cells, and B cells, which play a vital role in the host immune response (Trivedi and Adams, 2016). Gut bacteria regulate the maturation of the mucosal immune system, which in turn affects the composition of the gut microbiota (Shan et al., 2013). Studies have shown that microbes activated the immune cells to produce cytokines and initiated the host's immune response to balance the intestinal tolerance and immunity (Venkatesh et al., 2014; Rooks and Garrett, 2016). Thus, the homeostasis of the gut microbiota is essential for the maintenance of gut and liver metabolism and immune homeostasis (**Figure 1**). For example, intestinal symbiotic bacteria help induce a stable production of Th17 cells in the lamina propria of the small intestine and protect the intestinal mucosal barrier, and some bacteria also play an immunomodulatory role of macrophage polarization (Scott et al., 2018; Wang et al., 2020c).

Dysregulation of the gut microbiota leads to an inability of the intestinal mucosal barrier to resist an infection with pathogenic bacteria and facilitates the occurrence of diseases affecting the normal physiological function of the host. Meanwhile, gut metabolites, such as short-chain fatty acids (SCFAs), can also modulate local or systemic immune responses (Schulthess et al., 2019). Studies have demonstrated that SCFAs can affect the differentiation of B cells and maintain the homeostasis of intestinal T cells (Scott et al., 2018; Rosser et al., 2020). Butyric acid, a representative SCFAs, is necessary for the colonization of intestinal bacteria (Barcenilla et al., 2000).

Recent studies have suggested that yeast β-glucan, a polysaccharide produced by fungi, can contribute to the differentiation of Th2 cells and maintain the steady-state of the intestinal immune system (Cao et al., 2018). Additionally, the gut microbiota maintains the immune homeostasis of the liver through the gut-liver axis (Heymann and Tacke, 2016). The liver proposed as an important innate immune organ contains a large number of immune cells, such as NK, Kupffer, and T cells. The activation of Kupffer and T cells can trigger the innate and adaptive immune response when the liver is continuously stimulated by antigens, pathogens, and endotoxins produced by the gut microbiota. This process can affect the tolerance of liver immunity (Milosevic et al., 2019; Albhaisi et al., 2020).

Liver Disease

In recent years, the gut microbiota has received increasing attention because of its central role in host-microbiota interaction and its impact on liver diseases (Yang et al., 2020). A healthy gut microbiota can maintain liver metabolism and immune homeostasis. Meanwhile, this direct connection can cause several "negative effects." Bacteria-derived products or toxins can get into the liver and disrupt its homeostasis if the liver immune system is pre-imbalanced or the natural barrier of the intestine is disrupted (Schnabl and Brenner, 2014). A large number of intestinal-derived toxic products enter the liver and are recognized by specific receptors, such as TLR. This can promote an inflammatory response, direct liver cell death, or chronic liver damage (Seki and Schnabl, 2012).

Bile acids and many other bioactive substances released by the liver enter the gut to be processed and metabolized by intestinal bacteria. On the other hand, the liver receives 70% of

its blood supply from the gut via the portal vein, which contains metabolites, endotoxins, peptidoglycan and even microbiota. Thus, various types of liver cells, including hepatocytes, kupffer cells, hepatic stellate cells, sinusoidal cells, and biliary epithelial cells can respond to signals from the gut and affect liver function (Nicholson et al., 2012). The interaction between the gut microbiota and the liver involves multiple components, including metabolism, immunity, and neuroendocrine signals, and constitutes a complicated interaction network. The gut-liver axis plays an essential role in the pathogenesis of numerous liver diseases, such as alcoholic liver disease (ALD), chronic hepatitis, NAFLD, liver cancer, and drug-induced liver injury (Milosevic et al., 2019).

In general, increased intestinal permeability and bacterial translocation promote the arrival of microbial metabolites to the liver and aggravate the liver injury. This leads to dysfunctions in bile acid metabolism and intestinal peristalsis, systemic inflammation, and liver damage. The level of liver damage is closely related to the severity of intestinal disorders. Alteration of the bacteria that produce SCFAs, which dominate the composition of gut microbiota, has already been reported to affect intestinal homeostasis (Schulthess et al., 2019). Thus, a treatment based on microbial intervention is a promising method to improve the pathological process in the liver (Boursier et al., 2016).

At present, many studies have shown that supplementing with probiotics can balance the gut homoestasis, reduce intestinal bacterial translocation, inhibit liver inflammation, and improve acute liver damage **Table 1** (Van Nood et al., 2013; Huang et al., 2018; Le Barz et al., 2019). Recent studies have also demonstrated that differences of distribution, specific composition and metabolites produced by the gut microbiota constitute the risk for acute liver injury, the major ways for the gut microbiota participate in ALI (Gong et al., 2018; Dey, 2020). Thus, the specific mechanisms of gut microbiota in the onset and progression of ALI deserve to further study. Considering the different types of ALI, we summarized the currently reported four chemical-induced acute liver injury model that modulated by gut microbiota.

THE GUT MICROBIOTA AND APAP-INDUCED LIVER INJURY

Drug-induced liver injury (DILI) is the main cause of clinical liver injury in developed countries. For example, in the United States, nearly 46% of ALI cases were caused by overdose or abuse of N-acetaminophen (APAP). APAP has been the focus of the Food and Drug Administration (FDA) advisory committees over the past several decades (Bernal et al., 2015; Lee, 2017). The degree of liver injury is the determining factor in acute liver failure survival without transplantation. During the past few decades, around three-quarters of APAP-induced ALI patients survive with their naïve liver (Stravitz and Lee, 2019). Generally, APAP is a safe drug when used at therapeutic doses for the treatment of fever and pain (1–4 g/days) (Kaplowitz, 2004). Its safety margin is, however, relatively narrow (Maeda et al., 2020). A randomized study reported that a maximum dose of APAP for 5 days in healthy adults increased the level of serum transaminase (Watkins et al., 2006). Meanwhile, the susceptibility to APAP-induced liver injury may also be due to several risk factors, such as alcohol use, obesity, nutritional depletion, and the use of drugs that stimulate the cytochrome P450 (CYP) system (Bunchorntavakul and Reddy, 2013).

Typically, APAP is cleared in the liver primarily where it binds to O-sulfuric acid or glucuronic acid and is excreted into the bile or urine. At therapeutic doses, only a small amount of APAP is metabolized by the cytochrome P450 enzyme (CYPs) to N-acetyl-p-phenyl quinone imine (NAPQI). With high doses of APAP overdose or if the ability of glucuronide and sulfate esterification is saturated (Mitchell et al., 1974), CYPs (mainly CYP-2E1 and CYP-3A4 in mammals) become the major APAP metabolic enzyme and lead to the massive production of NAPQI, a highly active intermediate compound that leads to cytotoxicity (Lee et al., 1996; Laine et al., 2009). In the early stage, the majority of NAPQI is rapidly detoxified and excreted into the urine by binding with glutathione (GSH). Once the GSH storage is depleted, the remaining NAPQIs accumulate in hepatocytes and bind covalently to proteins sulfhydryl groups, producing harmful APAP protein adduct (APAP-ADs) that irreparably lead to liver cell necrosis (Jaeschke et al., 2012; Mcgill and Jaeschke, 2013).

N-acetylcysteine (NAC), the only approved therapeutic drug for APAP-induced hepatotoxicity, can protect the liver from damage by providing cysteine precursors and restore the hepatocytes GSH stores (Ferner et al., 2011). However, the treatment window for NAC is narrow and requires a controlled use of APAP (no more than 24 h). The accumulation of NAPQIs can destroy cytoplasmic membranes and induce severe mitochondrial dysfunctions, an overproduction of reactive oxygen species (ROS), ATP depletion, a fragmentation of nuclear DNA, and lipid peroxidation (Jaeschke et al., 2012; Ramachandran and Jaeschke, 2020). Therefore, massive hepatocellular necrosis subsequently leads to several damage-associated molecular patterns (DAMPs) releases, which activate macrophages and induce a sterile inflammation (Jaeschke and Ramachandran, 2020). Activated macrophages produce several chemokines and cytokines, which induce the intrahepatic aggregation of neutrophils, monocytes, and other immune cells and trigger intrahepatic and systemic inflammatory responses (Yang and Tonnesseen, 2019). Notably, sterile inflammatory responses not only clear necrotic cell debris and promote tissue repair, but also aggravate liver injury (Jaeschke and Ramachandran, 2020). Finally, when the degree of hepatocyte damage exceeds regeneration, the liver function rapidly fails.

The hepatotoxic effect of paracetamol is still considered to be the most important part of ALI. However, recent evidence suggests that changes in the gut microbiota, including their abundance, diversity, metabolites, intestinal permeability, and bacterial translocation also play profound roles in APAP-induced hepatotoxicity (Dey, 2020).

Several studies have shown that the presence of gut microbiota is important for APAP-induced hepatotoxicity. Jourova et al. found that the degree of APAP liver injury in germ-free (GF)

TABLE 1 | Application of probiotics in chemical-induced acute liver injury model.

References	Probiotic	Animal	Chemical	Protective mechanism
Jiang et al. (2021)	*Lactobacillus reuteri* DSM 17,938 (oral gavaged with 3×10^9 CFU daily for 7 days)	Sprague-Dawley rats	D-GalN, intraperitoneally injected of 1.1 g/kg body weight and sacrificed after injected 24 h.	Alleviate the disruption of the gut microbiota and metabolome; reduce the transcription of inflammatory factors in the liver
Saeedi et al. (2020)	*Lactobacillus rhamnosus* GG (oral gavage with $2x10^8$ CFU/100 μl HBSS daily for 14 days)	Germ-free C57BL/6 mice	APAP, oral gavage of 300 mg/kg bodyweight and mice were sacrificed after APAP gavage 24 h.	Production of 5-MIAA to activate Nrf2 in liver to protect against APAP induced oxidative liver injury
Neag et al. (2020)	MegaSporeBioticTM (MSB) (orally 1×10^9 CFU/rat through a feeding tube daily for 12 days)	Charles River Wistar white male rats	APAP, oral gavage of 2 g/kg bodyweight and mice were sacrificed after APAP gavage 48 h.	Reduced the pro-inflammatory cytokines (TNF-α, IL-1β), decreased the hepatocyte necrosis
Li et al. (2019a)	*Bifidobacterium adolescents* CGMCC15058 (gavage 3×10^9 CFU/ml PBS daily for 14 days)	Germ-free Sprague–Dawley (SD) rats	D-GalN, intraperitoneally injected of 1.1 g/kg body weight and sacrificed after 24 h.	Decreased levels of mTOR and the inflammatory cytokines TNF-α and IL-6; increased the anti-inflammatory cytokine interleukin-10
Li et al. (2019b)	*Bacillus cereus* (gavage at 3×10^9 CFU/ml PBS daily for 2 weeks)	Sprague-Dawley rats	D-GalN, intraperitoneal injection of 1.1 g/kg body weight and sacrificed after 24 h	Alleviating the inflammatory reaction, reinforcing gut barrier function; reshaping the gut microbiota
Grander et al. (2018)	*Akkermansia muciniphila* (oral gavage1.5×10^9 CFU/200 μl PBS daily for 2 days)	C57BL/6 mice	Alcohol, oral gavage of 6 g/kg bodyweight and mice were sacrificed after alcohol gavage 8 h	Reduced ethanol-induced hepatic injury, steatosis and infiltration of MPO^+ neutrophils
Wu et al. (2017)	*Akkermansia muciniphila* (oral gavage 3×10^9 CFU/200 μl PBS daily for 14 days)	C57BL/6 mice	Con A, 15 mg/kg injection through the tail vein and sacrificed after 8 h	Reduced inflammatory cytokines, cytotoxic factors and hepatocellular death; increased the diversity and reshaping the microbial community
Yu et al. (2017)	*Saccharomyces boulardii* (gavaged with 1×10^9 CFU/ml for 4 weeks)	BALB/c mice	D-GalN, intraperitoneally injected with 200 mg/kg body weight and sacrificed after 24 h.	Increased the relative abundance of *Bacteroidetes*; decreased the relative abundance of *Firmicutes* and *Proteobacteria*
Wang et al. (2016)	*Lactobacillus casei* Zhang (gavaged with 10^9 CFU/daily for 30 days)	Wistar rats	LPS / D-GalN, intraperitoneal injection of 50 μg/kg LPS and 300 mg/kg D-GalN and sacrificed after 8 h.	Modulation of the TLR-MAPK-PPAR-γ pathways to reduce pro-inflammatory cytokines and hepatic inflammation
Wang et al. (2012)	*Lactobacillus rhamnosus* GG (mixed with drinking water approximately 10^9 CFU /daily for 5 days)	C57BL/6N mice	Alcohol, oral gavage of 6 g/kg bodyweight and mice were sacrificed after alcohol gavage 1.5 or 6 h.	Activating HIF signaling to decrease the damage of alcohol-induced increased intestinal permeability and endotoxemia.

D-GalN, D-galactosamine; APAP, acetaminophen; 5-MIAA, 5-methoxyindoleacetic acid; Con A, Concanavalin A.

mice or mice cleared of gut microbiota was lower than in SPF mice. The expression of the P450 enzyme, which is involved in APAP metabolism in the liver, is closely linked to intestinal microorganisms (Jourova et al., 2020). The expression levels of CYP-1A2 and CYP-3A4 in the liver of GF mice were significantly lower than in SPF mice, which may explain the significant reduction of APAP-induced hepatotoxicity in GF mice (Jourova et al., 2020). Meanwhile, single-cell sequencing data showed that there were significantly fewer *LY6C*-positive monocytes and a lower proportion of activated non-parenchymal cells (hepatic stellate cells, sinusoidal endothelial cells, and Kupffer cells) in the liver of antibiotic-treated mice compared with SPF mice. The key mechanism depends on the clearance of intestinal microbiota, which dramatically reduces the microbial-associated molecular patterns (MAMPs) entering the liver via the portal vein. Blocking the TLRs-MYC signaling pathway of MAMPs can downregulate the activation of stellate, endothelial, and Kupffer cells and significantly decrease the expression of chemokines and pro-inflammatory mediators, thus reducing the intrahepatic inflammatory response in APAP-induced model (Kolodziejczyk et al., 2020). These results suggested that single cell sequencing is an important method to study the direct relationship between gut microbiota and liver.

Similarly, the change in diversity and abundance of the intestinal microbiota can also participate in the transformation and detoxification of APAP. Zheng et al. changed the composition of the gut microbiota in mice using vancomycin, which reduced the abundance of Gram-positive bacteria in the intestine and increased the level of 2-hydroxybutyric acid in the cecum and serum. The bioavailability of APAP was also decreased and the level of GSH in the liver was increased, which improved the APAP-induced liver injury in mice (Zheng et al., 2020). The pharmacokinetics of APAP is also affected by changing the composition of gut bacteria in mice. Several researchers showed that the degradation of APAP increased by 68% in

mice treated with *Lactobacillus reuteri,* while treatment with *Lactobacillus rhamnosus* did not show a similar effect (Kim et al., 2018).

The susceptibility to APAP toxicity varies considerably among different individuals. Endogenous p-cresol, a protein residue in the intestinal cavity, is mainly produced by intestinal microorganisms, such as *Clostridium difficile*. P-cresol absorbed into the liver is transformed into p-cresol sulfate by sulfotransferases in hepatocytes (Bone et al., 1976). Likewise, acetaminophen is a substrate of sulfotransferases and p-cresol can reduce the ability of sulfotransferases to sulfonate acetaminophen. The content of p-cresol varies greatly in different individuals, which may explain the different sensitivity of individuals to APAP hepatotoxicity (Clayton et al., 2009). Furthermore, individuals with intestinal dysbiosis are more sensitive to APAP-induced liver injury. Schneider KM et al. analyzed a cohort of 500,000 participants in the British Biobank and found that proton pump inhibitors (PPI) or long-term antibiotics (ABX) can cause intestinal microbial dysbiosis. The risk of ALF induced by APAP was significantly increased in participants with intestinal microbial dysbiosis (Schneider et al., 2020). Similarly, compared with wild-type mice, *Nlrp6*$^{-/-}$ mice (an intestinal dysbiosis model) also showed that microbial dysbiosis could aggravate APAP-induced liver injury. This phenotype was reproduced after fecal bacteria transplantation (Elinav et al., 2018; Schneider et al., 2020).

Microbial communities are altered by the circadian rhythm system and affect the host metabolism, energy homeostasis, and immune system (Teichman et al., 2020). Disruptions in the rhythm function of microbe-host interactions can seriously affect the pathology and severity of the disease (Bishehsari et al., 2020). Interestingly, changes in the gut microbial circadian rhythm can affect APAP-induced liver toxicity (Gong et al., 2018). 16S rRNA gene sequencing showed that changes in the daily rhythm were associated with changes in the relative abundance of gut microbiota. Compared with ZT0, the ratio of Firmicutes/Bacteroides in ZT12 was significantly reduced. The hepatotoxicity of APAP was more severe at night (ZT12) than in the morning (ZT0). This was likely due to the increased abundance of the metabolite 1-phenyl-1,2-propanedione (PPD), which can consume GSH in hepatocytes after being ingested by the liver through the portal vein at ZT12. Therefore, early consumption of GSH can cause the accumulation of a large amount of NAPQI in the liver of mice with an excessive APAP metabolism, which can aggravate the liver injury. The oral gavage of APAP weakens the intestinal mucosal barrier function during intestinal absorption and allows plasma albumin to seep into the intestinal cavity. Similarly, a higher intestinal permeability can also allow abundant harmful substances to enter the liver, which aggravates the inflammatory reaction and the hepatotoxicity of APAP (Schneider et al., 2020). Niu et al. confirmed that the disruption of the intestinal barrier integrity, which may be mediated by intestinal immune microenvironments, can aggravate APAP-induced hepatotoxicity (Niu et al., 2020).

A high concentration of APAP can rapidly induce the apoptosis of *Lgr5* $^{+}$ crypt basal stem cells in the small intestine. Although apoptotic cells are completely removed within 24 h, the potential consequence is a long-term defect in the intestinal barrier function (Chopyk et al., 2019). Apoptosis of *Lgr5*$^{+}$ crypt basal stem cells may partly explain why the low 30-days survival rate of ALF caused by APAP toxicity in liver transplant recipients compared with ALF caused by non-APAP-related causes (Cooper et al., 2009).

Probiotics restore the balance of the intestinal microbiota (symbiotic and pathogenic bacteria), maintain the integrity of the intestinal barrier, reduce the production of toxic products, and improve liver function. We wondered, however, how probiotics could affect the APAP-induced hepatotoxicity. It is a known fact that gut microbiota and its metabolites are involved in the regulation of oxidative stress and inflammation which play key roles in drug-induced hepatotoxicity (Jaeschke et al., 2004; Shehu et al., 2017). Many studies showed that, compared with GF mice, normal mice displayed an upregulation of the transcription factor Nrf2 in liver that improved the antioxidant and xenobiotic response to protect the liver from acute acetaminophen; this signaling upregulation is enhanced by supplementary of human commensal *Lactobacillus rhamnosus GG* (Saeedi et al., 2020). Meanwhile, Sharma et al. reported that probiotic *Enterococcus lactis IITRHR1* and *Lactobacillus acidophilus MTCC447* protect against APAP-induced liver injury by modulating the antioxidant capacity of the liver and the expression of key apoptotic/anti-apoptotic proteins (Sharma et al., 2011). MegaSporeBioticTM probiotic capsules are composed of a probiotic blend of spores from five Bacillus species that improve the histopathological hepatic injury and decreased the level of proinflammatory cytokines, indicating that *Bacillus spa* spores have a protective effect on acute hepatic injury induced by APAP (Neag et al., 2020). This data is significant for the treatment of ALI and we also need to further explore whether the pretreatment of probiotics could improve the detoxification ability and antioxidant capacity of hepatocytes.

THE GUT MICROBIOTA AND CON A-INDUCED AUTOIMMUNE HEPATITIS

Autoimmune hepatitis (AIH) is a complicated immune-mediated liver disease with a variable clinical phenotype that occurs worldwide. In the UK, the incidence rate of AIH reaches nearly 1.94 cases per 100,000 people (Webb et al., 2021). The histological diagnosis of AIH comprises interfacial hepatitis, increased serum alanine aminotransferase (ALT), increased aspartate aminotransferase (AST), elevated immunoglobulin G (IgG) levels, and the presence of autoantibodies (Floreani et al., 2018). Additionally, patients with acute severe autoimmune liver disease are more likely to develop acute liver failure and may need LT. However, autoimmune liver disease can still develop or recur in allografts with a 5-years recurrence rate of 36% to 68% after LT (Kerkar and Yanni, 2016). The mechanism of AIH recurrence after LT is still unclear. However, several extrahepatic factors may play an important role in the recurrence of AIH. Mounting evidence showed that the mechanisms of AIH were related to gut dysbiosis (Cai et al., 2017; Furukawa et al., 2020). Moreover, microbiome restoration therapies, such

as probiotics, prebiotics, and fecal microbiota transplantation (FMT), can effectively improve AIH (Allegretti et al., 2019; Zhang et al., 2020). The study of gut microbiota will provide new insights into the mechanism of AIH.

Due to the lack of widely accepted and valid mouse models for AIH, the research on the pathogenesis of AIH is still very limited. Concanavalin A (Con A) is a lectin originally extracted from jack-bean. In early 1992, it was first used to establish an immune hepatitis model (Tiegs et al., 1992). Con A-induced hepatitis is a typical chemical model that is used to investigate the cellular and molecular mechanism of immune-mediated liver injury (Tiegs et al., 1992; Fujita et al., 2016; Xu et al., 2018). The model is acute and the injury caused by Con A usually lasts for only 48 h. Con A can partly simulate the pathogenesis of human acute autoimmune liver diseases as it can rapidly induce the activation of natural killer T (NKT) cells and CD4 positive T cells in the mice liver and release a mass of cytokines to cause liver damage (Diao et al., 2004; Celaj et al., 2014). Here, we mainly summarize the role of gut microbiota in Con A-induced hepatitis and summarize the discovery and progress of immunotherapy related to the gut-liver axis.

NKT cell activation plays a critical role in Con A-induced hepatitis and the gut microbiota regulates the activation of NKT cells (Diao et al., 2004; Celaj et al., 2014). Wei et al. have shown that GF mice are not sensitive to Con A-induced liver injury when compared with SPF mice. This is mainly because NKT cells in the liver of GF mice are not activated after Con A treatment. Meanwhile, compared with GF mice, Con A treatment can significantly increase the circulation of LPS and the level of glycolipid antigen presented by CD1d in SPF mice. Intestinal microbial-derived antigens (glycolipids) are also important activators of liver NKT cells as they can activate them to mediate Con A-induced ALF (Wei et al., 2016).

Furthermore, Chen et al. showed that exogenous pathogenic bacteria exposed to the gut can exacerbate Con A-induced liver injury. This may be due to the increase of DCs activation, which subsequently augments the cytotoxicity of hepatic NKT cells against the liver parenchyma cells. In contrast, gentamicin treatment, which is bactericidal mainly against gram-negative (G^-) organisms in the gut, can alleviate Con A-induced ALI (Chen et al., 2014). Meanwhile, Celaj et al. demonstrated that differences in the gut microbiota could determine the sensitivity to Con A-induced acute liver injury. They found that mice from the Taconic Farms (TAC), and the Jackson Laboratory (JAX) exhibited different levels of liver damage induced by Con A. This difference in sensitivity is caused by the regulation of the Fas response pathway in the gut microbiota. Interestingly, this difference in susceptibility disappeared after co-housing (a way for minimizing the discrepancy of gut microbiota from the different background). By analyzing the fecal microbe by 16S rRNA gene sequencing, they found that among the identified OTUs, 8 genera abundance exhibited statistically significant differences in the two company's mice. Furthermore, they found the abundance of Ruminococcaceae was positively associated with the degree of liver injury (Celaj et al., 2014). Dopamine is an important neurotransmitter and also an immune modulator. It participates in the regulation of the T cell function through the D1-like receptor (Besser et al., 2005; Nakano et al., 2011). Xue et al. demonstrated that gut microbes can improve the synthesis of peripheral dopamine to inhibit the activation of hepatic iNKT cells and alleviate Con A-induced liver injury. Different with Wei et al. study in GF mice, the depletion of the gut microbiota by antibiotics reduced the synthesis of dopamine and exacerbated Con A-induced liver injury (Xue et al., 2018). These conflict phenotypes may be resulted from the difference of microbiota abundance between GF and antibiotics treated mice. Nobuhito et al. showed that inducing colitis with DSS for 7 days led to intestinal leakage, exposed intestinal microbes to the systemic immune cells and the liver through the portal vein, induced a systemic immune tolerance, and thereby reduced Con A-induced liver damage (Taniki et al., 2018).

Due to the high plasticity of the intestinal microbiota, the research of exogenous probiotics or prebiotics in autoimmune liver disease is progressing substantially. However, there are few reports about the impact of probiotics or prebiotics on Con A-induced immune liver injury. The gut microbiota may influence the susceptibility and severity of acute liver injuries caused by Con A in mice based on current evidence. Gabriela et al. reported that *Propionibacterium acidipropionici* CRL 1,198 decreased the proliferative effects of lectins in adenocarcinoma cells, inhibited the fermentative activity of colonic microbiota, and avoided undesirable lectin-epithelia-microbiota interactions (Zarate et al., 2017). As a promising probiotic with beneficial effects on liver diseases, *Akkermansia muciniphila* pre-treatment can also alleviate liver damage by altering transaminase activities and attenuate systemic inflammation by suppressing cytokines (including IFN-γ, IL-1β, IL-2, and IL-12p40) in Con A-induced liver injury. The beneficial effects of *A. muciniphila* were partly dependent on improved intestinal barrier and restored composition and function of gut microbiota (Wu et al., 2017).

Therefore, the study of the relationship between intestinal microorganisms and liver immune homeostasis is helpful for the diagnosis and treatment of acute autoimmune liver injuries.

THE GUT MICROBIOTA AND ALCOHOL-INDUCED ACUTE LIVER INJURY

Alcohol (ethanol) abuse is a common risk factor for multiple diseases including alcoholic liver diseases (ALD), cardiovascular diseases, and inflammatory bowel diseases (Connor et al., 2016). Every year, nearly 4% of adults get sick because of drinking and the harmfulness of alcohol even exceeds the perniciousness of smoking (Room et al., 2005). Alcohol is transformed by alcohol dehydrogenases and cytochrome CYP-2E1 to acetaldehyde in the endoplasmic reticulum of hepatocytes. Acetaldehyde is converted into acetic acid by acetaldehyde dehydrogenases and is finally excreted in urine (Arteel, 2003). However, the gut microbiota also have a crucial part to play in the metabolism of ethanol because a large number of bacteria can express alcohol dehydrogenases in the colon. Evidence

showed that a leaky gut, bacterial translocation, and intestinal inflammation modulate the susceptibility of acute ALD (Starkel et al., 2018).

Canesso et al. showed that compared with GF mice, administration of alcohol for 7 days can cause significant liver injury and increase the level of neutrophil infiltration and pro-inflammatory cytokines (CXCL-1 and interleukin (IL)-6) in SPF mice. This implied that the gut microbiota was directly or indirectly involved in acute ALD (Canesso et al., 2014). However, our data showed that a single gavage of a high concentration of alcohol (300 μl of 30% (vol/vol) EtOH at a dose of 3 g/kg) caused more severe liver damage, inflammation, hepatic steatosis, and higher levels of CYP-2E1 in GF mice than in SPF mice, which also indicated that the intestinal microbiota was directly or indirectly involved in alcohol-induced liver damage (Chen et al., 2015). According to these results, the time and concentration of alcohol exposure may explain the inconsistencies between the two programs.

To date, several studies showed that changes in the entero-hepatic axis, such as increased permeability of the intestinal barrier, a thinning of the protective mucosal layer, and changes in the gut microbiota, occurred after alcohol consumption (Carson and Pruett, 1996; Purohit and Brenner, 2006). *Akkermansia muciniphila*, a Gram-negative bacteria, can enhance mucus production to improve intestinal barrier function. Studies have documented that *A. muciniphila* abundance is significantly decreased in patients with alcoholic hepatitis and mice exposed to alcohol. Interestingly, the presence of alcohol did not affect the commensal *A. muciniphila* growth *in vitro* (Grander et al., 2018). The mechanism by which ethanol depletes *A. muciniphila* remains unclear. Lee et al. demonstrated that a low-dose (0.8 g/kg/days) of alcohol for 7 days can change the fecal microbiota composition, while fermented rice liquor can restore the microbial composition and inhibit intestinal inflammation (Lee et al., 2020).

The regulation of the gut microbiota appears to be a promising strategy to improve acute ALD (Vassallo et al., 2015). Pharmacological research showed that several drugs could regulate the composition of the gut microbiota and treat acute ALD. Audrey et al. proved that rhubarb extracts increased the abundance of mucus *A. muciniphila* and *Parabacteroides goldsteinii* by decreasing the activation of the TLR4 pathway and reducing the levels of inflammation and oxidative stress in the liver tissue, which improved acute liver injury caused by alcohol (30% w/v, 6 g/kg body weight) (Uesugi et al., 2001; Neyrinck et al., 2017). Meanwhile, there have been reports on the intervention of the gut microbiota in acute alcoholic liver disease. The supplementation of *Pediococcus pentosaceus* CGMCC 7,049 improved the intestinal barrier function and reversed gut microbiota dysbiosis by reducing the level of circulating endotoxin and proinflammatory cytokines (Jiang et al., 2020).

Animal studies have shown that probiotics and synbiotics improved gut microbiota disorders, enhanced the mucosal barrier, and protected hepatocyte from acute and chronic ethanol injury (Han et al., 2020; Lee et al., 2020). However, clinical studies data is still insufficient and we need further investigation in the future.

THE GUT MICROBIOTA AND D-GALACTOSAMINE-INDUCED ACUTE LIVER INJURY

D-galactosamine (D-GalN), a hexosamine derived from galactose, is a component of various glycoprotein hormones and a hepatotoxic agent. Intraperitoneal injection of D-GalN can cause diffuse hepatocyte necrosis and inflammation analogous to the changes of the liver pathology after clinical viral hepatitis (Keppler et al., 1968; Rahman et al., 2018). In general, the co-administration of a sublethal dose D-GalN and lipopolysaccharide (LPS) has been widely used in repetitive experimental animal models of fulminant liver failure in the clinic. The hepatotoxicity induced by D-GalN is mainly due to a decrease in the concentration of uridine diphosphate in hepatocytes and the methylation of ribosomal RNA, which can affect the normal translation of proteins. This results in the inhibition of RNA and protein synthesis and the disruption of the normal hepatocytes metabolism, which leads to cell necrosis and inflammatory infiltration (Clawson et al., 1990; Kmiec et al., 2000). It has been reported that D-GalN also affects the synthesis of cell membrane components and eventually causes hepatocyte death (Decker and Keppler, 1974).

We recently found LPS/D-GalN administration could rapidly change gut microbial function which may further influence intestinal soyasaponin II level. Soyasaponin II exerts anti-inflammatory effect by targeting Y-Box Binding Protein 1 and Nlrp3 inflammasome (Wang et al., 2020a). Beside our work, accumulating evidence suggests that D-GalN treatment not just induces hepatocyte damage but also destroys the gut microbiota homeostasis and the intestinal barrier structure by allowing MAMPs, such as LPS, to enter the liver and peripheral blood (Li et al., 2004). Conversely, pretreatment with probiotic *Lactobacillus reuteri* DSM 17938 can relieve the gut dysbiosis, reduce the transcription of inflammatory factors, and alleviate D-GalN-induced liver injury in rats (Jiang et al., 2021). Probiotic *Lactobacillus casei Zhang* can also reduce LPS/D-GalN-induced pro-inflammatory cytokines and hepatic inflammation through the modulation of the TLR-MAPK-PPAR-γ signaling pathways (Wang et al., 2016). Li et al. data showed that the oral gavage of *Bacillus cereus* for 15 days before D-GalN administration significantly improved liver injury and inflammatory processes by decreasing plasma endotoxin levels, reinforcing the gut barrier function, and improving the gut microbiota (Li et al., 2019b). Wang et al. demonstrated that Sprague-Dawley rats pretreated with *Lactobacillus helveticus* R0052 for 7 days showed a significant reduction in the levels of ALT, bilirubin, and total bile acid that were changed by D-GalN. Additionally, R0052 exhibits anti-inflammatory properties by down-regulating the transcription of Toll-like receptors, tumor necrosis factor-α, and nuclear factor-kappa beta (NFκβ) in liver tissues. R0052 can also improve intestinal lactic acid bacteria and Bacteroides (Wang et al., 2019). Similarly, *Bifidobacterium*

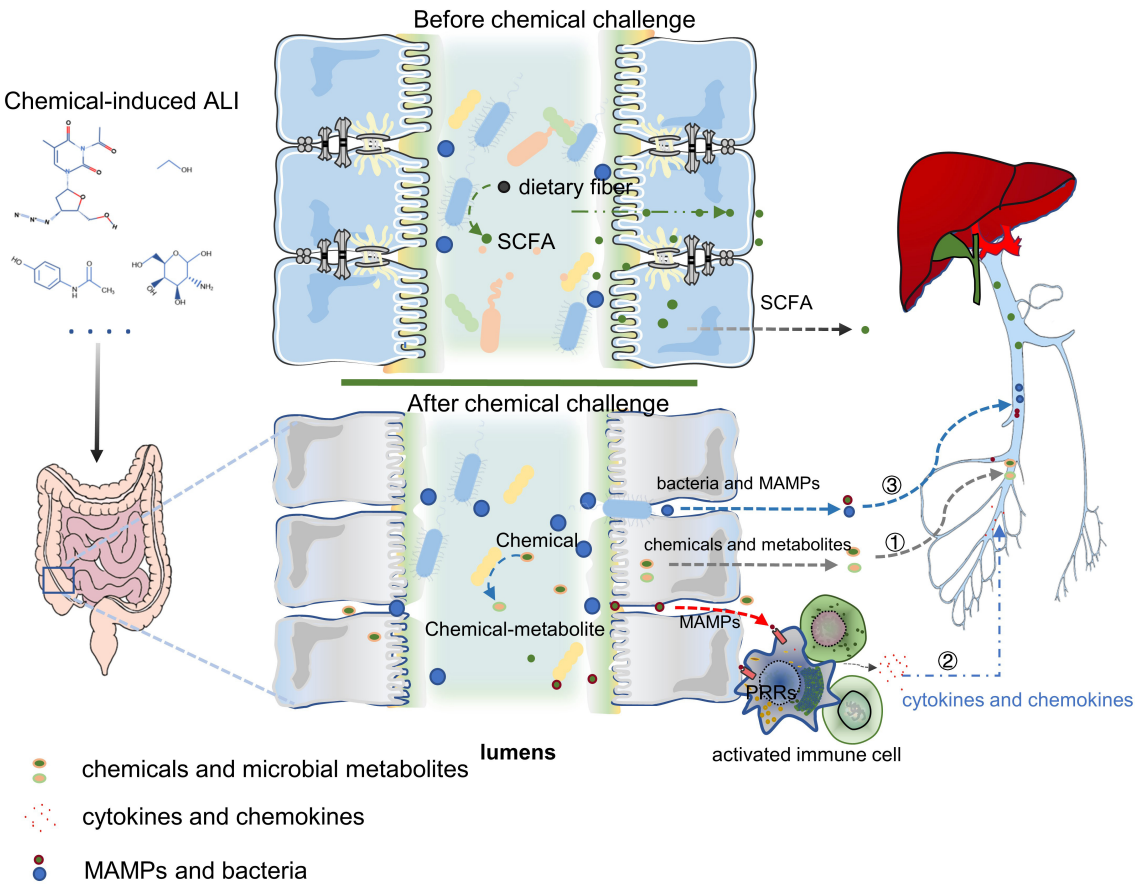

FIGURE 2 | The mechanism of gut microbiota in ALI can be summarized by three points: ① Chemicals transformation and metabolites of gut microbiota can influence the hepatocyte's ability to metabolize toxic substances and affect hepatic function; ② Gut harmful bacteria and MAMPs activate the systemic immune response, release a large number of chemokines and pro-inflammatory cytokines into the liver, leading to liver inflammation; ③ Intestinal mucosal barrier destroy (containing the weakening of the mucus barrier, the destruction of cell tight junction, and the necrosis of intestinal epithelial cells) increases bacterial translocation and the entry of MAMPs into the liver, which mediates liver damage. PRRs, Pattern Recognition Receptors. Figure was partly adapted from Smart Servier Medical Art (https://smart.servier.com/), which is licensed under a Creative Common Attribution 3.0 Generic License.

adolescents CGMCC15058, *Bifidobacterium longum* R0175, and *Saccharomyces boulardii* reduce the increase of cytotoxic factors and inflammatory cytokines in D-GalN-induced liver injury in rats (Yu et al., 2017; Li et al., 2019a; Wang et al., 2020b). In brief, D-GalN is a widely used model to examine the protective mechanism of exogenous probiotics, prebiotics on fulminant liver failure.

CONCLUSION

Acute liver injury is a life-threatening disease with various causes and rapid progress. The application of chemical-induced liver injury models is of great significance for the study of the pathophysiological mechanism of ALI. In recent years, with the development of rapid, sensitive, and cheap gene sequencing technology and omics technology, the gut microbiota was being found to play a key role in host liver immunity, metabolism, and detoxification. The role of gut microbiota in ALI has also been gradually revealed. In the chemical-induced ALI models, the main mechanisms of gut microbiota in regulating ALI can be summarized by three points (**Figure 2**). Firstly, the intestinal microbes directly affect the detoxification ability of hepatocyte through the changes of bacterial metabolites and the transformation of chemical toxins; secondly, microbes and MAMPs are exposed to the systemic immune cells through the damaged intestinal mucosal barrier. Then the immune cells were activated, which release a large number of chemokines and pro-inflammatory cytokines into the liver, and induce liver inflammation. Finally, gut microbiota dysbiosis or chemical poisons directly induced the intestinal mucosal barrier damage (including weakened mucus barrier, destructed cellular tight junctions and the necrosis of intestinal epithelial cells), increase bacteria and MAMPs translocation into the liver, and mediate liver injury. In future, using single-cell RNA sequencing might help to further study how the gut microbiota directly affects hepatocytes and liver non-parenchymal cells. Numerous studies

have shown that exogenous supplementation with prebiotics and probiotics can improve various chemical-induced ALI models. These data suggest that modulation of the gut microbiota for applying to clinical treatment of ALI is one possibility. The gut microbiota is a multifaceted community but nearly all of the research have focused on the role of bacterial disturbance in ALI models. The progress of fungi and viruses in this field is very limited. Future studies are required to better understand the systemic role of the gut microbiota; including viruses, fungi, and parasites, in the occurrence and development of ALI.

AUTHOR CONTRIBUTIONS

TC and RL draft the manuscript. PC edit the manuscript and supervised the work. All authors contributed to the article and approved the submitted version.

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Hepatic Branch Vagotomy Modulates the Gut-Liver-Brain Axis in Murine Cirrhosis

*Yuan Zhang[1], Jason D. Kang[1], Derrick Zhao[1], Siddartha S. Ghosh[2], Yanyan Wang[1], Yunling Tai[1], Javier Gonzalez-Maeso[3], Masoumeh Sikaroodi[4], Patrick M. Gillevet[4], H. Robert Lippman[5], Phillip B. Hylemon[1], Huiping Zhou[1] and Jasmohan S. Bajaj[6]**

[1] Division of Microbiology and Immunology, Central Virginia Veterans Health Care System, Virginia Commonwealth University, Richmond, VA, United States, [2] Division of Nephrology, Virginia Commonwealth University, Richmond, VA, United States, [3] Department of Physiology and Biophysics, Virginia Commonwealth University, Richmond, VA, United States, [4] Microbiome Analysis Center, George Mason University, Manassas, VA, United States, [5] Department of Pathology, Central Virginia Veterans Health Care System, Richmond, VA, United States, [6] Division of Gastroenterology, Hepatology, and Nutrition, Central Virginia Veterans Health Care System, Virginia Commonwealth University, Richmond, VA, United States

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***Correspondence:**
Jasmohan S. Bajaj
jasmohan.bajaj@vcuhealth.org

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Background: Cirrhosis and hepatic encephalopathy (HE) are linked with an altered gut-liver-brain axis, however, the relative contribution of hepatic vagal innervation is unclear. We aimed to determine the impact of hepatic vagotomy on the gut microbiome, brain, and liver in murine cirrhosis.

Methods: 10–15-week-old male C57BL/6 mice with and without hepatic vagotomy underwent carbon tetrachloride (CCl4) gavage for 8 weeks. Frontal cortex [inflammation, glial/microglial activation, BDNF (brain-derived neurotrophic factor)], liver [histology including inflammation and steatosis, fatty acid synthesis (sterol-responsive binding protein-1) SREBP-1, insulin-induced gene-2 (Insig2) and BDNF], and colonic mucosal microbiota (16srRNA microbial sequencing) were evaluated on sacrifice. Conventional mice with and without cirrhosis were compared to vagotomized counterparts.

Results: *Conventional control vs. cirrhosis*: Cirrhosis resulted in dysbiosis, hepatic/neuro-inflammation with glial/microglial activation, and low brain BDNF vs. controls. *Conventional control vs. vagotomy controls:* Vagotomized control mice had a lower colonic dysbiosis than conventional mice but the rest of the hepatic/brain parameters were similar. *Conventional cirrhosis vs. vagotomized cirrhosis:* After vagotomy + cirrhosis, we found lower dysbiosis but continuing neuroinflammation in the absence of glial/microglial activation vs. conventional cirrhosis. Vagotomy + Cirrhosis groups showed higher hepatic steatosis due to higher SREBP1 and low Insig2 protein and altered activation of key genes involved in hepatic lipid metabolism and inflammation. BDNF levels in the brain were higher but low in the liver in vagotomy + cirrhosis, likely a protective mechanism.

Conclusions: Hepatic vagal innervation affects the gut microbial composition, hepatic inflammation and steatosis, and cortical inflammation and BDNF expression and could be a critical modulator of the gut-liver-brain axis with consequences for HE development.

Keywords: vagotomy, pathobiont, inflammation, BDNF, hepatic encephalopathy, microbiota (16S)

INTRODUCTION

The gut-liver-brain axis mediates the development and progression of complications of cirrhosis, such as hepatic encephalopathy (HE) (Kang et al., 2016a; Ochoa-Sanchez and Rose, 2018). Most therapies for HE are focused on the modulation of the gut microbial milieu using laxatives and antibiotics (Vilstrup et al., 2014). In addition to the microbial metabolite and barrier impairment, gut-brain axis alterations could also be modulated through vagal innervation (Cryan et al., 2019). Prior murine studies have shown that transplanted stool from patients with cirrhosis, but not from healthy controls, results in neuro-inflammation in germ-free mice (Liu et al., 2020). In cirrhosis, the additional impact of hepatic failure and inflammation adds to the overall inflammatory milieu that can affect brain function (Shawcross et al., 2004, 2011). The hepatic branch of the vagus is associated with modulating metabolic signals between the brain and the liver (Pocai et al., 2005; Harada et al., 2014; Metz and Pavlov, 2018). The liver-brain connection through the hepatic vagus branch could be used to determine the relative contribution of the liver-brain aspect of the gut-liver-brain axis.

Our aim was to evaluate the effect of isolated hepatic vagotomy on gut microbiota, liver inflammation, and brain inflammation in the setting of murine cirrhosis.

Abbreviations: HE, hepatic encephalopathy; CCl4, carbon tetrachloride; BDNF, brain derived neurotrophic factor; MCP-1, monocyte chemoattractant protein-1; IL, interleukin; IBA1, ionized calcium-binding adaptor molecule 1; GFAP, glial fibrillary acidic protein; SREBP1, sterol regulatory element-binding protein 1; INSIG2, insulin induced gene 2; LEfSe, linear discriminant effect size.

MATERIALS AND METHODS

We used 10–12-week-old C57BL/6 male mice for this experiment (**Figure 1**). Cirrhosis was induced in a subgroup of mice using our protocol of CCl4 gavage for 14 weeks (Kang et al., 2016a). Another group of 10–12-week-old male C57BL/6 mice with isolated hepatic branch vagotomy were obtained from Charles River laboratories. After acclimatization at the VCU animal facility, the mice were then again divided into those followed as controls and those who received 14 weeks of CCL4 gavage. All mice were sacrificed at week 14 (n = 6 per group). At the time of sacrifice, we collected the small intestinal mucosa, large intestinal mucosa, liver, and frontal cortices from all mice. In the brain, we studied messenger

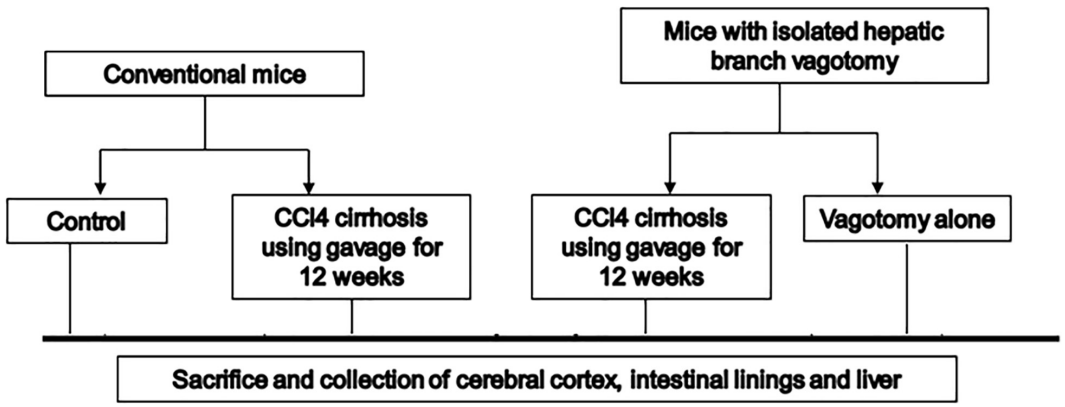

FIGURE 1 | Schema of the experiment. CCl4, carbon tetrachloride.

TABLE 1 | Real-time PCR Primers.

Name of gene	Genebank number	Forward primer	Reverse primer
BDNF	X55573	ACGGTCACAGTCCTAGAG	AGCCTTCCTTGGTGTAAC
Cd11b	NM_008401	CGGTAGCATCAACAACAT	GCATCAAAGAGAACAAGGT
Ces1g	NM_021456	CGCCAGAAGACTGAAGATGAGC	TGGTGCCTTTGGCAGCAACACT
Ces2	NM_133960.5	GCTCTCCAAGTGGCACATTTCC	CAAAGGCAACGTCATCACCATGG
CK-19	NM_008471	CGAGATTACAACCACTAC	GTTCTGTCTCAAACTTGG
Col1αa	NM_007742.4	ACATGTTCAGCTTTGTGGACC	TAGGCCATTGTGTATGCAGC
GFAP	NM_001131020	CAACCTGGCTGCGTATAG	CGAACTTCCTCCTCATAGAT
IBA-1	AB036423.1	GTCCTTGAAGCGAATGCTGG	CATTCTCAAGATGGCAGATC
IL-1b	NM_008361.4	GCAACTGTTCCTGAACTCAACT	GCCCAACATCTTTTGGGGTCCGTCAACT
LPL	NM_008509	GTCTAACTGCCACTTCAACC	CACCCAACTCTCATACATTCC
MCP-1	NM_011333	CTTCTGGGCCTGCTGTTCA	CCAGCCTACTCATTGGGATCA
Sirt1	NM_019812	TAGCACTAATTCCAAGTTCTATAC	TAACATCGCAGTCTCCAA
α-SMA	NM_007392.3	TGAAGAGCATCCGACACT	AGCCTGAATAGCCACATAC

RNA (mRNA) expression of (a) neuroinflammation [interleukin (IL)-1β, monocyte chemoattractant protein 1 (MCP1)]; (b) glial/microglial activation [ionized calcium-binding adaptor molecule 1 (IBA1) and glial fibrillary acidic protein (GFAP)]; (c) brain regeneration and plasticity [brain-derived neurotrophic factor (BDNF)]. The liver tissues were processed for histological analysis and isolation of total RNA and total protein lysate. The mRNA levels of the key genes involved in hepatic lipid metabolism and inflammation were measured by real-time quantitative PCR (RT-qPCR) (**Table 1**). The protein levels of SREBP-1 (Purified Mouse Anti-SREBP-1, from BD Pharmingen, Cat#557036) and Insig2 (Santa Cruz, Cat# sc-34823). Histological analysis of the hepatic inflammation, steatosis, fibrosis or cirrhosis was done by a pathologist in a blinded fashion with hematoxylin and eosin and trichrome staining using the NASH-Clinical Research Network criteria.

Large intestinal mucosal microbiota was analyzed using 16S ribosomal RNA (rRNA) sequencing after DNA was extracted (Gillevet et al., 2010).

Statistical analysis: We analyzed continuous data using unpaired *t*-tests/ANOVA for mean and SEM and Kruskal–Wallis/Mann–Whitney for comparing medians. For *in vivo* studies, in order to determine the group size, we will perform the power calculations to detect a 25% difference at a power of 0.8 and a confidence level of 95% for neuro-inflammation. Based on the data from an earlier mouse study with the standard deviation of 50% for each group, the group sizes equal to or greater than 6 are required. This was also based on our prior study of changes in gut microbiome affecting neuro-inflammation (Liu et al., 2020). Data are presented as mean ± SEM unless otherwise specified. We compared changes in microbial composition between mouse groups using linear discriminant effect size (LEfSe) (Segata et al., 2011) analysis.

Approvals were obtained from the Institutional Animal Care and Use Committee at VCU before study initiation. All animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals. All authors had access to the study data and had reviewed and approved the final manuscript.

RESULTS

There were no obvious changes in health appearance, weight changes or sickness behavior noted in any of the mice before the sacrifice.

Frontal Cortex Evaluation

Neuro-Inflammation

Two neuro-inflammatory markers (IL-6 and MCP-1) were significantly higher in conventional cirrhotic mice than controls (**Figure 2**). There was a significant increase in IL-1β and MCP-1 mRNA expression in mice with vagotomy compared to the controls, which marginally changed after the development of cirrhosis. Glial (GFAP) and microglial (IBA) markers were higher in cirrhotic mice without vagotomy. However, these changes were largely abrogated after vagotomy. BDNF (Cattaneo et al., 2016) was not affected by cirrhosis in the conventional state. Vagotomized mice showed increased frontal cortex expression and BDNF.

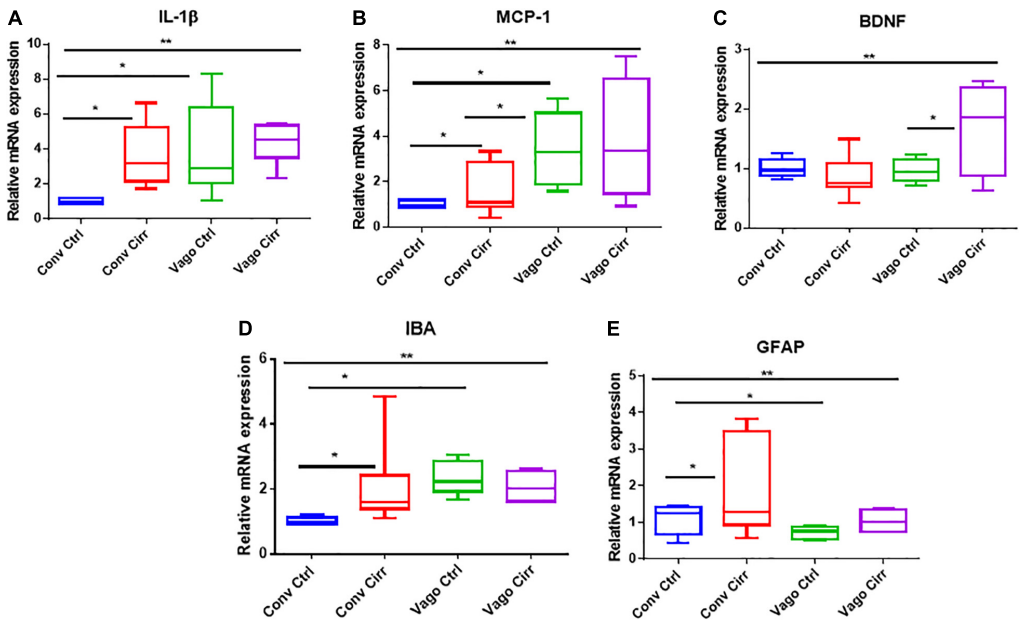

FIGURE 2 | mRNA expression in frontal cortices presented as median and 95% CI. **(A)** Interleukin 1 β, **(B)** Monocyte chemoattactant protein 1, **(C)** Glial fibrillary acidic protein and **(D)** ionized calcium-binding adaptor molecule 1. **(E)** Brain-derived neurotrophic factor. Conv, conventional; Ctrl, control; Vago-Ctrl, isolated hepatic branch vagotomy mice; Vago-Cirr, mice with CCl4 cirrhosis after isolated hepatic branch vagotomy. *p < 0.05–0.01, **p < 0.01–0.001, and ***p < 0.001 on Mann–Whitney or Kruskal–Wallis as appropriate.

TABLE 2 | LEfSe changes in colonic mucosal microbiota in Conventional mice and mice with Vagotomy.

LEfSe comparison	Higher in controls	Higher in cirrhosis
Control vs. Cirrhosis	Actinobacteria_Bifidobacteriaceae	Actinobacteria _Proprionibacteriaceae
	Bacteroidetes_Cryomorphaceae	Bacteroidetes_Marinilabiliaceae
	Bacteroidetes_Rikenellaceae	Cyanobacteri a_Chloroplast
	Firmicutes_Acidaminococcaceae	Firmicutes_St reptococcaceae
	Firmicutes_Heliobacteriaceae	Proteobacteri a_Enterobacteriaceae
	Firmicutes_Lachnospiraceae	Proteobacteri a_Burkholderiaceae
	Firmicutes_Peptostreptococcaceae	
	Tenericutes_Anaeroplasmataceae	
	Higher in controls	**Higher in vagotomy controls**
Control vs. Vagotomy control	Proteobacteria_Enterobacteriaceae	Firmicutes_Veillonellaceae
	Firmicutes_Erysipelothricaceae	Firmicutes_Lachnospiracae
	Firmicutes_Peptostreptococcaceae	Firmicutes_Ruminococcaceae
	Actinobacteria_Corynebacteriaceae	Firmicutes_ClostridialesIncSedXl
	Actinobacteria_Bifidobacteriaceae	Firmicutes_ClostridialesIncSedIV
	Actinobacteria_Coriobacteriacae	Actinobacteria_Propionibacteriaceae
	Bacteroidetes_Bacteroidaceae	Tenericutes_Aneroplasmataceae
	Bacteroidetes_Prevotellaceae	
	Bacteroidetes_Cryomorphaceae	
	Bacteroidetes_Marinilibiaceae	
	Bacteroidetes_Flammeovirgiaceae	
	Bacteroidetes_Flavobacteriaceae	
	Bacteroidetes_Porphyromonadaceae Verrucomicrobia_Verrucomicrobiaceae	
	Higher in cirrhosis	**Higher in vagotomy cirrhosis**
Cirrhosis vs. Vagotomy Cirrhosis	Cyanobacteria_Chloroplast	Bacteroidetes_Rikenellaceae
	Actinobacteria_Propionibacteriaceae	Bacteroidetes_Prolixibacteriaceae
	Bacteroidetes_Bacteroidaceae	Deferribacters_Deferribacteriaceae
	Bacteroidetes_Marinilibilaceae	Firmicutes_Lachnospiracae
	Firmicutes_Erysipelothricaceae	Firmicutes_Ruminococcaceae
	Proteobacteria_Enterobacteriaceae	Firmicutes_Veillonellaceae
		Firmicutes_Heliobacteriaceae
		Firmicutes_Acidaminococcaceae
		Firmicutes_Clostridiaceae
		Firmicutes_Defluviitaleaceae

Microbiota presented as Phylum_Family. LEfSe: Linear Discriminant Analysis Effect Size.

Mucosal Microbial Changes

An overview of microbial comparisons are shown in **Table 2** and **Figure 3**.

Conventional Controls vs. Conventional Cirrhosis Comparisons

As expected, conventional controls had higher potentially beneficial taxa such as Lachnospiraceae and Bifidobacteriaceae compared to conventional mice with cirrhosis that demonstrated greater pathobionts such as Enterobacteriaceae and Streptococcaceae.

Conventional Controls vs. Vagotomized Control Comparisons

After vagotomy, control mice had greater beneficial taxa belonging to Firmicutes, including Lachnospiraceae, Ruminococcaceae and Rikenellaceae, and lesser relative abundance of Enterobacteriaceae and Verrucomicrobiaceae compared to conventional control mice.

Conventional Cirrhosis vs. Vagotomy Cirrhosis Comparisons

Mice with cirrhosis after vagotomy had a higher representation of beneficial Firmicutes families compared to conventional cirrhosis.

Liver Findings

Histological Analysis

As expected, mice receiving CCl4 for 12 weeks developed cirrhosis on histology regardless of vagotomy. No changes in histology were seen at 12 weeks between conventional control and control vagotomized mice

(**Figures 4**, **5**). The inflammatory grade increased as expected with cirrhosis development, was lower in the vagotomized group, but the opposite was seen with the steatosis grade.

Liver Inflammation and Fibrosis

In addition to the histological inflammatory grade, there was a significant increase in mRNA expression of MCP-1, Cd112b, and CD63 in the mice that developed cirrhosis in the setting of vagotomy (**Figure 6**). BDNF expression increased in conventional mice with cirrhosis but not in vagotomized ones. In addition, the mRNA levels of Ck-19, α-SMA and Col1α were significantly upregulated in vagotomized cirrhotic mice.

Steatosis

Given the increase in steatosis with vagotomy cirrhotic mice, we examined the key genes involved in hepatic lipid metabolism using real-time RT-PCR and Western blot analyses. The RT-PCR further showed the upregulation of lipoprotein lipase (LPL) and downregulation of carboxylesterases, Ces1g and Ces2.

As shown in **Figure 7**, the nuclear form of SREBP1 protein levels were significantly increased in the vagotomy-cirrhosis group compared to the conventional cirrhosis group.

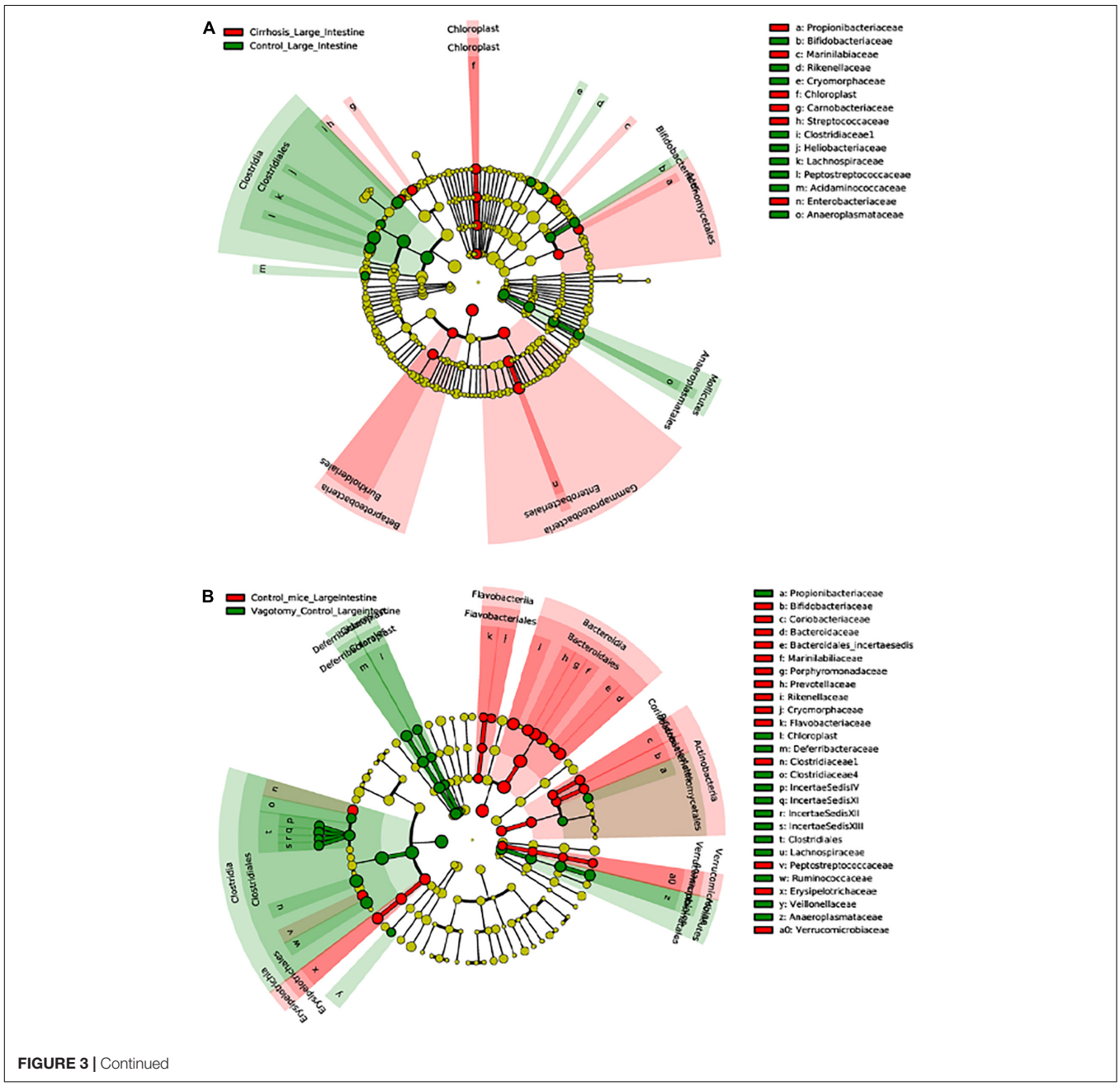

FIGURE 3 | Continued

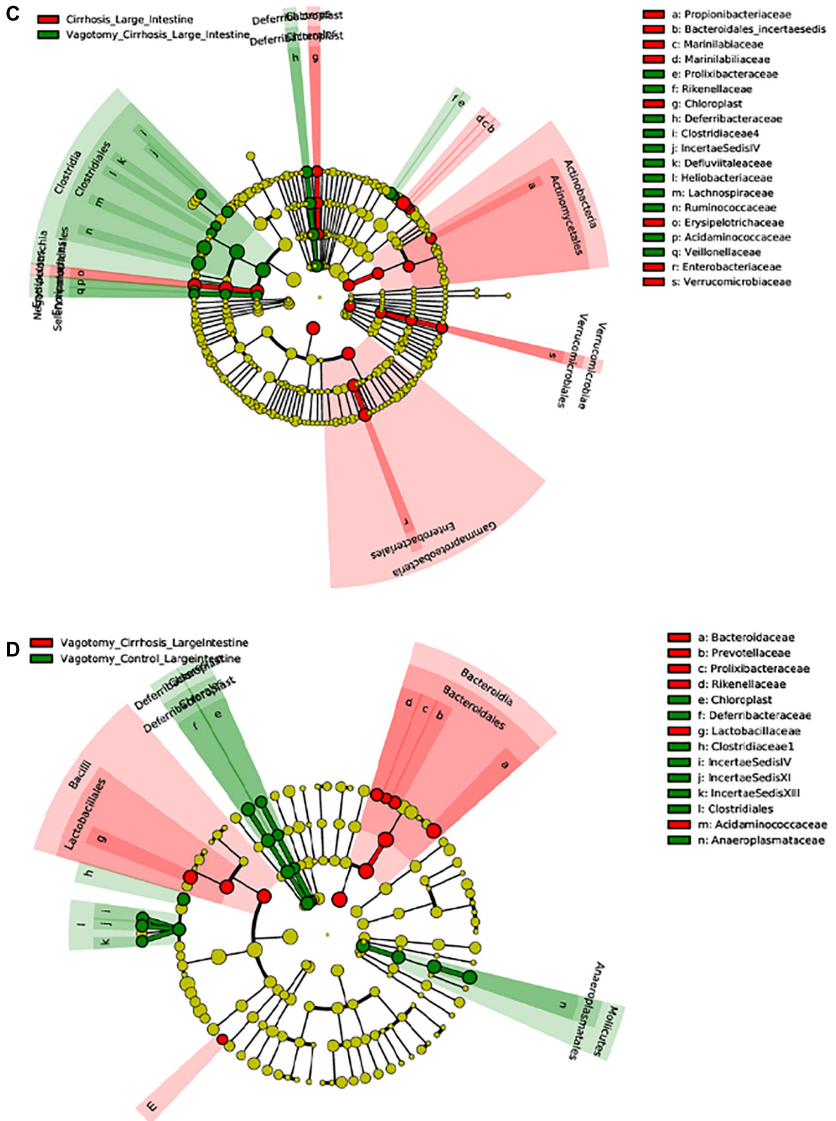

FIGURE 3 | Linear discriminant analysis Effect Size (LEfSe) cladograms of large intestinal mucosal comparisons. Significant differences are color coded. **(A)** Conventional controls vs. conventional cirrhosis. **(B)** Conventional controls vs. vagotomized controls. **(C)** Conventional cirrhosis vs. vagotomized cirrhosis. **(D)** Vagotomized controls vs. vagotomized cirrhosis.

Furthermore, the protein level of Insig 2 was significantly reduced in vagotomy cirrhotic mice.

DISCUSSION

Connections between the gut, liver, and brain are germane toward the development and progression of cirrhosis, but the role of hepatic vagal output requires greater clarification. Using a mouse model of hepatic branch vagotomy, we found that vagal output from the liver is associated with changes in gut microbial composition, neuro-inflammation, and plasticity, as well as hepatic steatosis and inflammation. These results demonstrate that hepatic vagal innervation can affect the gut-liver-brain axis in cirrhosis.

Although previous findings suggested that the hepatic branch of the vagus has a pivotal role in transmitting information from the liver to the brain, how this modulates the effects of cirrhosis remained largely unknown (Pocai et al., 2005; Harada et al., 2014). Mice with vagotomy showed lower dysbiosis with a higher relative abundance of potential autochthonous taxa such as Lachnospiraceaeae and Ruminococcaceae regardless of whether this was controls or in mice that developed cirrhosis. This interesting role of the hepatic vagus adds another dimension to the gut-liver axis in addition to the previously described factors such as intestinal barrier integrity, bile acid features and bacterial products. The data show

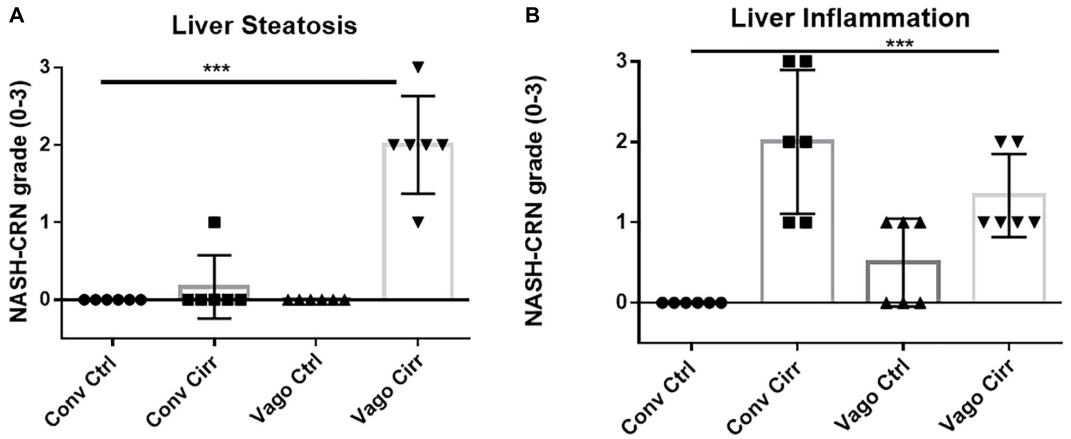

FIGURE 4 | Histological evaluation of steatosis and inflammation according to NASH-Clinical Research Network criteria. Conv, conventional; Ctrl, control; Cirr, CCL4 cirrhosis; VagoCtrl, isolated hepatic branch vagotomy mice; VagoCirr, Mice with CCl4 cirrhosis after isolated hepatic branch vagotomy. ***$p < 0.001$ on Kruskal–Wallis Individual mouse data and median 95% CI are presented. **(A)** Steatosis grade. **(B)** Inflammatory grade.

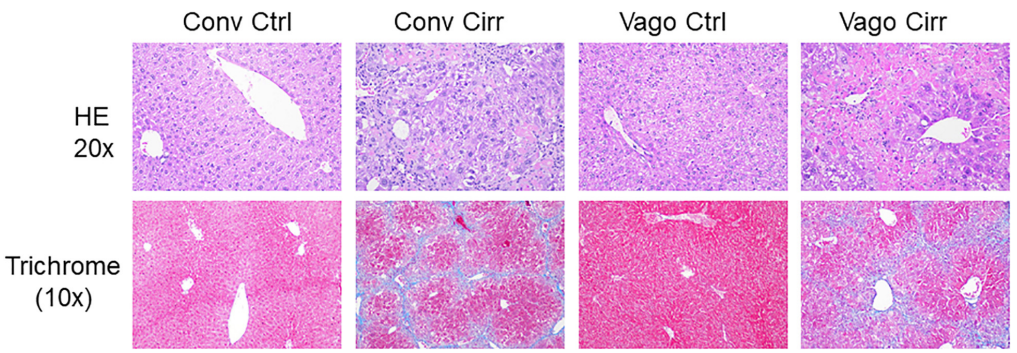

FIGURE 5 | Representative liver histological sections in H/E 20X and trichrome 10X showing normal histology in both control groups, cirrhosis on trichrome in both cirrhosis groups and steatosis in Vagotomized cirrhosis liver. Conv, conventional; Ctrl, control; Cirr, CCL4 cirrhosis; VagoCtrl, isolated hepatic branch vagotomy mice; VagoCirr, Mice with CCl4 cirrhosis after isolated hepatic branch vagotomy.

that vagal output from the liver enhances the overgrowth of potential pathobionts since vagotomy reduced dysbiosis in the mucosal microbiome. This is even more striking because hepatic branch vagotomy does not affect gastric acid secretion, intestinal motility or physical connections between the liver and the gut.

While the findings of lower dysbiosis after vagotomy seem to be at odds with the higher brain inflammation and higher liver steatosis, most of these changes only occurred after CCl4 gavage and were associated with differential changes in BDNF expression. However, some microbial taxa belonging to Ruminococcaceae that are typically lower in advancing cirrhosis, are actually associated with fibrosis and metabolic syndrome in patients with liver steatosis (Boursier et al., 2016; Lee et al., 2020). In addition, Verrucomicrobiaceae, which includes the beneficial taxon, *Akkermansia muciniphilia* were lower in vagotomized controls, which could also promote hepatic steatosis (Everard et al., 2013). Following this, we found higher hepatic steatosis in vagotomized cirrhotic mice, which had higher mucosal Ruminococcaceae. With the appearance of cirrhosis, these changes in Verrucomicrobia were not seen, likely due to the cirrhosis state reducing these organisms uniformly regardless of vagotomy.

Prior studies in vagotomized mice in the setting of NAFLD before cirrhosis have shown greater steatosis, but we extended these in a cirrhosis model of CCl4 gavage that typically does not demonstrate steatosis. While the exact mechanisms are unclear, there was an upregulation of the activated nuclear form of SREBP1 (nSREBP1) protein level in the liver after vagotomy. This increase in nSREBP1 was associated with a relative loss of Insig2 after vagotomy in the liver. Insig2 is an ER stress-responsive gene, which prevents the proteolytic processing of SREBP-1c from forming a maturing form, a critical transcriptional regulator of hepatic fatty acid metabolism (Takaishi et al., 2004). LPL is an important player in regulating lipid metabolism and energy balance. The upregulation of LPL has been reported to exacerbate liver fibrosis (Teratani et al., 2019). The carboxylesterases plays a critical role in hydrolyze a variety of xenobiotic and endogenous compounds

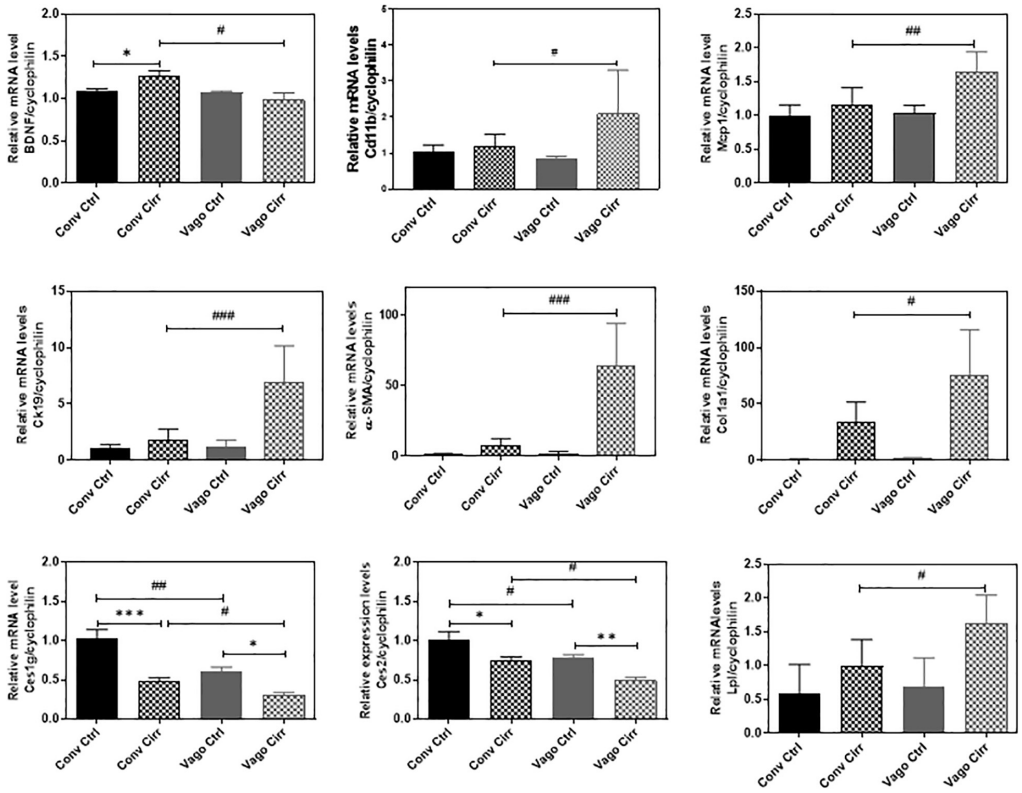

FIGURE 6 | Hepatic Expression of BDNF and Genes involved in fatty acid metabolism and inflammation presented as Mean ± SEM. Conv, conventional; Ctrl, control; Cirr, CCL4 cirrhosis; VagoCtrl, isolated hepatic branch vagotomy mice; VagoCirr, mice with CCl4 cirrhosis after isolated hepatic branch vagotomy. Comparisons using Mann–Whitney test, $^{\#}/^{*}p < 0.05$, $^{\#\#}/^{**}p < 0.01$, and $^{\#\#\#}/^{***}p < 0.0001$.

and including lipid esters. Six human CES genes have been identified. CES1 and CES2 are the two most prominent genes, which are mainly expressed in the gastrointestinal tract and liver. In mice, eight genes belong to Ces1 have been identified with relatively unique tissue expression patterns. Compared to other Ces1 family members, Ces1g is highly expressed in the liver and intestine (Lian et al., 2018). Previous studies have shown that deficiency of Ces1g or Ces2a was linked to metabolic diseases. It has been reported that that Ces1g suppresses the activity of Srebp1c promoter and enhances the degradation of Srebp1 (Xu et al., 2001). In addition, Ces1g inhibits Insig 1 degradation and *de novo* lipogenesis. Downregulation of Ces1g may attribute to activation of SREBP1c, leading to lipogenesis. Liver specific expression of Ces1g reduces hepatic steatosis (Bahitham et al., 2016). In addition, downregulation of Ces2 is associated with NASH disease progression and high-fat-diet-induced steatosis (Li et al., 2016). Hepatic CES2 plays a key role in fatty acid oxidation and inhibiting lipogenesis. We also found that the expression of Ces2 was downregulated in both conventional and vagotomy cirrhotic mice and vagotomy further inhibited Ces2 expression. Therefore, these could be the potential mechanisms behind the development of steatosis in the liver and lower inflammation after vagotomy and cirrhosis compared to the conventional animals (Gao et al., 2015; Amir et al., 2020).

Despite the increase in Ruminococcaceae in vagotomized animals that can promote steatosis, vagotomized animals did have higher relative abundances of other potentially beneficial taxa such as Lachnospiraceae and reduction in pathobionts like Enterobacteriaceae (Kang et al., 2016a,b, 2017). In prior studies of germ-free mice colonized with stools from differing human phenotypes, there was an increase in hepatic and frontal cortical neuro-inflammation in mice that received stool from patients with cirrhosis (Liu et al., 2020). Other studies demonstrated that susceptibility to alcohol-related liver disease was also modulated by the donor of the microbiota, whether it be a different mouse group or humans (Llopis et al., 2016; Cassard et al., 2017). Microglial and glial activation are usually associated with cirrhosis-related neuro-inflammation but in vagotomized mice, the expression of GFAP and IBA were abrogated even with cirrhosis. Therefore, factors other than dysbiosis and glial/microglial activation, such as systemic inflammation and BDNF may be associated with neuroinflammation in mice with hepatic branch vagotomy and cirrhosis (Shawcross et al., 2011; Gorg et al., 2015). BDNF is usually thought of as a primary neurotrophic molecule but of late studies have shown it to be an important part of the liver-brain axis (Pocai et al., 2005; Bercik et al., 2011; Cattaneo et al., 2016). The role of BDNF is complex since it can modulate insulin resistance and liver disease in animal models and is found

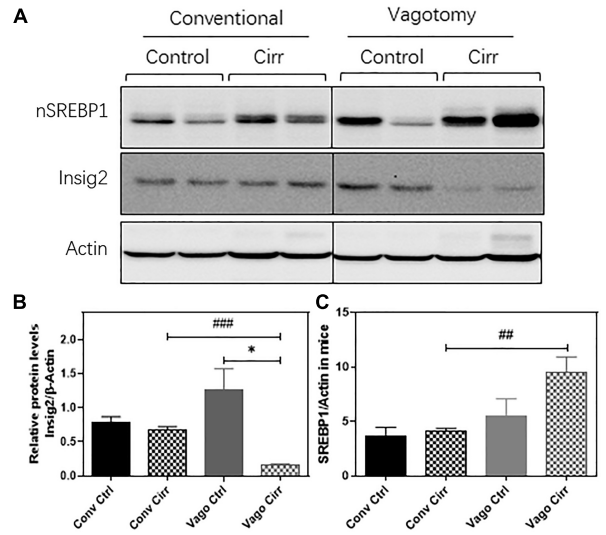

FIGURE 7 | Western Blot expression of Hepatic SREBP1 and Insig2 using Actin as the reference protein. **(A)** gels, **(B)** Mean ± SEM for SREBP1/Actin ratio, **(C)** Mean ± SEM for Insig2/Actin Ratio. Conv, conventional; Ctrl, control; Cirr, CCL4 cirrhosis; VagoCtrl, isolated hepatic branch vagotomy mice; VagoCirr, mice with CCl4 cirrhosis after isolated hepatic branch vagotomy. $^{###}p < 0.001$ and $^{##}p < 0.01$ between Conv Cirr and Vago Cirr, $^{*}p < 0.05$ between VagoCtrl and VagoCirr on Mann–Whitney tests.

in higher liver levels in those with cirrhosis and alcohol-induced injury (Teillon et al., 2010; Gao et al., 2015; Yang et al., 2017; Amir et al., 2020). However, BDNF is anti-inflammatory in the brain under most circumstances, including HE (Kang et al., 2016a; Dhanda et al., 2018). Our data showed that with the intact vagus, BDNF increased with cirrhosis, paralleling prior studies, but this circuit is broken with vagotomy, where cirrhosis does not lead to BDNF increase in the liver but does in the brain. This may be a protective mechanism since the reverse profile i.e., higher liver BDNF and lower brain BDNF is found in psychiatric disorders (Yang et al., 2017).

Ultimately, it is striking that most differentiators in the setting of vagotomy compared to conventional mice only occurred after the induction of cirrhosis through CCl4. Vagotomized control mice and conventional control mice were otherwise similar in most outcomes related to the brain and liver. Therefore, despite the relatively lower dysbiosis in vagotomized control mice, they were equally susceptible to CCl4-induced cirrhosis. Gavage with CCl4 typically induces toxic cirrhosis with dysbiosis and neuro-inflammation without the diversion of bile flow (Kang et al., 2016a). These interactions were enhanced in vagotomized animals, pointing to an important role of hepatic parasympathetic innervation in not only fatty liver as previously described but also in cirrhosis (Gao et al., 2015; Amir et al., 2020). Moreover, the decoupling of the gut-liver-brain axis found due to a lower dysbiosis but higher neuro-inflammation in vagotomized cirrhosis shows that the neuronal input from the liver may be an important way station in the gut-brain communication in cirrhosis. Vagotomy-induced lower hepatic and higher BDNF cortical expression also suggest that the neurotrophic factors may have a major role in the gut-liver-brain axis in cirrhosis (Amir et al., 2020).

Our study was limited since we used isolated hepatic vagotomy and not a more radical subdiaphragmatic approach (Bercik et al., 2011). However, the latter approach affects most gastrointestinal organs and can impact acid secretion and motility, all of which can confound the results by affecting microbiota independently. We used the entire frontal cortex given its involvement in HE but further work will be necessary to characterize the cell type in which expression of these neural plasticity and inflammatory markers occurs. We only used the CCl4 model *via* gavage because, unlike the bile duct ligated model, it does not result in microbiota change due to immediate bile diversion (Fouts et al., 2012; Dhanda et al., 2018). However, it does not cause major behavioral changes in mice, which is why we used inflammatory gene expression as the readout (Butterworth et al., 2009); future behavioral testing models are needed (DeMorrow et al., 2021).

We conclude that the parasympathetic innervation of the liver modulates hepatic steatosis, neuro-inflammation and dysbiosis even after the development of cirrhosis using CCl4 gavage. These data that vagal innervation of the liver plays an important role through modulation of BDNF in the gut-liver-brain axis, which has implications for the pathogenesis of cirrhosis and associated complications.

DATA AVAILABILITY STATEMENT

Data are available now at https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA735706.

ETHICS STATEMENT

The animal study was reviewed and approved by Virginia Commonwealth University IACUC.

AUTHOR CONTRIBUTIONS

JB and HZ conceptualized and were involved at all levels of the study. YZ, JK, DZ, SG, YW, and YT were involved in the animal handling, sacrifice, and experiments. JG-M and PH were involved in experiments design and manuscript revision. HL was involved in histological analysis. All authors contributed to the article and approved the submitted version.

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Characteristics of Gut Microbiota in Children With Biliary Atresia After Liver Transplantation

Wei Song[1,2], Li-Ying Sun[1,2,3], Zhi-Jun Zhu[1,2], Lin Wei[1,2], Wei Qu[1,2], Zhi-Gui Zeng[1,2] and Yun-Sheng Yang[4]**

[1] Liver Transplantation Center, National Clinical Research Center for Digestive Diseases, Beijing Friendship Hospital, Capital Medical University, Beijing, China, [2] Clinical Center for Pediatric Liver Transplantation, Capital Medical University, Beijing, China, [3] Department of Intensive Care Unit, Beijing Friendship Hospital, Capital Medical University, Beijing, China, [4] Department of Gastroenterology and Hepatology, The First Medical Center, Chinese PLA General Hospital, Beijing, China

Background and Aims: Biliary atresia (BA) is an idiopathic neonatal cholestasis and is the most common indication in pediatric liver transplantation (LT). Previous studies have suggested that the gut microbiota (GM) in BA is disordered. However, the effect of LT on gut dysbiosis in patients with BA has not yet been elucidated.

Methods: Patients with BA (n = 16) and healthy controls (n = 10) were recruited. In the early life of children with BA, Kasai surgery is a typical procedure for restoring bile flow. According to whether BA patients had previously undergone Kasai surgery, we divided the post-LT patients into the with-Kasai group (n = 8) and non-Kasai group (n = 8). Fecal samples were collected in both the BA and the control group; among BA patients, samples were obtained again 6 months after LT. A total of 40 fecal samples were collected, of which 16 were pre-LT, 14 were post-LT (8 were with-Kasai, 6 were non-Kasai), and 10 were from the control group. Metagenomic sequencing was performed to evaluate the GM.

Results: The Kruskal-Wallis test showed a statistically significant difference in the number of genes between the pre-LT and the control group, the pre-LT and the post-LT group ($P < 0.05$), but no statistical difference between the post-LT and the control group. Principal coordinate analysis also showed that the microbiome structure was similar between the post-LT and control group ($P > 0.05$). Analysis of the GM composition showed a significant decrease in *Serratia*, *Enterobacter*, *Morganella*, *Skunalikevirus*, and *Phifllikevirus* while short chain fatty acid (SCFA)-producing bacteria such as *Roseburia*, *Blautia*, *Clostridium*, *Akkermansia*, and *Ruminococcus* were increased after LT (linear discriminant analysis > 2, $P < 0.05$). However, they still did not reach the normal control level. Concerning functional profiles, lipopolysaccharide metabolism, multidrug resistance, polyamine biosynthesis, GABA biosynthesis, and EHEC/EPEC pathogenicity signature were more enriched in the post-LT group compared with the control group. Prior Kasai surgery had a specific influence on the postoperative GM.

Conclusion: LT partly improved the GM in patients with BA, which provided new insight into understanding the role of LT in BA.

Keywords: biliary atresia, liver transplantation, gut microbiota, metagenomic sequencing, infection

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Phillipp Hartmann, University of California, San Diego, United States

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Wei Gao, Tianjin First Central Hospital, China

***Correspondence:**
Li-Ying Sun
sunxlx@outlook.com
Yun-Sheng Yang
Sunnyddc@plagh.org

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INTRODUCTION

The gut microbiome plays an important role in human health and comprises about 10^{14} resident microorganisms, including bacteria, viruses, fungi, and archaea. Among these, bacteria are dominant (Lozupone et al., 2012). Gut microbiota (GM) provides energy and nutrients to intestinal epithelial cells by producing short-chain fatty acids (SCFAs). It can also promote gut immunity by acting on the toll-like receptor (TLR) (Singh et al., 2017). Inversely, it will lead to systemic infections when the microbiota balance is broken (Zhang et al., 2015). Accumulating literature suggests that GM imbalance occurs in many diseases such as liver disease (Miura and Ohnishi, 2014; Tilg et al., 2016), metabolic disease (Burcelin, 2017), cardiovascular disease (Ahmadmehrabi and Tang, 2017; Kazemian et al., 2020), inflammatory bowel disease (Nishida et al., 2018) and neurodegenerative disease (Quigley, 2017), and it plays a driving role in disease progression. By studying the role of GM in the management of illness, therapeutic measures have been developed to intervene in disease progression or complications, such as antibiotics, probiotics, prebiotics, and symbiotics.

Concerning end-stage liver disease, liver transplantation (LT) is the only cure. Biliary atresia (BA) is one of the most common indications for LT in children. BA is a devastating fibro-inflammatory disease that affects both extra- and intrahepatic bile ducts, its incidence ranges from 1/5,000 to 1/18,000. Prolonged neonatal jaundice, pale stools, and conjugated hyperbilirubinemia are the typical symptoms. Moreover, BA is a major cause of pediatric cholestasis (Feldman and Mack, 2015). Kasai surgery is typically performed to restore bile flow in early BA; however, up to 80% of patients will eventually require LT. Thus, Kasai is the standard first procedure for BA, and LT is reserved as a complementary and final therapy. Sun et al. (2017) demonstrated that LT significantly improved GM in adults with liver disease; GM disorders were also demonstrated in children with BA in our previous study. Nevertheless, the effect of LT on BA has not been described. Wang et al. (2016) summarized the impact of prior Kasai surgery on the clinical outcomes of LT and concluded that it might increase the risk of postoperative infection. However, the effect of prior Kasai surgery on the GM after LT is unknown.

Therefore, the present study aimed to investigate the effect of LT and describe the status of GM after LT in children with BA and the effect of prior Kasai surgery on the postoperative GM. Metagenomic sequencing was performed to evaluate the composition and function of the gut microbiome in feces.

Abbreviations: BA, biliary atresia; LT, liver transplantation; GM, gut microbiota; SCFAs, producing short-chain fatty acids; TLR, toll-like receptor; KO, KEGG ortholog; KEGG, Kyoto Encyclopedia of Genes and Genomes database; LEfSe, LDA effect size; LDA, Linear discriminant analysis; PCoA, Principal coordinate analysis; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, γ-glutaryltransferase; TBA, total bile acids; TBIL, total bilirubin; IL-2, interleukin-2; IL-4, interleukin-4; IL-6, interleukin-6; IL-10, interleukin; TNFα, tumor necrosis factor α; IFN-γ, interferon (IFN)-γ; PELD, Pediatric End-stage Liver Disease.

MATERIALS AND METHODS

Study Design

We collected stool samples from BA and healthy subjects at Beijing Friendship Hospital, Capital Medical University. Samples from BA patients were collected before and 6 months after LT. The point of post-LT timeframe was chosen to avoid immediate and transient postoperative complications (Jiang et al., 2018). Patients and controls were age-matched. Their dietary habits were similar. The following conditions in the enrolled group were met: (1) No digestive diseases within 1 month; (2) No antibiotics and probiotics within 1 month; and (3) age < 3 years. The diagnosis of BA was confirmed by intraoperative cholangiography and liver biopsy. The liver function index of controls was in the normal range. In patients with BA, preoperative data included age, body mass index (BMI), sex, pediatric end-stage liver disease (PELD) score, liver enzymes (ALT: alanine aminotransferase, AST: aspartate aminotransferase, ALP: alkaline phosphatase, GGT: γ-glutamyltransferase), and inflammatory cytokines (IL-2: interleukin-2, IL-4: interleukin-4, IL-6: interleukin-6, IL-10: interleukin, TNFα: tumor necrosis factor α, IFN-γ: interferon-γ), We examined liver function indexes and inflammatory factors again after LT. Metagenomic sequencing can analyze species composition and functional metabolism in greater depth.

Sample Collection and DNA Extraction

All samples were stored at –80°C within four hours of collection. Bacterial DNA was extracted using the QIAamp Fast DNA Stool Mini Kit (51604; Qiagen, Hilden, Germany). Ten micrograms of stool sample was weighed in a centrifuge tube, approximately 25 mg of pre-cooled submerged beads were added, and then 200 μL of acetonitrile/methanol (v/v = 8:2) solvent containing 10 μL of an internal standard for homogenous mixing was added and centrifuged at 13,500 rpm and 4°C for 20 min to remove proteins. After centrifugation, 10 μL supernatant was obtained, diluted with 90 μL of 1:1 acetonitrile/methanol (v/v = 80/20) and ultrapure water mixed solvent, shaken and centrifuged for analysis. The injection volume was 5 μL. The detailed extraction steps followed the QIAamp® Fast DNA Stool Mini Handbook instructions. The DNA concentration was measured with a NanoDrop (Thermo Fisher Scientific, MA, United States) and Qubit®2.0 (Invitrogen, Carlsbad, CA, United States), and the molecular size was estimated by agarose gel electrophoresis.

Metagenomic Sequencing

Following the Illumina TruSeq DNA Sample Prep v2 Guide (San Diego, CA, United States), we constructed the DNA paired-end libraries with an insert size of 500 bp for the 40 stool samples (16 from pre-LT, 14 from post-LT, and 10 from controls). The quality of all libraries was evaluated using an Agilent 2100 bioAnalyzer (Agilent Technologies, Wokingham, United Kingdom) and an Agilent 2100 DNA 1000 kit. All samples were subjected to 150-bp paired-end sequencing on an Hiseq X-ten platform (Illumina). Illumina raw reads were screened according to the following criteria: (1) adaptor contamination reads were removed; (2) reads

containing more than three ambiguous N bases were removed; (3) reads containing low quality (Q < 20) bases were trimmed; (4) reads containing less than 60% of high-quality bases (Phred score ≥ 20) were deleted.

Clean reads were subjected to bacterial genomes from the National Center for Biotechnology Information GenBank with SOAPaligner (version 2.21), and reads mapped to the host genome were abandoned.

For species classification, the NCBI database[1] (National Center for Biological Information) was used to align the clean reads with known bacteria, fungi, viruses, and archaea by SOAPaligner 2.21. With regard to the functional profiles, the non-redundant genes were annotated against the KEGG (Kyoto Encyclopedia of Genes and Genomes) database using BLAST (v. 2.2.28 +). When the assembled protein sequence was similar (score ≥ 60 and $E < 1e{-}5$) to a protein sequence in the database, the assembled protein was considered to play the same role as the database protein. The relative abundance of all orthologous genes was accumulated to generate the relative abundance of each KO (KEGG ortholog).

Statistical Analysis

Statistical analyses were completed using R v.3.3.1. The non-parametric Wilcoxon test and Kruskal-Wallis test were performed to analyze the different species or functional profiles. Differences were considered significant at $P < 0.05$ or a false discovery rate (FDR) < 0.1. Differences in taxa and functional profiles at various levels were evaluated by Wilcoxon rank testing and linear discriminant analysis (LDA) effect size (LEfSe), where a higher LDA score reflected a more prominent difference in abundance between the different groups. The LDA score cut-off of 2.0 indicated a significant difference. Spearman's rank correlation was used to examine associations among variables. Fishtaco was used to analyze the contribution of species to differential metabolic pathways.

[1]http://www.ncbi.nlm.nih.gov

RESULTS

Study Population

Forty stool samples were obtained from twenty-six participants enrolled in this study, including 16 pre-LT, 14 post-LT, and 10 control samples. In the post-LT group, two patients had diarrhea at the sampling time, which did not meet the inclusion criteria of this study, so their samples were not collected. All BA patients received orthotopic liver transplantation (OLT). After the operation, they were given immunosuppressive therapy with tacrolimus and methylprednisolone. In the first 5–7 days after LT, the patients received broad-spectrum antibiotics with cephalosporin to prevent infection. The clinical data of the enrolled individuals and demographic information are shown in **Tables 1**, **2**. The results of metagenomic sequencing and assembly data in each sample are listed in **Supplementary Table 2**.

Impact of LT on GM in BA Individuals

Principal coordinate analysis (PCoA) was performed to analyze GM composition structure between the three groups (pre-LT,

TABLE 1 | Demographic information and clinical data on patients with BA (pre-LT) and control individuals.

	BA (*n* = 16)	Control (*n* = 10)	*P*-value
Age, months, median (min, max)	7.8 (4.8, 21)	8 (5, 20)	>0.05
Gender			>0.05
Female, n (%)	10 (62.5%)	6 (60%)	
Male, n (%)	6 (37.5%)	4 (40%)	
BMI, kg/m^2, median (min, max)	16.94 (11.26, 21.33)	18.79 (15.85, 20.09)	> 0.05
Hepatic function, median (min, max)			
ALT, U/L	151.00 (21.00, 439.00)	14.85 (10.20, 20.10)	< 0.05
AST, U/L	211.90 (45.40, 724.30)	39.35 (27.20, 43.90)	< 0.05
ALP, U/L	610.5 (296.00, 1409.00)	236.20 (192.60, 317.00)	< 0.05
GGT, U/L	180.50 (26.00, 980.00)	11.30 (9.00, 16.30)	< 0.05
TBA, μmol/L	163.8 (57.00, 356.00)	1.30 (0.30, 4.70)	< 0.05
TBIL, μmol/L	278.50 (9.02, 898.13)	5.70 (2.50, 11.60)	< 0.05
Inflammatory cytokines (min, max)			
IL-2, pg/mL	73.63 (0.96, 262.02)	3.58 (0.96, 5.32)	< 0.05
IL-4, pg/mL	39.47 (3.60, 143.85)	2.22 (1.77, 11.09)	< 0.05
IL-6, pg/mL	70.87 (7.38, 233.53)	2.98 (1.23, 14.92)	< 0.05
IL-10, pg/mL	25.66 (2.56, 61.84)	0.86 (0.27, 4.28)	< 0.05
TNFα, pg/mL	31.55 (2.20, 117.86)	2.09 (1.11, 3.26)	< 0.05
IFN-r, pg/mL	85.38 (2.10, 341.05)	2.10 (2.09, 2.11)	< 0.05
PELD	19 (1, 39)	Not Applicable	–

BA, biliary atresia; LT, liver transplantation; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, γ-glutamyltransferase; TBA, total bile acids; TBIL, total bilirubin; IL-2, interleukin-2; IL-4, interleukin-4; IL-6, interleukin-6; IL-10, interleukin-10; TNFα, tumor necrosis factor α; IFN-γ, interferon-γ; PELD, Pediatric End-stage Liver Disease.

TABLE 2 | Characteristics of patients in the post-LT setting at the time of sample collection.

Variable	Value
Hepatic function, median (min, max)	
ALT, U/L	20.50 (11.00, 57.00)
AST, U/L	35.60 (23.00, 109.60)
ALP, U/L	266.50 (129.00, 510.00)
GGT, U/L	17.00 (10.00, 137.00)
TBA, μmol/L	9.24 (1.50, 20.30)
TBIL, μmol/L	8.45 (3.84, 18.54)
Inflammatory cytokines (min, max)	
IL-2, pg/mL	55.07 (17.84, 94.89)
IL-4, pg/mL	18.38 (6.02, 37.33)
IL-6, pg/mL	32.15 (7.93, 50.39)
IL-10, pg/mL	2.96 (1.09, 6.05)
TNFα, pg/mL	12.65 (6.85, 26.94)
IFN-r, pg/mL	15.84 (2.10, 72.22)
Sampling time (Since the last antibiotic use), days, median (min, max)	136 (125, 140)
Postoperative complications	
Infection	3
Portal thrombosis	1

BA, biliary atresia; LT, liver transplantation; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, γ-glutamyltransferase; TBA, total bile acids; TBIL, total bilirubin; IL-2, interleukin-2; IL-4, interleukin-4; IL-6, interleukin-6; IL-10, interleukin-10; TNFα, tumor necrosis factor α; IFN-γ, interferon-γ.

post-LT, and control group), which showed that the structure of the post-LT group was between the pre-LT group and the control group (**Figure 1A**). **Figure 1B** shows that the microbiome structure was similar between the post-LT and control group ($P > 0.05$). The Kruskal-Wallis test showed a statistically significant difference in the number of genes between the pre-LT and the control group, the pre-LT, and the post-LT group, but no statistical difference between the post-LT and the control group (**Figure 1C**). The ternary plot also indicated the GM composition differences between the three groups (**Figure 1D**). More species were seen in the control group than in the other two groups. Bacteroidetes and Firmicutes were enriched in the control group. Proteobacteria was predominant in the pre-LT group, and the post-LT group consisted of a mixture of control and pre-LT species. Taken together, LT significantly changed GM composition in patients with BA.

The Venn diagram demonstrates the shared and unique genera between the three groups (**Figure 2A**). Nine hundred and eighty-nine genera were shared among the three groups, while 83, 125, and 204 were unique in the control, pre-LT, and post-LT groups, respectively. Moreover, 1,129 genera were shared between the control and post-LT groups, and 1,022 genera were shared between the control and pre-LT groups. It also proved a shift in GM composition to the control group after LT surgery.

Next, LEfSe was performed to analyze the different species between the pre-LT and post-LT groups. It was shown that 26 and 54 genera were enriched in the pre-LT and post-LT groups, respectively. *Serratia*, *Enterobacter*, *Morganella*, *Skunalikevirus*, and *Phifllikevirus* were enriched in the pre-LT group, while *Roseburia*, *Blautia*, *Clostridium*, *Akkermansia*, *Ruminococcus*, and *Eubacterium* were enriched in the post-LT group (**Figure 2B** and **Supplementary Table 3**). Concerning functional profiles, the Wilcoxon test showed that M00004 (pentose phosphate pathway), M00007 (pentose phosphate pathway), M00019 (valine/isoleucine biosynthesis), M00165 (reductive pentose phosphate cycle), and M00376 (3-hydroxypropionate bi-cycle) were enriched in the pre-LT group. In contrast M00175 (nitrogen fixation), M00422 (acetyl-CoA pathway), and M00652 (vancomycin resistance) were enriched in the post-LT group (**Figure 3A** and **Supplementary Table 4**). An abundance clustering heat map of different modules shown in **Figure 3B**. In the pathways, lipid metabolism, amino acid metabolism, carbohydrate metabolism, energy metabolism, xenobiotics biodegradation and metabolism, and cell motility significantly increased after LT (**Supplementary Figure 1**). The contribution of GM to metabolic pathways indicated that *Serratia* spp., *Proteobacteria* bacterium CAG 139, *Enterobacter*, *Veillonella* sp. CAG 933, and *Streptococcus* spp. constituted in the pre-LT group (**Figure 4A**). These are all inflammatory pathogenic bacteria. The composition of GM and functional metabolism both improved after LT.

Difference in GM Between the Post-LT and Control Groups

Alpha diversity was represented by the Shannon–Wiener Index and Simpson Index, and beta diversity by PCoA; there was no significant difference between the two groups (**Figures 4B,C**).

LEfSe analysis was used to further identify different species between the two groups. At the genus level, there were 37 different species. *Enterobacteriaceae* bacterium 291 EBAC, *Oscillibacter*, *Klebsiella*, *Enterococcus*, *Epsilon15likevirus*, *Rouxiella*, *Bilophila*, and *Siphoviridae* were enriched in the post-LT group. In contrast, *Faecalibacterium*, *Blautia*, *Eubacterium*, *Clostridiales* bacterium VE202 03, and *Ruminococcus* were enriched in the control group (**Figure 5A** and **Supplementary Table 5**). There were 41 and 68 different species enriched in the post-LT and control group at the species level, respectively. *Shigella* sp. PAMC 28760, *Klebsiella* sp. KTE92, *Enterobacteriaceae* bacterium 291 EBAC, *Klebsiella pneumoniae*, Escherichia phage phiV10, *Klebsiella* sp. MS 92 3, *Klebsiella variicola*, *Klebsiella* sp. D5A, *Klebsiella* sp. KGM IMP216, *Klebsiella* sp. 4 1 44FAA, and Escherichia phage TL 2011b were enriched in the post-LT group, while *Faecalibacterium prausnitzii*, *uncultured Faecalibacterium* sp., *Roseburia* sp. CAG 100, *Bacteroides* sp. D2, butyrate-producing bacterium SS3 4, *Clostridiales* bacterium VE202 03, *Ruminococcus lactaris*, *Eubacterium eligens*, *Eubacterium eligens* CAG 72, and *Blautia* sp. CAG 37 were enriched in the control group (**Supplementary Figure 2** and **Supplementary Table 6**).

In functional metabolism, ABC transporters and Glycolysis/Gluconeogenesis were abundant in the post-LT group (**Supplementary Table 7**). LEfSe analysis showed that pathways related to lipopolysaccharide metabolism (M00064),

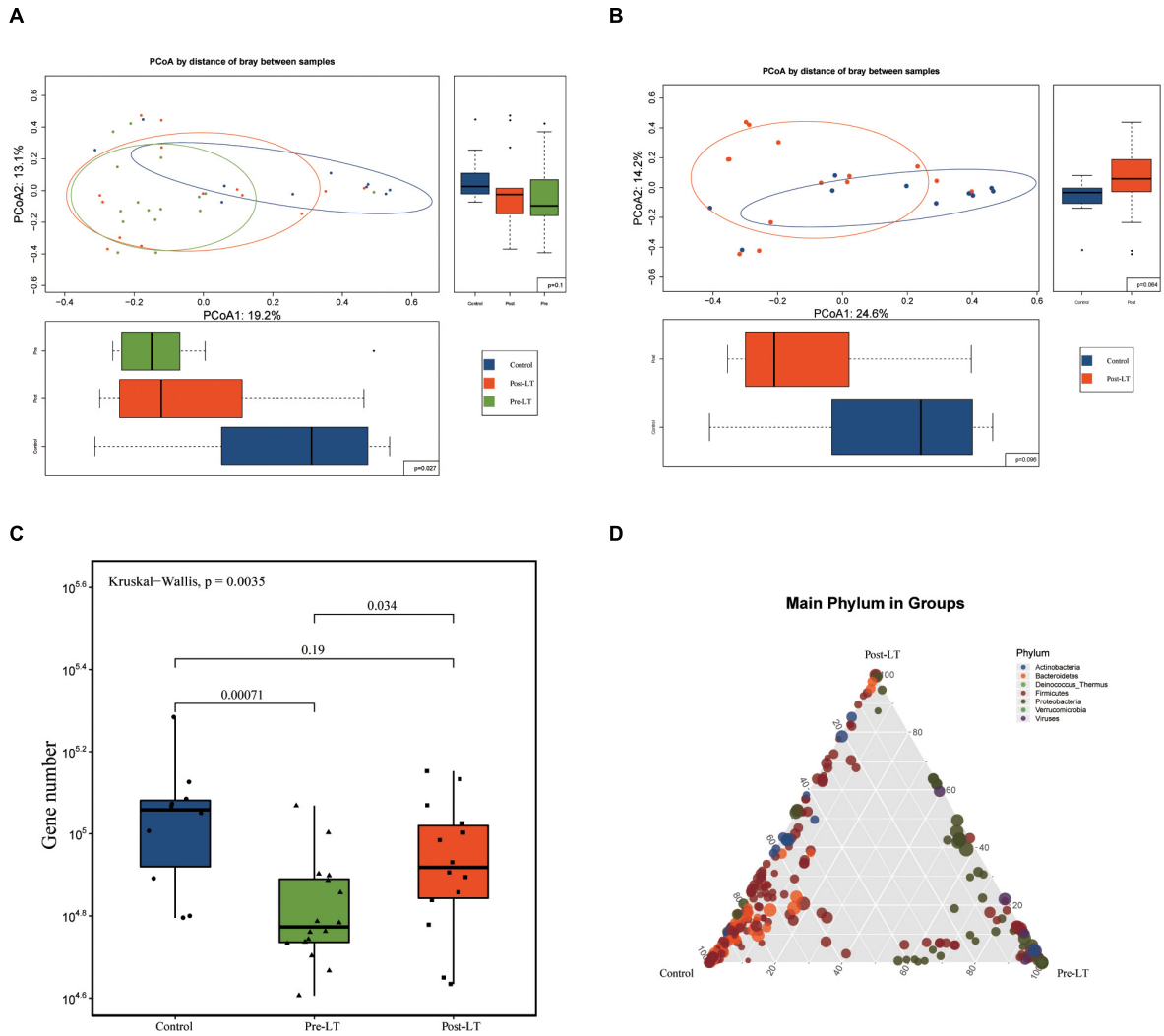

FIGURE 1 | (A) Structural changes in the gut microbiota in pre-LT, post-LT, and control individuals (principal coordinate analysis based on the Bray–Curtis distance of species abundance). **(B)** Structural changes in the gut microbiota in pre-LT and post-LT individuals (principal coordinate analysis based on the Bray–Curtis distance of species abundance). **(C)** Different gene numbers between the three groups (Wilcoxon rank-sum test). **(D)** Ternary plot of different species. The three vertices represent the three different groups, the points represent the different species at the species level, and the size of the points represent the average abundance in the three groups of samples. Points that are enriched in a certain color at a vertex indicate higher species abundance at the phylum level represented by the color in the grouping.

multidrug resistance (M00698), cysteine and methionine metabolism (M00034), polyamine biosynthesis (M00134), GABA biosynthesis (M00136), and EHEC/EPEC pathogenicity signature (M00542) were enriched in the post-LT group. In contrast, beta-lactam resistance (M00627), vancomycin resistance (M00652), methane metabolism (M00422), and lysine metabolism (M00527) were enriched in the control group (**Figure 5B**, and **Supplementary Table 8**). Fishtaco analysis indicated that the composition of the GM that drove the metabolic pathways was different between the two groups (**Figure 5C**). *Epsilon15likevirus, Shigella* sp. PAMC 28760, *Klebsiella* spp., *Escherichia* sp. 1 1 43, *Enterobacteriaceae* bacterium 291 EBAC, and *Bilophila wadsworthia* played major driving roles in the post-LT group. In contrast, *Roseburia* sp. CAG 100, *Roseburia hominis*, *Oscillibacter ruminantium*, *Lactobacillus mucosae*, *Flavonifractor* spp., and *Faecalibacterium prausnitzii* were the primary driving species in the control group. Thus, the significantly different species between the post-LT and control groups were investigated; however, the overall structure of the GM was similar. In addition, the functional pathway was different between the two groups.

Relationship Between GM and Clinical Indicators

The relationship between different species identified in the post-LT and control groups and clinical indicators were analyzed by Spearman rank correlation (**Figure 6A** and

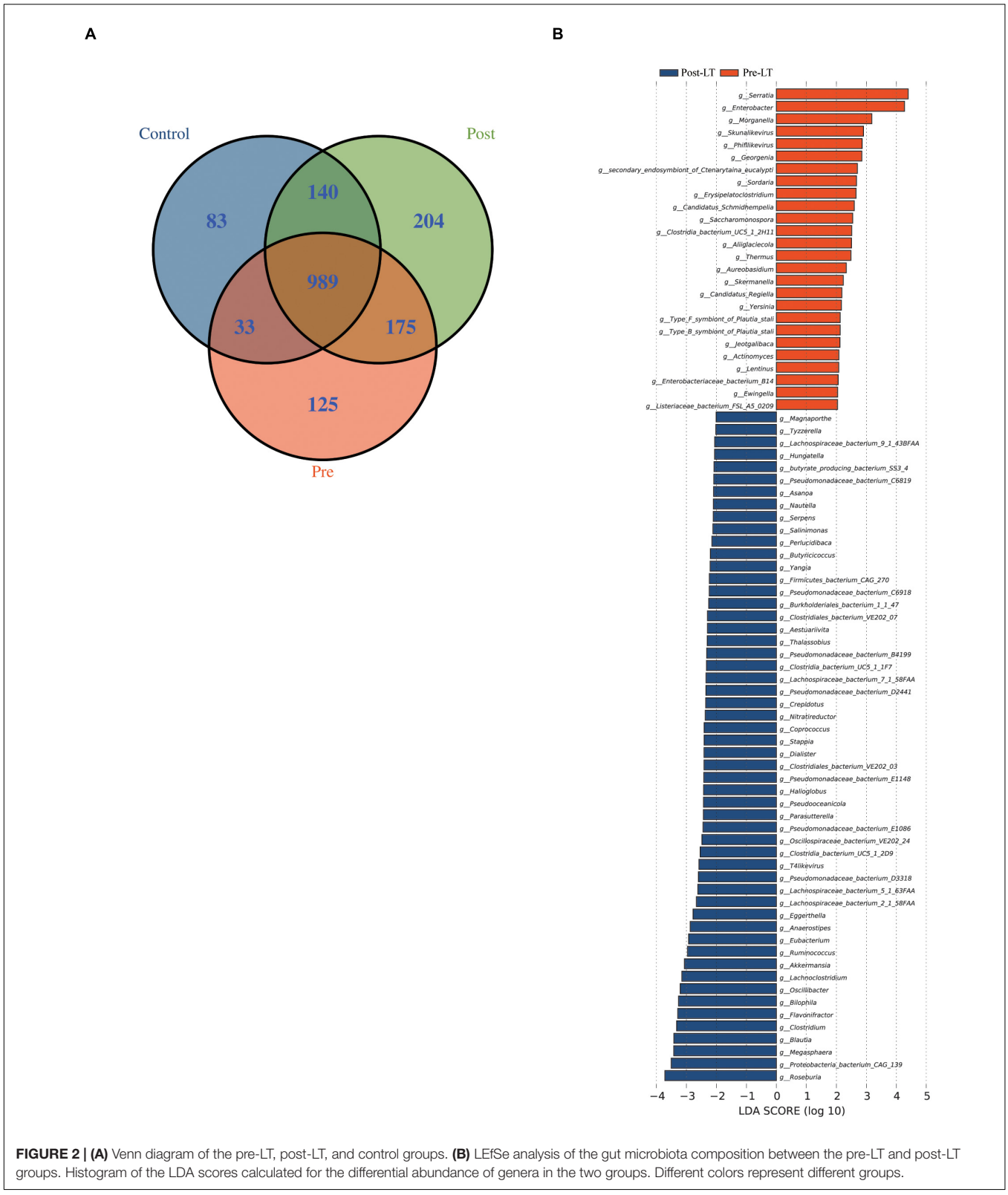

FIGURE 2 | (A) Venn diagram of the pre-LT, post-LT, and control groups. **(B)** LEfSe analysis of the gut microbiota composition between the pre-LT and post-LT groups. Histogram of the LDA scores calculated for the differential abundance of genera in the two groups. Different colors represent different groups.

Supplementary Table 9). It showed that *Erysipelatoclostridium* was positively correlated with ALT (P = 0.004, r = 0.71); *Eubacterium* sp. CAG 248 was negatively correlated with ALP (P = 0.004, r = −0.72); uncultured Faecalibacterium sp. was negatively correlated with GGT (P = 0.01, r = −0.65), while *Enterococcus* sp. GMD5E, *Enterococcus* sp. GMD3E,

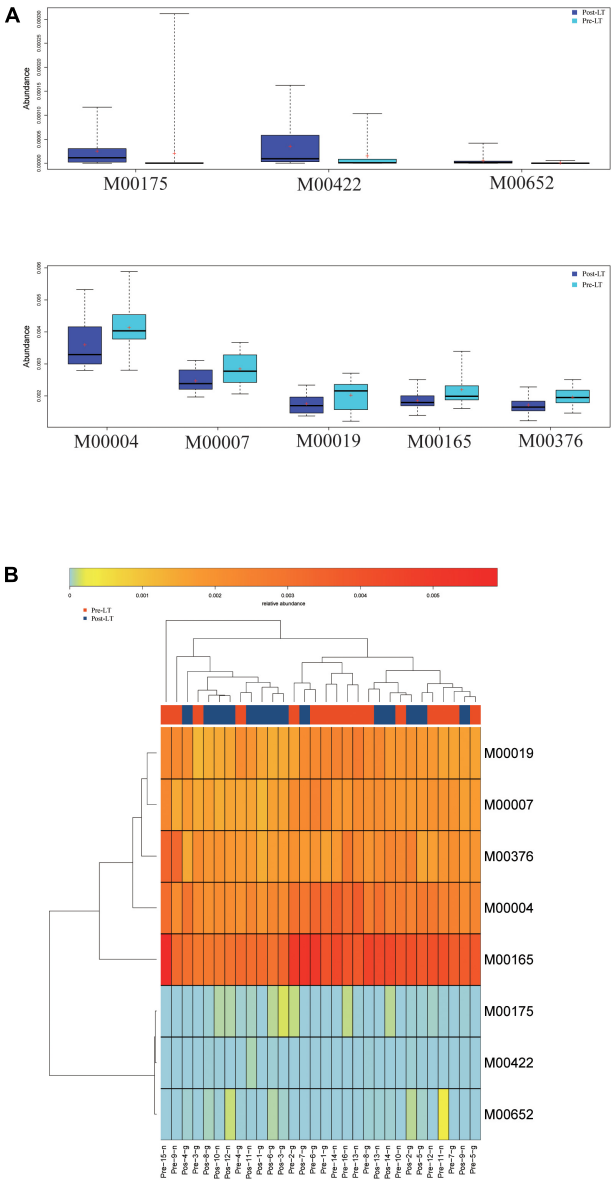

FIGURE 3 | (A) Functional profiles with significantly different abundances at the module level between the pre-LT and post-LT groups (Wilcoxon rank-sum test, $P < 0.05$). Different colors represent different groups. **(B)** Hierarchical clustering between differential modules and samples.

Enterococcus faecium, Escherichia phage TL 2011b, *Enterococcus* sp. GMD4E, and *Enterococcus* sp. GMD2E were all positively correlated with GGT ($P = 0.01$, $r = 0.64$; $P = 0.01$, $r = 0.66$; $P = 0.01$, $r = 0.66$; $P = 0.01$, $r = 0.65$; $P = 0.01$, $r = 0.66$; $P = 0.01$, $r = 0.66$, respectively); *Shigella* sp. PAMC 28760, *Citrobacter* sp. MGH100, and *Escherichia* sp. 1 1 43 were positively correlated with TBA ($P = 0.004$, $r = 0.73$; $P = 0.02$, $r = 0.61$; $P = 0.006$, $r = 0.71$, respectively), while uncultured Blautia sp., *Lachnospiraceae* bacterium TF01 11, *Eubacterium* sp. UNK MGS 26, *Ruminococcus bromii*, *Eubacterium ventriosum*, and *Eubacterium eligens* were negatively correlated with TBA ($P = 0.02$, $r = -0.6$; $P = 0.02$, $r = -0.61$; $P = 0.02$, $r = -0.63$; $P = 0.01$, $r = -0.67$; $P = 0.002$, $r = -0.76$; $P = 0.02$, $r = -0.65$, respectively). Taken together, liver functional indicators were positively correlated with species enriched in the post-LT group, while negatively correlated with the control group.

Effects of Kasai Procedure on Postoperative GM

According to whether patients had previously undergone the Kasai procedure, we categorized them into the with- and non-Kasai groups. In total, 8 and 6 children were categorized in the with-Kasai and non-Kasai groups. The results showed that GM overall structure and diversity were similar between the two groups. With regard to GM composition, the abundance of several species was different, *Lachnospiraceae* bacterium 5 1 63FAA, *Lachnospiraceae* bacterium CAG 25, *Firmicutes* bacterium CAG 270, *Firmicutes* bacterium CAG 227, *Weeksella*, *Moraxella*, and *Phaeodactylibacter* exhibited higher abundance in the with-Kasai group, while *Veillonella*, *Aerococcus*, and *Actinobacillus* did not (**Figure 6B** and **Supplementary Table 10**). In the functional profiles, cysteine and methionine metabolism (M00609) was enriched in the with-Kasai group, while lipopolysaccharide metabolism (M00064) was enriched in the non-Kasai group (**Figure 6C** and **Supplementary Table 11**). Taken together, these findings showed that a prior Kasai procedure had a certain effect on the GM after LT.

DISCUSSION

BA is the major cause of neonatal cholestasis, and LT is currently the standard treatment (Sun et al., 2013; Feldman and Mack, 2015; Kasahara et al., 2017). Previous studies have suggested that the GM was altered during LT in adults with liver disease and animal models (Wu et al., 2012; Bajaj et al., 2017; Sun et al., 2017; Tian et al., 2018). In addition, the characteristics of GM after LT in children with BA have not been reported. The present study aimed to analyze the characteristics of GM in children with BA during LT.

Compared with the GM in patients before LT, the abundance of SCFA-producing bacteria (e.g., *Roseburia*, *Blautia*, *Clostridium*, *Akkermansia*, *Ruminococcus*, and *Eubacterium*) increased after LT. However, it was still lower than that in the control group. It demonstrated that LT partially improved dysbacteriosis in children with BA. With regard to functional profiles, it found that *Serratia spp.*, *Proteobacteria* bacterium CAG 139, *Veillonella* sp. CAG 933, and *Streptococcus* spp. were predominant in pathogenic related metabolic pathways in the pre-LT group. After LT, the driving effect of these bacteria on metabolic pathways was reduced. Besides, lipid metabolism, amino acid metabolism, carbohydrate metabolism, energy metabolism, xenobiotics biodegradation and metabolism, and cell motility increased in the post-LT group. Therefore, functional metabolism also improved after LT. Among the contributing microorganisms, *Streptococcus* spp. and *Veillonella* spp. have been reported to be enriched in the intestinal tract and biliary tract in immune liver diseases (Wei et al., 2019;

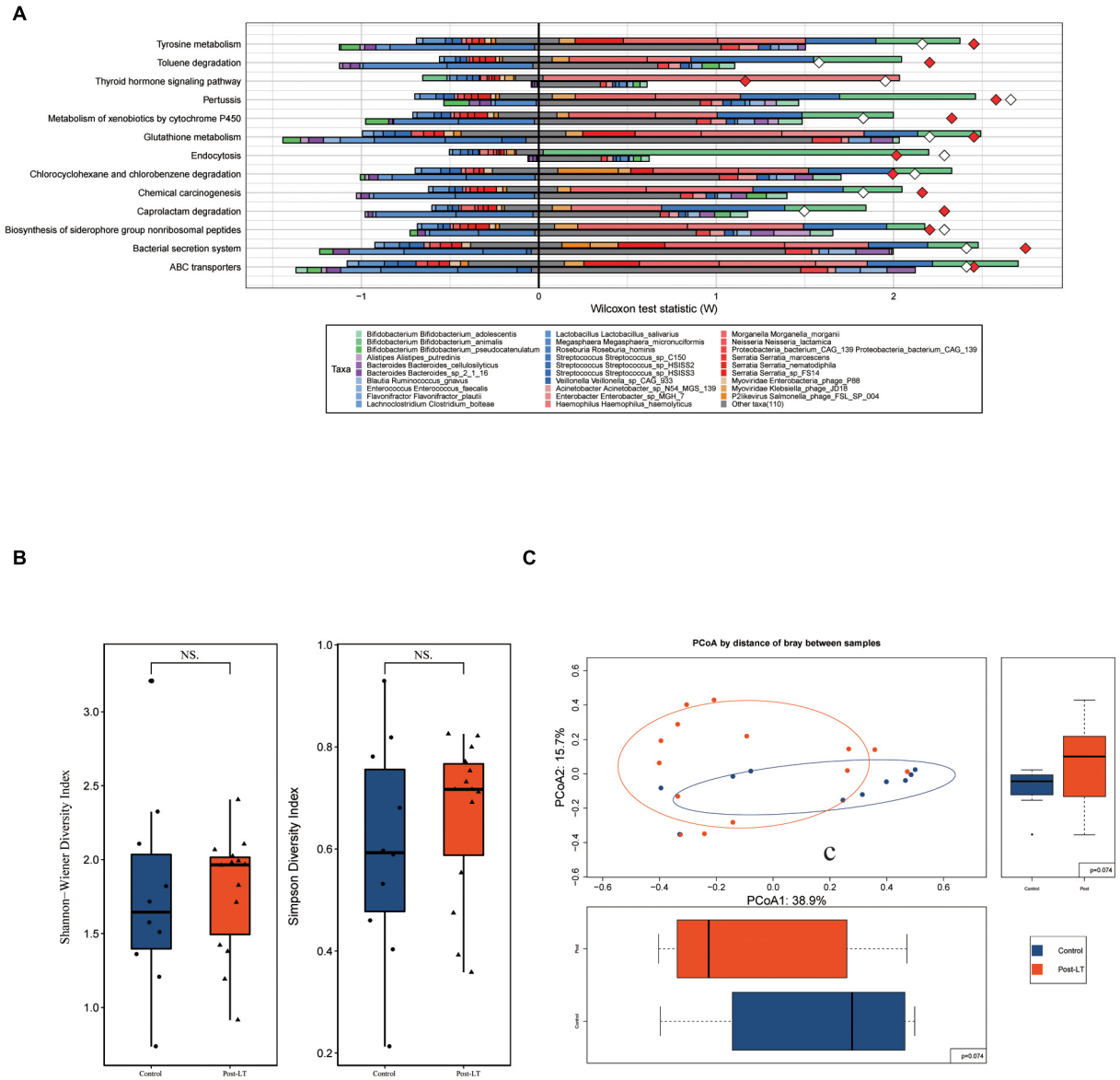

FIGURE 4 | (A) Fishtaco analysis of species contribution to metabolic pathways between the pre-LT and post-LT groups. The *x*-axis represents the Wilcoxon test statistic scores, and the *y*-axis the related functions. The driving factors for each differential function transformation were divided into four parts, represented by a histogram in two directions. Gut microbiota in the pre-LT group that drove the increase in the corresponding functional abundance (top right); Gut microbiota in the pre-LT group that inhibited the increase in the corresponding functional abundance (top left); Gut microbiota in the post-LT group that drove the increase in the corresponding functional abundance (bottom right); and gut microbiota in the post-LT group that inhibited the increase in the corresponding functional abundance (bottom left). Different color bars represent the corresponding species. The longer the bar, the greater the driving or inhibiting effect of the species on the corresponding function. **(B)** Alpha diversity between the post-LT and control groups (box plot of richness, Shannon–Wiener Index, and Simpson Index). **(C)** Beta diversity between the post-LT and control groups (principal coordinate analysis based on the Bray–Curtis distance of species abundance).

Abe et al., 2020; Liwinski et al., 2020), which are associated with the etiology of autoimmune liver disease and damage to the epithelial barrier (Kugathasan et al., 2017); thus, LT can reduce the occurrence of epithelial inflammation from the perspective of the gut flora. On the other hand, selecting appropriate antibiotics for these bacteria for clinical treatment is useful for the intestinal epithelial barrier's integrity and resisting inflammation.

Bile acids are critically important for maintaining a healthy gut microbiota. It interacts closely with the intestinal microbiota through the gut-liver axis. After LT, bile drainage into the intestine can reduce the pH of the intestinal environment, inhibit the growth of pathogenic bacteria, maintain the balance of intestinal microorganisms, and affect the composition of GM. Consistent with this, we observed that there was no statistical difference in either α or β diversity between the post-LT and

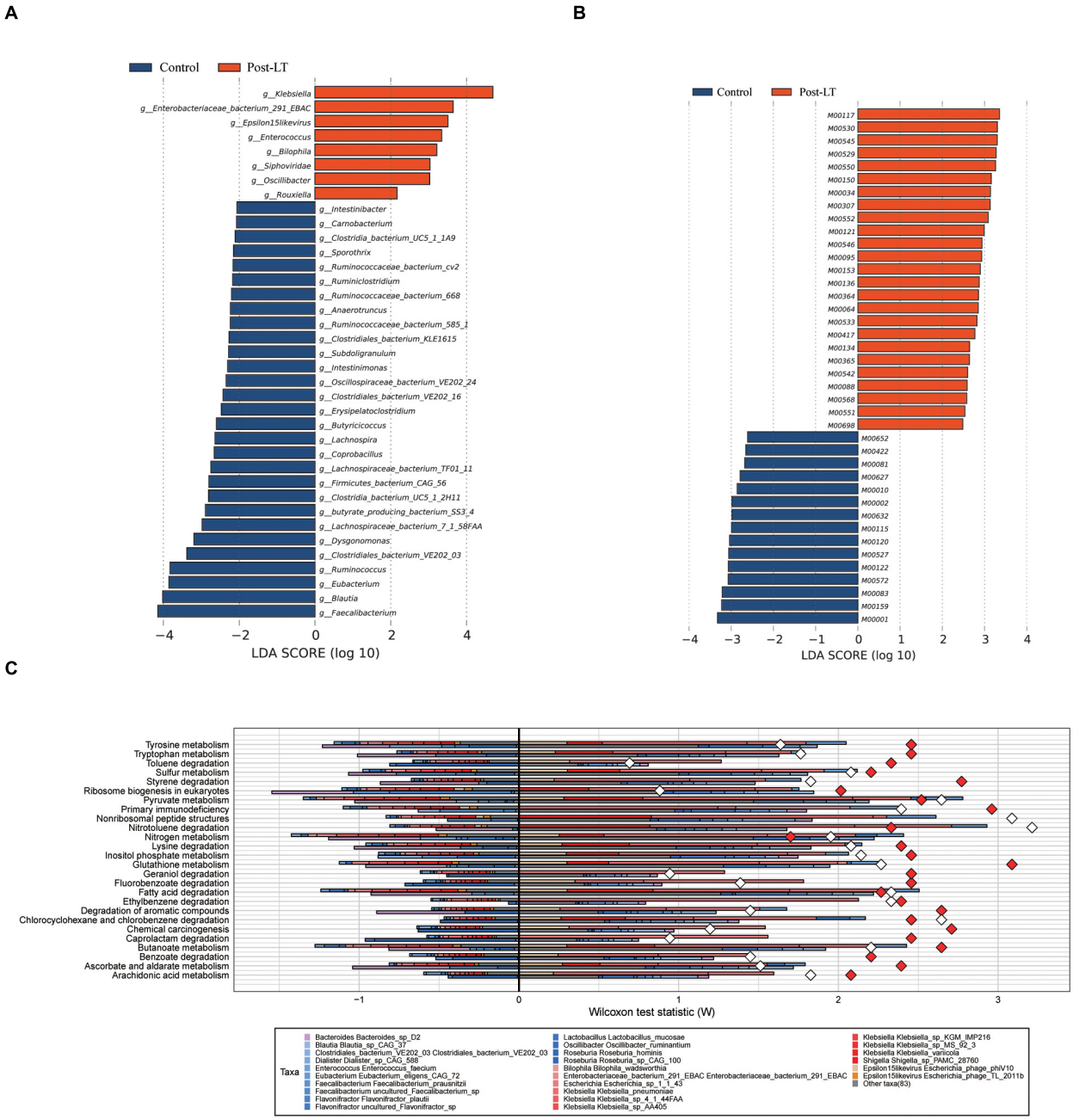

FIGURE 5 | (A) LEfSe analysis of gut microbiota composition between post-LT and control groups. **(B)** LEfSe analysis of functional profiles between the two groups. Histogram of the LDA scores calculated for a differential abundance of genera in the two groups. Different colors represent different groups. **(C)** Fishtaco analysis of species contribution to metabolic pathways between the post-LT and control groups. The *x*-axis represents the Wilcoxon test statistic scores, and the *y*-axis the related functions. The driving factors for each differential function transformation were divided into four parts, represented by a histogram in two directions. Gut microbiota in the post-LT group that drove the increase in the corresponding functional abundance (top right); Gut microbiota in the post-LT group that inhibited the increase in the corresponding functional abundance (top left); Gut microbiota in the control group that drove the increase in the corresponding functional abundance (bottom right); and gut microbiota in the control group that inhibited the increase in the corresponding functional abundance (bottom left). Different color bars represent the corresponding species. The longer the bar, the greater the driving or inhibiting effect of the species on the corresponding function.

control groups, which indicated that the composition of GM between the two groups was similar. Besides, to investigate whether the improvement in GM post-transplant is related to replacing the diseased liver or simply due to the establishment of bile flow to the intestine, we also compared the GM between the patients who gained successful bile flow (successful Kasai) and others were not (failed Kasai). We found that the abundances of 30 and 192 species were significantly increased after Kasai and LT

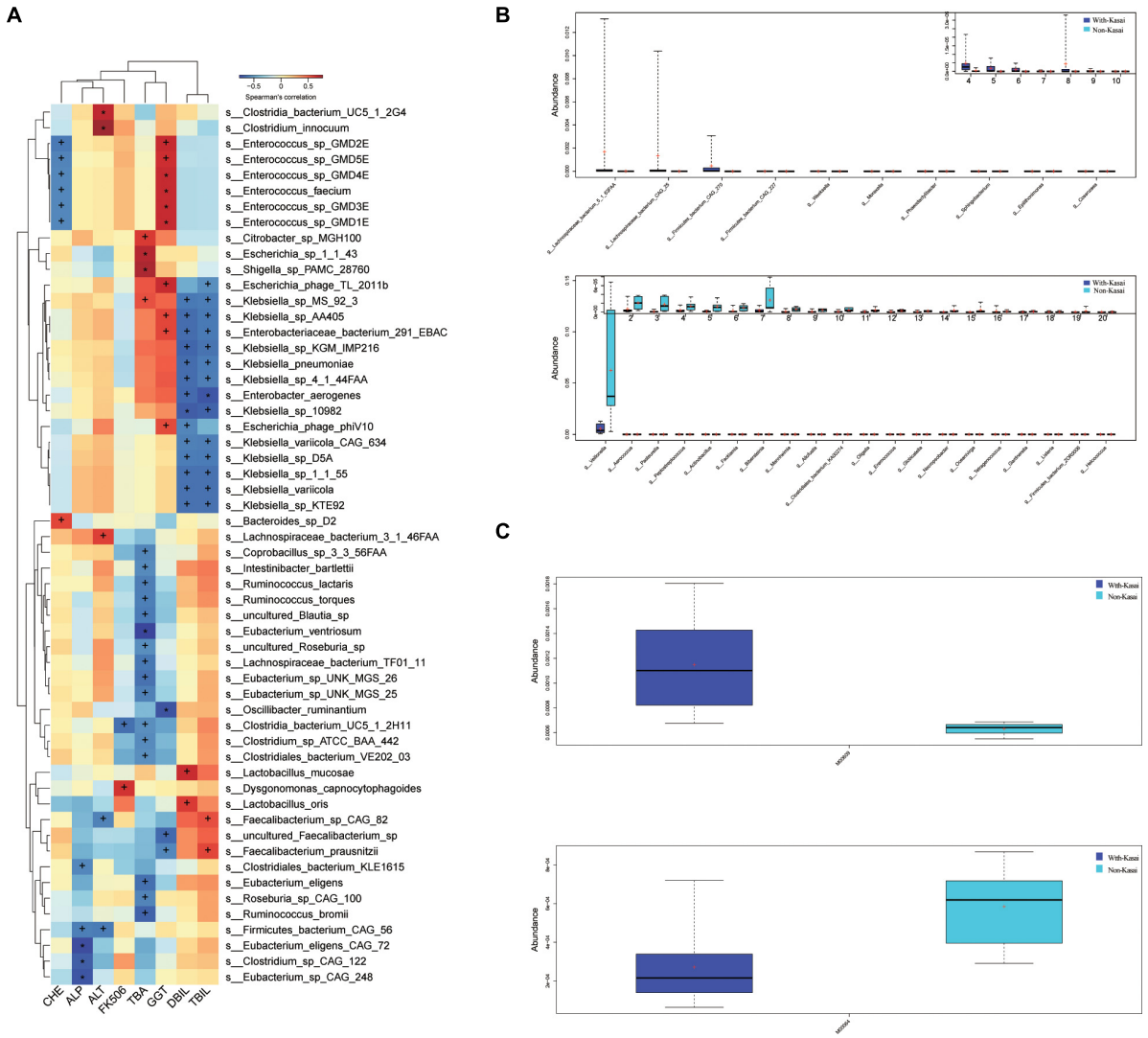

FIGURE 6 | (A) Spearman correlations between species and clinical indices. The *x*-axis represents the environmental factors, and the *y*-axis the species. The depth of the color indicates the correlation between species and environmental factors ($^{+}P < 0.05$, $^{*}P < 0.01$). Blue denotes a negative correlation and red a positive correlation. **(B,C)** The effect of prior Kasai surgery on the gut microbiota after liver transplantation. **(B)** Species with significantly different abundances at the genus level in the with-Kasai and non-Kasai individuals (Wilcoxon rank-sum test, $P < 0.05$). The upper panel shows the abundance ranking of species in the with-Kasai group, and the lower panel the abundance ranking of species in the non-Kasai group. The *x*-axis represents the different taxa, and the *y*-axis the abundance of taxa. Different colors represent different groups. Lines inside the boxes indicate medians, " + " indicated means. **(C)** Functional profiles with significantly different abundances at the module level between the two groups (Wilcoxon rank-sum test, $P < 0.05$). Different colors represent different groups.

($P < 0.05$, **Supplementary Table 13**), respectively. Of the species that increased after Kasai, five species also increased significantly after LT (*Acidaminococcus*, *Catabacter*, *Clostridiales* bacterium VE202 03, *Erysipelatoclostridium*, and *Flavonifractor*), and 21 species increased but did not reach statistical significance. These results suggested that the change of GM after LT was greater than that after Kasai. Moreover, most of the changes in GM after Kasai also occurred after LT. We believed that the changes in GM after LT were not caused by bile acid drainage alone. In the post-LT group, Proteobacteria was the dominant phylum, which contains many known human pathogens, such as Enterobacteriaceae, which are thought to be markers of inflammation, epithelial dysfunction, and disease (Litvak et al., 2017; Liwinski et al., 2020). In addition, *Klebsiella*, *Enterobacteriaceae* bacterium 291 EBAC, *Enterococcus*, and *Bilophila* were enriched in the post-LT group. *Bilophila* is a sulfate-reducing bacterium whose abundance leads to excessive hydrogen sulfide production in the gut, stimulating an inflammatory response (Ye et al., 2018). *Enterococcus* and *Klebsiella* have been detected in various liver diseases (Ray, 2017; Fukui, 2019; Liu et al., 2019; Nakamoto et al., 2019) and were associated with a predisposition to penetrating complications in pediatric patients (Steck et al., 2011). *Enterobacteriaceae* bacterium 291 EBAC is a member of Enterobacteriaceae, also associated with epithelial inflammation and functional disorders.

The number of SCFA-producing bacteria with anti-inflammatory effects was lower than that in the control group (Riviere et al., 2016). In early period of post-LT, recipients are at increased risk for developing infections. The use of antibiotic can deplete gut commensal bacteria, resulting in intestinal dysbiosis. Besides, immunosuppressive drugs may have caused the difference between the post-LT and control groups, and may affect the postoperative recovery of patients and increase the probability of complications. Additionally, Jiang et al. (2018) indicated that an optimal dosage of FK506 induced immunosuppression, normal graft function and stable gut microbiota following LT in rats. Therefore, regular detection of FK506 concentration is of positive significance for the homeostasis of intestinal flora. In addition, taking appropriate antibiotics or butyric acid may positively affect prognosis.

With regard to the functional profiles, ABC transporters and Glycolysis/Gluconeogenesis were abundant in the post-LT group. ABC transporters are an important cell-protective mechanism, which remove toxins and drugs from cells, and their enhanced metabolism may indicate intensive antibacterial ability in the intestine (Wang et al., 2019a; Ahmad et al., 2020). The metabolism of Glycolysis/Gluconeogenesis needs an oxygen-free environment, and its augmentation suggests that the anaerobic metabolism of sugar was active after LT (Wang et al., 2019b). Pathogen-related metabolism such as lipopolysaccharide metabolism and EHEC/EPEC pathogenicity signature were also abundant in the post-LT group. Lipopolysaccharide, also known as endotoxin, is the product of gram-negative bacteria, which can bind to TLR4 to initiate the inflammatory response (Cochet and Peri, 2017; Del Chierico et al., 2018). These results were in line with the bacterial composition in the post-LT group. Taken together, we believed that the changed functional profiles after LT are related to the different intestinal enrichment. Besides, GM are likely to affect the gut environment of hosts through itself composition and corresponding metabolism patterns and influence the development of disease.

Kasai operation could effectively delay the requirement of LT. Moreover, Li et al. (2019) had demonstrated that there was no significant difference in post-LT survival rate between the patients who had a prior Kasai and not. However, Urahashi et al. (2013) investigated that repeat Kasai might have a negative effect on patients who undergo LT for BA patients with potential lethal complications such as bowel perforation. Neto et al. (2015) also believed that a previous Kasai can increase post-LT complications as biliary complications and bowel perforations. Taken together, several studies have suggested that patients with a previous Kasai are at increased risk for bowel perforations, postoperative infection, and biliary complications from LT. Infection is a fatal complication after LT. Enterogenic infection represents a major complication in LT recipients (Zheyu and Lunan, 2006; Yuan et al., 2016; Mu et al., 2019); Therefore, we divided the post-LT group into two groups based on whether they had previously undergone the Kasai procedure to investigate the effect of Kasai surgery on the GM after LT. The results showed that some species and functional pathways were different between the two groups; however, whether this difference affects clinical outcomes deserves further study.

GM has been shown to play a unique role in the diversity of bile acids in bile acid pools by bile salt hydrolase and bile acid-inducible enzymes (Long et al., 2017; Ramirez-Perez et al., 2017; Mullish et al., 2018). *Eubacterium* and *Ruminococcus* have been proved to contain bile acid conversion enzymes (Jia et al., 2018); we found *Eubacterium* sp. UNK MGS 26, *Ruminococcus bromii*, *Eubacterium ventriosum*, and *Eubacterium eligens* associated with blood bile acids level according to Spearman analysis. These bacteria belong to *Eubacterium* and *Ruminococcus*, so they may also have the ability to convert bile acid.

In conclusion, we confirmed that LT could significantly improve the GM in children with BA and described the characteristics of GM after LT. It provides new insight into understanding the role of LT in BA.

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: NCBI SRA (https://www.ncbi.nlm.nih.gov/bioprojectPRJNA730640), it can be accessed with the BioProject identifier PRJNA730640.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Beijing Friendship Hospital, Capital Medical University (Approval ID: 2019-P2–131-02). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

WS: study design, data collection, analysis and interpretation of the data, and writing of the report. L-YS: study design, study supervision, and critical revision of the manuscript for important intellectual content. Z-JZ, LW, WQ, and Z-GZ: clinical treatment assistance. Y-SY: critical revision of the manuscript for important intellectual content. All authors read and approved the final manuscript to be submitted.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.704313/full#supplementary-material

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METHODS
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Amadis: A Comprehensive Database for Association Between Microbiota and Disease

Long Li[1,2†], Qingxu Jing[1,2†], Sen Yan[3], Xuxu Liu[1,2], Yuanyuan Sun[1,2], Defu Zhu[4], Dawei Wang[1,2], Chenjun Hao[1,2] and Dongbo Xue[1,2]*

[1] Department of General Surgery, The First Affiliated Hospital of Harbin Medical University, Harbin, China, [2] Key Laboratory of Hepatosplenic Surgery, Ministry of Education, The First Affiliated Hospital of Harbin Medical University, Harbin, China, [3] Department of Cardiology, The First Affiliated Hospital of Harbin Medical University, Harbin, China, [4] Family Medicine General Practice Clinic, The University of Hong Kong-Shenzhen Hospital, Shenzhen, China

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Edited by:
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***Correspondence:**
Dongbo Xue
xuedongbo@hrbmu.edu.cn

[†] These authors have contributed equally to this work and share first authorship

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The human gastrointestinal tract represents a symbiotic bioreactor that can mediate the interaction of the human host. The deployment and integration of multi-omics technologies have depicted a more complete image of the functions performed by microbial organisms. In addition, a large amount of data has been generated in a short time. However, researchers struggling to keep track of these mountains of information need a way to conveniently gain a comprehensive understanding of the relationship between microbiota and human diseases. To tackle this issue, we developed Amadis (http://gift2disease.net/GIFTED), a manually curated database that provides experimentally supported microbiota-disease associations and a dynamic network construction method. The current version of the Amadis database documents 20167 associations between 221 human diseases and 774 gut microbes across 17 species, curated from more than 1000 articles. By using the curated data, users can freely select and combine modules to obtain a specific microbe-based human disease network. Additionally, Amadis provides a user-friendly interface for browsing, searching and downloading. We hope it can serve as a useful and valuable resource for researchers exploring the associations between gastrointestinal microbiota and human diseases.

Keywords: gut microbiota, human diseases, network, database, bioinformatics

KEY POINTS

- Manually curated bioactive associations between gut microbiota and diseases are provided.
- Amadis provides custom network diagram construction function.
- Facilitation of exploring the bacterial community interactions during diseases is available.
- Facilitation of exploring the mechanism of gut microbes–diseases association from the "microbiota-gut-organ" axis perspectives is provided.
- Amadis provides a user-friendly website for browsing, searching and downloading the results.

INTRODUCTION

The human gut microbiome is a complex ecosystem (Weersma et al., 2020). Shaped by long-term coevolution, host-microbiota associations have been cultivated into mutually beneficial relationships. The microbiota provides hosts with genetic, transcriptomic and metabolic attributes that manipulate host biology in both beneficial and harmful ways. Over the past decades, it has been illustrated that microbial communities are important for human and environmental health. Members of the gut microbiota interact with their hosts, establishing advantageous relationships and influencing health throughout the life of the host.

Overwhelming evidence from sequence-based studies indicates that variations and changes in the composition of the gut microbiota influence normal human physiology and contribute to diseases ranging from Alzheimer's disease to diabetes mellitus. To gain a more comprehensive image of the functions performed by complex microbial communities (Lloyd-Price et al., 2019), it has become prevalent to deploy and integrate multi-omics (metatranscriptomics, metaproteomics, and metabolomics) technologies to reveal essential messages and the secrets hidden behind them (Garrett, 2020). Information gained by combining these different -omics measurements is leading to improved diagnostics, automated drug discoveries, optimized culture conditions for uncharacterized microbes and a better understanding of the functional processes occurring in the system (Jansson and Baker, 2016).

As a result, it has been experimentally validated that the gut microbiota is associated with a wide range of human diseases and communicates with distant organs, such as the lung, heart, liver, and kidney. The concept of the "microbiota-gut-organ" axis has emerged from a recent discovery and has risen as a major topic of research interest in biology. For example, clinicians are well aware of the benefit of rifaximin and rifaximin plus probiotics in the treatment of hepatic encephalopathy (Zuo et al., 2017). Alzheimer's disease (Paley et al., 2018), Parkinson's disease (Lai et al., 2018) and refractory epilepsy (Olson et al., 2018) are associated with the relative abundance of various bacterial divisions. The gut microbiota communicates with the CNS through neural (Li et al., 2018), endocrine (Benedek et al., 2017) and immune pathways (Amini-Khoei et al., 2019) and thereby influences brain function and behavior. Furthermore, modulation of the gut microbiota may be a tractable strategy for developing novel therapeutics for multiple human diseases. In 2013, Elsvan Nood et al. studied the effect of duodenal infusion of donor feces in patients with recurrent *Clostridioides difficile* infection (van Nood et al., 2013). Fecal microbiota transplantation (FMT) has been believed to be a reliable rescue treatment for recurrent *Clostridioides difficile* infection (R-CDI) with an ∼90% success rate (Poyet et al., 2019). In addition to FMT, the structure and composition of microbial communities could also be reprogrammed by medicines (Shi et al., 2018), probiotics (Qu et al., 2018), bacteriophages (Dong et al., 2020) or other means. With accumulating investigational trials and experiments in new areas, engineering the gut microbiota to treat disease is an emerging concept.

Currently, a robust collection of multi-omics data about gastrointestinal microbiota-disease associations have been generated in a short amount of time due to the rapid development of this field. However, these associations are scattered among many published studies. The large number of articles is troublesome for researchers who want to further explore the relationships between microbiota and diseases from a global view. More recently, researchers have endeavored to develop online repositories for storing and managing microbiota genomes, disease-related gene and protein signatures, metabolite signatures of diseases, and even providing analysis flows, such as the NCBI Taxonomy database (Federhen, 2012), GeneSigDB (Culhane et al., 2012), MetSigDis (Cheng et al., 2019), gutMDisorder (Cheng et al., 2020), and MEtaGenome Atlas (gutMEGA) (Zhang et al., 2021). Given that gut microbiota are in a complex symbiotic environment, only demonstrating relationships focusing on a single species or one disease has limited significance in revealing the role of intestinal microbiota in physiological and pathological processes. Larger datasets and comprehensive analysis functions will better help researchers explore the relationship between microbiota and human diseases. Showing the dysbiosis in various disease conditions is helpful to reveal the complex, interactive nature of trace microbiota changes in CNS diseases. Studying the pathogenicity mechanisms of a certain species of bacteria in a variety of diseases may identify new therapeutic targets. To date, this kind of analysis function has not been provided.

Therefore, we developed Amadis, a manually curated database that documents experimentally supported microbiota-disease associations and provides functional network visualization and topological analysis. The database is freely available at http://www.gift2disease.net/GIFTED. The current version of Amadis documents more than 20,000 associations between human diseases and gut microbes, curated from more than 1,000 articles. Terms of diseases and microbiota were organized according to International Classification of Diseases (ICD-10) (Hirsch et al., 2015), United States National Center for Biotechnology Information (NCBI) Taxonomy and Medical Subject Heading disease categories (MeSH). The microbiota-disease relationships are sorted by the way microorganisms interact with each other and with their hosts, such as the kinds of toxins produced by the microbes (Cougnoux et al., 2016), the microbiota's effects on host immune cells and their functional states (Feng et al., 2018) and the types of molecular modification (Armand et al., 2019) caused by dysbiosis of gastrointestinal microbiota in host cells. Additionally, we constructed network diagrams to depict the role of the microbiota in important microbiota-gut-organ axis. Amadis also provides a custom network diagram construction function. Users can select information about diseases, bacteria, genes, etc., of interest to build a relationship network. We expect Amadis to serve as a useful and valuable resource for researchers who seek to understand the functions and molecular mechanisms of GI microbiota involved in human diseases.

MATERIALS AND METHODS

Data Collection

The aim of the Amadis database is to provide comprehensive information about experimentally validated associations between gastrointestinal microbiota and human diseases. To achieve this, we obtained detailed information about microbiota-diseases relationships from published research articles. To ensure high-quality data collection, all Amadis entries about gut microbiota and disease were manually extracted from publications in a specific and precise manner as used for HMDD (Huang et al., 2019), Lnc2Cancer (Ning et al., 2016) and NSDNA (Wang et al., 2017). Up to April 2020, more than 4,000 papers potentially related to gut microbiota and human disease were selected by retrieving the PubMed database with a list of keywords. These keywords may be divided into two categories: (1) names of diseases and microbiota, such as Crohn's disease, non-alcoholic fatty liver disease, obesity, *Escherichia coli*, and *Akkermansia*; and (2) terms about pathogenic mechanisms, such as genotoxins, inflammatory responses and monocyte differentiation. Then, we retrieved the disease and microbiota names, intervention factors, experimental techniques (e.g., microarray, next-generation sequencing, gas chromatography-mass spectrometry), species of experimental animals, experimental samples (cell line, tissue, blood, and feces), expression patterns of dysregulated genes (upregulated and downregulated), dysbiosis of microbiota, hyperlinks to the PubMed database (PMID, publication year, title) and a brief functional description of microbiota from the original studies. Finally, all microbiota-disease associations and entries mentioned above were double-checked by different researchers.

Nomenclature Standardization and Classification

The current version of Amadis documents massive disease-microbiota data. Diverse descriptions of the microbiota and disease are used in research articles, thus we standardized these names. To better display data and facilitate accessibility of the database, we organized microbiota terminologies based upon the controlled vocabulary of the MeSH disease categories. Moreover, we integrated the raw taxa at the lowest classification level and obtained their high-level names from the NCBI Taxonomy. Then, we used several disease terminology systems, such as disease ontology (DO)[1], UMLS[2], and ICD-10[3], to describe diseases found to be related to microbiota. Diseases were sorted by 8 systems and 23 organs according to ICD-10. Moreover, since studies provide insight into the mechanisms contributing to microbiota-disease relationships, we classified entries by the mechanism, such as the kinds of toxins, the microbiota's effects on functional state of immune cells and the types of molecular modifications caused by dysbiosis of GI microbiota in host cells.

[1]http://disease-ontology.org

[2]http://umlsks.nlm.nih.gov

[3]http://www.cdc.gov/nchs

Network Development

With the data curated in Amadis, a network can be constructed to visualize the global relationships between diseases, gut microbiota and genes. We integrated the ECharts plugin software (V4.0)[4] into the Amadis framework to perform network visualization. For inputted diseases, microbiota or genes, Amadis will determine their neighbors and construct a biological network on the web page. In the network, diseases, gut microbiota, and genes are shown as different color nodes. According to the gene regulation pattern, Amadis will also construct different subnetworks in which upregulation is illustrated as red links and downregulation is illustrated as blue links. Amadis will perform a new search by clicking on each node in the network.

Database Framework and Web Interface

A user-friendly web interface was developed to present Amadis. All data were organized and managed by the MySQL data server. The web interface for browsing and searching was implemented by Java Server Pages (JSP). Apache Tomcat software (v7.0) was used for the http server.

RESULTS

Database Content

Amadis offers detailed information about each entry, such as the names of the microbiota and disease, the up-level names of microbiota, the target gene involved, the interventional factor, the evidence of the relationship, the simple type, the specific mechanisms, the change in microbiota compared with healthy controls, the metabolite type, the methods used in the studies, a summary of conclusions and the PMID of the corresponding literature reference. Since 16S rRNA sequencing provides genus-level information and metagenomic approaches can provide species-level information, Amadis integrates seven classification levels of information (**Figure 1A**), distributed in phylum (17.1%), class (1.9%), order (1.8%), family (9.3%), genus (43.1%), and species (26.7%). The genus and species levels occupy most of the data. Exploration of disease data indicated the significant effects of microbiota (**Figure 1B**). As shown in **Figure 1C**, compared with healthy donors, gut microbiota abundances in patients with diabetes mellitus changed dramatically, since it was associated with the greatest number of microbes at the genus level. In the data with experimental evidence, *Akkermansia muciniphila* arrested the progression of 13 diseases, showing potential benefits as a probiotic (**Figure 1D**). Among the data at the species level recorded by Amadis, most microbiota only changed in one disease (**Supplementary Figure 1**). These microbiota may therefore be used as biomarkers in the data with experimental evidence.

Network Visualization and Topological Analysis

To allow users to easily explore the relationship between gut microbiota and diseases on their own, we tried to provide

[4]http://echarts.apache.org/

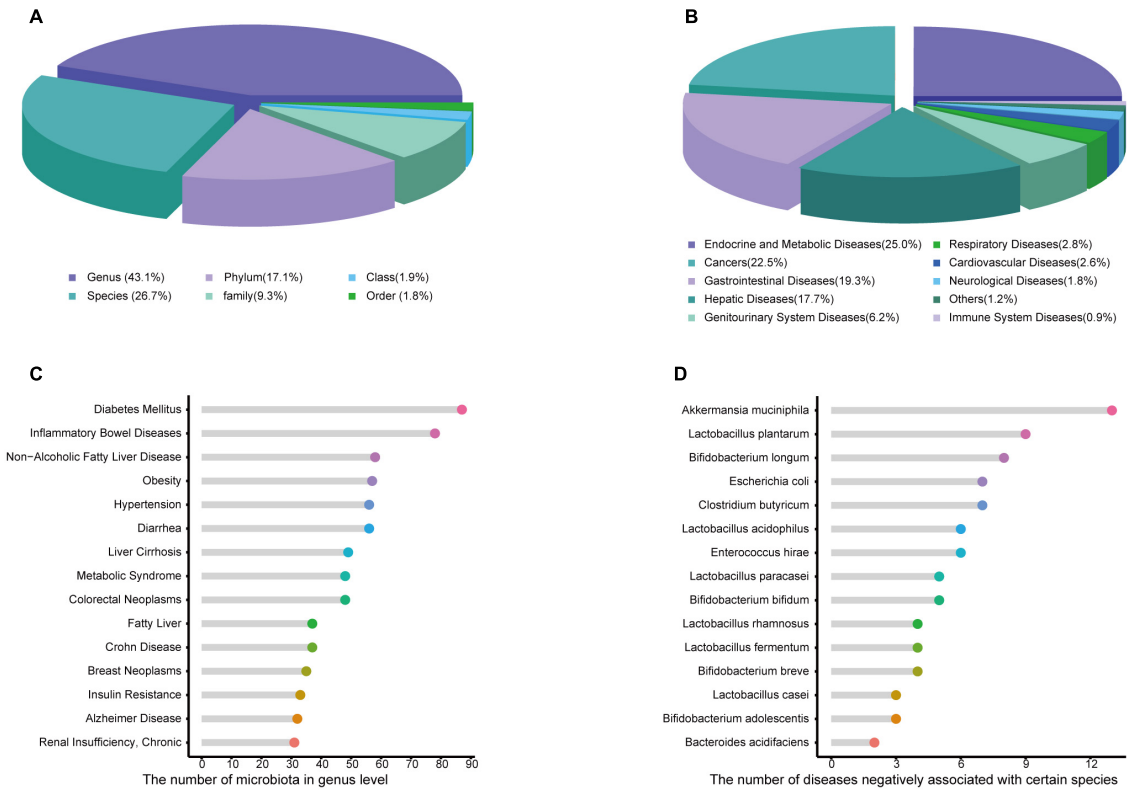

FIGURE 1 | (A) The distribution of gut microbiota in Human among different taxa. **(B)** Distribution of relationships based on diseases. **(C)** The number of microbiota related to single disease in genus level. **(D)** The number of diseases negatively associated with a single microbiota in species level.

the function of building a network diagram independently on the network page. To achieve this, we developed a network visualization interface using ECharts plugin software, where the global three-membered network and direct interactions of a certain microbiota, gene, or disease can be viewed (see Help page of Amadis for details). Users can select a variety of bacteria, diseases, and gene names included in the database and build a relationship network diagram based on the information provided in the database.

At present, it is accepted that a complex bidirectional communication system exists between the gastrointestinal tract and remote organ diseases. The term "microbiota-gut-organ axis" demonstrates the pivotal role of gut microbiota in maintaining local and systemic homeostasis. To better demonstrate the effect of the intestinal microbiota on these diseases and to discover "hub-microbiota" that may play a central role in diseases, we used the data obtained to construct comprehensive networks of bacteria, genetic information and all diseases in a single axis, such as the microbiota-gut-brain axis and the microbiota-gut-liver axis.

Database Utility

Homepage

Amadis provides a user-friendly web interface for an easy database query (**Figure 2**). To explore the Amadis database content rapidly, we have provided a quick search option on the home page of Amadis. Meanwhile, a comprehensive network analysis function was also provided. Users can obtain as many as 11 comprehensive analysis networks and gain a more comprehensive image of the diseases, gut microbiota, and genes involved in these networks.

Browsing

To clearly display the manually curated data, we developed a powerful browsing tool. There are five logical categories: microbiota, disease, organ, system and mechanism in the navigation bar on the left. Each category contains subcategories. Users can browse relevant entries in Amadis by clicking any drop-down menus or their submenus. Using "*Bacteroides*" as an example, the page of this entry displays the related disease, the sample type, the -omics technology, and the mechanism involved. Each entry contains detailed information, including the disease and microbiota names, the species involved, the association between the disease and microbiota (e.g., Microbiota promote disease progression and Microbiota inhibit disease progression), the experimental methods used (e.g., Transcriptomics and Real-time PCR), the detected tissue (e.g., brain and blood), the associated factors (e.g., levodopa and Interferon-Beta), the associated genes (e.g., MCAD and Fas), and a detailed description and corresponding literature (PMID and publication year).

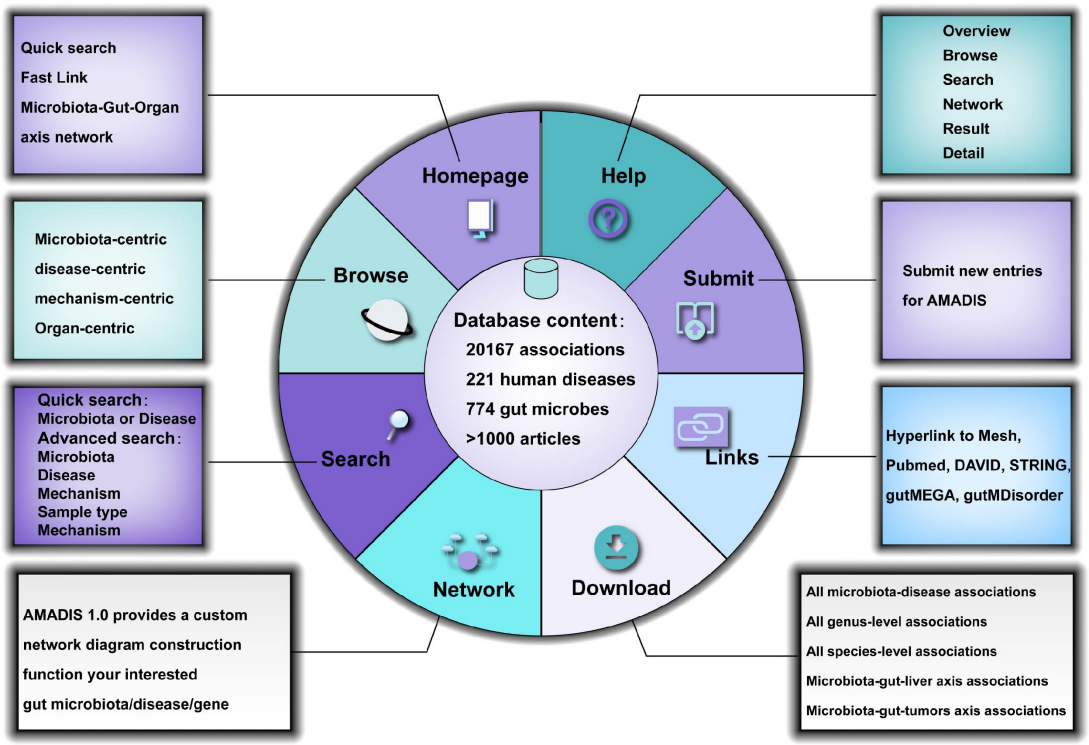

FIGURE 2 | Content and interface of Amadis.

Search Function

On the search page, four search fields are present that may be used together or separately: (1) microbiota, (2) disease, (3) sample type, and (4) mechanism. A user can query the database using standardized keywords, e.g., microbiota name, disease name, or sample type in the corresponding search fields. Amadis also offers a fuzzy search engine. The fuzzy search function allows users to retrieve entries by the name of a microbiota species, a disease or a sample type even when the query name is not perfectly clear. Once a certain query name is received, the system searches in the corresponding data field for terminology that contains the query words. The matching terminologies are listed as multiple hits in the pop-up result page. From these retrieved results, users can manually check to obtain the exact entry of interest through relevance to the query term.

Network Construction

In the lower right part of the homepage, we constructed multiple comprehensive analysis networks of the microbiota-gut-organ axis, such as the microbiota-gut-liver axis and microbiota-gut-brain axis. These network diagrams comprehensively demonstrate the role of microbiota in diseases of distant organs and provide reference materials for selecting research directions. On the network page, we provide a dynamic network construction method and allow users to freely select and combine modules to obtain their own network construction.

Users can select one or more bacterial species, diseases, and/or genes of interest to add to the column to be analyzed on the right. The information in the column can be deleted one by one or cleared all at once. After selecting the molecule, the user can click submit to start network construction and visualization. Clicking on a molecule in the network diagram will search for this molecule and display all entries.

Submit and Update

Amadis invites users to submit associations that are not documented in the database on the "Submit" page. To maintain the integrity of the database, we will conduct manual verification of the original publication(s) for data validation upon each submission from nonaffiliated researchers. Once approved by the review committee, the novel associations will be available to the public in the updated version.

Download and Help

As a publicly released scientific database, Amadis allows users to download all the obtained data on the "Download" page. In addition, detailed usage and guidelines of the database are available on the "Help" page.

DISCUSSION AND CONCLUSION

A growing number of people adhere to the idea that "we are what we eat." Nevertheless, for decades, investigators and clinicians have been trying to reveal the relationship between

intestinal microbiota and disease and to improve the diagnosis and treatment of disease. Researchers believe that some diseases are fundamentally not only genetic but also microbial diseases. However, accumulating experimentally supported associations are scattered in thousands of published studies. To provide a comprehensive resource of the functions of microbiota in human disease, several outstanding online repositories have been developed for storing microbiota-related data (van Nood et al., 2013; Benedek et al., 2017; Amini-Khoei et al., 2019).

In this study, we developed Amadis, a manually curated database that documents associations between human diseases and gut microbes, to better show the relationship between the gut microbiota and human diseases, to make full use of the massive amount of data accumulated in recent years, and to assist researchers in seeking novel insights. The database we developed not only obtains data from more than 1,000 articles but also conducts comprehensive analyses and constructs visual networks.

Serving as a useful and convenient resource, researchers could find some important implications behind a large, complex and integrated resource. By analyzing the network of the microbiota-gut-tumor axis, we found that *A. muciniphila* has an inhibitory effect on the development of six different tumors. *Akkermansia muciniphila* components, which are considered prebiotics, may be a new way to treat tumors. At the same time, studying the mechanism *A. muciniphila* uses to inhibit tumor progression may reveal new tumor treatment targets.

Among the dysregulated genes, there are several members of the CXCL family. By mining TCGA and Oncomine data, we found that the differences in the expression of CXCL family members in colorectal cancer, breast cancer and pancreatic cancer were remarkable. There were also significant differences in overall survival (OS) time and disease-free survival (DFS) time among people with different expression levels of CXCL family members. The abnormal expression of CXCL family members in a variety of tumors and their close relationship with OS and DFS time reveal the important role of this family in tumorigenesis and development. Moreover, the CXCL family is closely related to microbiota and may serve as a bridge between microbiota and diseases, especially cancers. Future research may clarify the relationship between the microbiota-CXCL family-diseases axis.

There are studies in the literature reporting that patients with rheumatoid arthritis (RA) have a significantly higher risk of coronary heart disease (CHD). To explore the role of microbiota in this situation, we can use the data documented in the database to construct a network of these two diseases to find microbiota whose abundance changes in both RA patients and CHD patients. By using the network analysis function, we found that the abundance of Blautia, Dorea, and Prevotella changed in both RA patients and CHD patients. These three genera may be involved in mediating the pathological process of CHD in patients with RA.

Overall, Amadis not only provides more than 20,000 manually curated microbiota associations with multi-omics data and experimental support but also offers global insights into microbiota functions in human diseases. Since it is the manually curated repository for annotating the function of gut microbiota, we believe that Amadis will serve as a useful resource for decrypting mechanisms, improving the diagnosis and treatment of human disease.

FUTURE DEVELOPMENT

The Amadis database represents one of the first steps in this project. Further extensions are under way. We will update the repository with experimentally supported association data every 2 months. As stated in the "data collection and database content" section, the microbiota–disease relationships documented in the current version were collected by indexing, cataloging, and searching of biomedical and health-related information on PubMed with a list of keywords referring to MeSH and the NLM-controlled vocabulary thesaurus. Furthermore, text-mining tools will be adopted to help us retrieve PubMed abstracts that potentially describe further microbiota-disease relationships. In addition, we are developing multi-omics profile- and interacting partner-based methods to predict novel microbiota-disease associations and expect to integrate these methods into the database in the future. These strategies will make the cataloging and searching processes more efficient and data content more comprehensive.

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.

AUTHOR CONTRIBUTIONS

LL: conceptualization, writing – original draft preparation, and writing – review and editing. QJ: methodology. SY: formal analysis. XL, DZ, YS, and CH: investigation. DW: supervision. DX: project administration. DX and LL: funding acquisition. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

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Global Analysis of Microbiota Signatures in Four Major Types of Gastrointestinal Cancer

Jihan Wang[1†], *Yangyang Wang*[2†], *Zhenzhen Li*[1], *Xiaoguang Gao*[2*] *and Dageng Huang*[3*]

[1] Institute of Medical Research, Northwestern Polytechnical University, Xi'an, China, [2] School of Electronics and Information, Northwestern Polytechnical University, Xi'an, China, [3] Department of Spine Surgery, Honghui Hospital, Xi'an Jiaotong University, Xi'an, China

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***Correspondence:**
Xiaoguang Gao
cxg2012@nwpu.edu.cn
Dageng Huang
345070558@qq.com

[†]These authors have contributed equally to this work

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The gut microbiota has been previously linked with tumorigenesis and gastrointestinal cancer progression; however, intra-tumor microbiota analysis has just emerged and deserves increasing attention. Based on the public databases of The Cancer Microbiome Atlas (TCMA) and The Cancer Genome Atlas (TCGA), this study identified the tissue/organ microbial signatures generated from 443 biosamples of four major gastrointestinal cancer types, including esophageal carcinoma (ESCA), which further includes esophageal adenocarcinoma (EAD) and esophageal squamous cell carcinoma (ESCC), stomach adenocarcinoma (STAD), colon adenocarcinoma (COAD), and rectum adenocarcinoma (READ). According to partial least squares discrimination analysis (PLS-DA), the profile differences in microbial communities between the tumor and normal samples were not particularly noticeable across the four cancer cohorts, whereas paired comparison analyses revealed several specific differences in bacteria between tumor and normal samples in the EAD, STAD, and COAD samples. The taxa classified from the phylum to genus level revealed a trend of distinguishable microbial profiles between upper and lower gastrointestinal tumors. The Bacteroidetes/Firmicutes ratio in lower gastrointestinal tract tumors was nearly three times that in upper gastrointestinal tract tumors. We also determined the relative tissue/organ-prevalent microbes for each of the four cohorts at the order and genus levels. Microbe *Alistipes*, *Blautia*, *Pasteurellales*, and *Porphyromonas* compositions were correlated with the clinical characteristics of patients with gastrointestinal cancer, particularly colorectal cancer. Taken together, our findings indicate that microbial profiles shift across different gastrointestinal cancer types and that microbial colonization is highly site-specific. Composition of specific microbes can be indicative of cancer stage or disease progression. Overall, this study indicates that the microbial community and abundance in human tissues can be determined using publicly available data, and provides a new perspective for intra-tissue/organ microbiota research.

Keywords: microbiota signature, gastrointestinal cancer, TCMA, TCGA, biomarker

INTRODUCTION

Gastrointestinal (GI) cancers are responsible for one-third of cancer mortality (1). According to statistics, an estimated 4.8 million new cases and 3.4 million related deaths of GI cancers occurred in 2018, accounting for 26% of all cancer incidence and 35% of cancer-related deaths. Approximately 8 in 100 men and 4 in 100 women are estimated to develop GI cancer before the age of 75, and more than half of new cases and related deaths occurred in Asia (2). Microorganisms, including bacteria, fungi, and viruses, have been described in terms of health and disease status (3–5). Nearly one-fifth of all cancers worldwide are linked with viral, parasitic, or bacterial infections; for instance, hepatitis B virus, human papillomavirus, and *Helicobacter pylori* are associated with hepatocellular carcinoma, cervical cancer, and stomach cancer, respectively (6). The human GI tract, in particular, harbors thousands of microbes. For example, the intestines have a dense community of approximately 10^{13} (7) microbes, whereas the stomach has the lowest microbial abundance due to its extreme acidity. These large numbers of microbial species constitute the microbiota, which refers to an ecological community of microbes that is found within a specific environment. The microbiota interacts with different types of host cells to modulate the organ microenvironment and to regulate physiological functions (8). Pathophysiological changes in cells and alterations in the microbial signature could have a significant impact on tumor occurrence and progression (9, 10), especially as microbial colonization is highly site-specific, allowing them to modulate the tumor microenvironment. The bacterial effects on cancer progression are related to the time and location of colonization (11), as well as on other pathogenic factors. In GI cancers, the microbiota has been recognized to be related to chemotherapy, radiotherapy, and immunotherapy efficacy (7, 8, 12, 13), indicating that the intestinal microbiota is a novel target to improve anti-tumor treatment (13). The presence of microbes within tumors and adjacent normal tissues may indicate disease progression and their potential roles in cancer pathogenesis (14–16). Understanding the alterations in the microbial community and abundance in GI organs thus aids in the study of GI cancer diagnosis and therapy.

Currently, the study of microbiota in life sciences has been greatly enhanced by advances in sequencing technology, accompanied by the application of multi-omics analysis (17). Intra-tumor microbiota analysis has recently emerged and has gradually increased in cancer studies (18–21). The Cancer Genome Atlas (TCGA) is a landmark cancer genomics program that sequenced and molecularly characterized 20,000 primary cancer and matched normal samples for 33 cancer types (22), which provides significant assistance in cataloguing and exploring cancer-causing genomic alterations and establishing a comprehensive "atlas" of cancer genomic signatures. Furthermore, the TCGA platform incorporates highly standardized clinical information regarding samples. Notably, the sequencing data in the TCGA offers a unique opportunity to study tissue/organ-related microbiota. Bioinformatics approaches authenticate microbiome research in the context of cancer-associated pathogenesis by using human sequencing data to characterize microbial profiles (bacterial, viral, or fungi). The Cancer Microbiome Atlas (TCMA, https://tcma.pratt.duke.edu) is a collection of curated, decontaminated microbial compositions of oropharyngeal, esophageal, GI, and colorectal tissues (23) based on samples from the TCGA database. At different taxonomic levels, the bacterial signatures of tumor and normal samples from patients with head and neck squamous cell carcinoma (HNSC), esophageal carcinoma (ESCA), stomach adenocarcinoma (STAD), colon adenocarcinoma (COAD), and rectum adenocarcinoma (READ) can be identified from TCMA, providing an excellent and powerful resource for studying the microbiome of GI cancers.

The objective of the current study was to investigate the microbiota profile in four major types of GI cancers, including ESCA, STAD, COAD, and READ. To identify the differences in microbial abundance between matched tumor-normal groups, the global microbiome signature at different taxonomic levels in both tumor and normal samples was analyzed. We also characterized the microbiome signature and identified relatively organ-prevalent microbes for each of the four GI cancer types to gain a better understanding of their similarities and heterogeneity based on their microbiome signatures. Furthermore, the correlation between specific candidate microbes and clinical variables of GI cancers was investigated by combining the TCMA microbial profile with the phenotype and survival data from TCGA. We believe that this is the first study to focus on the microbial composition of internal organs and their associations with four GI cancer types, which will provide evidence and a theoretical foundation for studying microbiome–host interactions and the role of the microbiome in digestive system malignant diseases.

MATERIALS AND METHODS

Data Acquisition From TCMA and TCGA

The microbial abundance profiles at different taxonomic levels were obtained from TCMA database for GI cancers including ESCA [specifically, including 20 tumors of esophageal adenocarcinoma (EAD), 40 tumors of esophageal squamous cell carcinoma (ESCC), and 22 normal samples], STAD (127 tumors of stomach adenocarcinoma and 39 normal samples), COAD (125 tumors of colon adenocarcinoma and 21 normal samples), and READ (45 tumors of rectum adenocarcinoma and 4 normal samples). TCGA includes biospecimens and the associated clinical information from human subjects under informed consent and authorization of local institutional review boards. We extracted the information about age, sex, race, tumor stage, and neoplasm histologic grade from the phenotype files, and about survival status and survival time from the survival files of TCGA data, we then integrated the microbial abundance profiles from TCMA and the clinical characteristics from TCGA for all the samples for further analysis. **Figure 1** depicts the study design and workflow. The clinical characteristics of the four types of GI tumors are summarized in **Table 1**.

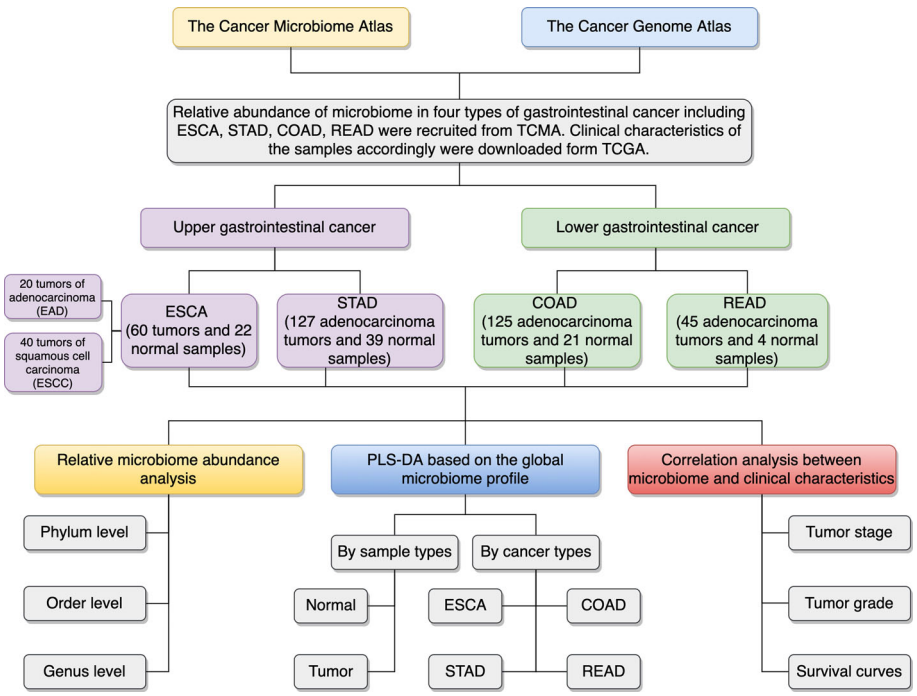


FIGURE 1 | Schematic overview of the study design and workflow. TCMA, The Cancer Microbiome Atlas; TCGA, The Cancer Genome Atlas; ESCA, esophageal carcinoma; EAD, esophageal adenocarcinoma; ESCC, esophageal squamous cell carcinoma; STAD, stomach adenocarcinoma; COAD, colon adenocarcinoma; READ, rectum adenocarcinoma; PLS-DA, partial least squares discrimination analysis.

Analysis of Global Microbiota Profiles at Various Taxonomic Levels

The global microbial abundance profiles at the phylum, order, and genus taxonomic levels were downloaded from the TCMA database. We performed partial least squares discrimination analysis (PLS-DA) to investigate the overall differences in microbiota profiles between the tumor and normal groups for each cancer type, as well as in the tumor samples among the four major GI cancer types.

Microbial Abundance Calculation and Analysis at Different Taxonomic Levels

Microbial abundance (percentage abundance) was calculated at the phylum, order, and genus taxonomic levels, and the microbiota profiles of the top five most abundant microbes at the phylum level and the top 10 most abundant microbes at the order/genus levels were summarized for further study. We used the paired two-tailed Student's t-test to compare microbial abundance in the tumor versus paired-normal samples, with $P < 0.05$ representing statistical significance. To examine the similarities and heterogeneities among the four types of GI cancer, a Venn diagram was drawn (http://bioinformatics.psb.ugent.be/webtools/Venn/) and bi-cluster analysis (using the "pheatmap" package in R version 4.0.2) based on microbiota profiles was performed.

Correlation Analysis of Microbial Abundance and Clinical Characteristics

Pearson correlation was performed in R version 4.0.2 using the cor.test () algorithm to analyze the correlation of specific microbial abundance and clinical characteristics, including tumor stage and histologic sample grade ($P < 0.05$). The Kaplan–Meier model from the survival and survminer packages in R version 4.0.2 was used for survival analysis based on microbial abundance. The microbial abundance values were divided into high (high) and low (low) groups based on median values, with $P < 0.05$ representing statistical significance.

RESULTS

Microbiota Profile Landscape of GI Cancers

Overall, we collected and integrated the microbiota profile and clinical characteristics of 443 GI cancer samples (including 357 tumor samples and 86 normal samples) from four cohorts. In total, 11 phyla, 38 orders, and 221 genera of microbial taxa were extracted from each sample from the TCMA database. First, we used a PLS-DA plot to compare the microbiota profile landscapes of tumor and normal samples from the same organ.

TABLE 1 | Clinical characteristics of the four types of gastrointestinal tumors in this study (derived from The Cancer Microbiome Atlas and The Cancer Genome Atlas databases).

Clinical characteristics	ESCA			STAD		COAD		READ	
	Tumor (EAD)	Tumor (ESCC)	Normal	Tumor	Normal	Tumor	Normal	Tumor	Normal
Age [median (min–max)]	72.75 (47–86)	59.45 (36–90)	77 (51–90)	68 (39–91)	72 (43–88)	69 (31–90)	68 (47–90)	66 (33–89)	58.5 (49–67)
Gender [number (%)]									
Male	15 (75.00)	36 (90.00)	15 (68.18)	82 (64.57)	24 (61.54)	61 (48.80)	10 (47.62)	22 (48.89)	1 (25.00)
Female	5 (25.00)	4 (10.00)	7 (31.2)	45 (33.86)	15 (38.46)	64 (51.20)	11 (52.38)	23 (51.11)	3 (75.00)
Race [number (%)]									
White	18	14	21 (95.45)	70 (55.12)	24 (61.54)	42 (33.60)	5 (23.81)	5 (11.11)	0 (0.00)
Black	0 (0.00)	2	0 (0.00)	2 (1.57)	1 (2.56)	3 (2.40)	0 (0.00)	1 (2.22)	1 (25.00)
Asian	1	24	0 (0.00)	14 (11.02)	1 (2.56)	21 (16.80)	0 (0.00)	0 (0.00)	0 (0.00)
Not reported	1	0 (0.00)	1 (4.55)	41 (32.28)	13 (33.33)	59 (47.20)	16 (76.19)	39 (86.67)	3 (75.00)
Tumor stage [number (%)]									
Stage I	6 (30.00)	2 (5.00)		21 (16.54)		27 (21.60)		10 (22.22)	
Stage II	4 (20.00)	26 (65.00)		40 (31.50)		47 (37.60)		17 (37.78)	
Stage III	6 (30.00)	9 (22.50)		33 (25.98)		35 (28.00)		12 (26.67)	
Stage IV	1 (5.00)	1 (2.50)		18 (14.17)		14 (11.20)		6 (13.33)	
Not reported	3 (15.00)	2 (5.00)		15 (11.81)		2 (1.60)		0 (0.00)	
Neoplasm histologic grade [number (%)]									
Grade X	11 (55.00)	4 (10.00)		0 (0.00)		Not		Not	
Grade 1	1 (5.00)	4 (10.00)		4 (3.15)		applicable		applicable	
Grade 2	4 (2.00)	23 (57.50)		43 (33.86)					
Grade 3	4 (2.00)	9 (22.50)		80 (62.99)					
Total [number (%)]	20 (100)	40 (100)	22 (100)	127 (100)	39 (100)	125 (100)	21 (100)	45 (100)	4 (100)

ESCA, esophageal carcinoma; EAD, esophageal adenocarcinoma; ESCC, esophageal squamous cell carcinoma; STAD, stomach adenocarcinoma; COAD, colon adenocarcinoma; READ, rectum adenocarcinoma.

The microbial profile could not well distinguish the sample type (tumor or normal) at the phylum, order, or genus levels, as shown in **Supplementary Figure 1**. We then focused on the hypothesis that microbial composition signatures are associated with different organs/tissues of GI tumors. As shown in **Figure 2**, we discovered that microbes have a highly organ-dependent signature. For example, the global microbiota profile of STAD is closer to that of ESCA (including EAD and ESCC) at the genus level, and samples from READ were nearly overlapped with the COAD group (**Figure 2A**). Furthermore, by combining ESCA and STAD samples as one type (upper GI tumor), and COAD and READ samples as another type (lower GI tumor), a clear distinction was found between upper and lower GI tumors, with the taxonomic rank ranging from the phylum to genus level (**Figure 2B**).

Microbiota Taxonomic Composition in GI Cancers

Bacteroidetes, *Firmicutes*, *Proteobacteria*, *Fusobacteria*, and *Actinobacteria* dominated the top 5 abundant taxa at the phylum level (**Figure 3A, B**). In the ESCA (including EAD and ESCC) tumor samples, *Bacteroidetes* (0.32), *Firmicutes* (0.34), *Proteobacteria* (0.16), *Fusobacteria* (0.08), and *Actinobacteria* (0.07) constituted nearly 97% of the microbiota phyla, the composition of which was similar to that of STAD samples: *Bacteroidetes* (0.29), *Firmicutes* (0.37), *Proteobacteria* (0.22), *Fusobacteria* (0.07), and *Actinobacteria* (0.03). Samples from the lower GI tumor had significantly higher levels of *Bacteroidetes* (0.53 for COAD, 0.51 for READ) and lower levels of *Firmicutes* (0.22 for COAD, 0.20 for READ). Thus, the lower GI tumor samples had a clearly higher *Bacteroidetes*/*Firmicutes* ratio compared to the upper GI tumor group (**Figure 3A**). However, the bi-clustering heatmap at the phylum level could not distinguish between the four cancer types (**Figure 3B**). The top 10 abundant microbiota taxa in each of the four cancer types were calculated and analyzed at the order and genus levels (**Figures 3C–F**). At the order level, half (5/10) of the most abundant microbiota were shared by all four cancer types (**Figure 3C**). Compared to the bi-clustering result at the phylum level, we observed a more obvious clustering trend at the order level, with ESCA clustering closer to STAD samples and READ clustering closer to the COAD group (**Figure 3D**). Furthermore, the difference in microbiome heterogeneity between the upper and lower GI tumors was more pronounced at the genus level. The composition of the top 10 abundant genera differed between the upper and lower GI tumors, as shown in **Figure 3E**. For instance, only two genera were shared by all four cancer types, the samples in the ESCA and STAD groups had five shared-genera, whereas the samples in the COAD and READ groups had six shared-genera. Furthermore, the organ-prevalent genera were identified relatively. The microbial *Capnocytophaga* presence ratios in the four cancer types were 18/60 (ESCA), 18/127 (STAD), 0/125 (COAD), and 0/45 (READ), respectively. *Helicobacter* genus had existence ratios of 5/60 (ESCA), 42/127 (STAD), 0/125 (COAD), and 0/45 (READ), respectively. The existence ratios for the *Faecalibacterium* genus were 0/60 (ESCA), 0/127 (STAD), 35/125 (COAD), and 11/45 (READ), respectively. *Porphyromonas*

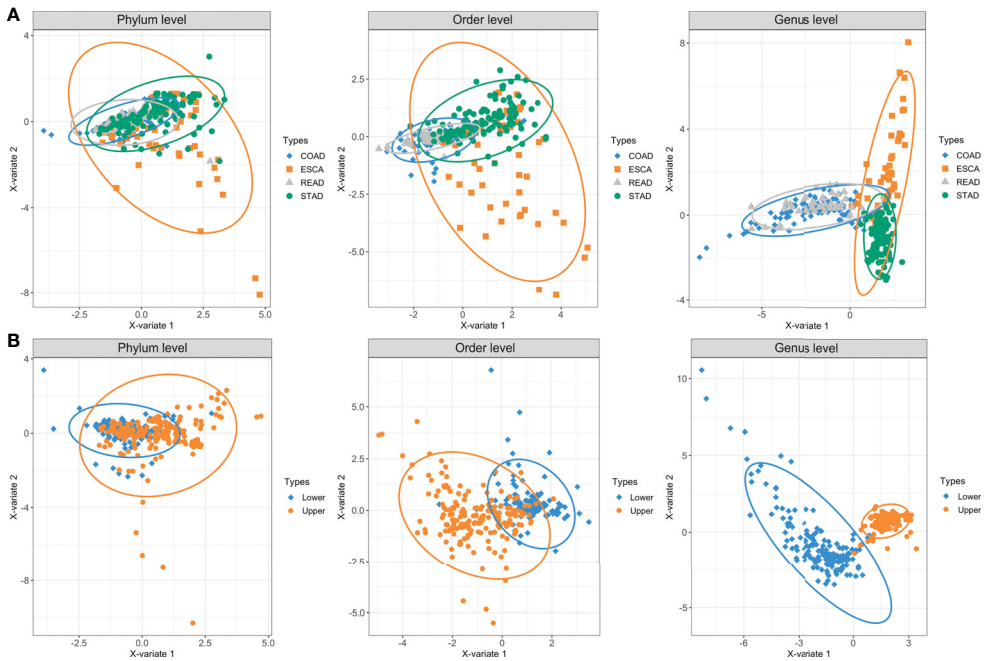


FIGURE 2 | PLS-DA plots based on the microbial profile of four GI cancer types. **(A)** PLS-DA plots of four cancer cohorts including ESCA, STAD, COAD, and READ at the phylum, order, and genus levels. **(B)** ESCA and STAD were further collected as upper GI tumors, whereas COAD and READ were collected as lower GI tumors. The light ellipse in each group represents the 95% confidence interval (CI) of the score calculated from the corresponding group. PLS-DA, partial least squares discrimination analysis; ESCA, esophageal carcinoma; STAD, stomach adenocarcinoma; COAD, colon adenocarcinoma; READ, rectum adenocarcinoma.

was found in nearly half of the READ samples (19/45), but less in the other three cancer types (6/60 for ESCA, 25/127 for STAD, 17/125 for COAD). Through the bi-clustering heatmap, we observed a relatively distinguishable pattern between upper GI and lower GI tumors based on their microbial profiles at the genus level (**Figure 3F**).

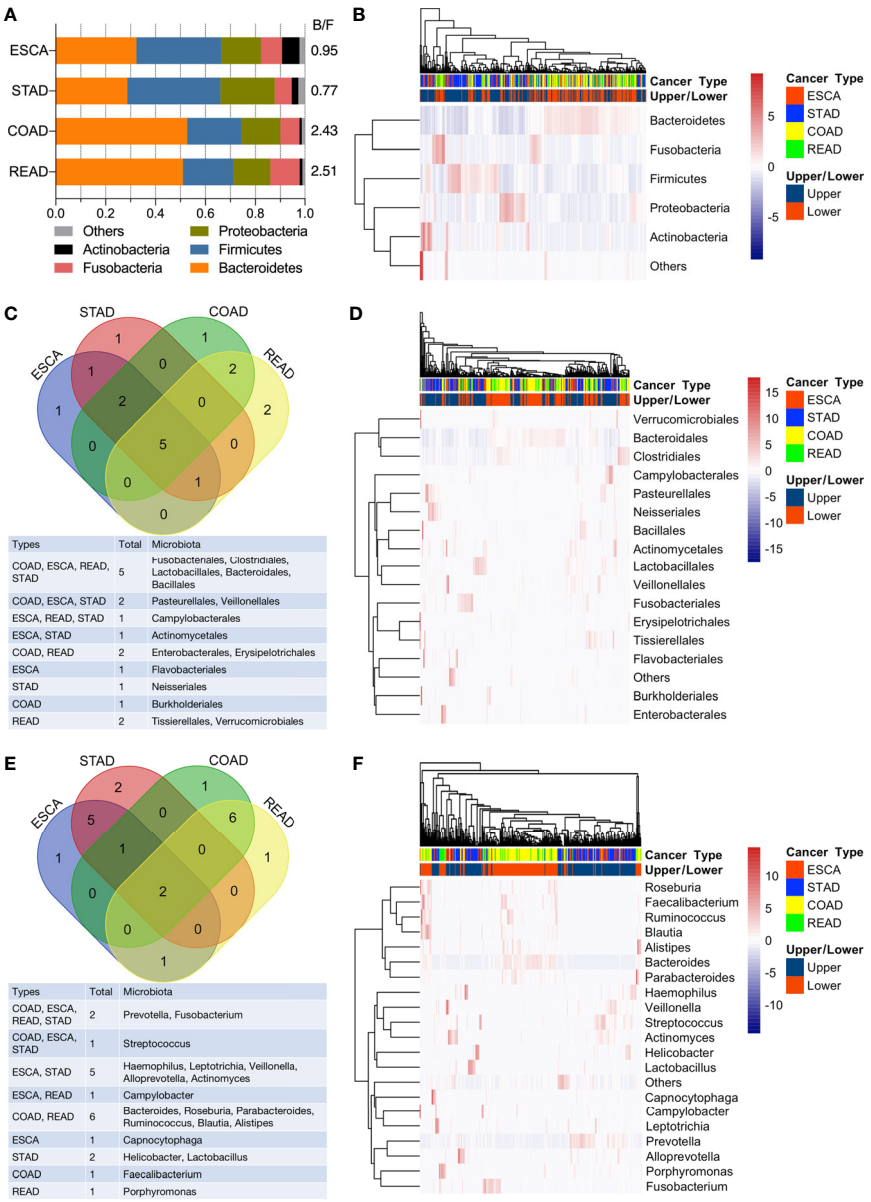
Furthermore, the most abundant microbial composition was used to analyze the differences in abundance between tumor and normal samples in the same organ. To obtain more accurate results, we used a two-tailed Student's t-test to compare the tumor and strictly paired normal samples for each cancer type. For ESCA, there were 18 and 4 paired tumor/normal samples for EAD and ESCC, respectively; for STAD, COAD, and READ, there were 38, 21, and 4 paired tumor/normal samples, respectively. **Figure 4** summarizes the statistically significant outcomes for the EAD, STAD, and COAD groups. There were no statistically significant differences between the tumor and paired normal samples for the ESCC and READ groups, possibly because of the small sample size of the two groups.

Microbiota Associated With Clinical Characteristics and Survival Status in GI Cancers

TCGA collects comprehensive clinicopathological annotation data, allowing researchers to investigate disease-related factors in cancer. After integrating the microbial abundance profile from TCMA and the clinical characteristics from TCGA, we investigated whether there were specific candidate microbial taxa that correlated with the clinical characteristics or survival status of GI cancers, as specific microbes have potential value as disease-related biomarkers. We discovered that the relative abundance of several microbial compositions was related to the overall survival rate or stage status in GI cancer patients, especially for COAD and READ (**Figure 5**). The high abundances of *Alistipes* and *Blautia* in tumor samples were correlated with better survival probability in patients with COAD ($P < 0.05$), and the relative levels of *Alistipes* and *Blautia* in the tumor were slightly decreased compared to their paired normal samples, but no significant difference was found (**Figures 5A, B**). Furthermore, the relative abundance of *Pasteurellales* was slightly increased (with no significant difference) in tumors compared with that in normal tissues and was positively correlated with COAD tumor stage ($P < 0.05$, **Figure 5C**). In READ, the relative abundance of *Porphyromonas* in tumors was elevated (with no significant difference) when compared with paired normal samples and was positively correlated with tumor stage ($P < 0.05$, **Figure 5D**).

DISCUSSION

Numerous studies have identified links between the microbiota and tumorigenesis and progression in various cancer types (24, 25). Until now, the majority of related studies have concentrated primarily on the role of the gut microbiota (GM) in disease. Several studies have recently characterized the profile of tissue-



Types	Total	Microbiota
COAD, ESCA, READ, STAD	5	Fusobacteriales, Clostridiales, Lactobacillales, Bacteroidales, Bacillales
COAD, ESCA, STAD	2	Pasteurellales, Veillonellales
ESCA, READ, STAD	1	Campylobacterales
ESCA, STAD	1	Actinomycetales
COAD, READ	2	Enterobacterales, Erysipelotrichales
ESCA	1	Flavobacteriales
STAD	1	Neisseriales
COAD	1	Burkholderiales
READ	2	Tissierellales, Verrucomicrobiales

Types	Total	Microbiota
COAD, ESCA, READ, STAD	2	Prevotella, Fusobacterium
COAD, ESCA, STAD	1	Streptococcus
ESCA, STAD	5	Haemophilus, Leptotrichia, Veillonella, Alloprevotella, Actinomyces
ESCA, READ	1	Campylobacter
COAD, READ	6	Bacteroides, Roseburia, Parabacteroides, Ruminococcus, Blautia, Alistipes
ESCA	1	Capnocytophaga
STAD	2	Helicobacter, Lactobacillus
COAD	1	Faecalibacterium
READ	1	Porphyromonas

FIGURE 3 | Analysis of microbial abundance in GI cancers. **(A)** The top five most abundant microbial phyla in the four cancer types; the B/F ratio represents the Bacteroidetes/Firmicutes ratio. **(B)** Bi-clustering heatmap based on the top five abundant microbial profiles at the phylum level. The horizontal axis represents the samples, and the vertical axis represents the microbial taxa. **(C)** Venn diagram of the top 10 abundant microbial compositions at the order level. **(D)** Bi-clustering heatmap based on the top 10 abundant microbial profiles at the order level. **(E)** Venn diagram of the top 10 abundant microbial compositions at the genus level. **(F)** Bi-clustering heatmap based on the top 10 abundant microbial profiles at the genus level.

resident microbiota in various cancers (6, 11, 18, 19, 26). Identification of microbial communities and abundance derived from human tissues and organs was significantly assisted by publicly available genome sequencing data in the TCGA database.

Herein, we described the global microbial signature associated with four major types of GI cancers by conducting a comprehensive analysis of the bacterial taxa in the TCMA. Overall, across the four cancer cohorts, the PLS-DA profile differences in the microbial communities between tumor and normal samples were not particularly noticeable. Despite this, the abundance of specific bacteria between strict paired tumor-normal samples were different. In the STAD cohort and EAD samples from the ESCA cohort, the relative abundance of *Firmicutes* in tumor samples was increased, whereas that of *Proteobacteria* was decreased significantly compared to that in normal samples. The link between *Helicobacter pylori* infection and gastric cancer has been well established. Noteworthy

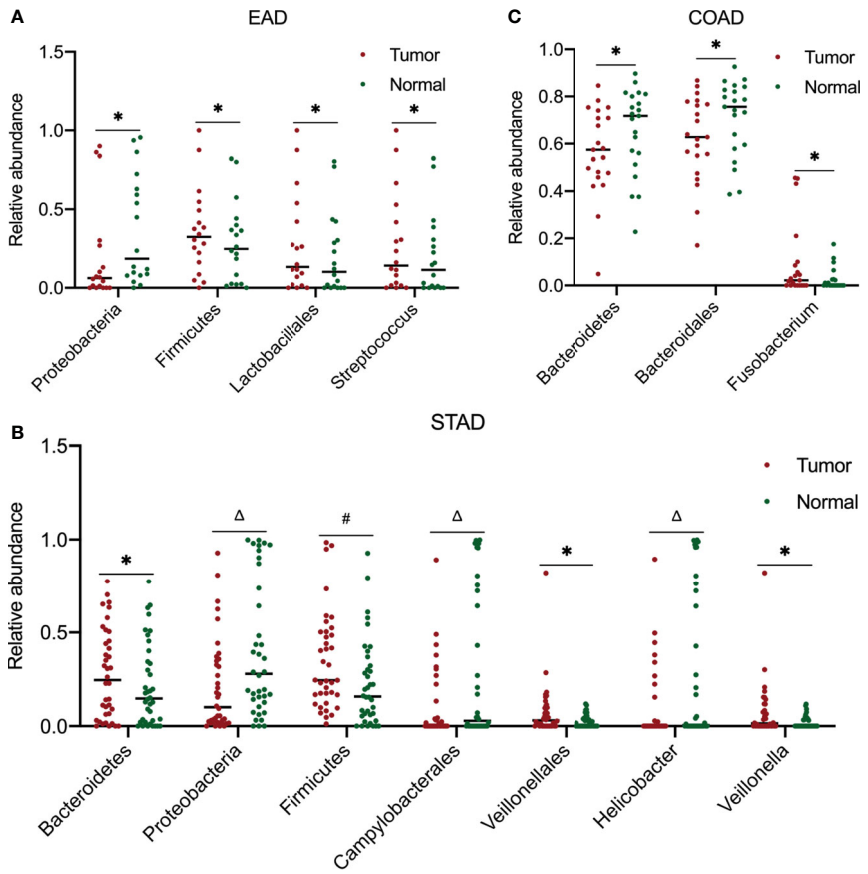


FIGURE 4 | Analysis of microbiota abundance differences between tumor and paired normal samples. In EAD **(A)**, STAD **(B)**, and COAD **(C)**, there were 18, 38, and 21 paired tumor-normal samples, respectively. For significance analysis, a paired two-tailed Student's t-test was used. $^{*}P < 0.05$, $^{\#}P < 0.01$, $^{\Delta}P < 0.001$ compared to the normal group. The differences between tumor and paired normal samples were not significant in ESCC and READ samples (data not shown). EAD, esophageal adenocarcinoma; ESCC, esophageal squamous cell carcinoma; STAD, stomach adenocarcinoma; COAD, colon adenocarcinoma; READ, rectum adenocarcinoma.

consistent with other studies (11, 27), the abundance of *Helicobacter* was higher, whereas that of *Veillonellales/Veillonella* was lower in the paired normal samples compared to tumor samples (38 paired of tumor/normal samples in this study) within the gastric cohort. In COAD, the tumor samples had significantly lower levels of *Bacteroidetes* and *Bacteroidales* and higher levels of *Fusobacterium* compared to their normal counterparts.

In our study, we observed distinct microbial profiles between the upper and lower GI tumors, as the taxa were classified from the phylum to genus level, whereas minor differences were found in the microbiota signature between ESCA and STAD in the upper GI tract and COAD and READ in the lower GI tract. As exploring tissue-resident microbiota profiles can help to identify predictive microbial biomarkers for a specific cancer type, we further concentrated on identifying and comparing the common and distinct microbial taxa in four GI cancer types. At the phylum level, *Bacteroidetes* and *Firmicutes* dominated the microbial composition in the ESCA and STAD cohorts of the upper GI tract, whereas *Bacteroidetes* dominated the lower GI tract samples of COAD and READ. At the order level, the four cancer cohorts shared half of the top 10 most detected microbial compositions. Furthermore, a trend of clustering was observed between ESCA and STAD, as well as between COAD and READ; the clustering phenomenon and differences in microbial profiles within groups were most visible at the genus level. According to our findings, only two common abundant microbial genera were detected in the four cancer types. Previous research has shown that the genera *Streptococcus*, *Lactobacillus*, *Veillonella*, and *Prevotella* predominate in the gastric microbiota (28), which is consistent with the current findings. The STAD group nearly overlapped the most abundant genus profile in ESCA samples. Several upper GI tract microbial genera (*Streptococci*, *Veillonella*, *Lactobacillus*) were reported in abundance in the microbial community coating the tongue (27), indicating that anatomically adjacent organs have relatively similar microbial signatures. The READ cohort had the most common abundant genera with the COAD cohort in the lower GI tract. Colorectal cancer (CRC) is closely correlated with dramatic changes in microbial composition, also known as

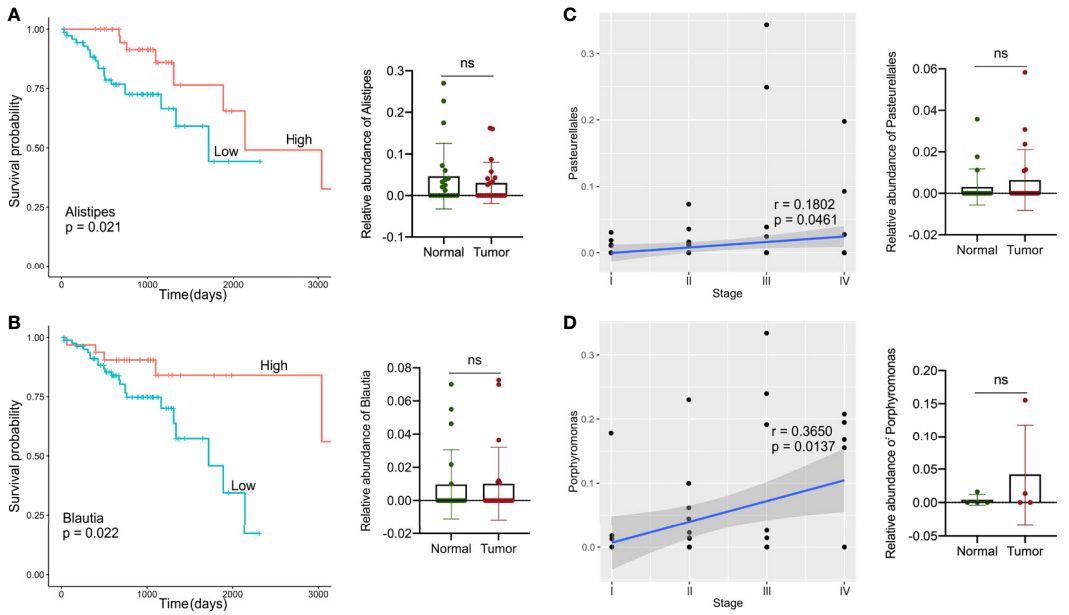


FIGURE 5 | Analysis of correlations between specific microbes and the clinical characteristics or survival status in GI cancers. **(A, B)** Kaplan–Meier survival curves based on microbial abundances of Alistipes **(A)** and Blautia **(B)**, and calculation of their abundance in COAD tumor and paired normal samples. **(C)** Correlation of Pasteurellales abundance with the tumor stage, and calculation of Pasteurellales abundance in COAD tumor and paired normal samples. **(D)** Correlation of *Porphyromonas* abundance with tumor stage, and calculation of *Porphyromonas* abundance in READ tumor and paired normal samples. COAD, colon adenocarcinoma; READ, rectum adenocarcinoma.

dysbiosis (29, 30). Evidence for important roles of *Fusobacterium nucleatum*, *Escherichia coli*, and *Bacteroides fragilis* as specific strains associated with CRC is also emerging (31). CRC-associated microbiota profiles differ from those found in healthy subjects; the microbiota composition in colorectal cancer in our study was similar to that found in other studies (32, 33). We also identified tissue/organ-specific flora. For example, *Capnocytophaga* and *Helicobacter* were only found in the ESCA and STAD cohorts. It is known that *Helicobacter pylori* is a major etiological factor in the development of upper GI tract conditions (34), and its infection in the stomach is a risk factor for STAD prognosis (35). On the contrary, *Faecalibacterium* was found only in CRC samples.

Finally, we examined the relationship between candidate microbes and clinical variables in patients after combining the microbiome profile from TCMA and clinical characteristic information from TCGA for all samples, focusing on factors such as tumor stage, histologic grade, and overall survival status. In general, we discovered more microbial correlations with CRC clinical characteristics than with upper GI cancers. For example, the abundance of *Alistipes* and *Blautia* was moderately decreased in tumors compared to that in the paired normal samples, and their high level indicated a better survival probability in patients with COAD. The composition of *Pasteurellales* and *Porphyromonas* was related to the tumor stage status of COAD and READ, respectively. Recently, there has been contradictory evidence indicating the two-sided effects of *Alistipes* on health. *Alistipes* may confer protective effects against diseases such as liver fibrosis, colitis, and cardiovascular disease (36). Other studies have found *Alistipes* to be pathogenic in colorectal cancer (33, 36, 37), which contradicts the results of the current study and requires further clarification. In a study on mucosa-adherent microbiota, *Blautia* was found to be lower in patients with CRC than in healthy controls (38). Several studies (39, 40) have found high levels of *Porphyromonas* in colorectal cancer, which is consistent with our findings. The results indicate that a novel approach to microbial-based cancer discrimination and prognosis prediction may provide significant future value to patients.

Our study has some limitations; the small size of paired tumor-normal samples weakened the power of the comparison study, particularly in the READ cohort and the ESCC subgroup of ESCA. Further, the clinicopathological data in this study need to be supplemented and completed to obtain more comprehensive results regarding the relationship between GI cancers and the microbiota. Besides, the current study is more of an observational research, and interference study is essential and need to be conducted in the future to eliminate the false correlation drawing from bioinformatics data.

CONCLUSION

In this study, we characterized the microbiota signatures of four major GI cancer types: ESCA (including EAD and ESCC), STAD, COAD, and READ. Taken together, our findings indicate that microbial profiles differ noticeably between upper and lower GI tissues/organs, and that microbial colonization is

relatively site-specific. Several candidate microbial biomarkers can be predictive of tumor stage and cancer prognosis. This approach confirms the ability to identify the microbial community and abundance in human tissues based on publicly available genome sequencing data, helps to discover prognostic species, and enables systematic matched microbe-host multi-omic analyses, which provides a new perspective for intra-tissue/organ microbiota research and will help guide future studies of the microbiome's role in human health and disease.

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 authors.

AUTHOR CONTRIBUTIONS

JW designed, performed the research, and co-wrote the manuscript. YW and XG contributed to data and statistical analysis. ZL co-wrote the manuscript. DH supervised the research. 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/fonc.2021.685641/full#supplementary-material

Supplementary Figure 1 | PLS-DA plots based on microbial profile of tumor and normal samples from the same organ. PLS-DA plots at phylum, order, and genus levels of tumor and normal samples from ESCA **(A)**, STAD **(B)**, COAD **(C)**, and READ **(D)**.

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Changes and Correlations of the Intestinal Flora and Liver Metabolite Profiles in Mice With Gallstones

Yang Chen[1,2†], Qiang Wang[1†], Wenqi Gao[1], Biao Ma[1], Dongbo Xue[1] and Chenjun Hao[1]**

[1] Department of General Surgery, The First Affiliated Hospital of Harbin Medical University, Harbin, China, [2] Department of General Surgery, Heilongjiang Provincial Hospital, Harbin, China

There is increasing appreciation for the roles of the gut-liver axis in liver and gall diseases. Specific gut microbes are associated with susceptibility to gallstone diseases, while the relationship between intestinal flora and liver metabolism in the formation of gallstones remains unclear. In this study, an experimental group of model mice was given a lithogenic diet, and a control group was given a normal diet. Both groups were fed for 8 weeks. Integrating 16S rRNA gene sequencing and non-targeted metabolomics to explore the impact of the lithogenic diet on intestinal flora and liver metabolism, Spearman correlation analysis reveals the network of relationships between the intestine and liver. Our findings showed that the gut microbiome and liver metabolome compositions of the test group were significantly changed compared with those of the normal group. Through our research, biomarkers of gallstones were identified at the phylum (5), class (5), order (5), family (7), and genus levels. We predicted the function of the differential flora. We analyzed the liver metabolism of mice with gallstones paired with their flora, and the results showed that there were 138 different metabolites between the two groups. The metabolic pathways enriched by these differential metabolites are highly consistent with the functions of the disordered flora. We focused on an analysis of the relationship between deoxycholic acid, asymmetric dimethylarginine, glucosamine, tauroursodeoxycholic acid, and the disordered flora. This provides a basis for the establishment of the intestine-liver axis in gallstone disease. This research provides a theoretical basis for the research and development of probiotics and prebiotics.

Keywords: gallbladder stones, intestinal flora, liver metabolic disorders, intestinal liver axis, dysbacteriosis

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Ravichandra Vemuri, Wake Forest Baptist Medical Center, United States

****Correspondence:***
Dongbo Xue
xuedongbo@hrbmu.edu.cn
Chenjun Hao
haochenjun1986@163.com

[†]These authors share first authorship

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INTRODUCTION

Gallstones are very common worldwide and affect 10–20% of the global adult population (Lammert et al., 2016). Recently, due to improved lifestyles and increased consumption of fat- or cholesterol-rich diets, the prevalence of gallstone disease has increased rapidly. Currently, the treatment for gallstone disease remains predominantly invasive; however, cholecystectomy potentially generates health problems, including intestinal dysfunction (Di Ciaula et al., 2019) and even increased colon cancer risk (Chen et al., 2014, 2020; Shabanzadeh et al., 2017). Therefore, future efforts should focus on preventive strategies to prevent the formation of gallstones. Hepatic hypersecretion of

cholesterol caused by genetic and dietary factors is the main reason of the formation of cholesterol gallstones. Intestinal factors leading to the formation of cholesterol gallstones include reduced absorption of bile salt and increased absorption of cholesterol. The intestinal flora, as an intestinal factor, plays an important role in the formation of gallstones.

Accumulating evidence suggests that the intestinal flora affects liver and gall diseases by regulating the gut-liver axis (Jia et al., 2019; Lang et al., 2020). Compositional changes in the fecal bacterial microbiome of patients with GSD have been reported in several cross-sectional studies (Wu et al., 2013; Wang et al., 2020). Ruminococcus gnavus is a marker for distinguishing gallstone patients from healthy controls (Wang et al., 2020). Gallstone patients had higher levels of 7alpha-dehydroxylating bacteria than patients without gallstones (Wells et al., 2000). All strains of 7alpha-dehydroxylating bacteria appear to belong to the genus Clostridium (Wells et al., 2000; Begley et al., 2005), and the abundance of Clostridium also increases in mice with gallstones (Wang et al., 2017, 2020). The intestinal flora cooperates with the liver to regulate bile acid and fat metabolism (Sayin et al., 2013), which can in turn contribute to the development of gallstones.

Researchers have collected bile and serum samples from patients with gallstones (Sharma et al., 2017; Molinero et al., 2019; Petrov et al., 2020) to explore the formation of gallstones from the perspective of metabolomics. Patients with gallstones had different bile metabolic profiles compared with individuals without hepatobiliary disease (Molinero et al., 2019). Deoxycholic acid is a metabolite of the genus Clostridium, and high levels of deoxycholic acid in bile feces are associated with an increased risk of cholesterol gallstone disease (Molinero et al., 2019; Wang et al., 2020). Trimethylamine-N-oxide (TMAO) is a microbial-dependent metabolite that increases the expression of abcg5/8 in the liver to increase the secretion of cholesterol in hepatocytes and then promote the formation of gallstones (Chen et al., 2019). The liver is an important organ for bile acid production and metabolism of cholesterol and lipids, which are all related to the formation of gallstones. Therefore, liver metabolomics is an important aspect of our exploration of gallstone formation.

To the best of our knowledge, few studies have examined liver metabolomics in gallstone patients or mice, while no study has examined the association between the intestinal flora, liver metabolomics and gallstones. Therefore, in our study, gallstones in model mice were induced by lithogenic diet (LD), and 16S rRNA gene sequencing and LC/MS-based metabolomics were applied to provide more information on the interplay between the intestinal flora and liver, with the purpose of identifying the possible mechanism of gallstone formation in the flora-gut-liver axis.

MATERIALS AND METHODS

Animal Experiments and Sample Collection

All experimental protocols were approved by the Ethics Committee of Harbin Medical University. Adult male C57BL/6J mice (3 weeks old, Liaoning Changsheng Biotechnology Co., Ltd, Liaoning, China) were housed in a controlled environment (12-h light-dark cycle) in terms of temperature (18–24°C) and humidity (50–60%). Each group was fed the indicated diet and water ad libitum. The mice were given 1 week to adapt to the new environment, followed by 8 weeks of dietary intervention. Twenty mice were randomly divided into the control group (group C) fed a normal diet and the test group (group T) fed a lithogenic diet (LD, containing 1.25% cholesterol and 0.5% cholic acid). After the 8 weeks of dietary intervention, we collected feces at 7 o'clock every morning. We used cotton swabs to stimulate the end of the rectum in mice to promote defecation. We collected mouse feces in sterile tubes and immediately froze them in a −80°C refrigerator. Mice were euthanized after an 8-h fast. To reduce the effects of postmortem delay on liver metabolites, all samples were collected and frozen at −80°C within 10 min after death.

Gallstone Examination and Liver Histology

To observe the effects of lithogenic diet-induced gallstone phenotypes, gallstone formation was evaluated macroscopically, and gallbladder bile was examined by polarizing light microscopy without a cover slip. The liver was removed and fixed in 10% formalin and then processed and embedded into wax blocks. After sectioning, the liver sections were stained with hematoxylin and eosin (HE) to examine hepatic steatosis.

DNA Extraction and Intestinal Flora 16S rRNA Sequencing

Total genomic DNA of samples was extracted using the MagPure Soil DNA LQ Kit (Magen, Guangzhou, China). The concentration of DNA was verified with a NanoDrop (Thermo Fisher, United States) and agarose gel electrophoresis. Bacterial DNA was amplified with primers targeting the V3–V4 regions (5′-TACGGRAGGCAGCAG-3′, 5′-AGGGTATCTAATCCT-3′). Amplicon quality was visualized using gel electrophoresis, purified with AMPure XP beads (Agencourt), and amplified for another round of PCR (Bio-Rad, United States). After purification with AMPure XP beads again, the final amplicon was quantified using a Qubit dsDNA assay kit (Life Technologies, California, United States). Equal amounts of purified amplicons were pooled for subsequent sequencing by the Illumina MiSeq platform (Illumina, California, United States) by OE Biotech (Shanghai, China).

Raw sequencing data were in FASTQ format. The raw data were treated and processed using the QIIME software package (version 1.8.0). Clean reads were subjected to primer sequence removal and clustering to generate operational taxonomic units (OTUs) using Vsearch software with a 97% similarity cutoff. The representative read of each OTU was selected using the QIIME package. All representative reads were annotated and BLASTed against the Silva database Version 123 using the RDP classifier (confidence threshold was 70%).

Liver Metabolomic Analyses

Extraction and sample preparation were performed according to a protocol described previously (Sun et al., 2021). Non-targeted metabolite analysis was performed by OE Biotech (Shanghai, China). A Dionex Ultimate 3,000 RS UHPLC system fitted with a Q Exactive quadrupole Orbitrap mass spectrometer equipped with a heated electrospray ionization (ESI) source (Thermo Fisher Scientific, Waltham, MA, United States) was used to analyze the metabolic profiling in both ESI-positive and ESI-negative ion modes. An ACQUITY UPLC HSS T3 (1.8 μm, 2.1 mm × 100 mm, Waters, United Kingdom) was employed in both positive and negative modes. The binary gradient elution system consisted of (A) water (containing 0.1% formic acid, v/v) and (B) acetonitrile (containing 0.1% formic acid, vol/vol). The initial composition was 95% A and 5% B. Separation was achieved using the following gradient: 5% B, 0.01–2 min; 5–30% B, 2–4 min; 30–50% B, 4–8 min; 50–80% B, 8–10 min; 80–100% B, 10–14 min. The composition was held at 100% B for 2 min; 100–5% B, 15–15.1 min; 5% B, 15.1–16 min. The QC samples were injected at regular intervals (every 10 samples) throughout the analytical run to provide a set of data from which repeatability could be assessed. The acquired LC-MS raw data were analyzed by progenesis QI software (Non-linear Dynamics, Newcastle, United Kingdom). Metabolites were identified by progenesis QI (Waters Corporation, Milford, United States) Data Processing Software.

Methods of Statistical Analysis

The Wilcoxon rank sum test was applied to assess the significant differences in α diversity between two compared groups, and β diversity was visualized by principal coordinates analysis (PCOA) using weighted Unirac distance matrix data. LEfSe differences between two groups were tested for significance using the Kruskal-Wallis sum-rank test, and biological significance was subsequently investigated using a set of pairwise tests among groups with the Wilcoxon rank-sum test. Finally, linear discriminant analysis (LDA) was used to obtain the final differential species, followed by the Wilcoxon rank-sum test. The metagenomes of the intestinal flora were imputed from 16S rRNA sequences using PICRUSt. The predicted KEGG database[1] results were counted at three levels according to the Wilcoxon algorithm. Multivariate statistical analysis first uses unsupervised principal component analysis (PCA) to observe the population distribution among samples and the stability of the whole analysis process and then uses orthogonal partial least squares analysis (OPLS-DA) to distinguish the overall differences in metabolic profiles among groups and find the metabolites that are different between groups. In this study, the default 7-round cross validation was applied, with one seventh of the samples excluded from the mathematical model in each round to guard against overfitting. The screening standard for differential metabolites was that the first principal component in OPLS-DA model was VIP>1, *T*-test $p<0.05$. Pathway enrichment analysis of differential metabolites was performed by using the KEGG database. In addition, hypergeometric tests were used to identify pathway entries that were significantly enriched in significantly differentially expressed metabolites compared with the overall background, and pathways with $p < 0.05$ were considered significantly enriched. The correlation between the gut microbiome and liver metabolome was analyzed using Spearman correlation tests (Student's *t*-test, $p < 0.05$, $|$ correlation coefficient$|$ > 0.3). All analyses and graphics were performed using QIIME (version 1.8.0), R software (version 3.6.2), and R software (version 3.5.1). All values are presented as the means ± SEM. The results with $p < 0.05$ were considered statistically significant.

[1] http://www.genome.jp/kegg

RESULTS

Gallbladder Lithogenesis Rate and Liver Pathological Changes

After 8 weeks of dietary intervention, as expected, cholesterol gallstones were observed in all mouse gallbladders of the T group, and cholesterol crystals were also confirmed by polarized-light microscopy (lithogenesis rate 100%; **Figure 1A**), while no cholesterol gallstones or crystals were found in the C group either by gross observation or polarized-light microscopy (lithogenesis rate 0%; **Figure 1B**). Pathological sections stained with H&E showed that there were larger lipid droplets, lamellar necrosis and inflammatory cell infiltration in the liver cells of the T group mice, but they did not exist in the C group mice (**Figure 1C**). The results show that mice with gallstones have fatty liver. The two groups of mice did not exhibit any changes in body mass from 3 to 10 weeks (**Figure 1D**), and the food intake and water intake of the two groups of mice did not change significantly (**Figure 1E**). This shows that the lithogenic diet does not cause differences in body weight, food and water intake.

Effects of LD Interventions on Intestinal Flora

The alpha diversity of the intestinal flora in gallstone model mice was significantly reduced. The significant decrease in the Chao1 index of the gallstone group indicates that the gut microbial abundance of gallstone mice was significantly reduced (**Supplementary Figure 1A**), but the significant increase in the goods coverage index indicates that the biodiversity was reduced (**Supplementary Figure 1B**). The results of the animal model are consistent with the changes in the intestinal flora of patients with gallstones. The species accumulation curve tends to flatten gradually, indicating that the sampling is sufficient, and the samples can reflect the richness of species (**Supplementary Figure 1C**). The rank abundance curve reflects the abundance and uniformity of species in the sample (**Supplementary Figure 1D**). Compared with the control group, the beta diversity of the intestinal flora of gallstone model mice was significantly different.

Beta diversity is the degree of diversity between biological environments, that is, the comparison of differences between samples in different groups. These differences are compared based on the similarity of OTU sequences or the structure of the community. The results show that there are significant

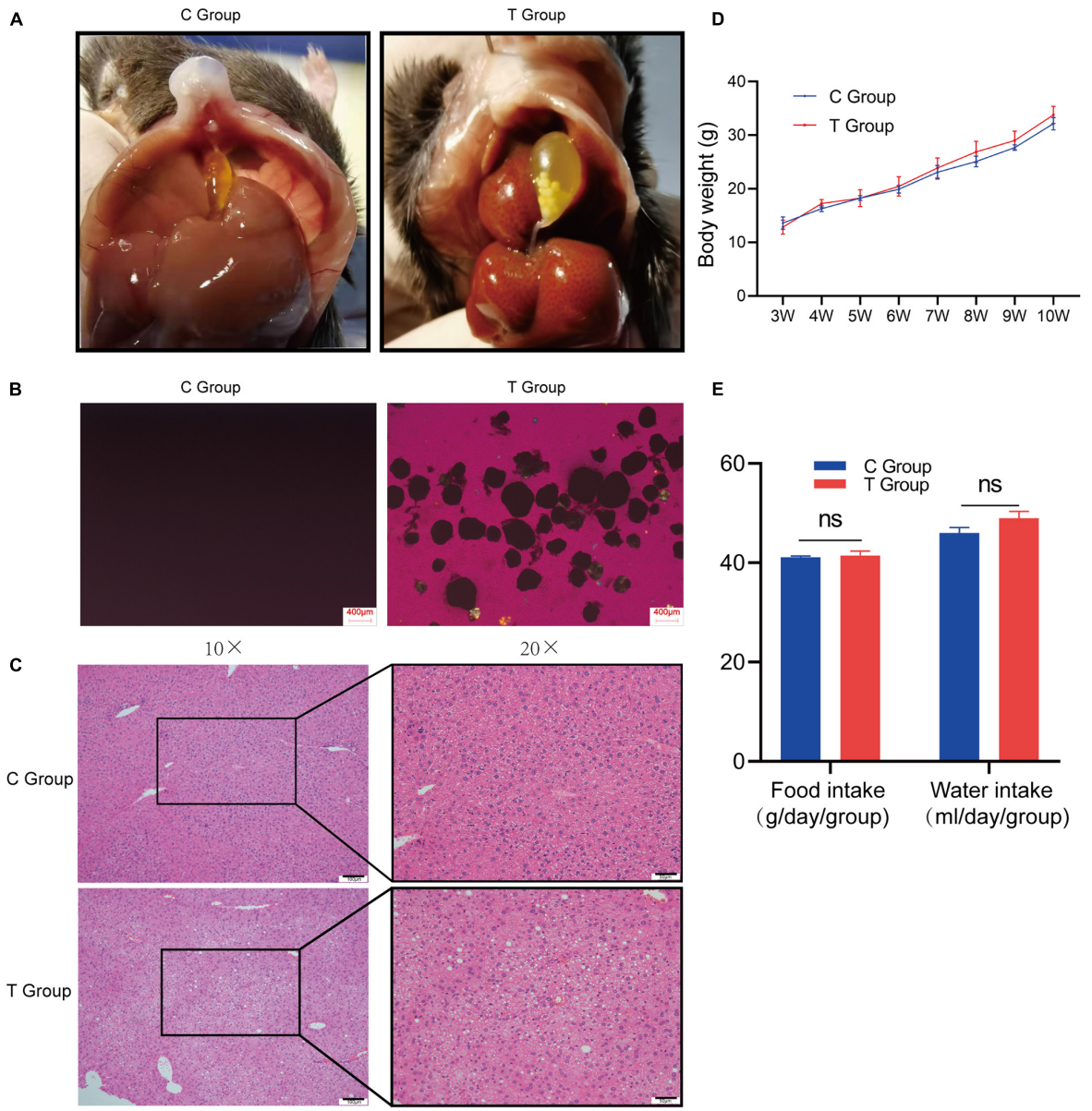

FIGURE 1 | Gallstone formation and liver pathology of C57BL/6J mice after 8 weeks of dietary intervention. **(A)** Macroscopic appearances of the gallbladders of T group and C group mice. **(B)** Polarized light microscopy of gallbladder bile of T group and C group mice. **(C)** Severe steatosis occurred, along with inflammatory cell infiltration and hepatocyte necrosis in the liver cells of the T group, and liver cells were well formed in the C group. **(D)** Changes in body weight of mice from 3 weeks of age to 10 weeks of age. The figure shows that the body weight of the two groups of mice is not statistically significant. **(E)** Calculate the daily food intake and water intake of the two groups of mice. The results showed that there was no statistical difference in the food intake and water intake of the two groups of mice.

differences in the distribution and structure of intestinal flora between gallstone mice and normal mice. We used Bray-Curtis distance analysis (**Supplementary Figure 2A**), NMDS analysis (**Supplementary Figure 2B**), UPGMA repeated sampling reliability analysis (**Supplementary Figure 2C**), PCA (**Supplementary Figure 2D**), PCoA analysis (**Supplementary Figure 2E**) and sample hierarchical cluster analysis (**Supplementary Figure 2F**) to compare the flora of the C group and T group. The results show that there are significant differences in the distribution and structure of intestinal flora between gallstone mice and normal mice. The above data fully confirm that there is a serious imbalance in the gut microecology of gallstone mice.

To more clearly describe the changes in the intestinal flora of mice with gallstones, we made a comparison at the phylum, genus, and species levels. At the phylum level, we found that Proteobacteria, Actinobacteria, Deferribacteres, and Firmicutes were significantly increased in gallstone mice, while Bacteroidetes, Tenericutes, and Elusimicrobia were significantly reduced in gallstone mice (**Figures 2A,B**). At the genus level,

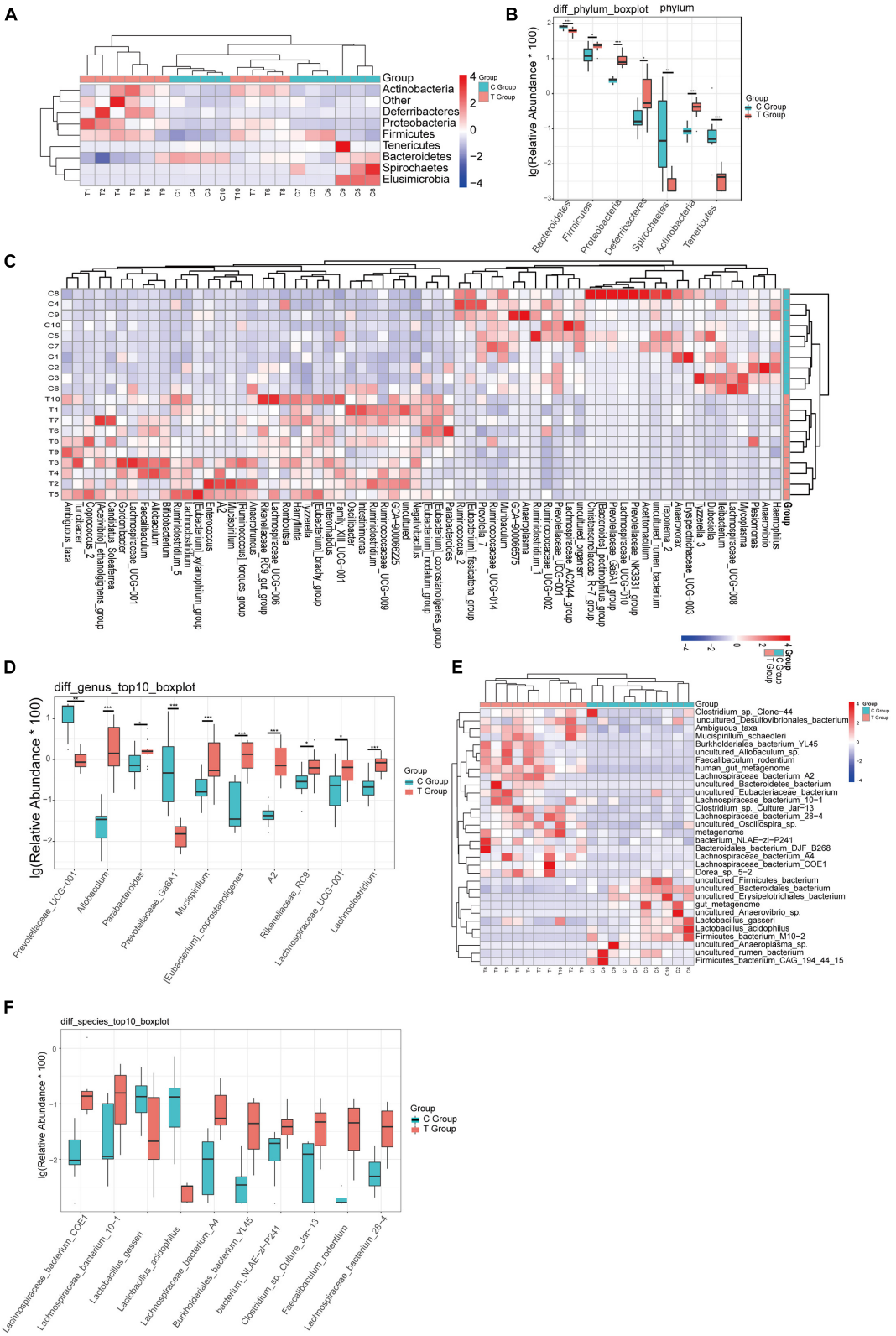

FIGURE 2 | The difference in bacterial flora between the gallstone group and the control group was analyzed at different levels. **(A,B)** Analysis of changes in flora at the phylum level. **(C,D)** Analysis of changes in flora at the genus level. **(E,F)** Analysis of changes in flora at the species level. (Wilcoxon rank-sum test, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$).

36 genera were significantly increased in gallstone mice, and 30 genera decreased significantly (**Figure 2C**). Eight of the top ten bacterial genera with the highest abundance of bacteria increased significantly in mice with gallstones (**Figure 2D**). At the species level, 21 species increased significantly in gallstone mice, and 11 species decreased significantly (**Figure 2E**). **Figure 2F** shows the top ten species, 8 of which were significantly increased in gallstone mice.

To identify the specific communities and specific biomarkers in gallstone mice, we compared the compositions of the intestinal flora between the two groups using LEfSe analysis (**Figures 3A,B**). In total, LEfSe analysis revealed 33 discriminative features [phylum (5), class (5), order (5), family (7), and genus (11)]. At the phylum level, the abundance of Firmicutes was significantly enriched in the T group (LEfSe: $p<0.05$, LDA>2), followed by Deferribacteres and Proteobacteria, whereas the abundance of Bacteroidia and Spirochaetia was enriched in the C group. At the family level, T group-enriched species included Tannerellaceae, Deferribacteraceae, Ruminococcaceae, Erysipelotrichaceae, and Desulfovibrionaceae, while Prevotellaceae and Spirochaetaceae were prevalent in the C group. At the genus level, 8 genera with significantly greater species abundances, including Ambiguous_taxa, Parabacteroides, Mucispirillum, A2, Eubacterium__coprostanoligenes_group, Allobaculum, and Faecalibaculum, were observed in the T group rather than in the C group, while Treponema_2, Prevotellaceae_UCG_001, and Prevotellaceae_Ga6A1_group were enriched in the C group.

To characterize the intestinal flora functional alterations after LD dietary intervention, we predicted functional composition profiles based on 16S rRNA sequencing data by performing phylogenetic reconstruction of unobserved states (PICRUSt) analysis. There were 215 different KEGG terms between the two groups. **Figure 3C** shows the top 50 with the most significant differences. The results suggested that many KEGG pathways, including ABC transporters, secondary bile acid biosynthesis, primary bile acid biosynthesis, lipid biosynthesis proteins, galactose metabolism, and glycerophospholipid metabolism, were significantly modulated after LD dietary intervention and were related to the formation mechanism of gallbladder stones. Some of the pathways were supported by subsequent LC/MS-based metabolomics analyses.

Effects of LD Interventions on Liver Metabolomic Profiles

To evaluate changes in liver metabolic profiles caused by different dietary interventions, we performed non-targeted metabolomics profiling of paired liver samples, and 1271 compounds were identified. PCA (R2X = 0.546) and PLS-DA (R2X = 0.57, R2Y = 0.965, Q2 = 0.679) were performed to characterize the global metabolomics differences between the two groups. The results showed that the liver metabolism of gallstone mice was significantly different compared to that of the control group (**Figures 4A,B**). Next, supervised orthogonal partial least squares analysis (OPLS-DA) was used to distinguish the overall differences in metabolic profiles among groups and find the metabolites that were different between groups. As shown, the T group and C group could be separated into distinct regions according to their metabolic differences [OPLS-DA models: R2Y = 0.992 and Q2(cum) = 0.799 (**Figure 4C**)]. To prevent overfitting of the model, sevenfold cross validation and 200 response permutation tests (RPTs) were used to evaluate the quality of the model (OPLS-DA validation models: R2Y = 0.908 and Q2 = −0.461), showing that the OPLS-DA model possessed a satisfactory fit with good predictive power. The Splot-OPLS-DA chart shows the most significant metabolites, including taurohyocholate, PE and PC (**Figure 4D**).

To discover the different metabolites of biological significance, a combination of multidimensional analysis and single-dimensional analysis was applied. Finally, a total of 138 significantly changed metabolites (VIP $>$ 1.0 and $p < 0.05$, **Supplementary Material 3**) were successfully identified between the two groups, as shown in the heatmap (**Figures 4E,F**). Further stratified analysis by metabolite categories showed that the 138 metabolites could be classified mainly into carboxylic acids and derivatives (31), steroids and steroid derivatives (24), organonitrogen compounds (17), glycerophospholipids (12), fatty acyls (12), pyrimidine nucleosides (9), purine nucleotides (7), benzene and substituted derivatives (6), organic sulfuric acids and derivatives (3), tetrapyrroles and derivatives (2), sphingolipids (2), other small molecules (11), and unclassified compounds (2). Compared with the C group, the levels of 57 metabolites increased in the T group. Among these metabolites, amino acids, peptides, and analog compounds increased in the T group, whereas bile acids, alcohols and derivative compounds decreased in the T group. We noticed that these metabolites included 3a,6b,7b-trihydroxy-5b-cholanoic acid, deoxycholic acid 3-glucuronide, chenodeoxycholic acid, nutritionolic acid, deoxycholic acid, tauro-b-muricholic acid, 2-propyl-2,4- pentadienoic acid, alpha-muricholic acid, glycerophosphocholine, galactonic acid, mesobilirubinogen, and N-alpha-acetyllysine. However, the concentration of primary bile acids (cholic acid, glycocholic acid, taurocholic acid) decreased in the T group. **Supplementary Material 3** shows all of the different metabolites.

To further clarify the changes in liver metabolic pathways in mice with gallstones, we performed KEGG functional enrichment analysis on the differential metabolites. We show the top 20 metabolic pathways that are most meaningful (**Figures 5A,B**). We noticed that ABC transporters, glycerophospholipid metabolism, primary bile acid biosynthesis, galactose metabolism and aminoacyl-tRNA biosynthesis and the FoxO signaling pathway are related to the formation of gallstones.

The Potential Link Between Intestinal Flora Imbalance and Liver Metabolism Changes Is Related to the Formation of Gallstones

To investigate the extent to which the altered gut microbiome was associated with liver metabolites in the host, we used Spearman's correlation analysis to determine the covariation between the differential intestinal flora (52) and top 100

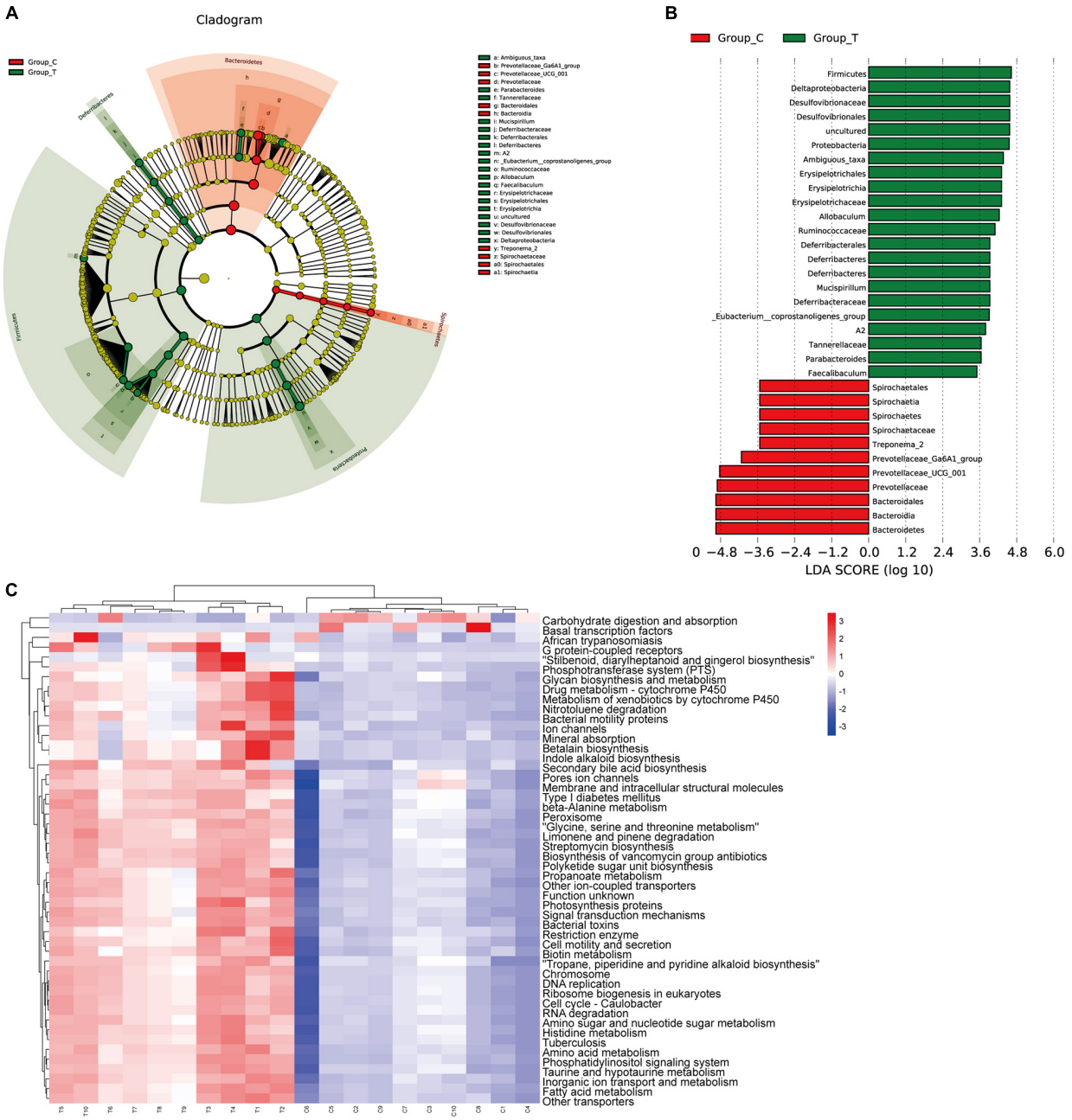

FIGURE 3 | LEfSE analysis and PICRUSt analysis results. **(A)** The LEfSe cladogram in green shows the taxa enriched in the T group, and that in red shows the taxa enriched in the C group. The diameter of nodes is directly proportional to the relative abundance. **(B)** Taxonomic cladogram obtained using linear discriminant analysis (LDA) effect size (LEfSe) analysis and Mann–Whitney *U*-tests of the 16S sequences. LEfSe identified the taxa with the greatest differences in abundance between the T group and C group. At the phylum, class, order, family and genus levels, the control-enriched taxa are indicated by a positive LDA score (red), and T group-enriched taxa are indicated by a negative score (green). Only taxa meeting a significant LDA threshold value of > 2 are shown. **(C)** PICRUSt analysis results of predicted functional pathways in the gut microbiota.

metabolites (**Supplementary Material 4**), which is presented in a heatmap (**Figure 6A**). A significant correlation (Student's *t*-test, $p < 0.05$, $|$ correlation coefficient$| > 0.3$) was identified between the changes in the gut microbiome and liver metabolite profiles. We focused on metabolites that are closely related to the formation of gallstones, including deoxycholic acid, asymmetric dimethylarginine, glucosamine and tauroursodeoxycholic acid. Deoxycholic acid (DCA) is elevated in the livers of mice in group T and is positively correlated with certain bacterial genera (Clostridium_sp, Allobaculum, Enterorhabdus, Faecalibaculum, Bifidobacterium, [Eubacterium]_coprostanoligenes_group, [Acetivibrio]_ethanolgignens_group, A2, Candreatus_Sobaclea).

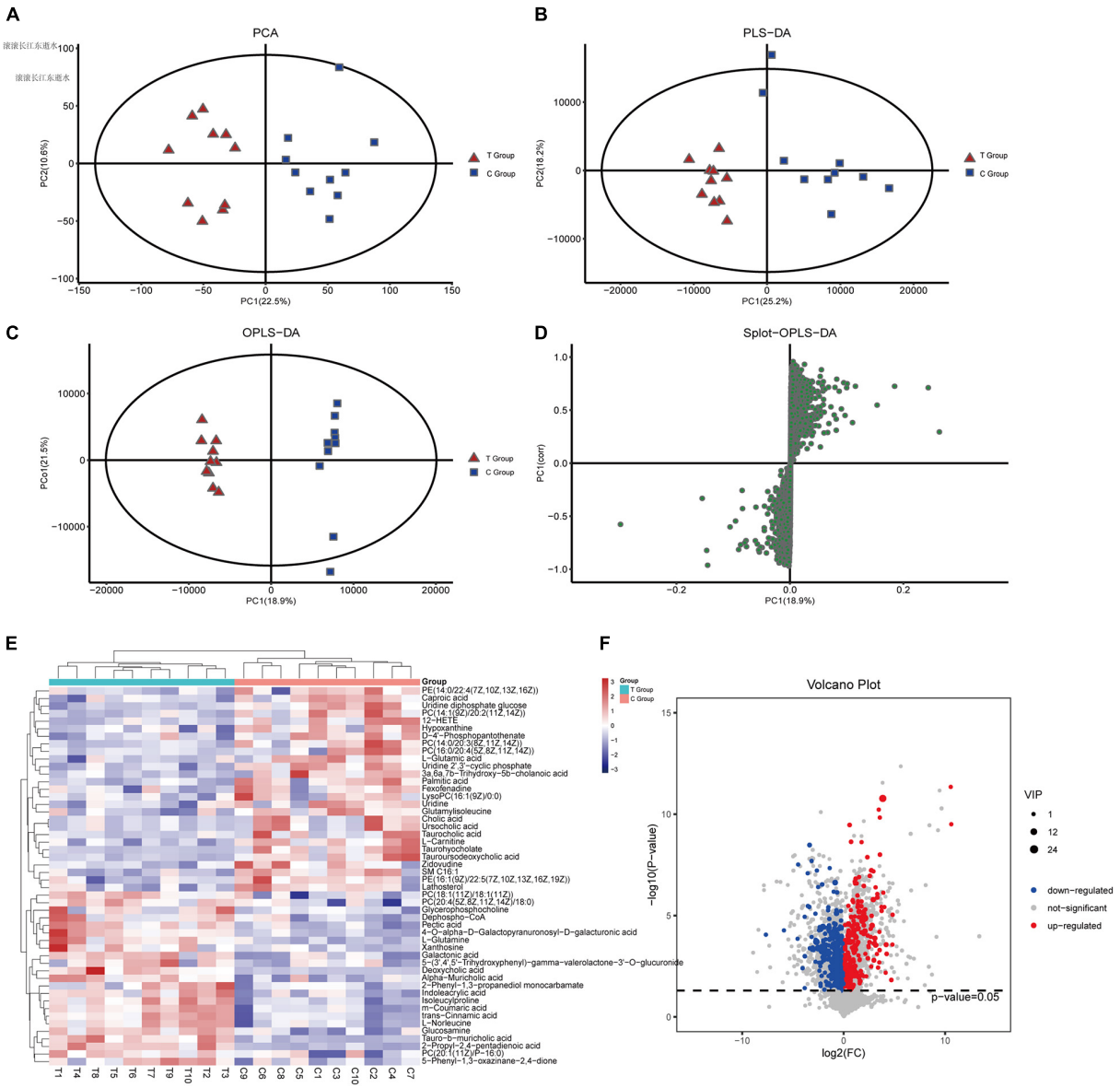

FIGURE 4 | Effects of dietary interventions on liver metabolomic profiles. **(A)** PCA score plot of T and C groups. **(B)** PLS-DA score plot of T and C groups. **(C)** OPLS-DA score plot of T and C groups. **(D)** Plot based on PLS-DA score. **(E,F)** Significantly different metabolites and the expression levels of the top 50 differentially expressed metabolites with VIP values were analyzed by hierarchical clustering (VIP $>$ 1, $p < 0.05$).

However, there was a negative correlation with other bacterial genera (Ileibacterium, Prevotellaceae_Ga6A1_group, Erysipelotrichaceae_UCG-003, Hemophilus, Tyzzerella_3, uncultured_rumen_bacterium) (**Figure 6B**). Asymmetric dimethylarginine increased in the T group and had a positive correlation with Family_XIII_UCG-001. Family_XIII_UCG-001 also increased significantly in the gallstone group (**Figure 6A**). Glucosamine has a positive correlation with A2 Intestinimonas, Ruminiclostridium, Oscillibacter, Bifidobacterium, Tyzzerella, GCA-900066225, Ruminococcaceae_UCG-009, [Ruminococcus]_torques_group, Coprococcus_2, Harryflintia, and Candidatus_Soleaferrea. However, it had a negative correlation with Prevotellaceae_UCG-001 and Prevotellaceae_Ga6A1_group (**Figure 6C**). Tauroursodeoxycholic acid was significantly reduced in mice with gallstones. TUDCA has a negative correlation with Muribaculum, Treponema_2, [Bacteroides]_pectinophilus, and Tyzzerella_3. Turicibacter, Tyzzerella, [Ruminococcus]_torques_group and TUDCA were negatively correlated and were significantly increased in the gallstone group (**Figure 6D**). **Supplementary Material 5** shows the correlation analysis between different species and different metabolites at the species level. In general, these results indicate that changes in intestinal flora are related to changes in liver metabolites. We speculate that the interaction

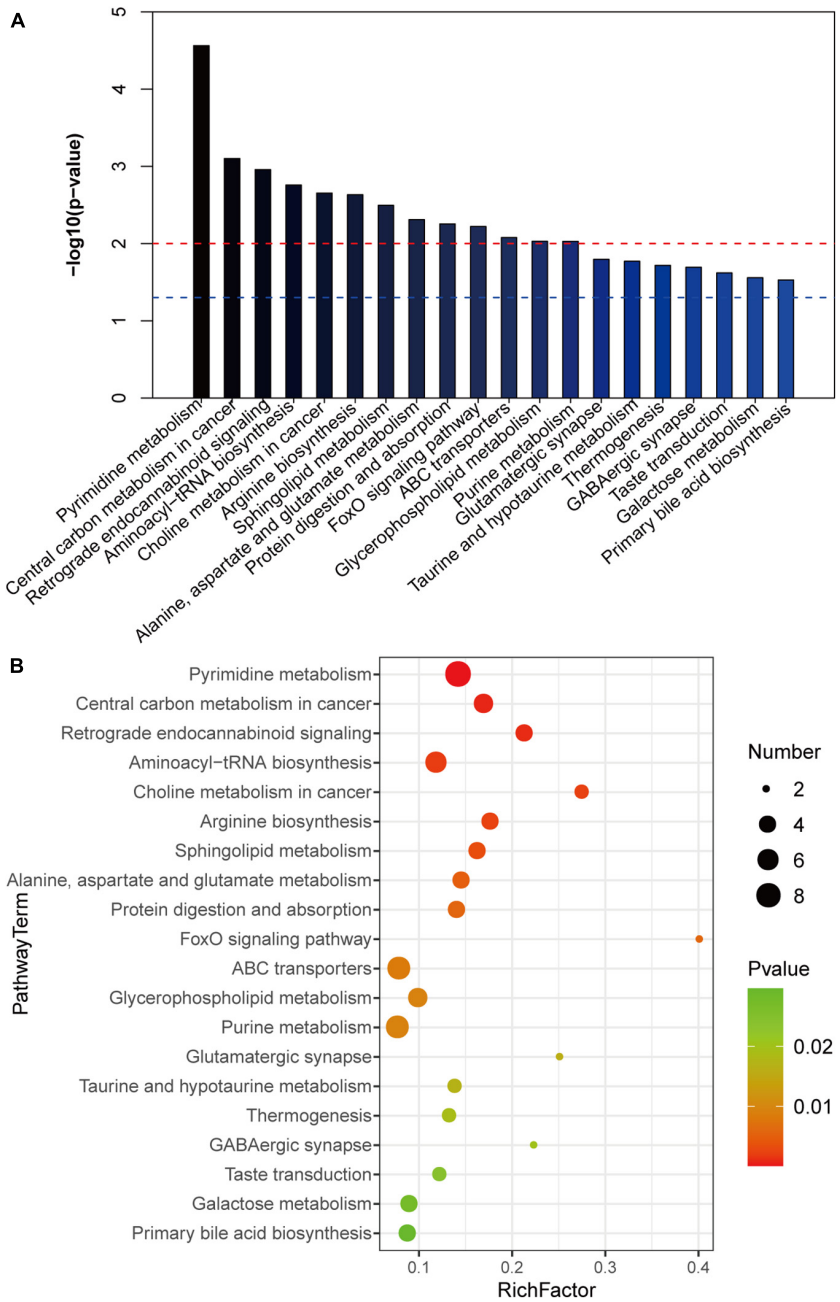

FIGURE 5 | KEGG analysis of differential metabolites. **(A,B)** The top 20 metabolic pathways with the highest enrichment of differential metabolites ($p < 0.05$).

between these two factors may be an important target for driving or preventing gallbladder stones.

DISCUSSION

Numerous studies have demonstrated that the gut microbiome and its metabolites are widely involved in communication between the gut and the liver. Due to liver and intestinal flora coparticipation in the process by which bile acid and lipid metabolism regulate host immunity, the gut-liver axis has been found to be involved in the pathogenesis of liver and gall diseases such as non-alcoholic fatty liver disease (Boursier et al., 2016), primary biliary cholangitis (Tang et al., 2018), and liver cancer (Ma et al., 2018). The intestinal flora are associated with susceptibility to gallstone diseases (Wang et al., 2017, 2020). Our research results show that the intestinal flora of gallstone mice is seriously dysregulated. This finding is similar to previous studies investigating the gut microbiota of both gallstone patients (Wu et al., 2013; Wang et al., 2020) and mice (Fremont-Rahl et al.,

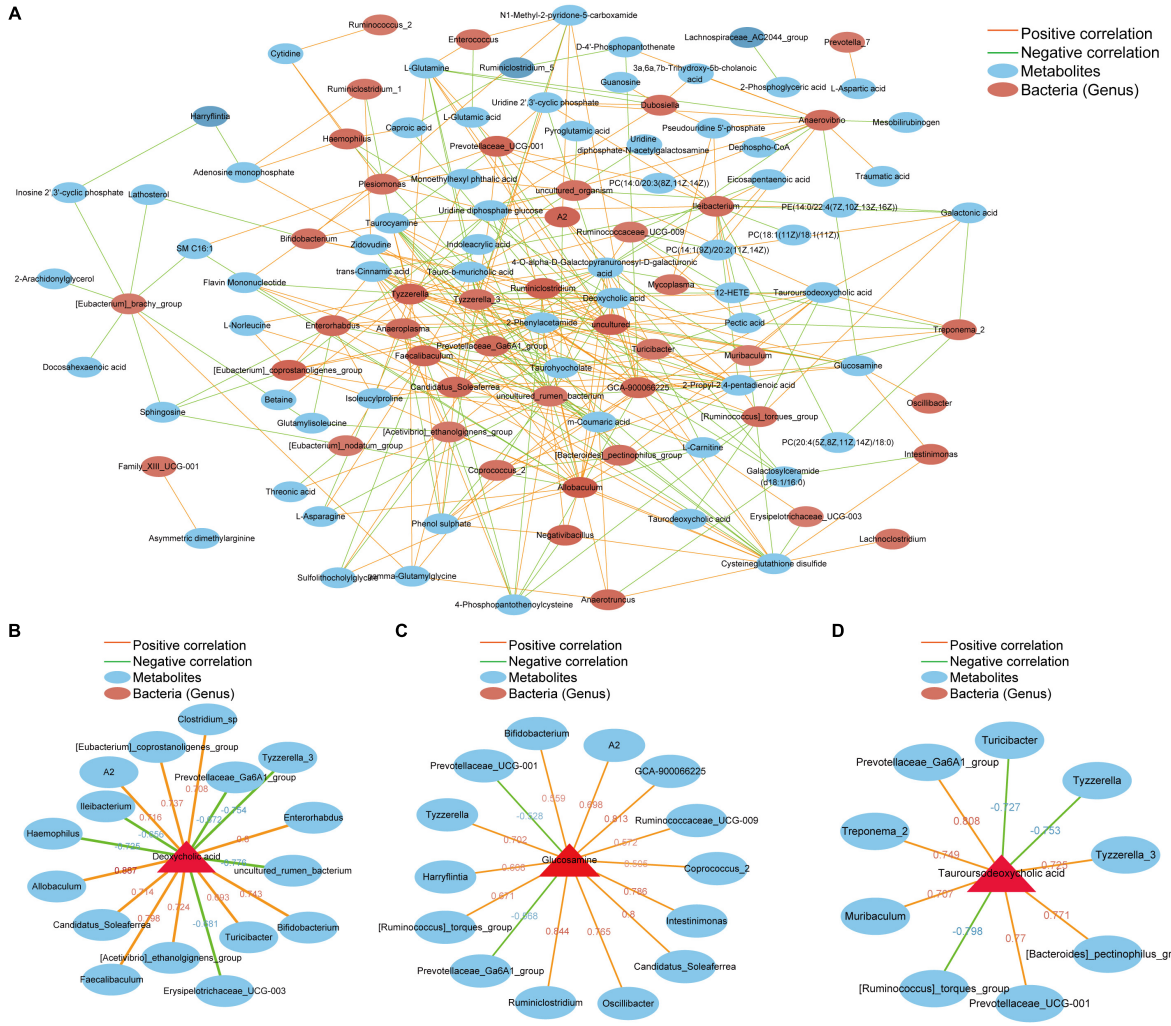

FIGURE 6 | Potential correlations between the gut microbiota and liver metabolites. The red line represents a positive correlation, and the green line represents a negative correlation. The thickness of the line represents the correlation coefficient. **(A)** Correlation between 52 different intestinal bacterial genera and the top 100 metabolites. **(B)** Bacteria that are positively or negatively related to deoxycholic acid. **(C)** Bacteria that are positively or negatively related to glucosamine. **(D)** Bacteria that are positively or negatively related to taurodeoxycholic acid. (Student's *t*-test, $p < 0.05$, Spearman correlation analysis, | correlation coefficient| ≥ 0.6).

2013). The results of the study showed that liver metabolism in mice with gallstones was also severely disturbed. We conducted a detailed analysis of these two factors.

In our study, deoxycholic acid (DCA) was significantly higher in the T group mice than in the C group mice. Studies have shown that DCA has a positive correlation with an increased prevalence of cholesterol gallstones (Thomas et al., 2005). DCA, as an FXR agonist (Wahlstrom et al., 2016), increases human gallbladder cholesterol saturation and bile acid hydrophobicity, and both decrease cholesterol solubility in bile and increase the risk of gallstone formation (Heaton, 2000). Members of the genus Clostridium show 7α-dehydroxylation enzymatic activity, which can convert host primary bile acids into secondary bile acids such as DCA (Berr et al., 1996). In our study, the abundance of Clostridium sp. in the T group was higher than that in the C group, and the abundance was positively correlated with DCA. Several studies have reported that patients or mice with gallstones have an increased relative abundance of the genus Clostridium (Wells et al., 2000; Wang et al., 2017). In addition, we found that DCA interacts and has a positive correlation with Allobaculum, Enterorhabdus, Faecalibaculum, Bifidobacterium, [Eubacterium]_coprostanoligenes_group, [Acetivibrio]_ethanolgignens_group, A2, Candidatus_Soleafer rea and Turicibacter but a negative correlation with Ileibacterium, Prevotellaceae_Ga6A1_group, Erysipelotricha ceae_UCG-003, Hemophilus, Tyzzerella_3, and uncultured_rumen_bacterium. Interestingly, as a probiotic, Bifidobacterium plays a therapeutic role in many diseases. Studies have shown that Bifidobacterium exerts a serum cholesterol-lowering effect and prevents hypercholesterolemia (Jones et al., 2013; Urdaneta and Casadesus, 2017). However, our study found that there was a significant increase in

bifidobacteria in the gut of mice with gallstones. It is suggested that excessive probiotics (Bifidobacterium) may be related to the formation of gallstones. In fact, Clostridium, Bifidobacterium, and Enterorhabdus are involved in the metabolism of bile acids, and excess bile acids in the intestine disrupt enterohepatic circulation and may be involved in the formation of gallstones (Grigor'eva and Romanova, 2020).

Asymmetric dimethylarginine (ADMA), the methylated derivative of L-arginine, is a differential metabolite highly expressed in the T group. Increasing evidence has shown that the serum ADMA level has a positive correlation with insulin resistance (IR). Hepatic IR was found to be an independent risk factor for GSD (Biddinger et al., 2008; Chang et al., 2008); therefore, we infer that ADMA plays a promoting role in the process of gallstone development. ADMA is an endogenous inhibitor of nitric oxide (NO) synthetase, and endothelial dysfunction due to reduced bioavailability of nitric oxide (NO) is a risk factor for atherogenesis (Qin et al., 2021), myocardial infarction (Mannino et al., 2019), and chronic kidney disease (Shah et al., 2021). At the same time, studies have shown that GB hypomotility plays a key role in gallbladder (GB) normal relaxation (Chen et al., 1997; Swartz-Basile et al., 2000), while GB hypomotility is also a required factor for gallstone pathogenesis. Therefore, whether ADMA is friend or foe of gallstone formation requires further testing. In this study, ADMA was positively correlated with Family_XIII_UCG-001. Little information in the literature on Family XIII UCG-001 has shown that it is correlated with depression-like behavior in mice (Tian et al., 2019). The relationship between Family XIII UCG-001 and hepatic IR needs to be further tested.

Our study showed that glucosamine was a significantly elevated differential metabolite in the T group. Glucosamine is a precursor in the synthesis of mucin (Feller et al., 1990; Tailford et al., 2015). Mucin is well known to protect intestinal mucosal barrier function in the intestine, and abnormal mucin secretion appears in many tumor diseases and serves as a marker (Grondin et al., 2020; Paone and Cani, 2020). Previous studies have shown that mucin-4 is involved in gallstone formation (Di Ciaula et al., 2018; Hu et al., 2021). Specific bacteria and microbial products such as LPS can stimulate mucin secretion (Paone and Cani, 2020). Our research found that A2, Intestinimonas, Ruminiclostridium Oscillibacter, Bifidobacterium, Tyzzerella, GCA-900066225, Ruminococcaceae_UCG-009, [Ruminococcus]_torques_group, Coprococcus_2, Harryflintia, and Soleaferrea_idatus had a positive correlation with glucosamine. These bacterial genera are significantly increased in the gut of mice with gallstones. We speculate that these bacterial disorders may increase the production of glucosamine in the liver, thus creating conditions for the synthesis of mucin. This will undoubtedly accelerate the formation of gallstones. Interestingly, we found that two genera (Prevotellaceae_UCG-001 and Prevotellaceae_Ga6A1_group) had a negative correlation with glucosamine. They are all from the family Prevotellaceae and are reduced in the gallstone group. This suggests that the decreases in Prevotellaceae_UCG-001 and Prevotellaceae_Ga6A1_group may be related to the increase in glucosamine. These genera may have the potential to act as probiotics to prevent gallstones. Studies suggest that Tyzzerella accumulates in the intestines of obese children (Jaimes et al., 2021) and hyperlipidemic rats (Zhang et al., 2020). We hypothesized that intestinal flora are involved in the synthesis of mucin precursors, thereby affecting gallstone formation.

Tauroursodeoxycholic acid (TUDCA), which is a hydrophilic bile acid that inhibits intestinal inflammation, improves intestinal barrier function, and reduces the inflammation of the liver caused by LPS in blood, was significantly elevated in the C group (Wang et al., 2018). TUDCA can reduce the formation of gallstones by improving intestinal flora disorder in HFD-fed mice and inhibiting lipid absorption, intestinal cholesterol absorption and synthesis in the small intestine (Lu et al., 2020). Our results show a positive association between TUDCA and Prevotellaceae_UCG-001 (**Figure 6D**). A study showed that Prevotellaceae_UCG-001 was significantly enriched after inulin treatment, which helped to improve glucose and lipid metabolism (Song et al., 2019). Prevotellaceae, as a potential probiotic genera, is believed to be associated with the synthesis of short-chain fatty acids (SCFAs) (Xie et al., 2020; Zhu et al., 2020), which are important fuel for intestinal epithelial cells and are known to strengthen gut barrier function (Parada Venegas et al., 2019). The lack of SCFAs weakens their protective effect on the intestinal mucosal barrier, which may lead to increased levels of enterogenous endotoxin, further proving that the reduction in SCFA-producing bacteria may play an important role in the pathogenesis of NAFLD (Shen et al., 2017). Previous studies have shown that abnormal intestinal mucosal barrier function probably induces the formation of gallstones through a bacterial translocation mechanism (Su et al., 2009). We hypothesized that Prevotellaceae may play a protective role in inhibiting the formation of gallstones by regulating bile acid composition and protecting the intestinal barrier. Whether Prevotellaceae can be used as a non-invasive observation index for the detection of litholytic drugs remains to be further verified. In addition, we found that TUDCA had a negative correlation with Muribaculum, Treponema_2, [Bacteroides]_pectinophilus, and Tyzzerella_3. These bacteria were reduced in the gallstone group, and whether they can affect TUDCA as a target for preventing gallstones needs further exploration. Turicibacter, Tyzzerella, [Ruminococcus]_torques_group and TUDCA were negatively correlated, and they were significantly increased in the gallstone group. These bacteria may cause a decrease in TUDCA.

We noticed that the function of the differential flora was consistent with the metabolic pathway enriched by the liver's differential metabolites, for example, taurine and hypotaurine metabolism, beta-alanine metabolism, pantothenate and CoA biosynthesis, D-glutamine and D-glutamate metabolism, purine metabolism, pyrimidine metabolism, glycerophospholipid metabolism, primary bile acid biosynthesis, alanine, aspartate and glutamate metabolism, glutamatergic synapse, aminoacyl metabolism, tRNA biosynthesis, ABC transporters, galactose metabolism, pentose and glucuronate interconversions. All of the pathways were supported by subsequent LC/MS-based metabolomics analyses. This suggests that the disordered flora caused liver metabolic disorders through these metabolic pathways, leading to gallstone formation.

CONCLUSION

In summary, this study confirmed that mice with gallstones have severe intestinal flora imbalance, and these disordered flora will not recover without intervention. This becomes an important driving factor for the formation of gallstones. Through our research, biomarkers of gallstones were identified at the phylum (5), class (5), order (5), family (7), and genus levels. We predicted the functions of the differential flora, which provides an important reference for further intervention in the disease from the perspective of the flora. At the same time, we analyzed the liver metabolism of gallstone mice paired with their flora, and the results showed that there were 138 different metabolites between the two groups. We performed KEGG enrichment analysis on differential metabolites, and we were surprised to find that the metabolic pathways enriched by these differential metabolites were highly consistent with the function of the disordered flora. Therefore, we conducted an association analysis of the two factors, and we constructed a network of relationships between disordered bacteria and disordered liver metabolites. This provides a basis for the establishment of the intestine-liver axis in gallstone disease. Furthermore, this research provides a theoretical basis for the research and development of probiotics and prebiotics. We focused on the analysis of the relationship between deoxycholic acid, asymmetric dimethylarginine, glucosamine, tauroursodeoxycholic acid, and the disordered flora. It is important that DCA is a contributing factor to the formation of gallstones, but Bifidobacterium, a well-known probiotic, also has a positive correlation and increases significantly in the gallstone group. The Family XIII UCG-001-ADMA axis may promote the occurrence and progression of gallstones. In the pathogenesis of gallstones, glucosamine is a risk factor that increases in the gallstone group, while TUDCA is a protective factor that decreases in the gallstone group. Interestingly, we found that Prevotellaceae decreased in the gallstone group and had a positive correlation with glucosamine but a negative correlation with TUDCA. Therefore, we speculate that Prevotellaceae can be used as a probiotic to treat gallstones.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the NCBI BioProject accession number is: PRJNA736820 and in metabolights accession number is: MTBLS2945.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Harbin Medical University.

AUTHOR CONTRIBUTIONS

YC and QW participated in the analysis of data, writing of the manuscript, and specimen collection. WG and BM participated in data collection and analysis. DX and CH participated in the study conception and supervision and data analysis and manuscript editing. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

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Supplementary Figure 1 | Changes in gut microbiota diversity and composition after dietary intervention. **(A)** The CHAO1 index of gallstone model mice was significantly reduced. **(B)** The Good's coverage index of gallstone model mice increased significantly. **(C)** The Specaccum species accumulation curve tends to gradually flatten, indicating that samples are sufficient, and its samples can reflect the richness of species. **(D)** Rank abundance curve reflects the abundance and uniformity of species in samples.

Supplementary Figure 2 | Beta diversity is the degree of diversity between biological environments. **(A)** According to the distance matrix obtained by the Bray-Curtis distance algorithm, the matrix is clustered hierarchically, and the distance of the sample branches can be clearly seen. The bluer the color, the closer the distance between the samples, and the higher the similarity; the redder the color, the further the distance. **(B)** NMDS analysis. The abscissa (NMDS1) and the ordinate (NMDS2) are two sorting axes. Each point in the figure represents a sample. The same color is the same group. Similar samples will gather together. If the difference between the samples is large, the distance is far away. **(C)** UPGMA repeated sampling reliability analysis. The red part represents a reliability of 75–100%; the yellow part is 50–75%; the green part is 25–50%; the blue part is less than 25%. **(D)** PCA: The more similar the sample composition, the closer the distance reflected in the PCA chart; the more different the sample composition, the farther the distance reflected in the PCA chart. Based on the analysis results of the Bray-Curtis distance matrix, PCoA can be used to observe the differences between individuals or groups. Each point in the figure represents a sample. The same color is the same group. If the samples of the same group are closer together and further away from other groups, this shows that the grouping effect is good. **(F)** Sample hierarchical cluster analysis: The closer the branch distance, the more similar the samples.

Supplementary Table 1 | An excel sheet of all differential metabolites detected in liver data (Wilcoxon rank-sum test, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$).

Supplementary Table 2 | Data table of the relationship between sequencing and differential metabolites (Wilcoxon rank-sum test, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$; Spearman correlation analysis, $|\text{correlation coefficient}| \geq 0.6$).

Supplementary Table 3 | Correlation analysis between different species and different metabolites at the species level (Wilcoxon rank-sum test, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$; Spearman correlation analysis, $|\text{correlation coefficient}| \geq 0.6$).

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REVIEW
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Dysbiosis, Host Metabolism, and Non-communicable Diseases: Trialogue in the Inborn Errors of Metabolism

Chiara Montanari[1†], Sara Parolisi[2†], Elisa Borghi[3], Lorenza Putignani[4], Giulia Bassanini[3], Juri Zuvadelli[5], Cristina Bonfanti[6], Albina Tummolo[7], Carlo Dionisi Vici[8], Giacomo Biasucci[9], Alberto Burlina[10], Maria Teresa Carbone[2‡] and Elvira Verduci[1,3‡] on behalf of SIMMESN Working group for gut microbiota in inborn errors of metabolism*

[1]Department of Pediatrics, Vittore Buzzi Children's Hospital, University of Milan, Milan, Italy, [2]UOS Metabolic and Rare Diseases, AORN Santobono, Naples, Italy, [3]Department of Health Science, University of Milan, Milan, Italy, [4]Department of Diagnostic and Laboratory Medicine, Unit of Microbiology and Diagnostic Immunology, Unit of Microbiomics and Multimodal Laboratory Medicine Research Area, Unit of Human Microbiome, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy, [5]Clinical Department of Pediatrics, ASST Santi Paolo e Carlo, San Paolo Hospital, University of Milan, Milan, Italy, [6]Rare Metabolic Disease Unit, Pediatric Department, Fondazione MBBM, San Gerardo Hospital, Monza, Italy, [7]Metabolic Diseases and Clinical Genetics Unit, Children's Hospital Giovanni XXIII, Bari, Italy, [8]Division of Metabolism, Bambino Gesù Children's Hospital, Rome, Italy, [9]Department of Paediatrics & Neonatology, Guglielmo da Saliceto Hospital, Piacenza, Italy, [10]Division of Inborn Metabolic Diseases, Department of Diagnostic Services, University Hospital of Padua, Padua, Italy

Inborn errors of metabolism (IEMs) represent a complex system model, in need of a shift of approach exploring the main factors mediating the regulation of the system, internal or external and overcoming the traditional concept of biochemical and genetic defects. In this context, among the established factors influencing the metabolic flux, i.e., diet, lifestyle, antibiotics, xenobiotics, infectious agents, also the individual gut microbiota should be considered. A healthy gut microbiota contributes in maintaining human health by providing unique metabolic functions to the human host. Many patients with IEMs are on special diets, the main treatment for these diseases. Hence, IEMs represent a good model to evaluate how specific dietary patterns, in terms of macronutrients composition and quality of nutrients, can be related to a characteristic microbiota associated with a specific clinical phenotype ("enterophenotype"). In the present review, we aim at reporting the possible links existing between dysbiosis, a condition reported in IEMs patients, and a pro-inflammatory status, through an altered "gut-liver" cross-talk network and a major oxidative stress, with a repercussion on the health status of the patient, increasing the risk of non-communicable diseases (NCDs). On this basis, more attention should be paid to the nutritional status assessment and the clinical and biochemical signs of possible onset of comorbidities, with the goal of improving the long-term wellbeing in IEMs. A balanced intestinal ecosystem has been shown to positively contribute to patient health and its perturbation may influence the clinical spectrum of individuals with IEMs. For this, reaching eubiosis through the improvement of the quality of dietary products and mixtures, the use of pre-, pro- and postbiotics, could represent both a preventive and therapeutic strategy in these complex diseases.

Keywords: microbiota, inborn errors of metabolism, diet, gut-liver axis, non-communicable diseases, enterophenotype

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Suman K. Ray, Independent Researcher, India

***Correspondence:**
Elisa Borghi
elisa.borghi@unimi.it

[†]These authors have contributed equally to this work and share first authorship

[‡]These authors have contributed equally to this work and share last authorship

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INTRODUCTION

The gut microbiota takes an active part in the flux and in the disease phenotype (Putignani et al., 2019) and can be influenced, especially in pediatric life, by many factors, including genetic variation, diet, lifestyle, antibiotics, xenobiotics, and infectious agents (Putignani et al., 2014; Lynch and Pedersen, 2016). The host-associated gut microbiota, which is composed of around 10–100 trillion microorganisms (Turnbaugh et al., 2007), contributes to maintaining human health by providing unique metabolic functions to the human host (Zhang et al., 2000). Functional maturation of the gut microbiota takes place during the first 3 years of life (Han, 2014; Lynch and Pedersen, 2016) and plays an important role in extracting energy from food fibers and complex carbohydrates that are not digested in the upper portion of the human digestive tract (Gill et al., 2006). The gut microbiota is also involved in stimulating and promoting the maturation of the immune system and in facilitating the resistance against pathogens (Martin et al., 2013; Sommer and Bäckhed, 2013; Lynch and Pedersen, 2016). It interacts with the host by influencing immunity, metabolism, health status (Tremaroli and Backhed, 2012) and the association between a wide range of diseases and dysbiosis has long been well known (Guinane and Cotter, 2013).

Inborn errors of metabolism (IEMs) represent a complex system model, mainly due to genetic mutations affecting a single gene but which are also influenced by different determinants, both internal and external, contributing to the disease phenotype (McCabe, 2019). External factors such as diet, lifestyle, and antibiotics are the main environmental constraints regulating human metabolism.

A novel-challenge to approach IEMs is to consider the metabolic flux as part of a dynamic system, moving from a static concept of biochemical and genetic defects to a novel way. The key point is to evaluate the metabolic flux in a real-time manner to assess the actual metabolome dynamics. Omics sciences (e.g., genomics, metagenomics, transcriptomics, proteomics, metaproteomics, metabolomics, meta-metabolomics, and interactomics) allow to stratify in a system biology approach, related-multi-omics data within layers describing the entire process of disease phenotyping (Lanpher et al., 2006; Putignani et al., 2016).

The cornerstone therapy of many IEMs is dietary treatment, based on strict regimens that must be maintained lifelong, therefore making this disease category an ideal model to evaluate how specific dietary patterns can be related to a characteristic hallmark of the microbiota ("enterophenotype"). Interestingly, recent studies have shown a high rate in the variability of the intestinal ecosystem in relation to different dietary patterns (Putignani and Dallapiccola, 2016), making the use of advanced technologies applied to microbiota study an effective approach to define the "core" bacteria profiles associated with different dietary patterns (Harmsen et al., 2000; Zimmer et al., 2012). Diets excessively rich or poor in some nutrients, a common characteristic of dietary intervention in several IEMs, can promote a possible state of dysbiosis with potential systemic effects (Colonetti et al., 2018). Considering that the gut microbiota may influence some specific clinical phenotypes, this paper aims at exploring the possible links between IEMs, with external modulating factors, with the gut microbial community, and the health status of affected individuals (Reijngoud, 2018), also in relation to the eventual presence of non-communicable diseases (NCDs). Moreover, we want to discuss the potential contribution of gut microbiota in the challenging research of new treatments for these complex diseases.

DIETARY TREATMENT IN IEMS AND GUT MICROBIAL PROFILE

Few studies available in the current literature report the composition of gut microbiota in IEMs, in particular in phenylketonuria (PKU) and glycogen storage disease (GSD) in relation to diet composition.

In PKU (OMIM261600), an inherited metabolic disease determined by the impaired activity of the enzyme phenylalanine hydroxylase, with a consequent phenylalanine (Phe) accumulation in the blood, a special "low phe intake" diet should be started as early as possible in the first weeks of life to hamper neurological damages (Singh et al., 2014; van Spronsen et al., 2017). Dietary treatment consists of three pillars: (i) natural protein restriction, (ii) Phe-free-L-amino acid supplements, and (iii) low protein foods. Indeed, the special low protein products, which are variants/substitutes of some common "natural" foods, such as bread pasta), are rich in sugars to ameliorate the palatability, determining a daily glycemic index and glycemic load in PKU patients higher than the normal values (Moretti et al., 2017). Phe-restricted diet shows similarity with the Mediterranean diet -high vegetable and fiber intake- that has been reported to promote growth of beneficial bacteria and the increase of their derived metabolites (Tosti et al., 2018).

However, the quality of carbohydrates intake is a key factor in determining the gut microbial composition and the short-chain fatty acid (SCFA) production, since different bacteria possess different metabolic pathways to break down sugars (Hamaker and Tuncil, 2014). To date, few studies have analyzed the intestinal microbiota in subjects with PKU, and data are summarized in **Supplementary Table S1** in supplemental materials.

Verduci et al. (2018) highlighted in PKU children a low gut microbial biodiversity, generally considered the first distinctive sign of intestinal dysbiosis (Mosca et al., 2016). Moreover, shifts in microbiota composition lead to a different microbial metabolite production, mainly affecting the SCFAs profile (Scott et al., 2013). The most relevant differences between PKU and mild hyperphenylalaninemia (MHP) children microbiota concern the Firmicutes phylum, with a consequent reduction of total fecal SCFAs and butyrate. Furthermore, the PKU gut microbiota was enriched in the genera *Blautia* and *Clostridium* and depleted in the genus *Faecalibacterium* as well as in *Lactobacillus* spp. (Pinheiro de Oliveira et al., 2016; Bassanini et al., 2019). Also in adult PKU patients, the gut microbiota, recently investigated by Mancilla et al. (2021), showed a similar pattern with an enrichment in *Clostridium* and a depletion in *Faecalibacterium*, but with a less pronounced abundance of *Blautia* spp.

Glycogen storage diseases are hereditary metabolic disorders caused by the deficiency of enzymes involved in glycogen metabolism. In particular, GSD Ia and Ib, are hepatic GSDs due to defects in the glucose-6-phosphatase complex, sharing hepatomegaly and fasting hypoglycemia as the main clinical abnormalities. The aim of treatment is to prevent hypoglycemia and secondary metabolic complications (Kishnani et al., 2014). Dietary therapy is based on frequent meals, with a high intake of complex carbohydrates, restriction of sugars, and a lower amount of lipids compared to the general population. Part of carbohydrates is supplied by uncooked cornstarch (UCCS) that patients take at regular intervals several times a day, and also during the night, to maintain euglycemia (Heller et al., 2008; Kishnani et al., 2014). Given the peculiarity of the GSD-I diet, an impact on gut microbiota can be expected. Indeed, a GSD-linked gut dysbiosis has been recently reported in two works, summarized in **Supplementary Table S2** in supplemental materials. The main reported characteristics in GSD patients are a reduction in intestinal microbiota richness and differences in taxa relative abundances compared with the control group, with a dramatic increase in Proteobacteria at the expense of Firmicutes and Bacteroidetes, the two main phyla generally colonizing healthy subjects' gut microbiota (Colonetti et al., 2019; Ceccarani et al., 2020). Furthermore, the beneficial genera *Faecalibacterium* and *Oscillospira* were significantly reduced, while the Proteobacteria abundance was characterized by a high presence of *Enterobacteriaceae* family, within which *Escherichia* spp. was found significantly increased, with a pro-inflammatory activity capable of further triggering intestinal imbalance. At metabolic level, SCFAs quantification showed a significant increase of fecal acetate and propionate in GSD subjects (Ceccarani et al., 2020). Based on these findings, gut dysbiosis might contribute to several comorbidities of the GSD phenotype, including obesity, inflammatory bowel disease (IBD), and chronic liver disease. Other GSD patients, like GSD-III patients, may require a hyperproteic and hyperlipidic diet, similar to a ketogenic diet, with a lower use of UCCS. However, no sufficient data about the microbiota composition are available neither in GSD-III patients nor in other GSDs types (Ceccarani et al., 2020).

MANAGEMENT-RELATED FACTORS THAT (POTENTIALLY) AFFECT THE GUT MICROBIOTA IN IEMS

As previously described, the bioavailability of dietary substrates affects the gut microbial composition and leads to a different production of microbial metabolites (Verduci et al., 2018). Beyond the dietary pattern, the type and the quality of specific nutrients used in the treatment of IEMs can be a potential influencing factor able to further modify the gut microbiota composition.

Starting from the few available studies, we have summarized in **Table 1** the possible alterations of the gut microbiota related to (i) the use of special dietary products, such as low-protein foods (LPs; Wang et al., 2019) and protein substitutes (Davila et al., 2013; Sawin et al., 2015; Lin et al., 2017; van Wegberg, 2017; Yang and Liao, 2019), (ii) the use of specific nutrients, such as UCCS (Kishnani et al., 2014; Colonetti et al., 2019), medium chain triglycerides (MCTs; Bach and Babayan, 1982; Decuypere and Dierick, 2003; Das et al., 2010; Zentek et al., 2013), and long-chain polyunsaturated fatty acids (LC-PUFAs; Pinheiro de Oliveira et al., 2016; Pu et al., 2016; Costantini et al., 2017; Watson et al., 2018; Bassanini et al., 2019), or (iii) the exclusion/reduction of specific nutrients, as breast milk (Coppa et al., 2006; MacDonald et al., 2006; Hascoet et al., 2011; Videhult and West, 2016) and (iv) the utilization of special nutrition mode, as tube feeding (O'Keefe, 2010; Takeshita et al., 2011; Burlina et al., 2018).

Chronic use of drugs in IEM patients may represent another important factor influencing the composition of the gut microbiota. The disease itself, or specific comorbidities related or not to metabolic control, can require pharmacological treatment. It is documented that drug-induced shifts in the composition of gut microbiota can be also associated with disease progression (Sayers et al., 2018). To date, many studies investigated the role of drugs, especially antibiotics, on gut microbiota but few data have been collected on this aspect in IEMs. We summarized in **Table 2** the possible microbiota profile modifications in relation to the use of specific medications, as allopurinol (Kishnani et al., 2014; Yu et al., 2018; Ceccarani et al., 2020), antibiotics (metronidazole; Baumgartner et al., 2014; Jakobsson et al., 2010) and the supplementation with L-carnitine (Tang et al., 2013; Wilson et al., 2016; Chen et al., 2019; Vernocchi et al., 2020).

THE ROLE OF GUT IMMUNE SYSTEM IN INFLAMMATION

A dynamic interplay between the host and his gut microbiota seems to be a key point to achieve and maintain the immune homeostasis. Providing pivotal molecular signals through microbial surface antigens and host metabolites, a consequent maturation of immune tissues and a coordinated immune response take place (Rooks and Garrett, 2016). A functional characterization of microbiome is nowadays possible by means of the high-throughput DNA sequencing technologies. Indeed, these methods have paved the way for many descriptive studies on microbial ecology and have better investigated the interaction between microbial metabolism and host development. Since the gut microbiota plays a crucial role in the host immune system, alterations in its composition and function can trigger a large number of physiological processes, such as low-grade inflammation and excess lipid accumulation, contributing to the development of metabolic perturbations. The patho-mechanisms of inflammatory diseases are still under intense investigation but it seems clear that gut microbiota changes could be involved in the onset and can influence the severity of disease, through a different crosstalk between the host and its microbiota (Webb et al., 2016). Recently, the role of single species in regulating inflammation stage and metabolic disfunctions has been based on complete genome sequencing

TABLE 1 | Potential effects of dietary factors on gut microbiota in IEMs.

Dietary factors	Potential effects on microbiota
(i) Special dietary products	
Low-protein foods (LPs)	Low protein foods are starches that constitute the major intake of carbohydrates in patients with amino acid metabolism diseases:
	– enriched in sugars: ↑ glycemic Index and glycemic load → reduction of SCFAs production inm phenylketonuria (PKU) patients (Moretti et al., 2017; Verduci et al., 2018);
	– variable proportion of amylose and amylopectin (not known) and a variable content of soluble fiber. Starches with a high amylose content and soluble fiber could increase the production of SCFAs (Wang et al., 2019).
Protein substitutes	In patients with aminoacidopathies, most of the protein requirement is guaranteed by free amino acids (free-AAs) formulas. AAs act as a supplemental substrate for SCFA production by the gut microbiota; AAs metabolites impact the gut epithelia physiology, both in a beneficial (e.g., butyrate and indole) and deleterious (e.g., ammonia) way (Davila et al., 2013; Lin et al., 2017); Glutamate increases microbial diversity and promote gut colonization by *Faecalibacterium prausnitzii* and *Roseburia* (Yang and Liao, 2019)
	In PKU treatment, an alternative of Phe-free-AA supplements can be represented by the glycomacropeptide -GMP-, a low-Phe protein source derived from whey protein (van Wegberg et al., 2017). GMP supplementation → anti-inflammatory effect, ↓ *Desulfovibrio* spp.; ↑ production of SCFAs (Sawin et al., 2015)
(ii) Specific nutrients	
Uncooked cornstarch (UCCS)	UCCS is normally used in the dietetic treatment of glycogen storage disease (GSD) I for maintaining blood glucose concentration in the desirable range (Kishnani et al., 2014). UCCS's overload in GSD type I patients→ reduction of the fecal pH with alteration in the relative abundances of fermenting bacteria species and reduction of SCFAs (Colonetti et al., 2019)
Medium chain triglycerides (MCTs)	MCTs are widely used in the dietary treatment of some metabolic diseases, like in long-chain fatty acid (LCFA) oxidation disorders or in the deficiency of the carnitine system (Bach and Babayan, 1982), as well as in the ketogenic diet and recently also in GSDs (Das et al., 2010).
	In animal models MCTs prevent LPS-mediated endotoxemia improving the intestinal barrier integrity and affecting the gut microbial composition in terms of Gram-positive and Gram-negative ratio (Decuypere and Dierick, 2003)
	In weaning piglets, a supplementation of medium-chain fatty acids (MCFAs) seems to increase the abundance of *Escherichia*, *Hafnia*, *Shigella* and *Clostridia* (Zentek et al., 2013)
Long-chain Polyunsaturated Fatty Acids (LC-PUFAs)	Early supplementation with LC-PUFAs and DHA is strongly recommended in PKU patients (Giovannini et al., 2012).
	PUFAs are able to restore a eubiotic status, after dysbiosis, in animal model;
	increase the production of SCFAs acting as prebiotics for Bacteroidetes and butyrate-producing bacteria belonging to the Lachnospiraceae family (Costantini et al., 2017)
	A mixed DHA/EPA supplement on healthy subjects seems to:
	↑ *Clostridiaceae*, *Sutterellaceae* and *Akkermansiaceae*
	↑ *Bifidobacterium* ssp. and *Oscillospira* spp.
	↓ *Coprococcus* spp. and *Faecalibacterium* spp.
	(Watson et al., 2018)
	DHA-enriched high oleic canola oil supplementation in individuals at risk for metabolic syndrome seems to:
	↓ *Faecalibacterium* spp. (Pu et al., 2016).
	These changes is common observed in phenylketonuric cohorts that are supplemented with DHA patients (Pinheiro de Oliveira et al., 2016; Bassanini et al., 2019)
(iii) Exclusion/reduction of specific nutrients	
Lower consumption of breast milk	Early dietetic intervention in IEMs often consists in the total exclusion of breastfeeding from the diet (e.g., in galactosaemia) or in a limited intake in most diseases (MacDonald et al., 2006).
	→ reduction of *Bifidobacteria* and *Lactobacillus* (Coppa et al., 2006; Hascoet et al., 2011; Videhult and West, 2016)
(iv) Special nutrient feeding	
Tube feeding	Tube feeding is commonly used as a treatment strategy for children with methylmalonic acidemia (MMA) and propionic acidemia (PA; Burlina et al., 2018)
	Enteral tube feeding can disrupt the oral indigenous microbiota (Takeshita et al., 2011) and in PA/MMA creates a permissive environment for *Clostridioides difficile* infection (O'Keefe, 2010);
	With enteral tube feeding, there is also the difficulty to maintain an adequate fluid intake → possible constipation

derived from shotgun metagenomic data that allow characterizing, at species- and strain-level, the enteric microbial communities and key metabolic features (Gophna et al., 2017). Indeed, the effects of microbiota on the pathogenesis of the "metabolic syndrome/disease" are not only related to the specific microorganisms' composition, but also to the microbial molecules produced. Specifically, microbial fermentation and production of SCFAs has been shown to be different in some metabolic disorders, with a different potential role in immune system modulation (Webb et al., 2016). SCFAs are produced by the saccharolytic fermentation of non-digestible carbohydrates that escape digestion and absorption in the small intestine, and they are mainly represented by acetate, propionate and butyrate, followed by formate and lactate. Minor amounts of branched chain fatty acids (BCFAs), such as iso-butyrate and iso-valerate, are produced through the protein-derived branched chain amino

TABLE 2 | Potential effects of use of specific medications on the gut microbiota in IEMs.

Medication	Potential effects on microbiota
Allopurinol	Gout can be a long-term complication in GSDs patients related to the high levels of uric acid. Although a good metabolic control may prevent hyperuricemia, allopurinol treatment is recommended to prevent gout attacks (Kishnani et al., 2014).
	In hyperuricemic rates was observed:
	↑ *Bifidobacterium* and *Collinsella*
	↓ *Adlercreutzia* and *Anaerostipes*
	(Yu et al., 2018)
	Microbiota of GSD patients taking allopurinol showed a slight increase of *Bifidobacterium* (Ceccarani et al., 2020)
Metronidazole	In PA/MMA an option to reduce intestinal propionate synthesis is the administration of metronidazole (25% of propionate is produced in the gut by anaerobic fermentation; Baumgartner et al., 2014)
	→ reduction of species biodiversity and total bacterial population (long term risk of drug resistance; Jakobsson et al., 2010)
L-carnitine	L-carnitine is commonly used in many IEMs to balance the primary or secondary carnitine deficiency.
	It is metabolized into trimethylamine (TMA) by several gut microbes, and converted in the liver to trimethylamine N-oxides (TMAO; Tang et al., 2013; Chen et al., 2019; Vernocchi et al., 2020), that decreases the transport of reverse cholesterol and bile acid synthesis → possible atherosclerosis mediator (Koeth et al., 2013; Wilson et al., 2016)

acids (BCAAs; Russell et al., 2011). SCFAs represent an important source of energy for both intestinal epithelial cells and gut microbiota itself, and maintain the mucosal immunity by fortifying the intestinal epithelium functions. SCFAs also play a crucial role on host immune system functions. They act as histone deacetylase inhibitors (Waldecker et al., 2008), promoting an anti-inflammatory cell phenotype and the immune homeostasis, and they function on G protein-coupled receptors (Brown et al., 2003). The gut microbiota also produces aryl hydrocarbon receptor (AHR) ligands and polyamines (Rooks and Garrett, 2016). The transcription factor AHR has a confirmed role in xenobiotic metabolism and in regulating mucosal immune responses. Li et al. (2011) demonstrated in mice that an increased severity of dextran sodium sulfate-induced colonic inflammation was observed in the absence of AHR ligands while, by supplementing mice diet with synthetic AHR ligands, they achieved an attenuation of the inflammatory status. Indeed, polyamines are primarily involved in the maintenance of intestinal barrier integrity by producing intercellular junction proteins, and many studies have highlighted their role in regulating immune responses (Zhang et al., 2000), and in modulating systemic and mucosal adaptive immunity (Minois et al., 2011). Moreover, the gut microbiota releases formylated peptides that act on G-protein-linked surface receptors, which are expressed on neutrophils and macrophages that recognize gut microbial products and stimulate reactive oxygen species (ROS) production responsible for an increase in cell oxidative stress (Marciano and Vajro, 2017).

THE CROSS-TALK BETWEEN GUT AND LIVER

During the past decade, it has become clear that microbial ecology can be involved in the development of a pro-oxidative and pro-inflammatory state (Luca et al., 2019). Oxidative stress along with chronic inflammatory conditions pave the way for the development of several metabolic diseases such as obesity and insulin resistance (Wellen and Hotamisligil, 2005; Heilbronn and Campbell, 2008; Cani and Delzenne, 2009; Pedersen et al., 2016; Rani et al., 2016).

The ability of the gut microbiota to affect gastrointestinal homeostasis, predisposing to chronic inflammation and modulating other metabolic functions of the host, is mediated by the gut-liver axis (Nieuwdorp et al., 2014). The liver and the intestine talk and influence each other through a bi-directional communication (**Figure 1**), mediated by biliary tract, portal vein and systemic mediators (Tripathi et al., 2018; Albillos et al., 2020). Bile acids (BAs) and other bioactive factors produced by the liver affect the gut microbiota by controlling unrestricted bacterial overgrowth and maintaining intestinal eubiosis *via* nuclear receptors such as farnesoid X receptor (FXR) and G protein coupled bile acid receptor (GPBAR1; also known as TGR5; Inagaki et al., 2006; Albillos et al., 2020).

On the other hand, the metabolites produced by the host and by the intestinal bioreactor do not limit their effect on the intestinal wall, but, moving through the portal vein, affect the liver and other organs and systems by entering into the circulation (Stärkel and Schnabl, 2016; Tripathi et al., 2018). This dual exchange operates in healthy conditions and plays a fundamental role in pathological contexts (Grenham et al., 2011).

Dysbiosis has been demonstrated to favor oxidative stress and to affect the immunological and inflammatory status of the host (Luca et al., 2019). In particular, it is considered the major driver of increased intestinal permeability (Henao-Mejia et al., 2012; Leclercq et al., 2012). Impaired integrity of gut barrier shifts BAs and choline metabolism, with translocation of microorganisms and their metabolites to the liver through the portal system, resulting in increased circulating levels of lipopolysaccharide (LPS; Ridlon et al., 2014; Mouzaki et al., 2016; Arab et al., 2017). Bacterial LPS endotoxins act as important signaling molecules that trigger and maintain oxidative stress and a low-tone inflammatory state and, by activating pattern recognition receptors such as Toll-like receptors (TLR), causing an innate immune response, insulin resistance and liver damage, thus transferability of disease *via* the microbiota (Uesugi et al., 2001; Cani and Delzenne, 2007; Csak et al., 2011; Anand et al., 2016).

Cani et al. (2007) defined the inflammatory state often associated with metabolic syndrome like "metabolic endotoxemia." This systemic inflammation is thought to occur as bacteria translocate through the gastrointestinal tract, increasing circulating levels of LPS (Tripathi et al., 2018). Higher circulating levels of LPS have been observed in obese subjects with insulin resistance compared with matched controls (Karlsson et al., 2013). An important link between gut microbiota, intestinal permeability and inflammation is represented by *Akkermansia muciniphila*, a Gram-negative anaerobe whose reduced abundance has been associated with compromising gut barrier integrity, increased inflammation and with both alcoholic and nonalcoholic liver damage (Everard et al., 2013; Borgo et al., 2017; Grander et al., 2018). Among other factors, SCFAs might also modulate the immune response by reducing intestinal permeability (Donohoe et al., 2011). An inadequate SCFAs production by gut microbiota leads to increased intestinal permeability, exacerbating metabolic endotoxemia and subsequent low-grade inflammation. This evidence is also supported by the observation of Everard et al. (2013): administration of propionate-producing bacteria *A. muciniphila* is able to partially reverse the obesity-associated low-grade of inflammation and subsequent insulin resistance induced by translocation of LPS from the intestine to the portal vein in mice, without affecting food intake.

DOES IEM'S MICROBIOTA SHARE CHARACTERISTICS WITH GUT MICROBIOTA OF INFLAMMATORY DISEASES?

In this section, we aim at comparing the different profiles and at describing the possible pro-inflammatory profile of gut microbiota in PKU and in GSD, mainly in GSD-Ia and GSD-Ib, exploiting inflammatory-related known signatures by decision support systems (DSSs). However, evidence of intestinal inflammation has not been demonstrated in PKU patients and thus it is not listed amongst the characteristic clinical features of the disease.

On the other hand, a dysbiosis in phenylketonuric patients has been recorded, showing some characteristics shared with an inflamed gut microbiota. An increase in pro-inflammatory genera, such as *Escherichia* and *Blautia*, and a depletion in

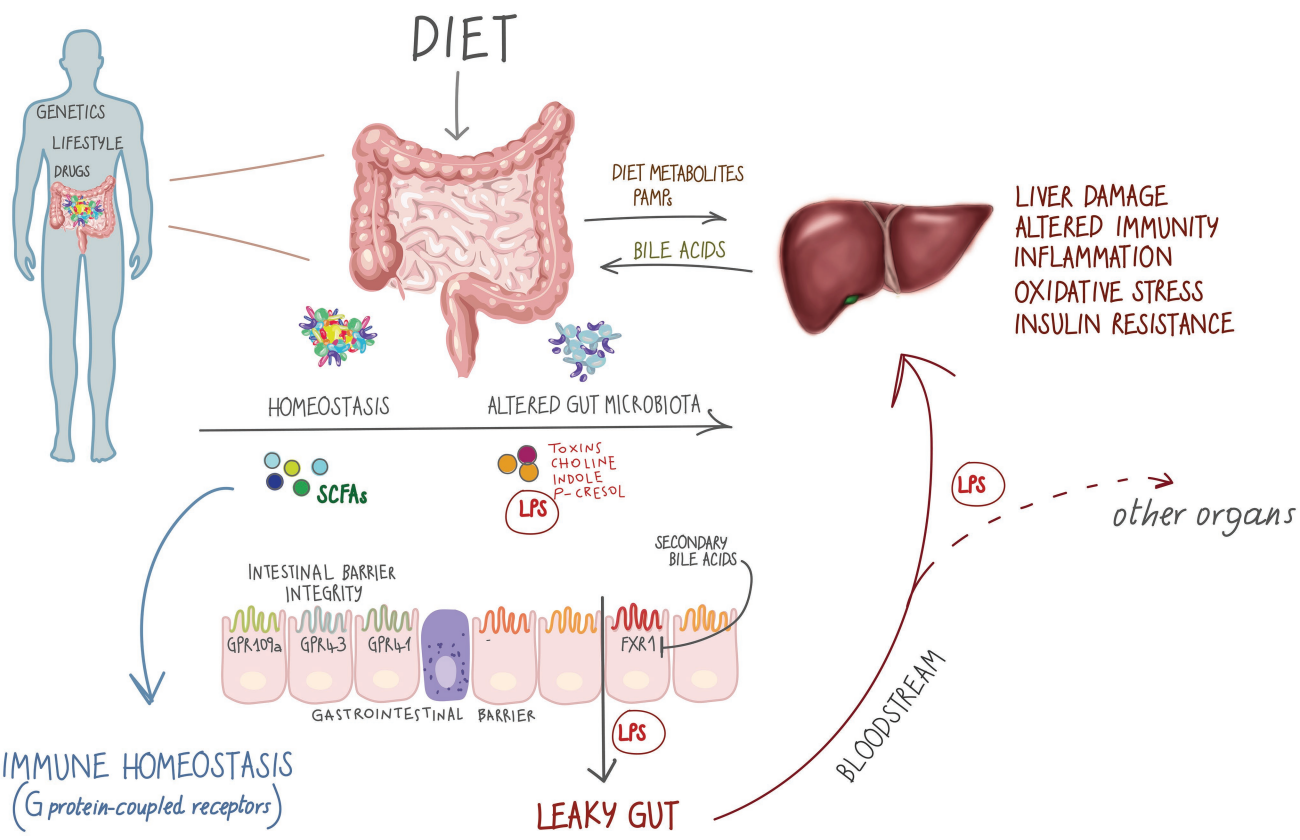

FIGURE 1 | The gut-liver axis and metabolic diseases. Metabolic diseases affect the gut-liver axis through exposome factors such as diet, xenobiotics, sedentary lifestyle as well as genetic treats. On the other hand, in inborn errors of metabolism (IEMs), special diets represent an important modulator of microbiota, impacting the gut-liver axis by altering microbial metabolites production. short-chain fatty acids (SCFAs) interact with metabolite-sensing G protein-coupled receptors GPR4, GPR43 and GPR109A promoting gut homeostasis (Macia et al., 2015) that has a central role in many human body functional axes. Alterations in the microbial community might promote a leaky gut condition, by altering gut barrier permeability and allowing translocation of whole bacteria and metabolites, such as lipopolysaccharide (LPS), further affecting the gut-liver axis and participating in non-communicable disease development.

beneficial genera, such as *Faecalibacterium*, *Roseburia*, and *Akkermansia* represent the microbial profile observed in PKU patients. Indeed, *Escherichia* spp. is a Gram-negative microorganism, displaying the LPS on the outer membrane (Hotamisligil, 2006; Bassanini et al., 2019), while *Blautia* stimulates pro-inflammatory cytokines secretion by host cells (Tuovinen et al., 2013). Pinheiro de Oliveira et al. (2016) highlighted a PKU microbiome involvement in LPS biosynthesis, suggesting a potential role of the gut microbiota in promoting gut inflammation.

Faecalibacterium is considered a biomarker of intestinal wellness due to its activity in anti-inflammatory molecules production, specifically mediated by the inhibition of the NF-κB pathway (Bassanini et al., 2019), and by SCFAs production. Therefore, the decrease in *Faecalibacterium* abundance and in other beneficial bacteria is associated with low amounts of total SCFAs and, in particular, butyrate (Verduci et al., 2018; Bassanini et al., 2019).

The functional predictive analysis of PKU microbiota suggest how some bacterial functions were underrepresented with a potential influence on starch and sucrose metabolism, glycolysis/gluconeogenesis, as well as biosynthesis of some amino acids, while the overrepresented taxa in PKU subjects were involved in LPS biosynthesis. The functional prediction seems to support the existing correlation between the dietary pattern in PKU, the gut microbiota composition, and the altered glucose metabolism, which in turn envisage a potential influence on the overweight status and obesity onset, promoted in a pro-inflammatory milieu (Verduci et al., 2020).

The gut microbiota in GSD patients has been poorly investigated so far (Colonetti et al., 2019; Ceccarani et al., 2020), and seems to be characterized by a significantly lower biodiversity compared with healthy controls that could indicate a first marker of inflammation. Both studies also showed an enrichment in Proteobacteria and, in particular, *Enterobacteriaceae*, a finding consistent with the dysbiosis reported in IBD patients. The two GSD-I subtypes differ for some clinical features. GSD-Ib patients typically develop neutrophil dysfunction, predisposing them to IBD. Although limited to a very few cases, IBD was recently reported also in GSD-Ia (Lawrence et al., 2017; Carnero-Gregorio et al., 2019), highlighting a potential contribution of GSD-I associated gut microbiota abnormalities in the development of intestinal inflammation. Indeed, *Enterobacteriaceae* exert pro-inflammatory activity both locally, at the gastrointestinal mucosa level, and systemically in genetically predisposed individuals, as GSD-Ib patients. Colonetti et al. (2019) also found an increased *Escherichia/Shigella* abundance. Moreover, the Veillonellaceae family was increased both in GSD patients (Ceccarani et al., 2020) and patients with IBD, probably participating in the pro-inflammatory status. Another pro-inflammatory characteristic in GSD cohorts is determined by the *Faecalibacterium* and *Oscillospira* genera depletion. *Faecalibacterium* spp., as mentioned above, has the ability to produce anti-inflammatory metabolites and to reduce the severity of chemically-induced inflammation in murine models (Sokol et al., 2008; Martin et al., 2014). This genus is considered one of the main butyrate producers found in the intestine (Barcenilla et al., 2000; Duncan et al., 2002). Butyrate can reduce intestinal mucosa inflammation by inhibiting NF-κB transcription factor activation (Inan et al., 2000), upregulating PPARγ (Schwab et al., 2007) and inhibiting interferon gamma release (Klampfer et al., 2003). Therefore, both GSD and PKU certainly share the characteristics of an inflamed intestine, with a potential pro-inflammatory role of their microbiota, then more or less evident clinical manifestations.

IEMS AND NON-COMMUNICABLE DISEASES: WHAT'S THE ROLE OF MICROBIOME?

Non-communicable diseases are mainly represented by cardiovascular diseases (heart impairment, stroke, etc.) and diabetes as a consequence of a "metabolic syndrome," characterized by overweight/obesity, hypertension, raised blood sugar, insulin resistance and raised cholesterol (World Health Organization, 2021). Dementia, cancers, chronic respiratory diseases, autoimmune diseases and chronic kidney disease (CKD) are also enlisted in NCDs. They are generally reported as the leading cause of mortality in the world. Common and modifiable risk factors underlying most NCDs are tobacco, pollution, harmful use of alcohol, unhealthy diet, and physical inactivity (Peters et al., 2019).

Nowadays the IEMs are clinically well investigated and an earlier diagnosis is possible especially thanks to the expanded newborn screening. Therefore, an early treatment could guarantee an improvement of the *quod vitam* prognosis of these patients.

Life expectancy of these patients has markedly improved, allowing us to evaluate the possible onset of long-term comorbidities, such as NCDs, and trying to understand the causes with the aim of preventing them.

The lifelong special diet of people affected by IEMs may induce changes in the microbiota towards a pro-inflammatory profile with a consequent difference in the SCFAs production while the clinical expression of the disease itself could be directly responsible for altering the composition of the microbiota. Therefore, we could hypothesize a synergistic phenomenon, where two or more actors, like the dietary treatment, chronic use of drugs or supplementations, and the disease phenotype, may shape a typical microbiota signature, with a consequent dysbiotic status. Accumulating evidence indicates that an altered microbial community is correlated with a number of pathologies (Sekirov et al., 2010), ranging from metabolic (Sanmiguel et al., 2015; Tang et al., 2017) to immunologic and psychic diseases (Dinan and Cryan, 2017). Specifically, dysbiosis seems to contribute to the development of NCDs including type 2 diabetes, fatty liver disease and obesity (Tomasello et al., 2016). Hence, we need to address several questions: what long-term effects could have the observed dysbiosis in IEMs? Can a different intestinal microbiota increase the risk of NCDs in this population?

Up to date, there are still sparse studies in the literature that have tried to investigate these aspects in IEMs. Nevertheless, major findings are summarized in the following sections.

Phenylketonuria

The quality of dietary carbohydrates of PKU children could directly affect the abundance of beneficial bacteria such as *Faecalibacterium*. In agreement with the data described so far, Fava et al. (2013) highlighted that a diet rich in carbohydrates with a high glycemic index leads to a reduction in the abundance of *Faecalibacterium prausnitzii* in subjects at risk of developing metabolic syndrome. Furthermore, some studies reported a reduction in the proportion of *Roseburia* spp. and *F. prausnitzii* in subjects with type 2 diabetes mellitus. These taxa are considered among major butyrate-producing bacteria and a reduced production of butyrate is reported to be associated with the development of insulin resistance both in humans and in the animal model (Qin et al., 2012; Karlsson et al., 2013). In PKU patients the different dietary pattern, focused on limiting Phe consumption, could be the cause of an unbalanced ratio Firmicutes/Bacteroidetes (Verduci et al., 2020). The balance of these two phyla ratio has an important role for obesity and higher body mass index (BMI), since a decreased ratio is associated with an increase in energy harvest, resulting in weight gain (Ley et al., 2005). Several studies reported that BMI and childhood obesity are influenced by ratio Firmicutes/Bacteroidetes (Indiani et al., 2018). Therefore, the modified microbiota of patients with PKU could potentially lead to a worse glycemic control, with a consequent insulin resistance, and weight gain (Verduci et al., 2020).

Since a different microbiota with a pro-inflammatory profile seems to predispose to NCDs in PKU subjects, probably due to a systemic pro-inflammatory state and an altered gut-liver axis, some studies reported a higher prevalence of overweight or increased fat mass (FM) in older subjects on dietary treatment, particularly in women (Acosta et al., 2003; Belanger-Quintana and Martinez-Pardo, 2011; Burrage et al., 2012; Aldamiz-Echevarria et al., 2014; Verduci et al., 2016; Sena et al., 2020). In particular, Scaglioni et al. investigated childhood overweight in a longitudinal observational study including 97 hyperphenylalaninemic children. They observed that 24.7% of children were overweight at the age of 8 years with an earlier BMI rebound than non-overweight children and a higher BMI from the age of 1 year (Scaglioni et al., 2004). An increase of triglyceride-glucose index (TyG index) in children with PKU compared to age and sex matched healthy controls was also observed (Moretti et al., 2017). The TyG index, calculated as Ln [TG (mg/dl) × glucose (mg/dl)/2], is considered a marker of low-grade inflammation and peripheral insulin resistance (Er et al., 2016). Moretti et al. (2017) showed a positive correlation between the TyG index and the glycemic load in PKU, that is higher than the normal values, strengthening the hypothesis of a possible link between the quality of carbohydrates and the predisposition to the development of metabolic disorders. In children with PKU an increase in the consumption of rapidly absorbed carbohydrates was observed, escaping the intestinal microbial fermentation with a consequent increase in the glycemic index and glycemic load (Bassanini et al., 2019).

Couce et al. studied the glucose metabolism in a cohort of 83 PKU and MHP patients and found increased fasting insulin levels in individuals with PKU compared to MHP subjects. Carbohydrate intolerance and insulin resistance were more evident in adults and in overweight patients. Patients treated with tetrahydrobiopterin (BH4) have been shown to have lower insulin and HOMA-IR levels, but the study was biased by the lack of a healthy control group (Couce et al., 2018).

A recent study showed that the cardiovascular phenotype of adult PKU patients is characterized by traditional cardiovascular risk factors, high levels of inflammatory and oxidative stress markers, endothelial dysfunction and vascular stiffness (Azabdaftari et al., 2019).

Morion Deon et al. reported an increased urinary oxidative stress parameter with a decreased urinary antioxidant capacity in PKU treated patients, associated with an increase of proinflammatory cytokines' plasmatic levels, as interleukin-6 and interleukin-1. Specifically, urinary isoprostanes (oxidative metabolites, result of a damaged lipid oxidation) resulted positively correlated with interleukin-1 suggesting an enhanced inflammatory process in PKU patients, associated with lipid damage. In PKU patients it seems that both a restricted diet and the Phe metabolites, with an excessive production of reactive species, could impact on the oxidative stress (Deon et al., 2015). Moreover, Schulpis et al. (2005) reported a moderate hyperhomocysteinemia in PKU patients on a strict diet, with a possible endothelial activation and arteriopathy.

Overall, these data suggest that PKU patients may be vulnerable to a higher risk of obesity, insulin resistance and its complications, in a context of low-grade inflammation and enhanced oxidative stress with an increased cardiovascular risk, regardless of the cause.

Glycogen Storage Disease-I

In hepatic GSDs, IBD, and liver disease are common features and they are part of the clinical expression of the pathology itself. Hepatocytes are sensitive to microbial products that may trigger an inflammatory immune response with systemic effects (Colonetti et al., 2019). GSD-I patients are at high risk for developing insulin resistance, traditionally attributed to nutritional "overtreatment" (Rossi et al., 2018). Especially in the past, in the attempt to avoid hypoglycemic crisis, there was a tendency to administer a high proportion of carbohydrates, sometimes exceeding the patient needs, with a consequent long-term risk of weight gain and hyperinsulinism with insulin resistance (Kishnani et al., 2014). Rossi et al. sustain that mitochondrial dysfunction has been implicated in the development of IR. The abnormalities in plasma acylcarnitines and urine organic acids found in GSD-I patients are indicative of a mitochondrial impairment, probably due to a possible distress on the intermediary metabolism as a consequence of the block of gluconeogenesis and glycogenolysis block. The oxidative stress seems to be related to higher insulin serum levels and other

insulin resistance indexes, especially in GSD-Ia, and with altered lipid profile (Rossi et al., 2018).

Inflammatory bowel disease is a phenotypic expression of the disease in GSD-Ib, probably related to the neutrophil dysfunction, although it has also been described in GSD-Ia patients (Lawrence et al., 2017; Carnero-Gregorio et al., 2019).

Tomasello et al. reported the oxidative stress among the possible pathogenetic mechanisms of IBD. The increase of ROS could arise from an incomplete reduction of oxygen, related with dysbiosis, probably through a positive feedback mechanism, where oxidative stress prompts the gut inflammation exacerbating the ROS production and subsequent tissue damage (Tomasello et al., 2016).

Other Diseases

In branched-chain amino acid disorders, i.e., organic acidurias (OA), and urea cycle disorders (UCD), few data are available in literature and the studies are mainly focused on body composition. Compared to the controls or reference values, a normal or increased FM has been reported in both children and adults affected by UCD and OA (Manoli, 2016; Evans et al., 2017).

An Italian observational study examined 17 adult UCD and OA patients on a low protein diet and detected a BMI >25 in 40% of the subjects analyzed, and an increased FM compared to normal values (Gugelmo et al., 2020). In UCD the few available data demonstrated an energy intake lower than the recommended value with a negative correlation between the percentage of FM and the total protein intake (Hook et al., 2016; Evans et al., 2017). Brambilla et al. (2019), in a more recent study, evaluated the resting energy expenditure in argininosuccinic aciduria (ASA) and in the other UCD, demonstrating that ASA had a resting energy expenditure (from indirect calorimetry, IC-REE) of 88% of the value predicted by the FAO and Harris-Benedict equations, whereas in the other UCD it was similar to the one expected. Low IC-REE was associated in ASA patients with increased prevalence of pathological waist circumference-to-height ratio, hypertension, hypertriglyceridemia and low HDL-cholesterol. No significant differences in body composition parameters were observed between the two groups. Definitely ASA patients have higher risk of obesity and increased cardiovascular risk. No data about microbiota in UCD are available, but it could be interesting to evaluate the presence of bacterial strains associated with proinflammatory patterns (Brambilla et al., 2019).

In OA branched-chain amino acid disorders and homocystinuria, an oxidative stress and mitochondrial dysfunction has been reported, as the effect of toxic metabolite accumulation, with significant multi-organ damage. The imbalanced ratio between the production and the adequate use of energy may provoke an inefficient cell metabolism as a feature in diseases like IEMs, underlining a link between hypertension, obesity, dyslipidemia and mitochondrial impairment (Ray and Mukherjee, 2021).

Although the prevalence of NCDs in IEMs is variable and the scientific evidence is still incomplete, we believe that IEMs deserve attention in this field. IEMs are predominantly monogenic disorders, but their phenotypic presentation is complex and heterogeneous as they can be the result of either a toxic accumulation of metabolites or deficiency in end products, going to impact different physiological systems (Kirby et al., 2020) also through an imbalance of oxidative state (Ray and Mukherjee, 2021).

Both the metabolic dysfunction and the dietary regimen are likely responsible for alterations in gut microbiome composition in IEMs patients. Indeed, dysbiosis, which determines a pro-inflammatory state alongside a condition of increased oxidative stress found in these patients, could be cumulative and concatenated risk factors. The contribution of gut microbiome in causing NCDs is still unclear, but of course an improvement of microbiota composition in anti-inflammatory and anti-oxidative direction can reduce the risk, since dysbiosis could move the clinical phenotype to further exacerbation as a potential patho-genetic mechanism, and it can also represent a preventive action in relation to NCDs (Kirby et al., 2020).

Hence, IEMs must be rethought and rearranged in a framework of complexity that cannot fail to take into account the intestinal microbiota and its dialog with other systems of the body through the gut-liver axis. There are still questions and hypotheses waiting to be confirmed or disapproved by future studies, such as: where in the pathophysiological chain gut dysbiosis is located? What its long-term effects may be? Can intestinal microbiota be a target in the treatment of IEMs? In our opinion, long-term studies designed in adult patients will be needed to better investigate eventual effects of dysbiosis in IEMs. This is the challenge for us in the scientific world, representing a completely open field of exploration that may allow a better care of the patient with IEMs.

POTENTIAL THERAPEUTIC APPROACHES TARGETING THE GUT-LIVER AXIS IN IEMS

In the light of data reported so far, we would like to underline the importance of the patient's general health status, in the context of the periodical evaluation of patients affected by IEMs. It is essential to point out the crucial role of the nutritional status assessment, as well as of monitoring the key clinical and biochemical signs of an eventual onset of a metabolic comorbidity to prevent and reduce the NCDs risk and optimize the long-term health outcome in IEMs. It becomes necessary for a "best clinical practice patient-personalized" to promote a healthy food consumption, within the different dietary patterns indicated for the disease, and to encourage daily physical exercise. Therefore, we believe that an important step in the nutritional and clinical management of patients affected by IEMs could focus on improving dietary products and mixtures commonly used in IEMs, for example proposing a change in the quality of the low protein foods by a careful selection of starches and consequently modifying the quantity of soluble fibers, or paying attention to a the possible "overtreatment" with an excessive intake of carbohydrates to avoid hypoglycemia.

Moreover, a supplementation with pro-, pre-, or postbiotics, within or without dietary products and mixtures, could be an important improvement of the quality of the diet.

Functional Foods (Pre-, Pro-, Post-biotics)

Manipulating the microbiota composition is one of the potential implications of the growing evidence of microbiome alterations in IEMs. The so-called functional foods encompass a group of food products with a potentially positive effect on health (Altveş et al., 2020). Probiotics are "live microorganisms that administered in adequate quantities confer a health benefit on the host." Prebiotics are non-digestible compounds whose positive effects are due to promoting the growth of a selected number of bacteria in the gut. Postbiotics are bacterial or metabolic products produced by probiotic microorganisms that have a biologic activity in the host (Tsilingiri et al., 2012; Tsilingiri and Rescigno, 2013).

To date, the role of functional foods in IEMs has been poorly investigated, in particular no evidences are available so far on a possible role of postbiotics in the context of IEMs and the majority of studies focus on the use of pre/probiotics in conditions which share physiopathological aspects, dietetic approach and/or secondary multiorgan involvement, paving the way to their possible application to IEMs. The potential impact of pre/probiotics treatment of PKU, the most common disorder among IEMs, found a greater development. MacDonald et al. (2011) investigated the possible beneficial effects of prebiotic oligosaccharides (scGOS/lcFOS), commonly present in breast milk, added in the amino acid mixture for PKU newborns. The supplemented AA mixture was able to keep *Bifidobacteria* and *Lactobacilli-Enterococci* levels at values comparable to healthy newborns. In the last decade, emphasis has been given to the role of GMP in the PKU diet, as naturally poor in Phe and more palatable than amino acid formulas. The gut microbiome plays an important role not only in amino acid metabolism, but also in carbohydrate and vitamin pathways, influencing the physiology of liver, brain and GI tract (Colonetti et al., 2018). This leads us to speculate that the microbiome modification may provide potential advantages in the management of many IEMs. Tang et al. (2017) reported on the synergistic effects of *Lactobacillus plantarum* S58 (LP. S58) and hull-less barley β-glucan (β-G) on lipid accumulation in mice fed with a high-fat diet. LP.S58 and β-G synergistically attenuated lipid accumulation by activating AMPK signaling and regulating the gut microbiota. In another recent study of Danhong et al. (2021), a supplement of probiotics is defined as a promising strategy for NAFLD and obesity treatment. Authors investigated the separated and combined effects of Bifidobacteria and resveratrol against obesity and NAFLD and concluded that a combination with a prebiotic substrate may improve the effects of probiotics. Probiotics, including *Lactobacillus rhamnosus* (LGG strain), have been shown to have several beneficial effects on the intestinal function by normalizing the dysbiotic microbiota (Resta-Lenert and Barrett, 2003; Bruzzese et al., 2004; Sartor, 2004; Ewaschuk and Dieleman, 2006; Versalovic, 2007; Marciano and Vajro, 2017). In particular, LGG has also been reported to reduce intestinal oxidative stress (Tao et al., 2006). Forsyth et al. (2009) showed that daily LGG treatment significantly improved severity of alcoholic steatohepatitis (ASH) and alcohol-induced gut leakiness, reduced markers of intestinal and liver oxidative stress and inflammation, and normalized the gut barrier function, preventing liver disease in a rat model of alcohol induced leaky gut and steatohepatitis. In pediatric patients with NAFLD, the subsequent changes in gut microbiome composition allowed a NAFLD-linked microbiota profiling (Del Chierico et al., 2017) and to design a tailored probiotics treatment (Putignani et al., 2016; Nobili et al., 2018, 2019). Rodríguez-Cerdeira et al. (2019) showed how the administration of a mixture of *Lactobacilli*, *Bifidobacteria*, and *Streptococcus thermophilus* in a young GSD-I woman can modify gut microbiota and improve the patient's quality of life in terms of ameliorating irritable bowel symptoms. Regular administration of the probiotic mixture led to an increase of Bacteroidetes, *Clostridium leptum* and *Eubacterium* and to a decrease in *Enterobacteriaceae* (*Escherichia*, *Klebsiella*, *Proteus*; Carnero-Gregorio et al., 2019). In OA prebiotics could also play an important role. Burlina et al. supposed that prebiotics that lower pH in the gut microbiota environment could potentially decrease propionate production so stimulating the activity of lactate and the predominant acetate-producing species could reduce propionate production. Studies in healthy adults have shown that FOS alone stimulates subsequent cross-feeding, i.e., metabolism of lactate to butyrate (Belenguer et al., 2006; Rios-Covian et al., 2015). As the properties of butyrate are closer to propionate, in the human gut microbiota, this finding needs careful consideration in the development of prebiotic strategies for the management of PA/MMA. Other studies in healthy infants have shown that prebiotics containing GOS/FOS (9:1) stimulate acetate and lactate production, while suppressing propionate and butyrate production (Oozeer et al., 2013). A controlled study in healthy infants showed that GOS/FOS mixture added to infant formula stimulated the growth of *Bifidobacteria* and increased the overall metabolic activity of intestinal microbiota, resulting in higher acetate and lower propionate levels (Knol et al., 2005). By lowering pH, the prebiotic mixture can make the gut less hospitable to pathogens and decrease production of propionate (Belenguer et al., 2007). Therefore, the efficacy of any potential approach to reduce propionate production, such as the prebiotic administration of GOS and FOS, should be tested in patients with PA/MMA.

There is no direct evidence of the possible role of functional foods in lysosomal storage diseases (LSD). Recent studies show the role of microbiome modulation in slowing the progression of end stage-renal failure, a complication and frequent cause of death of Fabry disease. Vaziri et al. (2013) demonstrated the proliferation of dysbiotic bacteria in patients with CKD, which translocate in the systemic circulation through the impaired intestinal barrier. A recent randomized, double-blind, placebo-controlled trial on CKD patients, following a 6-month probiotic therapy, reported a significant reduction in the serum levels of endotoxin and pro-inflammatory cytokines, an increase of the anti-inflammatory cytokine IL-10, and a preservation of residual kidney function (Wang et al., 2019). The potential role of prebiotic use to slow down the CKD in OA such as methylmalonicacidemia, should take into account a possible effect in increasing propionic acid production and should be limited to non-propiogenic

compounds (Burlina et al., 2018). The same mechanism of pathological translocation of commensal bacteria has been described also in several myopathies (Du Preez et al., 2018). The consequent altered immune response and systemic chronic inflammation, a common feature of inherited metabolic myopathies such as Pompe disease, can speculate a role of manipulating the altered microbiome also in this condition. Many studies suggest that increasing gut bacterial SCFAs production may positively affect skeletal muscle mass and physical function in humans (Lustgarten, 2019). In terms of bacterial species that may positively impact muscle mass, *Lactobacillus casei* or *Bifidobacterium longum* demonstrated to increase the muscle mass/body weight ratio (Ni et al., 2019). Overall, the use of functional foods in IEMs may represent a potential approach to all the above-mentioned conditions. As the administration of the probiotic *Lactobacillus reuteri*, engineered to express a phenylalanine lyase gene from the cyanobacteria *Anabaena variabilis*, demonstrated its efficacy in reducing Phe levels in PKU mice, representing a potential safe approach to PKU patients, the creation of genetically modified probiotics able to normalize defective metabolic pathways or controlling multiorgan complications, may represent a future therapeutic approach also to many other IEMs.

CONCLUSION

The next challenges of current microbiome research should be to identify the mechanisms by which metabolic exchanges drive the diet-microbiome-pathophysiology interactions in IEMs, characterizing the gut microbiota in IEM patients beyond DNA-based composition analysis and incorporate other "omics" technologies, such as (meta)transcriptomics, (meta)proteomics, and (meta)metabolomics. The application of shotgun approaches in metagenomics pipelines will substantially contribute on the deep characterization at species and strain levels of microorganisms, particularly involved in metabolism regulation, such as SCFA and ethanol producers.

Furthermore, a more specialized microbiomics, based on shallow shotgun and trans kingdom metagenomics will improve the clinical microbiology dedicated to the understanding of microbiome in metabolic diseases, including not only bacteriome, but also virome, protozoa, and metazoan (i.e., parasitome) reservoirs, including their metabolic relationships. The new approach will be based on an "agnostic" view to characterize, through the different taxa, all possible microorganisms.

It will be necessary to turn attention to the intestinal ecosystem as a set of microbial metabolic interactions rather than to single isolated biotic factors (Burlina et al., 2018) in the framework of clinical DSSs driven by advanced bioinformatics and artificial intelligence-based pipelines and to study their possible long effects in IEMs.

Finally, modelling the intestinal microbiota through non-pharmacological nutritional treatments, based on a healthy diet and the use of specific pre-, pro-, and postbiotics, may represent an innovative approach of "precision medicine" in microbiomics focused on restoring microbiota balance in IEMs, thus significantly improving the health status of these subjects.

SIMMESN (ITALIAN SOCIETY FOR THE STUDY OF INBORN ERRORS OF METABOLISM AND NEONATAL SCREENING) WORKING GROUP FOR GUT MICROBIOTA IN INBORN ERRORS OF METABOLISM

Rita Barone, Giulia Bassanini, Giulia Bensi, Giacomo Biasucci, Cristina Bonfanti, Elisa Borghi, Alberto Burlina, Maria Teresa Carbone, Chiara Cazzorla, Serena Gasperini, Giuseppe Giordano, Diego Martinelli, Cettina Meli, Chiara Montanari, Francesco Porta, Sara Parolisi, Lorenza Putignani, Francesca Righetti, Albina Tummolo, Elvira Verduci, Carlo Dionisi Vici and Juri Zuvadelli.

AUTHOR CONTRIBUTIONS

MC and EV conceptualized and reviewed the article. CM and SP collected all the data. CM, SP, GBa, JZ, CB, and AT drafted the manuscript. EB and LP critically revised the manuscript. CV, GBi, and AB gave the final approval. All authors contributed to the article and approved the submitted version.

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Microbiome in Eosinophilic Esophagitis—Metagenomic, Metatranscriptomic, and Metabolomic Changes: A Systematic Review

Jordan D. Busing[1], *Matthew Buendia*[2], *Yash Choksi*[3,4], *Girish Hiremath*[2] *and Suman R. Das*[3,5]*

[1] Department of Pediatrics, Vanderbilt University Medical Center, Nashville, TN, United States, [2] Division of Pediatric Gastroenterology, Hepatology, and Nutrition, Monroe Carrell Jr Vanderbilt Children's Hospital, Nashville, TN, United States, [3] Department of Medicine, Vanderbilt University Medical Center, Nashville, TN, United States, [4] Tennessee Valley Health System, Veterans Affairs, Nashville, TN, United States, [5] Department of Otolaryngology and Head and Neck Surgery, Vanderbilt University Medical Center, Nashville, TN, United States

Background: Our understanding of human gut microbiota has expanded in recent years with the introduction of high-throughput sequencing methods. These technologies allow for the study of metagenomic, metatranscriptomic, and metabolomic bacterial alterations as they relate to human disease. Work in this area has described the human gut microbiome in both healthy individuals and those with chronic gastrointestinal diseases, such as eosinophilic esophagitis (EoE).

Objectives: A systematic review of the current available literature on metagenomic, metatranscriptomic, and metabolomic changes in EoE was performed.

Methods: This review was performed following the PRISMA guidelines for reporting systematic reviews and meta-analyses. All relevant publications up to March 2021 were retrieved using the search engines PubMed, Google Scholar, and Web of Science. They were then extracted, assessed, and reviewed. Only original studies published in English were included.

Results: A total of 46 potential manuscripts were identified for review. Twelve met criteria for further review based on relevance screening and 9 met criteria for inclusion, including 6 studies describing the microbiome in EoE and 3 detailing metabolomic/tissue biochemistry alterations in EoE. No published studies examined metatranscriptomic changes. Samples for microbiome analysis were obtained via esophageal biopsy ($n = 3$), esophageal string test ($n = 1$), salivary sampling ($n = 1$), or stool specimen ($n = 1$). Samples analyzing tissue biochemistry were obtained via esophageal biopsy ($n = 2$) and blood plasma ($n = 1$). There were notable differences in how samples were collected and analyzed. Metabolomic and tissue biochemical alterations were described using Raman spectroscopy, which demonstrated distinct differences in the spectral intensities of glycogen, lipid, and protein content compared to controls. Finally, research in proteomics identified an increase in the pro-fibrotic protein thrombospondin-1 in patients with EoE compared with controls.

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***Correspondence:**
Suman R. Das
suman.r.das@vumc.org

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Conclusions: While there are notable changes in the microbiome, these differ with the collection technique and method of analysis utilized. Techniques characterizing metabolomics and tissue biochemistry are now being utilized to further study patients with EoE. The lack of published data related to the human microbiome, metagenome, metatranscriptome, and metabolome in patients with EoE highlights the need for further research in these areas.

Keywords: eosinophilic esophagitis, metagenomics, metatranscriptomics, metabolomics, omics, metaproteomics, microbiome

INTRODUCTION

Eosinophilic esophagitis is a chronic, food, and/or aeroallergen-mediated inflammatory disease (Liacouras et al., 2011; Wechsler and Bryce, 2014; Davis and Rothenberg, 2016). It is characterized clinically by symptoms of esophageal dysfunction such as vomiting, abdominal pain, and dysphagia. The diagnosis is confirmed by the presence of intense eosinophilic inflammation (> 15 eosinophils per high-power field [eos/hpf]) in esophageal mucosal biopsies (Liacouras et al., 2011). The prevalence of EoE is estimated to be 1 in 2000 in the United States. The disease adversely affects the quality of life of patients and imposes a substantial financial burden on the healthcare system (Jensen et al., 2015; Jensen and Dellon, 2018).

The current disease paradigm is that a combination of genetic predisposition, dysregulated immunity, and environmental factors contribute toward the development of EoE (Lehman and Lam, 2019). While substantial progress has been made in understanding the role of genetics and immune response, there is growing interest on the impact of environmental factors on the development and progression of EoE. Among environmental factors, early studies on the microbiome in EoE have focused on characterizing which microbes are more common in disease, but more recent studies have investigated the function of those microbes, how they interact with the host machinery, and which substrates are transformed during cellular and biochemical metabolism.

In recent years, advancements and increased access to high-throughput sequencing technologies have expanded our understanding of the role of the human microbiome in various disease states. These approaches have also facilitated metagenomic and metatranscriptomic investigations of the interactions between host tissues and their microbial communities. Metagenomics is the study of the collective genetic material of the human microbiome (Bikel et al., 2015). This process maps genes to characterize the putative functional pathways which allows insight into the abundance and genetic potential within the microbial community present. Metagenomics, while powerful, doesn't discriminate between live and dead bacteria. A sample can still possess DNA from bacteria regardless of whether that bacteria is currently living or not. Metagenomics is limited to sequencing DNA that is merely present, but that does not necessarily provide insight into which bacterial are alive and active in the sample. Metatranscriptomics describes whole-genome analysis and mapping of the expressed pathways, which allows for determination of which microorganisms are actively involved in the disease phenotype. Similarly, metabolomics is the study of small molecules produced by cells (Patti et al., 2012). The presence and alterations of microbial metabolites such as lipids, carbohydrates, amino acids can provide direct insight into biochemical alterations which lead to phenotypic presentation of disease. The use of these technologies allows for the expansion of the central dogma of molecular biology—DNA(genomics) to RNA(transcriptomics) to protein (proteomics) to metabolite (metabolomics)—to better understand the epigenetic and post-translation modification (Patti et al., 2012). Thus, metagenomic sequencing, along with metatranscriptomics and metabolomics (multi-omics), can help characterize the functional relevance of bacterial gene expression, while also potentially providing insight into the mechanistic role of the microbiome in EoE. In this state-of-the art review, we aim to summarize what is known about the microbiome, metagenomics, metatranscriptomics, and metabolomics in EoE.

METHODS

Our methods adhere to the guidelines established by Preferred Reporting Items for Systematic Review and Meta-Analyses (PRISMA) (Page et al., 2021). To identify relevant studies, we conducted a search in PubMed, Google Scholar, and Web of Science. To optimize the search, no date limits were imposed. The last search was performed on March 1st, 2021. A combination of search terms such eosinophilic esophagitis/oesophagitis (EoE), microbiome (or microbiota), human microbiome (or microbiota), genome (or genomics), metagenomics (or metagenome), transcriptome (or transcriptomics), metatranscriptome (or metatranscriptomics), or metabolome (or metabolomics) were used.

We included publications which detailed original data and descriptions of the terms mentioned above. To ensure quality, non-original articles, non-human studies, and abstract-only publications were excluded. Additionally, studies published in languages other than English were also excluded.

Two authors (M.B., J.B.) evaluated articles for eligibility and quality. Each person extracted data independently. Variables that were sought were obtained using a standard form that was designed to collect title, author, publication year, country of origin, study type, specific aims, research methods, and conclusions. Risk of bias was assessed independently by two

investigators (M.B., J.B.) Any disagreement between authors in data abstraction or bias was resolved by discussion with the senior authors (S.D. and G.H.) and review of the publications. This approach allowed for the minimization of bias.

RESULTS

Our search yielded a total of 46 studies, all of which were reviewed by abstract and title. There were 34 articles excluded following initial screening. Articles were excluded following screening because they described another disease process ($n = 6$), were not original data/were systematic reviews ($n = 14$), or described non-relevant aspects of EoE ($n = 14$). After the initial screening process, a total of 12 articles were sought for retrieval. Publications that were not in English language or had no relevant outcomes were excluded, leaving a total list of 9 articles for inclusion (**Figure 1**). The results of the studies are summarized in **Figure 2**. Each study is outlined in **Table 1**.

Microbiome, and Eosinophilic Esophagitis

Currently, culture-independent sequencing utilizing amplicon-based 16S ribosomal RNA (a highly conserved region amongst bacteria) is the most common form of molecular sequencing (Li et al., 2018). Bacterial 16S ribosomal RNA (rRNA) genes generally contain nine "hypervariable regions" (V1-V9) that are used for species identification (Chakravorty et al., 2007). While 16S rRNA sequencing has limitations, this method utilizing hypervariable regions V1-V9 is a cost-effective, efficient, and unbiased method which captures the microbial community structure and composition and has been utilized in most of the previous work done examining the esophageal, oral, and stool microbiome in patients with EoE. Using oral and esophageal swabs and subsequent 16S sequencing with V1 to V2 primers, Benitez et al. showed that patients with active EoE have a distinct esophageal microbiome as compared with non-EoE controls. This study included 68 subjects ages 2–18 years old. They demonstrated that the normal esophageal microbiome is dominated by *Firmicutes* species. However, when compared with non-EoE controls, patients with active EoE had increased abundance of *Neisseria* and *Corynebacterium*, both Proteobacteria. Interestingly, patients with inactive EoE did not have significant differences compared to healthy controls. Additionally, they showed that enrichment of the bacterial genuses *Granulicatella* and *Campylobacter* occurred with re-introduction of highly allergenic foods (dairy, wheat, nuts, eggs, soy, and shellfish) to both the active and inactive EoE cohorts' diet (Benitez et al., 2015). This group also examined the oral and esophgeal microbiome to determine if the oral microbiome was like that of the esophageal microbiome, and thus could serve as a less invasive surrogate for study of the esophageal microbiome. They show a modest, but significant correlation between these two environments. This correlation was unaffected by disease status. They conclude, however, that the oral microbiome was unchanged in patients with active, inactive, and healthy controls and thus their data did not recommend using oral samples in place of esophageal samples for disease monitoring (Benitez et al., 2015).

In a different study, Harris et al. used the esophageal string test to sample esophageal luminal secretions and then performed subsequent 16s rRNA sequencing using V1 to V2 primers to study the esophageal microbiome of patients with EoE and GERD compared to healthy controls (Harris et al., 2015). This study included both children and adults and the majority of patients were Caucasian males. They found a significant increase in the relative abundance of *Haemophilus* in patients with untreated EoE compared to healthy controls. The abundance of *Haemophilus* was reduced to levels similar to that in controls and patients with GERD when the histologic remission of EoE was achieved through either swallowed steroids or dietary measures. This highlighted that treatment can play a role in altering the microbial community of the esophagus in patients with EoE.

In a follow-up study to their 2015 study, Benitez et al. used both 16S rRNA gene and internal transcribed spacer (ITS) sequencing to examine the effect of topical swallowed corticosteroids on esophageal bacterial and fungal populations in EoE (Benitez et al., 2021). This study included 69 children with EoE, 33 with active EoE and 36 with inactive EOE. There were 10 healthy controls. They found that *Streptococcus*, *Prevotella*, and *Alloprevatella* dominated the esophageal microbiota in all studied patients, including controls. Both active and inactive EoE patients had decreased abundance of *Alloprevatella* when compared to non-EoE controls. There was a stepwise increase in the abundance of *Haemophilus* from control to inactive to active EoE. This is also the first group to examine the fungal component of the microbiome present in EoE, including patients being treated with topical swallowed steroids. *Candida*, *Cladosporiaceae*, and *Malassezia* were the most common fungal taxa in all groups. Other important findings included an increased proportion of *Candida* in non-EoE controls compared to steroid-naïve EoE subjects and changes in the fungal community following treatment with topical swallowed steroids—namely, significant increase in *Candida* in inactive EoE patients treated with topical swallowed steroids (TSS) when compared with those not treated with TSS (Benitez et al., 2021).

In 2018, Grussell et al. utilized traditional culture driven results of brush samplings and mucosal punch biopsies from the oral cavity and esophagus instead of molecular sequencing to compare microbiota in adult patients with GERD and EoE to healthy controls (Norder Grusell et al., 2018). This group found alfa-streptococci was the most common group in all three patient populations and patients with EoE had significantly more diversity compared to healthy controls. Additionally, they confirm findings by Harris et al. and Benitez et al. with increased abundance of *Haempholius* in patients with EoE compared to individuals with GERD and healthy controls (Norder Grusell et al., 2018).

More recently, Hiremath et al. used 16s rRNA sequencing utilizing the V4 region to characterize the salivary microbiome in children with EoE (Hiremath et al., 2019). They found *Streptococcus* was more abundant with active EoE vs. non-EoE controls. *Haemophilus* was more abundant in active EoE vs. inactive EoE and positively correlated with esophageal endoscopic and histologic disease activity. This increase in *Haemophilus* with active EoE mirrors previous findings (Harris

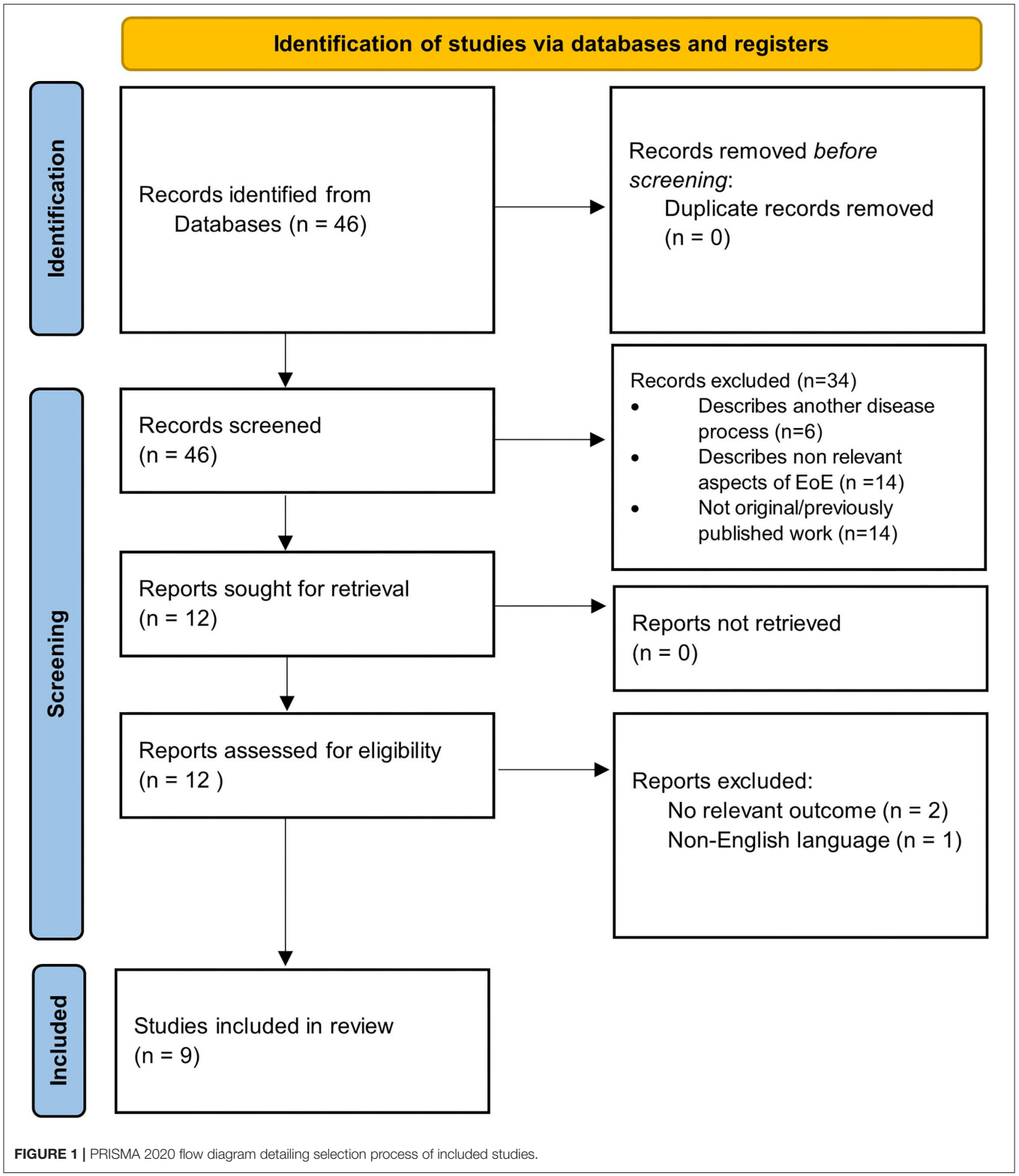

FIGURE 1 | PRISMA 2020 flow diagram detailing selection process of included studies.

et al., 2015). They also note a trend toward lower microbial richness and alpha diversity in children with EoE (Hiremath et al., 2019).

Finally, Kashyap et al. studied the stool microbiome in patients with EoE (Kashyap et al., 2019). This group used 16s rRNA sequencing utilizing the V4 region to compare the stool

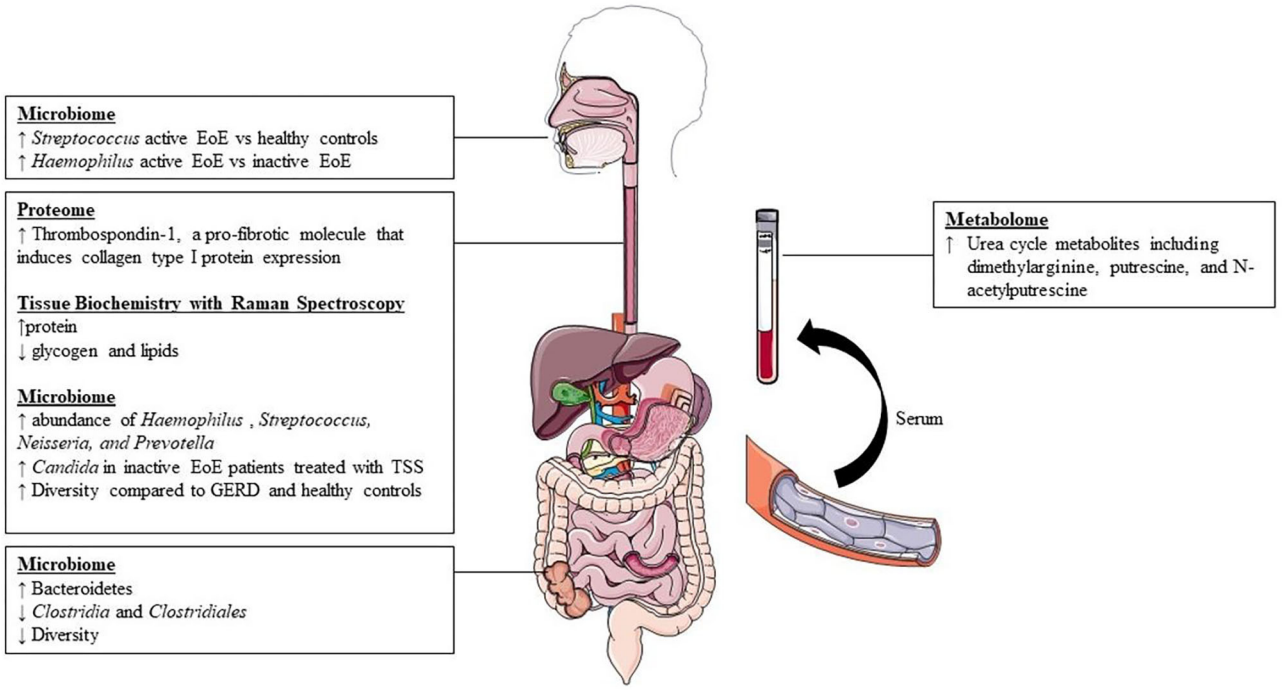

FIGURE 2 | Summary of available findings from literature.

microbiota of 12 patients with EoE to 12 healthy controls. This group found significant decreases in *Clostridia* and *Clostridiales* in patients with EoE. They also note decreased stool microbial diversity in patients with EoE compared to controls.

Transcriptomics, Metatranscriptomics, and Eosinophilic Esophagitis

Studies describing the EoE transcriptome have allowed researchers to further understand disease mechanisms of EoE. They are discussed here in brief to highlight how the transcriptome has contributed to the understanding of EoE and further display how further understanding of the metatranscriptome could be important. Blanchard et al. first utilized whole-genome wide transcript oligonucleotide-based DNA microarray chips with esophageal biopsies to define the EoE transcriptome (Blanchard et al., 2006). They subsequently identified 574 transcripts—colloquially known as the EoE transcriptome—that were expressed differently in children ages 2–17 with EoE compared to healthy controls. These altered genes have varying function and play a role in several areas including immunity, inflammatory response, barrier function, atopy, and eosinophilia (Sherrill et al., 2014). Wen et al. expanded further on this work and developed an EoE molecular diagnostic panel by utilizing quantitative PCR on fixed paraffin embedded esophageal biopsy samples (Wen et al., 2019). This panel utilized 96 genes to accurately identify EoE in adults and children with 96% sensitivity and 98% specificity. Importantly, this the EoE diagnostic panel was able to differentiate active EoE vs. controls, EoE in topical steroid remission vs. controls, and EoE vs. GERD (Wen et al., 2013).

Genes involved in epithelial barrier dysfunction and T helper type-2 mediated immune dysregulation are thought to be central to the pathogenesis of EoE (Lyles and Rothenberg, 2019). Current genes implicated in epithelial barrier dysfunction include CAPN14, DSG1, FLG, and SPINK5, and SPINK7. Additionally, CCL26, involved in eosinophil chemotaxis, and TSLP, involved in dendritic cell chemotaxis, have been implicated in T helper type-2 mediated immune dysregulation (Lyles and Rothenberg, 2019).

More recent work by Wen et al. has focused on single cell RNA sequencing to investigate resident esophageal T-cells in patients with EoE compared to healthy individuals and those with EoE in remission (Wen et al., 2019). Important findings include identification of eight T-cell subclasses that are increased in active EoE inflammation, including the two most highly upregulated populations: Treg cells (FOXP3+) and effector Th2-like (GATA3+) cells.

While much work has been completed to describe transcriptomic changes in EoE, briefly highlighted above, little has been done to characterize the metatranscriptome. Specifically, the expression profile of microorganisms which are altering host biology has been understudied and is necessary before metatranscriptomic analysis can be done. Metatranscriptomic data is a powerful tool that would improve our understanding of how previously identified microbiota in active EoE might alter the functional profile of the esophageal epithelial gene expression.

TABLE 1 | Summary of studies applying microbiome analysis in eosinophilic esophagitis.

Study	Study population	Methods	Findings
Microbiome			
Benitez et al. (2015)	• Sample size: $n = 68$ • Oral swabs and esophageal biopsies from 33 EoE patients and 35 controls	• 16S rRNA gene sequencing (V1 to V2)	• Higher abundance: *Streptococcus*, *Neisseria*, and *Prevotella*
Benitez et al. (2021)	• Sample size: $n = 79$ • Esophageal biopsies from 69 EoE patients (36 inactive, 33 active) and 10 controls	• 16S rRNA gene sequencing (V1 to V2) and internal transcribed spacer (ITS) sequencing	• EoE patients: decreased abundance of *Alloprevatella* • Increase in the abundance of *Haemophilus* to inactive to active EoE. • Increase in *Candida* in inactive EoE patients treated with TSS when compared with those not treated with TSS.
Harris et al. (2015)	• Sample size: $n = 70$ • Esophageal string test from 37 children with EoE, 8 with GERD, and 25 controls	• 16S rRNA gene sequencing (V1 to V2)	• Significant increased *Haemophilus* in patients with EoE that returns to controls when disease is controlled.
Hiremath et al. (2019)	• Sample size: $n = 35$ • Saliva samples from 26 children with EoE and 19 controls	• 16S rRNA gene sequencing (V4 region)	• Higher abundance: *Streptococcus* was more abundant with active EoE vs. non-EoE controls, *Haemophilus* was more abundant in active EoE vs. inactive EoE • Diversity: no significant difference
Norder Grusell et al. (2018)	• Sample size: $n = 27$ • Oral punch biopsies and brush samplings from the oral cavity (as well as brush samplings and endoscopy biopsies of upper and lower esophagus) of 17 patients with GERD and 10 with EoE	• Culture	• Higher abundance: *Streptococcus* (viridians) was the most common bacteria in both groups • Diversity: decreased in GERD vs. EoE
Kashyap et al. (2019)	• Sample size: $n = 20$ • Stool bacterial DNA from 12 patients with EoE and 12 controls	• 16S rRNA gene sequencing (V4 region)	• Significant decreases in *Clostridia* and *Clostridiales* in patients with EoE. • Diversity: decreased in EoE
Proteomics and tissue biochemistry			
Hsieh et al. (2021)	• Sample size $n = 10$ • Esophageal biopsy from 5 healthy donors and 5 with therapy refractory EoE.	• Esophageal biopsy with fibroblasts placed on autologous or non-autologous decellularized ECM	• Increased hrombospondin-1, a pro-fibrotic molecule that induces collagen type I protein expression, is increased in patients with EoE compared to controls.
Hiremath et al. (2020)	• Sample size $n = 24$ • Children with active EoE ($n = 8$) and inactive EoE ($n = 6$) and non-EoE controls ($n = 10$)	• Utilized Raman Spectroscopy to profile and compare esophageal samples	• Raman peaks attributable to glycogen content was lower in children with active EoE compared with that in non-EoE controls • Protein intensity was higher in children with aEoE compared with that in non-EoE controls. • Raman peaks attributable to glycogen and lipid inversely correlated with eosinophilic inflammation and basal zone hyperplasia.
Metabolomics			
Moye et al. (2018)	• Sample size $n = 24$ • Children with EoE ($n = 7$) and children on PPI ($n = 11$, 4 with EoE) comparted to healthy controls not on PPI ($n = 6$)	• Blood sample profiling using the subclasses: amino acids, tricarboxylic acid cycle, acetylation, and methylation. 48 metabolites measured in total.	• Increased urea cycle metabolites including dimethylarginine, putrescine, and N-acetylputrescine in patients with EoE

Metabolomics and Tissue Biochemistry

Our understanding of the pathogenesis of EoE remains incomplete due to limitations in characterizing not only the microbiome, but also the biomolecular and biochemical alterations that are present in the esophageal epithelium of these patients (Hiremath et al., 2020). Several groups have recently described the protein expression pattern in patients with EoE.

Hiremath et al. utilized Raman spectroscopy and proteomic analysis of esophageal mucosa to demonstrate that patients with EoE had distinct differences in spectral intensities of glycogen, lipid, and protein content compared to controls (Hiremath et al.,

2020). The findings were able to use Raman spectroscopy to reliably distinguish between controls, active EoE, and inactive EoE. Specifically, peak intensity for glycogen was decreased and peak intensity for proteins increased for patients with active EoE compared to healthy controls. Additionally, peak intensity for lipids was higher in children with inactive EoE compared to those with active disease. This group hypothesized that a decrease in glycogen and lipids in the epithelium of patients with active disease could be due to increased uptake of glycogen by eosinophils and increased amounts of undifferentiated epithelial cells (i.e., basal cell hyperplasia) with decreased cytoplasmic glycogen volume. They postulate that lipid content could be higher in children with inactive EoE because of the underlying mucosal healing process. Finally, they hypothesized that increased protein content in EoE could be related to increased chemokines and cytokines mediating inflammation.

Hsieh et al. recently described unique changes to the extracellular matrix proteome in patients with EoE (Hsieh et al., 2021). This group isolated fibroblasts from 5 children with active EoE and 5 healthy controls and utilized extracellular matrix (ECM) from both groups to culture these fibroblasts. The goal of this study was to determine how the extracellular matrix of patients with EoE can alter the function of normal fibroblasts. Fibroblasts from healthy controls that were cultured on ECM from patients with active EoE demonstrated higher levels of type 1 collagen and α-smooth muscle actin when compared to control fibroblasts cultured on autologous ECM. The authors then analyzed both sets of ECM and subsequently demonstrated that thrombospondin-1, a pro-fibrotic molecule that induces collagen type I protein expression, is increased in patients with EoE compared to controls. This work highlights how further insight into proteomics can further our understanding of disease specific long-term sequelae.

Work has also been completed to not only catalog which proteins are differentially expressed in EoE, but also to describe whether there are differences in measurable metabolites. In 2019, Moye et al. was the first group to describe key blood plasma metabolite changes in children with EoE compared to healthy controls (Moye et al., 2018). They found differences in metabolites between healthy controls and patients with EoE. Notably, they identified key differences in patients with EoE both on and off proton pump inhibitor therapy. Based on their work, they suggest dimethylarginine, putrescine, and N-acetylputrescine as potential biomarkers for EoE.

DISCUSSION

In this review, we summarize the published data related to human microbiome and EoE and highlight what is known about the metagenome, metatranscriptome, and metabolome. Our major conclusion is that there is significantly more work needed in these areas to characterize the complex role of the gut microbiome in EoE. At this time, transcriptomics (tissue microarray, bulk RNA sequencing, and single cell RNA sequencing) has been done, but independent of the gut microbiome. There is no published work to our knowledge integrating the immune response, gene expression, and microbiome. Similarly, little work has been done regarding metabolomics and metaproteomics—advances in these fields could give insight into potential non-invasive disease monitoring or therapeutic targets.

The goal of the study of the human microbiome is to characterize the microbial community, its interaction with the host, and its role in human health and disease. To date, 16S RNA sequencing is the most common method for studying the human microbiome. This method utilizes highly conserved bacterial regions to identify bacterial RNA within a sample and hypervariable region to identify and quantify different species within a sample (Bikel et al., 2015). This method is utilized for two primary reasons—it is fast and cost effective. However, 16S RNA sequencing is not without its limitations in characterizing the microbiota in a sample (Li et al., 2018). First, adequate sequencing is limited by primer selection, which can alter the apparent abundance of specific communities within a given sample (Bikel et al., 2015). Second, external factors such as reagent contamination and varying amplification cycling conditions can affect results (Salter et al., 2014; Eisenhofer et al., 2019; Stinson et al., 2019). Third, 16S sequencing is limited to using only known primers resulting in the potential for excluding unknown sequences and thus excluding bacterial species present in a sample (Ross et al., 2012).

While these initial studies have provided important preliminary data, they demonstrate the limitations of 16S rRNA sequencing in patients with EoE and highlight the varying sampling methods used for analysis. Given the complexity of the host microbiome, differences in sampling and analysis lead to further difficulties deriving meaningful information from these studies. In the six studies above, there were various methods utilized to characterize the EoE microbiome. First, both culture independent with 16S rRNA sequencing and traditional culture driven methods were utilized. These methods can produce different results from the same sample. Not all bacteria can be cultured using traditional media, leading to exclusion of bacterial species. An advantage of culture driven data vs. culture independent approaches is that advanced sequencing techniques utilizing rRNA can potentially amplify dead bacteria and skew results toward bacteria that are not actually active in a sample (Norder Grusell et al., 2018). Theoretically, this could amplify bacteria that play no active role in the disease process but are identified because of the sequencing method selected. Second, in studies that used 16s rRNA, two groups used primers from the V4 region while three groups used primers V1-V2. This difference can lead to skewed identification based on primer selection; however, there is no consensus on which hypervariable region is most appropriate for use in EoE. Different primers are selected for a variety of reasons, including cost and lab availability. Finally, the sampling sites and methods of these studies varied. These include traditional esophageal biopsy, esophageal string test, saliva sampling, and stool collection. There is no consensus on the most accurate way to collect microbial data from esophageal tissue, but esophageal biopsy obtained via endoscopy has traditionally been the gold standard. It is possible that different sampling techniques utilized could play a role in varying results outlined above. For example, the

researchers that utilize the string test for sampling provide strong evidence that this test accurately captures esophageal microbial composition when compared with biopsy results.

While 16S sequencing is a valuable tool for quantifying the composition and abundance of bacteria in a sample, it does little to describe the function of individual bacteria within a microbial community. The full genetic make-up and function of a microbial community can be described better through the study of metagenomics, metatranscriptomics, and metabolomics. Metagenomics characterizes the gene content and functional potential of a microbial sample. Metatranscriptomics describes the functional genetic profile of a particular sample at a given point in time by characterizing the mRNA expression of the microorganisms in the sample. In other words, as Li et al. state, metagenomics and metatranscriptomics characterize a bacterial community by identifying "who they are" and what they do" (Li et al., 2018).

Metabolomics is the study of the down-stream effect of this genetic material that identifies the metabolites produced by a microbial community. Metabolomics describes a high-throughput technique for directly measuring biochemical activity by monitoring the substrates and biochemical products produced during cellular metabolism (Patti et al., 2012). Taken together, these techniques can fully characterize a microbial community by describing the microbial species present, ascertaining differentially expressed transcripts, and defining the metabolites produced as a result of altered gene expression.

These advances in sequencing techniques are not without limitations. Human DNA can interfere with the ability to correctly sample the microbiome and is highly dependent on the site from which the sample is taken (Bikel et al., 2015). This leads to potential for large sequences of DNA to be eliminated because they are derived from the host instead of the microbial community, which is wasteful and can be cost prohibitive (Bikel et al., 2015). Outside of cost, other relevant challenges also include lack of adequate reference databases, inability to differentiate active vs. inactive members, and sequencing such a large array of microorganisms (Shakya et al., 2019).

One challenge in the use of advanced sequencing techniques is interpreting the large volumes of data produced and attempting to determine how that data can be used to advance real life implications of disease. While the amount of information derived from study of the -omics can seem daunting, others have shown how integration of these techniques can directly lead to important discoveries. For instance, van Dam et al. gained valuable insight into gene expression of *M. tuberculosis* by utilizing omics co-expression networks that would have not been possible by focusing on only one type of analysis in isolation (Van Dam et al., 2014).

While there has been work done to characterize the microbiome in patients with EoE, little work has been done in these fields. Study of advanced genomics in EoE represents a substantial knowledge gap which needs to be filled in the future to advance the characterization and treatment of EoE.

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.

AUTHOR CONTRIBUTIONS

MB and JB contributed toward literature search, identifying relevant publications, data extraction, interpreting the results, drafted the manuscript, and designed the tables and figures. SRD, GH, and YC contributed toward conceiving and designing the study, literature search, identifying relevant publications, data extraction, interpreting the results, drafted the manuscript, and designing the tables and figures. All authors contributed to the article and approved the submitted version.

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REVIEW
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Metabolic Influences of Gut Microbiota Dysbiosis on Inflammatory Bowel Disease

*Salma Sultan[1], Mohammed El-Mowafy[2], Abdelaziz Elgaml[2,3], Tamer A. E. Ahmed[1,4], Hebatoallah Hassan[1,5] and Walid Mottawea[1,2]**

[1] Faculty of Health Sciences, School of Nutrition Sciences, University of Ottawa, Ottawa, ON, Canada, [2] Department of Microbiology and Immunology, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt, [3] Department of Microbiology and Immunology, Faculty of Pharmacy, Horus University, New Damietta, Egypt, [4] Department of Cellular and Molecular Medicine, Faculty of Medicine, University of Ottawa, Ottawa, ON, Canada, [5] Department of Biotechnology, Institute of Graduate Studies and Research, Alexandria University, Alexandria, Egypt

Inflammatory bowel diseases (IBD) are chronic medical disorders characterized by recurrent gastrointestinal inflammation. While the etiology of IBD is still unknown, the pathogenesis of the disease results from perturbations in both gut microbiota and the host immune system. Gut microbiota dysbiosis in IBD is characterized by depleted diversity, reduced abundance of short chain fatty acids (SCFAs) producers and enriched proinflammatory microbes such as adherent/invasive *E. coli* and H_2S producers. This dysbiosis may contribute to the inflammation through affecting either the immune system or a metabolic pathway. The immune responses to gut microbiota in IBD are extensively discussed. In this review, we highlight the main metabolic pathways that regulate the host-microbiota interaction. We also discuss the reported findings indicating that the microbial dysbiosis during IBD has a potential metabolic impact on colonocytes and this may underlie the disease progression. Moreover, we present the host metabolic defectiveness that adds to the impact of symbiont dysbiosis on the disease progression. This will raise the possibility that gut microbiota dysbiosis associated with IBD results in functional perturbations of host-microbiota interactions, and consequently modulates the disease development. Finally, we shed light on the possible therapeutic approaches of IBD through targeting gut microbiome.

Keywords: IBD, gut microbiota, dysbiosis, SCFAs (short chain fatty acids), metabolome

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***Correspondence:**
Walid Mottawea
wmottawe@uottawa.ca

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INTRODUCTION

Inflammatory Bowel Disease (IBD) is a chronic and remitting disorder characterized by relapsing episodes of gastrointestinal inflammation (Ramos and Papadakis, 2019). IBD is comprised of two major classes; ulcerative colitis (UC) and Crohn's disease (CD). The inflammation in UC is restricted to the mucosal layer, leading to superficial damage of the bowel wall, while CD is associated with inflammation of all layers of the bowel wall (Kobayashi et al., 2020). Epidemiological studies showed that IBD is spreading widely throughout the world leading to a public health challenge worldwide (Zheng H. et al., 2017; Windsor and Kaplan, 2019; Kaplan and Windsor, 2021). It is widely accepted that the IBD pathogenesis results from an interplay between gut microbiota and the host immune system with the predisposition of genetic susceptibility and environmental factors (Ma et al., 2019; O'Donnell et al., 2019; Ramos and Papadakis, 2019; Pang et al., 2021). The

microbiome-immune system interaction leads to improper immune activation responsible for the clinical and endoscopic observations in IBD patients (Ramos and Papadakis, 2019; Zheng et al., 2020).

The human gastrointestinal tract comprises very rich and diverse microbial community that includes more than 100 trillion microorganisms (Thursby and Juge, 2017). The gut microbiome plays an important role in human health and in the development of several chronic diseases. The altered composition of the gut microbiota (dysbiosis), in addition to the disturbance of the metabolic harmony of such microbial community plays a crucial role in the pathogenesis of IBD (Nishida et al., 2018; Zuo and Ng, 2018; Khan et al., 2019). Indeed, recent advances in molecular biology techniques together with improving the usability of microbial databases led to comprehensive characterization of microbial communities and revealing the association between gut microbiota dysbiosis and IBD (Wright et al., 2015; Lloyd-Price et al., 2019; Loeffler et al., 2020; Ryan et al., 2020). However, the actual contributions of this dysbiosis to the inflammation and the cause/effect relationship between gut microbe and IBD are still unclear.

In this review, we outline the recent findings that correlate gut microbiota dysbiosis and metabolic dysfunctionality to IBD. We highlight the metabolic pathways by which microbial dysbiosis could contribute to the inflammation seen in IBD. We also consider the connection among different metabolic pathways in relation to disease progression. In addition, we clarify the strategies of manipulating gut microbiota to promote gut health in IBD.

NORMAL GUT MICROBIOTA

Our gut is populated by a complex and dynamic microbial ensemble, which is considered an additional organ of the human body and collectively known as the gut microbiota. This cohort consists mainly of bacteria with low percentage of archeae, eukaryotic and viral members (Qin et al., 2010). Using fecal samples from 124 Europeans and Illumina-based metagenomic sequencing, the gut microbial gene catalog was estimated to be 100–150-fold that of human genes with 99.1% of bacterial origin. The bacterial species that compose the entire community were calculated to range from 1,000 to 1,150 prevalent species with at least 160 species per individual (Qin et al., 2010).

Studying mucosal and luminal microbiota structure via sequencing *16S rDNA* clones has revealed that approximately 90% of gut microbes are related to main two phyla; Firmicutes (51%) and Bacteroidetes (48%), with the remaining 10% distributed among Proteobacteria, Actinobacteria, Fusobacteria, and Verrucomicrobia (Eckburg et al., 2005). At lower levels, the majority of the detected Firmicutes were related to the Clostridia class, with the vast majority classified under clostridial clusters IV, XIVa, and XVI. Bacteroidetes, on the other hand, has showed less diversity than Firmicutes with 67% of its sequences classified under 3 main phylotypes*B. vulgatus*, *Prevotellaceae*, and *B. thetaiotamicron*. *B. thetaiotamicron* was the only common Bacteroidetes among all tested individuals. Other low abundant species related to other phyla included Proteobacteria species (*Sutterellawadsworthensis*, *Desulfomonaspigra*, and *Bilophilawadsworthia*), Actinobacteria genera (e.g., *Actinomyces*, *Collinsella*, and *Bifidobacterium*) and the mucin-degrading Verrucomicrobia species, *Akkermansiamuciniphila* (Eckburg et al., 2005). Along with bacteria, the most common coexisting fungal genera in the gut are *Saccharomyces*, *Candida*, *Galactomyces*, *Pleospora*, *Bullera*, *Aspergillus*, *Trametes*, *Sclerotinia*, *Penicillium*, and *Rhodotorula*, while virome mainly exists in the form of bacteriophages (Ungaro et al., 2019a; Beheshti-Maal et al., 2021).

The diversity and the composition of gut bacteria vary along the length of the gastrointestinal tract (GIT) with a gradual increase in bacterial load and diversity from the stomach and duodenum (10^2 CFU/gm content) to the colon (10^{11}–10^{12} CFU/gm content) (Eckburg et al., 2005; Eckburg and Relman, 2007; Frank et al., 2007; Sartor, 2008). The composition also varies at different intestinal locations starting with aerobic *Streptococcus* and *Lactobacillus* species in the duodenum and ending with strict anaerobes such as *Bacteroides*, *Bifidobacterium* and clostridial clusters in the colon (Frank et al., 2007). This difference in composition and diversity may be attributed to nutritional substrate availability, oxygen content, luminal acidity, and other physiological and immunological conditions at different parts of the GIT.

In addition to the stable differences in the composition at different intestinal locations, the gut bacteria are known to, significantly, vary among individuals (Eckburg et al., 2005). Furthermore, gut bacteria have been shown to be in continuous temporal variation within the same individual as revealed by monitoring the gut microbiota structure of two subjects for 15 months (Caporaso et al., 2011). These temporal variations could be attributed to small perturbations in some environmental factor, such as a short-term change in diet, a gastrointestinal infection, or due to a reversible change in the immunological state of the host (Caporaso et al., 2011; Lozupone et al., 2012). However, the gut microbiota tolerates these temporal changes and returns to its original structure once all physical and physiological conditions of the gut return to the normal state, which is known as resilience of the gut microbial ecosystem (Lozupone et al., 2012). Contrary to this, gut microbiota can lose this resilience by exposure to certain permanent factors, such as broad spectrum antibiotics (Lozupone et al., 2012), chronic diseases [e.g., Diabetes (Qin et al., 2012), IBD (Sartor, 2008)], obesity (Ray, 2012), or with age (Claesson et al., 2011). With regards to age, gut microbiota begins to colonize the intestine immediately after birth with a few aerobes (Enterobacteria, *Staphylococcus,* and *Streptococcus*), which gradually are replaced by anaerobic bacteria to reach the same complexity as mentioned above for adults by the first year of life (Palmer et al., 2007). However, differences between children and adults are still significant even after the first year of life. For example, children 1–7 years of age have lower fecal microbiota diversity with higher abundance of Enterobacteria than adults (Hopkins et al., 2001). Also, a more recent comprehensive study has indicated a substantial difference in elderly people when compared to the established

adult pattern (Claesson et al., 2011), with Bacteroidetes as the predominant phyla and lower abundance of Firmicutes. This age-related pattern has been confirmed previously by comparative assessment of Firmicutes/Bacteroidetes ratio at different ages by quantitative PCR (Mariat et al., 2009). Also, individual bacterial species such as *Escherichia coli*, *Enterococci*spp., *Bacteroides*spp., *Bifidobacterium* spp., and lactobacilli have been demonstrated to exhibit specific age-related profiles in adults and elderly subjects (Woodmansey, 2007; Enck et al., 2009). Yatsunenko et al. (2012) linked the microbial diversity to both age and geographical location. They surveyed the gut microbiome structure in the stool of 314 Americans, 114 Malawians, and 100 Amerindians at different ages. They reported that the inter-subject variability is higher in the early stages of life compared to the adult microbiome with a progressive increase in microbial diversity with age (Yatsunenko et al., 2012). The authors also illustrated that the infant microbiome is dominated by Bifidobacteria and reaches an adult-like composition by the age of three (Yatsunenko et al., 2012). Moreover, the composition of the gut microbiota was more similar between Malawians and Amerindians and its diversity was higher than the American's reflecting the association between geography and gut microbiota diversity.

ROLE OF GUT MICROBIOTA IN HUMAN HEALTH

Many reports have described humans as a superorganism that live in symbiosis with different microbes within various parts of the body. This ecosystem offers many benefits to the human host that are essential for good health. The gut harbors the greatest human microbial assembly (Sultan et al., 2020; El-Mowafy et al., 2021). The first role of the gut bacteria involves its metabolic capacity to process undigestible food particles such as complex carbohydrates, plant glycans, choline and bile acids (Tremaroli and Backhed, 2012). The microbial processing of indigestible polysaccharides generates beneficial short chain fatty acids (SCFAs) such as butyrate, acetate and propionate, which represent around 90% of SCFAs produced in the human gut (Cummings and Macfarlane, 1991). Butyrate is considered the major source of energy to the colonocytes. For example, the colonocytes of germ-free mice develop impaired mitochondrial respiration and increased autophagy in comparison with conventionally raised mice, and these findings have been reversed by adding butyrate to germ free colonocytes (Donohoe et al., 2011). On the other hand, acetate and propionate exert extra intestinal roles, where they act as metabolic substrates for lipogenesis and gluconeogenesis (Bergman, 1990; Tremaroli and Backhed, 2012). The major producers of SCFAs include the genus *Bacteroides*, Clostridium clusters IV and XIVa and *Bifidobacterium* (Cummings and Macfarlane, 1991; Louis et al., 2010; Martens et al., 2011). While *Eubacterium rectale*, *Roseburiafaecis*, *Eubacterium hallii,* and *Faecalibacterium prausnitzii* are the major butyrate producers in the gut as revealed by investigating the butyryl-CoA:acetate CoA-transferase gene (Louis et al., 2010), *B. thetaiotamicron* and *B. ovatus* showed a high genomic content of carbohydrate active enzymes (CAZymes) that enable them to metabolize indigestible plant and host glycans (Martens et al., 2011). This might explain the predominance of Bacteroidetes in the gut of rural Africans who have a mainly plant-based diet (De Filippo et al., 2010). The highest percentage of SCFAs in the large bowel is seen in the cecum and proximal colon, and decreases gradually toward the distal colon (Cummings and Macfarlane, 1991). This gradient may be explained by the higher prevalence of substrates in the proximal colon, which decreases progressively toward the rectum (Cummings and Macfarlane, 1991). The majority of the SCFAs produced are absorbed by the gut or delivered to peripheral tissues such as the liver and muscles (Bergman, 1990; Cummings and Macfarlane, 1991). Hence using feces to measure the gut metabolites may be inappropriate.

Gut microbiota, via various mechanisms, contributes to intestinal epithelial integrity. The first of these factors is the production of SCFAs, notably butyrate. Butyrate is a main modulator of mucin release, which acts as the first barrier against gut microbial invasion (Barcelo et al., 2000). In addition, butyrate controls gene expression of the colonocytes either via inhibition of histone deacetylase (HDAC) or through binding to G-protein coupled receptors (GPR41 or GPR43) (Tremaroli and Backhed, 2012). For example, sodium butyrate up-regulates the expression of the tight junction proteins and their mRNA via the inhibition of HDAC (Bordin et al., 2004). Furthermore, intra-rectal delivery of *C. tyrobutyricum* to immunocompetent and immunodeficient pathogen free mice showed a protective effect against dextran sulfate sodium (DSS)-induced colitis. This is mediated by inducing the expression of zonula occludens (ZO)-1 tight junction proteins, as well as MUC-2 mucin both of which are directly related to the luminal level of butyrate (Hudcovic et al., 2012). In addition to SCFAs, other microbial components contribute to the epithelial integrity. Germ free mice showed a thinner mucus layer relative to microbiota colonized mice, and this was corrected following exposure to LPS or peptidoglycan (Petersson et al., 2011). Recently, it has been shown that gut microbiota, also, induces intestinal mucosal endothelial and mesenchymal cells via stimulation of toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD) like receptor pathways (Schirbel et al., 2013). Together, this emphasizes the critical role of gut microbiota or their metabolites in maintaining the integrity of the intestinal barrier.

In addition to the development of tissues and cells, the gut microbiota is responsible for the shaping and maturation of the immune system. Germ free mice showed defective gut associated lymphoid tissues (GALT), fewer and smaller Peyer's patches, less cellular mesenteric lymph nodes, less cellular lamina propria, lower expression of TLRs and class II major histocompatibility complex (MHC II) molecules, and finally reduced intraepithelial lymphocytes and $CD4^+$ T cells in comparison to conventional mice. Additionally, gut colonization with microbes corrected some of these deficiencies (Lee and Mazmanian, 2010). In relation to specific pathogen free mice, germ free mice also showed accumulation of invariant natural killer T cells (iNKT) in the lamina propria of the colon and lung, which resulted in higher mortality rates (Olszak et al., 2012). However, the exposure of neonatal germ free mice, but not adults, to

commensal bacteria was protective against iNKT-accumulation and its undesirable consequences (Olszak et al., 2012). Likewise, mice who received $CD4^+CD62L^+$ lymphocytes from germ free mice developed colitis faster than mice who received the same regulatory T cells (Tregs) from conventionally housed mice (Strauch et al., 2005). This suggests a critical role of the gut microbiota in the development of the intestinal immune system. This gut immune maturation is dependent on the host-specific microbiota. Colonization of germ free mice with either human or rat microbiota resulted in fewer intestinal T cells and dendritic cells, and lower antimicrobial peptide expression relative to germ free mice colonized with a murine microbiota, and humanized mice were more susceptible to salmonella infection (Chung et al., 2012). Both humanized mice and the mice colonized with a murine microbiota showed similar bacterial diversity at higher taxonomic levels, but they harbored different species (Chung et al., 2012). This indicates that each host selects a specific microbial consortium that shapes its immune system and maintains intestinal health.

GUT MICROBIOTA DYSBIOSIS IN IBD

As mentioned above, our gut is populated with a complex and dynamic ecosystem, which under normal circumstances is characterized by a stable structure at various intestinal segments in everyone. Any alteration of this consortium may disrupt its functionality and eventually, a diseased state will appear. The dysbiosis of the intestinal microbiota is well reported in different diseases such as irritable bowel syndrome (IBS), obesity, diabetes, and IBD (Shanahan, 2007; Saulnier et al., 2011; Hansen et al., 2012; Qin et al., 2012; Everard et al., 2013). The relationship between IBD and gut microbiota dysbiosis was first established by studying animal models of colitis. Germ free IL $10^{-/-}$ mice do not develop colitis unless they are colonized by enteric bacteria (Schwerbrock et al., 2004). Garrett et al. (2007) were able to clearly demonstrate that the alteration of the gut microbiota composition could induce colitis in immunocompetent mice. They reported that deficiency of T-bet, a transcriptional factor that is important for gut homeostasis, resulted in microbial population shifts into a colitogenic community. This colitogenic microbiota was able to drive the intestinal inflammation in genetically intact mice (Garrett et al., 2007). Many molecular studies have illustrated the changes in gut microbial composition of IBD patients in comparison with non-IBD controls. The gut microbiota of individuals with IBD is characterized by low microbial diversity (Ott et al., 2004; Andoh et al., 2012), a reduced abundance of *Bifidobacterium* spp. (Joossens et al., 2011; Andoh et al., 2012), *Lactobacillus* spp. (Ott et al., 2004), and *F. prausnitzii* (Sokol et al., 2009; Joossens et al., 2011; Andoh et al., 2012) and a higher abundance of pathobionts such as adherent/invasive *E. coli* (Darfeuille-Michaud et al., 2004; Sokol et al., 2006a) and *C. difficile* (Rodemann et al., 2007), resulting in lower SCFA concentrations compared with healthy individuals (Huda-Faujan et al., 2010).

Studies using *16S rDNA* sequencing have shown a decrease in the diversity of gut biota in IBD mucosal specimens (Baumgart et al., 2007; Frank et al., 2007). Using single strand confirmation polymorphism (SSCP) fingerprint, based on *16S rRNA* showed that the diversity decreased by 50 and 30% in CD and UC, respectively (Ott et al., 2004). Frank et al. (2007) illustrated this imbalance by sequencing SS-rRNA clones from 190 biopsies. In their study, the *Lachnospiraceae* family of Firmicutes and Bacteroidetes were depleted in IBD subjects, with a relative increase of Proteobacteria, Actinobacteria, and Bacillus subgroups of Firmicutes. FISH analysis, on the other hand, has illustrated an increase in the relative abundance of Bacteroidetes and a low abundance of some butyrate producing bacteria such as *F. prausnitzii*in mucosal IBD specimens (Swidsinski et al., 2005; Sokol et al., 2008b, 2009). Using 454 pyrosequencing of the *16S rRNA* V5 and V6 regions extracted from the fecal materials of concordant and discordant twins, ileal CD, colonic CD and healthy individuals were differentiated from each other according to their microbial profile (Willing et al., 2010). However, the authors were not able to discriminate between UC and healthy subjects by following the same approach (Willing et al., 2010). Colonic CD was characterized by higher Firmicutes (mainly *Faecalibacterium*, and *Ruminococsulus*), *Bifidobacteriaceae* (*Bifidobacterium*), *Coriobacteriaceae* (*Collinsella*), and *Aneroplasmataceae*. Conversely, ileal CD showed depletion of *Ruminococaceae* family, especially *Faecalibacterium*, and *Collinsella* with higher abundance of Proteobacteria due to the increase of the *Enterobacteriaceae* family (Willing et al., 2010). For UC, Willing et al. (2010) were able to identify few differences such as depletion of *Prevotella*, *Streptococcus*, and *Asteroleplasma*. In contrast, other study was able to discriminate between UC and healthy individuals by calculating the mean of *16S rDNA*-clone libraries taken from sigmoid colon biopsies of 62 individuals (Lepage et al., 2011). The microbiota dysbiosis of UC was characterized by less diversity, fewer *Lachnospiraceae* and *Ruminococcacea* families with higher abundance of Proteobacteria and Actinobacteria (Lepage et al., 2011). These two studies confirm the association between IBD and gut microbiota dysbiosis. However, the discrepancy between them, regarding the UC microbiota structure, underlines the importance of the sampling approach in these types of studies (Mottawea et al., 2019).

For pediatric IBD, some studies have monitored the gut microbiota in IBD compared to healthy controls. Conte et al. (2006) described the alteration in microbiota composition along the length of the intestine in pediatric IBD patients in comparison to control subjects. They used the conventional culture-based techniques and *16S rRNA*-based real time PCR for quantifying the mucosa associated bacteria at the ileum, caecum, and rectum of 42 subjects. One important observation of this study is that, in contrast to adult IBD, *B. vulgatus* was found at lower abundance in patients with IBD compared to controls. This supports the idea that pediatric IBD is a unique form of IBD. Moreover, a study investigating the relative abundance of 9 bacterial groups using real-time PCR showed higher number of *E. coli* and lower number of *F. prausnitzii* in children with CD in comparison to control subjects (Schwiertz et al., 2010). Furthermore, microbiota diversity in pediatric IBD patients was shown to differ from that of adults (Cucchiara et al., 2009).

However, these studies solely examined the dominant bacterial groups by applying conventional culture-based techniques and simple molecular methodology. Since it is widely accepted that 80% of gut microbiota are unculturable (Eckburg et al., 2005), a comprehensive molecular survey of the gut microbiota in pediatric IBD is necessary. Two studies have applied a high throughput molecular approach to characterize gut microbiota in pediatric IBD (Hansen et al., 2012; Papa et al., 2012). The first study applied synthetic learning in microbial ecology (SLiME) analytic approach of 454 pyrosequencing data obtained from fecal samples of 91 individuals and other published datasets. They were able to differentiate between children with IBD and healthy individuals or those with other diseases based on their microbiota composition (Papa et al., 2012). The drawback of this study is that they relayed upon stool samples for their analysis, and it is known that fecal materials contain different microbiota when compared to mucosa associated bacteria. Moreover, Hansen et al. (2012) reported that the microbiota diversity is lower in CD but not in UC relative to control subjects. Pairwise comparison among the 3 groups identified only 7 differentially abundant taxa. One important result is that *F. prausnitzii* was highly abundant in CD compared to controls, which is the reverse of what has been previously documented in adults with IBD (Sokol et al., 2008b; Hansen et al., 2012), indicating once again the unique microbiota composition of pediatric IBD. Using intestinal mucosal colonoscopic washes, Mottawea et al. (2016) have reported dysbiosis of gut microbiota in new onset pediatric IBD with enrichment of H_2S producing bacteria. Overall, these studies support the association between intestinal microbiota imbalance and pediatric IBD at different ages. Nevertheless, the cause/effect relationship between these conditions and gut microbiota is still unclear.

Generally, microbiota dysbiosis is significantly greater in patients with CD than with UC (Pascal et al., 2017), where the microbial community stability and diversity is significantly lower in case of CD than in UC (Ryan et al., 2020). In case of CD a specific microbial signature that is comprised of eight groups is well reported. Eight groups of microorganisms involving *Anaerostipes*, *Methanobrevibacter*, *Faecalibacterium*, an unknown *Peptostreptococcaceae*, *Collinsella*, an unknown *Christensenellaceae*, and *Escherichia*, *Fusobacterium* could be utilized to differentiate between CD from non-CD, where, the first six groups are relatively low and the latest two groups are relatively high in case of CD (Pascal et al., 2017; Ryan et al., 2020). Moreover, it is noteworthy fecal (stool) and mucosal sampling is critical during the determination of the microbial dysbiosis in IBD (Lo Presti et al., 2019). The IBD stool samples are characterized by reduced diversity of microbiota in comparison to IBS and healthy population (Mei et al., 2021). IBD cases are characterized by decreased Verrucomicrobia and Bacteroidetes than healthy population (Lo Presti et al., 2019; Mei et al., 2021). On the other hand, in case of IBS Bacteroidetes are increased in comparison to healthy population. Moreover, IBD are reported to harbor less population of Bacteroidetes and Verrucomicrobia, and higher abundance of Actinobacteria in comparison to IBS (Lo Presti et al., 2019; Ryan et al., 2020; Mei et al., 2021). *Lactobacillus*, *Ruminococcus,* and *Streptococcus* are significantly higher in IBD than healthy population, while *Oscillospira*, *Lachnospiraceae*, *Ruminococcaceae,* and *Rikenellaceae*as well as *Akkermansia muciniphila* are diminished in IBD (Lo Presti et al., 2019; Ryan et al., 2020; Mei et al., 2021). In case of IBS, *Pseudomonas* and *Lactococcus* are decreased in comparison to healthy population, while *Parabacteroides distasonis*is relatively increased (Forbes et al., 2016; Lo Presti et al., 2019). By comparing IBS and IBD, *Rikenellaceae*, *Bacteroides*, *Butyricimonas*, *Oscillospira*, *Mogibacteriaceae*, *Anaerostipes*, *Barnesiellaceae*, *Roseburia*, *Parabacteroides*, *P. distasonis,* and *A. muciniphila* were more abundant in IBS than in IBD, while *Granulicatella* was relatively decreased (Dziarski et al., 2016; Lo Presti et al., 2019; Cuffaro et al., 2020). Similarly, to fecal samples, mucosal samples exhibited reduced microbiota diversity passing from healthy population to IBS to IBD (Lo Presti et al., 2019). Generally, there is no significant difference in microbiota population between inflamed and not-inflamed tissue samples of IBD. On contrary, the microbiota of inflamed mucosa of IBD patients exhibited low abundance of Firmicutes and Bacteroidetes and higher abundance of Proteobacteria in comparison to healthy population (Zuo and Ng, 2018). The abundance level of *Enterobacteriaceae* was significantly increased and the abundance levels of *Lachnospiraceae*, *Ruminococcaceae*, *Rikenellaceae*, *Bacteroides*, *Coprococcus*, *F. prausnitzii,* and *P. distasonis* were diminished in IBD inflamed mucosa compared to healthy population (Lo Presti et al., 2019; Aldars-García et al., 2021).

GUT MYCOBIOME DYSBIOSIS IN IBD

In addition to dysbiosis of bacteria, the dramatic changes that occur in the fungal community named as "mycobiome" is relatively important during IBD (Liu et al., 2020). Mechanistically, it appears that fungi may paly crucial role in the progression of IBD through either affecting the gut microbiota composition or increasing the production of pro-inflammatory cytokines (Iliev and Leonardi, 2017). In addition, mycobiome dysbiosis is a well-reported case in IBD (Beheshti-Maal et al., 2021). It is reported that fungal diversity is higher in CD patients than in healthy controls (Ott et al., 2008).

An increased ratio of Basidiomycota/Ascomycota is a characteristic feature of IBD (Sokol et al., 2017). Qiu et al. (2017) reported higher abundances of *Aspergillus*, *Wickerhamomyces*, *Candida,* and *Sterigmatomyces* and lower abundances of *Alternaria*, *Penicillium*, *Exophiala*, *Emericella*, *Acremonium*, *Epicoccum,* and *Trametes* in patients suffering from UC in comparison to healthy controls. However, there was no significant association between *Basidiomycota*/*Ascomycota* ratio and the increased levels of pro-inflammatory cytokines. Moreover, *Candida* spp., particularly *C. albicans*, is significantly increased in patients suffering from CD or general IBD (Li et al., 2014; Chehoud et al., 2015; Sokol et al., 2017). It is reported that specific-pathogen-free (SPF) Clec7a$^{-/-}$ mice exhibit more severe colitis symptoms when colonized with the pathogenic fungus, *C. tropicalis* compared to uncolonized Clec7a$^{-/-}$ mice or colonized wild type mice (Iliev et al., 2012; Tang et al., 2015).

These studies confirm the crucial link between mycobiome dysbiosis, especially *Candida* spp. and IBD (Li et al., 2019). Li et al. (2014) illustrated higher levels of *Candida* spp. in the inflamed mucosa of IBD patients than in healthy population. In addition, Standaert-Vitse et al. (2009) illustrated higher colonization of familial CD patients by *C. albicans*. Moreover, other reports reported high levels of both *C. albicans* and *C. glabrata* in CD patients (Liguori et al., 2016; Sokol et al., 2017). In addition, Kowalska-Duplaga et al. (2019) documented the high abundance of *Candida* in CD patients, which was decreased by therapeutic intervention, especially with anti-TNF-α treatment.

Besides *Candida* spp., *Malasseziarestricta*, which is a skin normal fungus, significantly increases in patients suffering from CD (Limon et al., 2019). This fungus was found to aggravate the colitis in mouse models via mechanisms demanding on a protein included in antifungal immunity; CARD9 (Limon et al., 2019). Moreover, depletion of *Saccharomyces cerevisiae* is reported in IBD patients (Sokol et al., 2017). Tiago et al. (2015) depicted the protective effects of *S. cerevisiae* UFMG A-905 in mice suffering from UC. In addition, Sivignon et al. (2015) reported that adherent-invasive *E. coli* (AIEC)-induced ileal colitis can be reduced by *S. cerevisiae* CNCM I-3856 in a murine model. However, on the other hand, Chiaro et al., 2017 demonstrated that *S. cerevisiae* worsens the disease condition in a murine model of colitis (Chiaro et al., 2017).

The crosstalk between fungi and bacteria may be a pivotal concern in IBD patients. However, in pediatric IBD patients there was no significant correlation between leading fungal species with particular bacterial taxa and it has been hypothesized that fungal dysbiosis may be a result of or a cause for the gut bacterial dysbiosis (Chehoud et al., 2015). On the other hand, previous report showed a positive correlation between *C. tropicalis* and both *E. coli* and *Serratia marcescens* (Hoarau et al., 2016). It is noteworthy that the correlation between mycobiota-bacterial dysbiosis with the disease severity in IBD patients may afford evidence for the crucial role for gut mycobiome during IBD.

GUT VIROME DYSBIOSIS IN IBD

In addition to microbiota and mycobiota, gut virome, which is comprised of viruses infecting both prokaryotes and eukaryotes, constitute a large portion of gut microbiome (Manrique et al., 2016; Lin and Lin, 2019). Bacteriophages are the major components of the enteric virome (Clooney et al., 2019). Generally, in patients suffering from IBD, alteration of the virome reflects microbiota dysbiosis (Clooney et al., 2019). One of the first studies, which reported virome dysbiosis in IBD patients revealed higher abundance of bacteriophages infecting *Alteromonadales*, *Clostridiales*, and *Clostridium acetobutylicum* as well as *Retroviridae* family in IBD patients in comparison to healthy population (Pérez-Brocal et al., 2015). It is well reported that Caudovirales phage families, including *Siphoviridae*, *Myoviridae,* and *Podoviridae*, are significantly enriched in IBD subjects (Wagner et al., 2013; Norman et al., 2015; Duerkop et al., 2018). A previous study illustrated the high abundance of Caudovirales phage in children suffering from IBD (Fernandes et al., 2019). On the other hand, another study reported a decreased diversity and consistency of Caudovirales that is directly connected to the inflammation degree of the intestine in UC patients (Zuo et al., 2019). Moreover, phages infecting *Enterobacteria* and *Escherichia* were reported to be abundant in UC patients (Zuo et al., 2019). A recent study established in germ-free mice showed that certain phages including *Escherichia*, *Lactobacillus,* and *Bacteroides* infecting phages as well as phage DNA exacerbate gut inflammation and contribute to IBD pathogenesis through increased production of IFN-γ via a TLR9-dependent pathway (Gogokhia et al., 2019).

In addition to bacteriophages, phages infecting and incorporating into eukaryotic cells are relatively important and have been related to IBD pathogenesis due to their ability of integration in human genome and affecting physiological state of the intestinal cells (Gloor et al., 2010; Beller and Matthijnssens, 2019; Santiago-Rodriguez and Hollister, 2019). Previous reports conducted on patients suffering from UC revealed higher abundance of *Pneumoviridae* and lower abundance of *Anelloviridae* compared to healthy population (Zuo et al., 2019). On the other hand, a study that was established on a small cohort of patients suffering from UC and CD depicted the higher abundance of *Herpesviridae* compared to healthy control (Wang et al., 2015). Other studies demonstrated that infection with Norovirus contributes to intestinal inflammation and can increase the rate of colitis incidence (Cadwell et al., 2010; Basic et al., 2014). Moreover, recent studies depicted the high abundance of *Hepeviridae* and *Hepadnaviridae* in the intestinal mucosa of patients suffering from CD and UC, respectively (Ungaro et al., 2019b). Despite all these studies, the actual role of virome dysbiosis during IBD has not been fulfilled yet, even with some reports revealing that intestinal inflammation may be initiated by eukaryotic viruses. By detecting and identifying viruses that infect IBD patients during the early stages of intestinal inflammation, it will be promising to establish a complete correlation between virome and disease progression.

Major dysbiosis of gut microbiota, mycobiome, and virome during IBD from different studies is summarized in **Table 1**. Moreover, the contribution of this dysbiosis to worsening the condition and progression of the disease is illustrated in **Figure 1**.

DYSFUNCTIONALITY OF GUT MICROBIOTA IN IBD

Two metagenomic studies tried to link the dysfunctionality of the gut microbiome and IBD via two different approaches (Greenblum et al., 2012; Morgan et al., 2012). First, Greenblum et al. (2012) treated the gut microbiota as one single organism and constructed the metabolic network of the entire community and compared the odd ratios, of the topology of different metabolic pathways, genes and biological processes of obese and IBD cases against healthy individuals. This approach yielded an association between IBD and genes involved in NO_2 production and metabolism of choline and p-cresol (Greenblum et al., 2012). In contrast to the metagenomic analysis of the community as a single supra-organism, Morgan et al. (2012)

TABLE 1 | Major dysbiosis of gut microbiome during IBD.

Bacteriome dysbiosis					
Microorganism	**Disease subtype**	**Dysbiosis**	**Model**	**Specimen**	**References**
Bifidobacteria, Firmicutes, *F. prausnitzii*	In both CD and UC	Decrease	Human	Mucosa	Sokol et al., 2008a
Enterobacteriaceae (AIEC, pathogenic *E. coli* B2+D group)		Increase			
Bifidobacteria, Lactobacilli		Decrease		Stool	
Enteroadherent *E. coli* associated with CD or UC		Increase			
Bacteroidetes, Cyanobacteria, *Bacteroides*, *Flavobacterium*, *Oscillospira*	In both CD and UC	Decrease	Human	Stool	Santoru et al., 2017
Firmicutes, Proteobacteria, Verrucomicrobia, Fusobacteria, *Escherichia*, *Faecalibacterium*, *Streptococcus*, *Sutterella*, *Veillonella*		Increase			
Proteobacteria (non-jejuni *Campylobacter*)	CD	Increase	Human	Stool or Mucosa	Zhang et al., 2014
Proteobacteria (*E. coli*)	CD	Increase	Human	Mucosa	Martin et al., 2004
Yersinia enterocolitica, *Bacteroides vulgatus*, *Helicobacter hepaticus*, Mycobacterial species	CD	Increase	Human	Stool	Khan et al., 2019
Firmicutes	In both CD and UC	Decrease	Human	Mucosa	Walker et al., 2011
Bacteroidetes		Increase			
Enterobacteriaceae	CD	Increase			
Anaerostipes, *Methanobrevibacter*, *Faecalibacterium*, an unknown *Peptostreptococcaceae*, *Collinsella*, an unknown *Christensenellaceae*	CD	Decrease	Human	Stool	Pascal et al., 2017
Escherichia, Fusobacterium		Increase			
Firmicutes, Bacteroidetes, and *Lachnospiraceae*	In both CD and UC	Decrease	Human	Mucosa	Frank et al., 2007
Proteobacteria and the Bacillus subgroup of Firmicutes		Increase			
Clostridium IXa and IV groups, *Bacteroides*, Bifidobacteria	In both CD and UC	Decrease	Human	Mucosa	Fava and Danese, 2011
Sulfate-reducing bacteria, *Escherichia coli*		Increase			
Enterobacteriaceae (*E. coli*)	CD	Increase	Human	Stool	Seksik et al., 2003
Bifidobacteria	CD	Decrease	Human	Stool	Favier et al., 1997
Dialister invisus, Clostridium cluster XIVa, *Faecalibacterium prausnitzii*, *Bifidobacterium adolescentis*	CD	Decrease	Human	Stool	Joossens et al., 2011
Ruminococcus gnavus		Increase			
F. prausnitzii	CD	Decrease	Human	Stool	Fujimoto et al., 2013
Bacteroides species, *Eubacterium* species, *Lactobacillus* species	In both CD and UC	Decrease	Human	Mucosa	Ott et al., 2004
Proteobacteria, *Enterobacteriaceae*		Increase			
Firmicutes	In both CD and UC	Decrease	Human	Stool or Mucosa	Morgan et al., 2012
Ruminococcus gnavus, Enterobacteriaceae: Escherichia/Shigella		Increase			
Clostridium leptum group (IV), and *Faecalibacterium prausnitzi*	In both CD and UC	Decrease	Human	Mucosa	Vrakas et al., 2017
Bacteroides		Increase			
Bacteroides fragilis	CD	Increase	Human	Stool	Walters et al., 2014

(Continued)

TABLE 1 | (Continued)

Microorganism	Disease subtype	Dysbiosis	Model	Specimen	References
Enterobacteriaceae	In both CD and UC	Increase	Human	Stool	Jacobs et al., 2016
Faecalibacterium and *Roseburia*		Decrease			Willing et al., 2010
	CD		Human	Stool	
Enterobacteriaceae, *Ruminococcusgnavus*		Increase			
Lactobacillus, Bifidobacteria	In both CD and UC	Decrease	Human	Mucosa	Verma et al., 2010
Bacteroides, *Ruminococcus*		Increase			
F. prausnitzii, Prevotella copri	In both CD and UC	Decrease	Human	Stool	Halfvarson et al., 2017
Enterobacteriaceae, *Ruminococcus*		Increase			
Roseburia hominis, *Faecalibacterium prausnitzii*	UC	Decrease	Human	Stool	Machiels et al., 2014
Firmicutes, Erysipelotrichales, Bacteroidales, Clostridiales		Decrease			Gevers et al., 2014
	CD		Human	Mucosa	
Enterobacteriaceae, *Pasteurellacaea*, *Veillonellaceae*, *Fusobacteriaceae*		Increase			
Firmicutes, *F. prausnitzii*, Bacteroidetes	In both CD and UC	Decrease	Human	Mucosa	Tong et al., 2013
Proteobacteria		Increase			
F. prausnitzii	In both CD and UC	Decrease	Human	Stool or mucosa	Wang et al., 2014
Bifidobacteria, *Lactobacillus* group		Increase			
Enterobacteriaceae	CD	Increase	Human	Stool	Seksik et al., 2003
Firmicutes	TNBS colitis	Decrease	Animal (rats and mice)	Mucosa	Ettreiki et al., 2012
Bacteroidetes, *Bacteroides*, *Enterobacteriaceae*		Increase			
Helicobacteraceae, *Mucispirillum, Desulfovibrio*	Experimental colitis	Increase	T-bet(−/−), Rag2(−/−) mice	Mucosa	Rooks et al., 2014
Enterobacteriaceae and AIEC	Experimental colitis	Increase	IL-10(−/−) mice	Mucosa	Arthur et al., 2012
Akkermansia muciniphila, *Bacteroides distasonis*, *Enterobacteriaceae*, *Clostridium ramnosum*	DSS-colitis	Increase	Animal (mice)	Mucosa	Håkansson et al., 2015
Porphyromonas genera, *Bacteroides*	Experimental colitis	Increase	APC(Δ468) IL-10(−/−) mice	Mucosa	Dennis et al., 2013
Mycobiomedysbiosis					
Microorganism	**Disease**	**Dysbiosis**	**Model**	**Specimen**	**References**
Alternaria, *Penicillium*, *Exophiala*, *Emericella*, *Acremonium*, *Epicoccum,* and *Trametes*	UC	Decrease	Human	Mucosa	Qiu et al., 2017
Aspergillus, *Wickerhamomyces*, *Candida,* and *Sterigmatomyces*		Increase			
Candida spp., particularly *C. albicans*	CD or general IBD	Increase	Human	Stool or Mucosa	Li et al., 2014
Basidiomycota/Ascomycota ratio, *Candida* spp., particularly *C. albicans*	In both CD and UC	Increase	Human	Stool	Sokol et al., 2017
Candida spp., particularly *C. albicans*	CD	Increase	Human	Stool	Standaert-Vitse et al., 2009; Sokol et al., 2017; Kowalska-Duplaga et al., 2019
Candida spp. particularly C. glabrata	CD	Increase	Human	Mucosa	Liguori et al., 2016
Candida spp. *C. albicans*, *C. tropicalis*	Experimental colitis	Increase	Clec7a$^{-/-}$ mice	Mucosa	Dalmasso et al., 2006; Rodríguez-Nogales et al., 2018

(Continued)

TABLE 1 | (Continued)

Virome dysbiosis					
Microorganism	**Disease**	**Dysbiosis**	**Model**	**Specimen**	**References**
Malasseziarestricta	CD	Increase	Human and animal (mice)	Mucosa	Zheng et al., 2015
Bacteriophages infecting Alteromonadales, Clostridiales, and *Clostridium acetobutylicum* as well as Retroviridae family	CD	Increase	Human	Stool or Mucosa	Pérez-Brocal et al., 2015
Caudovirales phage families, including *Siphoviridae*, *Myoviridae,* and *Podoviridae*	Experimental colitis	Increase	Animal (mice)	Mucosa	Galtier et al., 2017
Caudovirales phage	In both CD and UC	Increase	Human	Stool	Fernandes et al., 2019
Caudovirales phage, Anelloviridae	UC	Decrease	Human	Stool	Zuo et al., 2019
Enterobacteria and *Escherichia* infecting phages, Pneumoviridae		Increase			
Escherichia, *Lactobacillus,* and *Bacteroides* infecting phages as well as phage DNA	Experimental colitis	Increase	Animal (mice)	Mucosa	Virgin, 2014
Caudovirales phage families, including *Siphoviridae*, *Myoviridae,* and *Podoviridae*	In both CD and UC	Increase	Human	Stool	Lim et al., 2015
Caudovirales phage	CD	Increase	Human	Mucosa	Kernbauer et al., 2014
Herpesviridae	In both CD and UC	Increase	Human	Mucosa	Wang et al., 2015
Norovirus	Experimental coltits	Increase	Animal (mice)	Mucosa	Basic et al., 2014
Herpesviridae	CD	Increase	Human	Mucosa	Ungaro et al., 2019b
Hepadnaviridae	UC	Increase	Human	Mucosa	Ungaro et al., 2019b

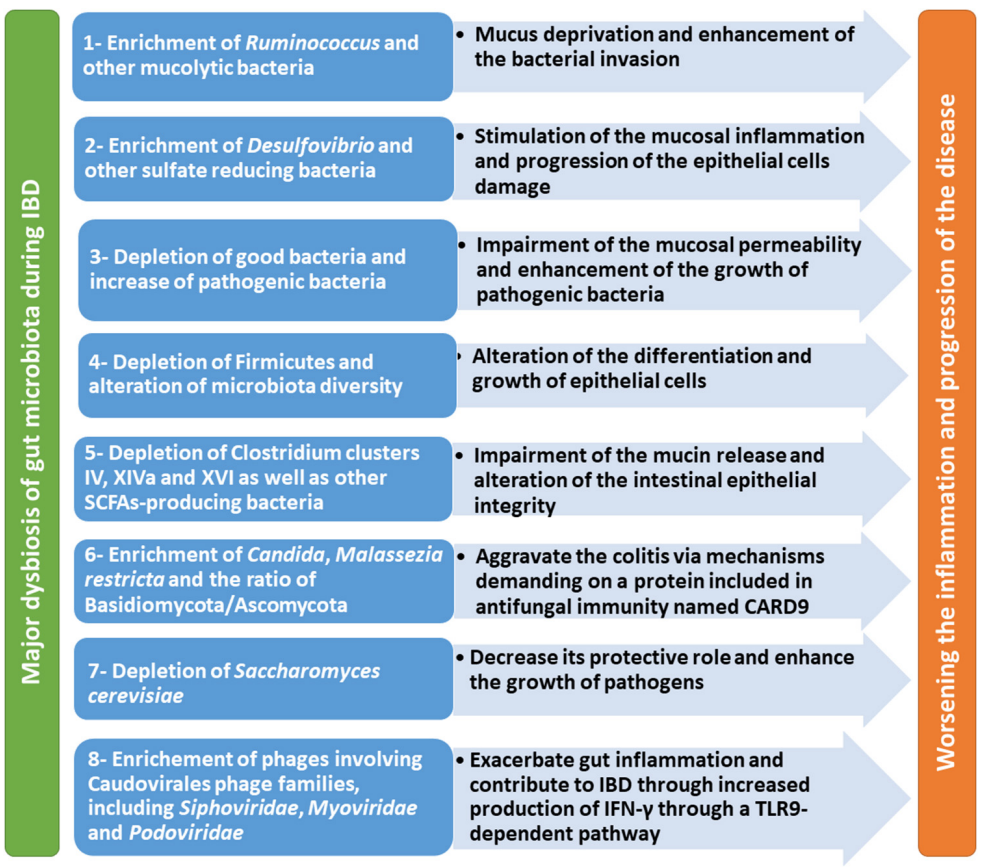

FIGURE 1 | Major dysbiosis of gut microbiome during IBD and its impact on worsening the condition and progression of the disease.

identified the microbiota composition of each disease phenotype. Then, they extracted the genome of the microbiota composing each community and constructed a gene catalog for each phenotype, followed by genes and pathway assessment using sparse multivariate analysis (Morgan et al., 2012). They also confirmed their findings by shotgun metagenomic analysis of 4 CD and 7 healthy fecal microbiota (Morgan et al., 2012). They found an IBD-associated enrichment of genes involved in glutathione transport and metabolism, sulfur amino acid metabolism, redox homeostasis, mucin degradation, secretion systems, adhesion, and invasion as well as a depletion of genes involved in the biosynthesis of SCFAs, some nucleotides and amino acids (Morgan et al., 2012). Recently, an interesting study identified a network of bacterial metabolite interactions including sulfur metabolism as an important player correlated to CD activity (Metwaly et al., 2020). To better understand the dysfunctionality of gut microbiota in IBD, we will discuss the IBD-associated metabolic shift in more detail.

Hydrogen Sulfide Metabolism

Gut microbiota generate hydrogen sulfide (H_2S) via 2 different biochemical pathways. First, sulfate reducing bacteria (SRB) couple the reduction of sulfate as a terminal electron acceptor to the oxidation of H_2 or other organic compounds such as lactate as an electron donor during their anaerobic respiration (Carbonero et al., 2012). The second pathway is followed by bacteria that ferment sulfur containing amino acids. These bacteria might depend on desulfhydrases such as cysteine desulfhydrase or other enzymes, which have the ability to metabolize cysteine (Awano et al., 2005). Sulfate reducing bacteria mainly include members of Deltaproteobacteria in addition to *Desulfotomaculum*, *Desulfosporosinus*, *Thermodesulfobacterium*, and *Thermodesulfovibrio* genera (Blachier et al., 2010). Using multiplex PCR to identify fecal-SRB isolates, luminal SRB are dominated by *Desulfovibriopiger*, *D. fairfieldensis*, and *D. desulfuricans* (Loubinoux et al., 2002). A diversity analysis of *Desulfovibrio* using the dissimilatory sulfite reductase (*dsrAB*) gene confirmed the predominance of *D. piger* with the detection of a new unclassified SRB (Scanlan et al., 2009). Another study identified 8 sulfate and sulphite reducing bacteria in addition to the sulphite reducing bacterium, *Bilophilawadsworthia* via 454 pyrosequencing of *dsrAB* gene fragment (Jia et al., 2012). The identified bacteria include four known species; *D. piger*, *D. vulgaris*, *Desulfovibrio* sp. *NY682,* and *D. desulfuricans F28-1*, and four new species highly similar to *D. desulfuricans* F28-1 (93% *dsrAB*sequence similarity); *D. oxamicus* (84% identity), *Desulfotomaculum* sp. *Lac2* (80% identity), and *D. simplex* (88% identity) (Jia et al., 2012). H_2S producing microbiota through fermentation of amino acids include, for example, *Fusobacterium nucleatum*, Atopobium spp., *Gemella sanguinis*, *Micromonas micros*, *Streptococcus* spp., *Actinomyces* spp., *Eubacterium* spp., *Veillonella* spp., *Bulleidiamoorei.*, *Prevotella* spp., *Campylobacter* spp., and *Selenomonas* spp. (Washio et al., 2005). Mottawea et al. (2016) have reported the enrichment of H_2S producers in children with CD with one H_2S generating organism; *A. parvulum*, has proinflammatory characteristics in IL10$^{-/-}$ mice. Recently, an interesting study identified a network of bacterial metabolite interactions including sulfur metabolism as an important player correlated to CD activity (Metwaly et al., 2020). The authors showed that CD patients with active disease are enriched in members belonging to *Enterococcus*, *Fusobacterium*, *Haemophilus*, *Megasphaera*, and *Campylobacter*, while *Roseburia*, *Christensenellaceae*, *Oscillibacter*, and *Odoribacter* are enriched in CD patients with inactive disease (Metwaly et al., 2020). Another report detected two dominating sulfate-reducing bacteria morphotypes that differ in colonial size and quantitate in the feces of healthy and patients with colitis. In the feces of healthy individuals, 93% of sulfate-reducing bacteria of morphotype I prevailed (*Desulfovibrio*) while morphotype II included only 7% (*Desulfomicrobium*); in the feces of patients with colitis, the ratio of these morphotypes was 99:1, respectively (Kushkevych et al., 2021). In addition to microbiota-released H_2S, it can also be synthesized endogenously by intestinal colonocytes from L-cysteine via two main enzymes; cystathionine β-synthase and cystathionine γ-lyase (Shibuya et al., 2013). Two additional pathways (3-mercaptopyruvate sulfurtransferase in combination with cysteine aminotransferase and 3-mercaptopyruvate sulfurtransferase coupled with D-amino acid oxidase) were identified in H_2S production, peripherally (Shibuya et al., 2013; Guo et al., 2016). Normally, the luminal H_2S concentration of the human large intestine is 1.0–2.4 mmol/L (Macfarlane et al., 1992), while the concentration in the fecal contents ranges from 0.17 to 3.38 mmol/kg (Florin, 1991; Magee et al., 2000). Taking into consideration the lipid solubility and the passive diffusion of H_2S through the intestinal mucosa, these concentrations are underestimated.

At lower concentrations (<1 mM), H_2S is considered a cytoprotective metabolite that induces some cellular anti-inflammatory responses. These cellular responses include, for example, prevention of caspase activation and apoptotic cell death (Rose et al., 2005), inhibition of leukocyte adhesion to vascular endothelium, which decreases infiltration of neutrophils and lymphocytes (Zanardo et al., 2006), induction of cyclooxygenase-2 (COX-2) expression (Wallace et al., 2009), and promotion of neutrophil apoptosis (Mariggio et al., 1998). The later was contradicted by Rinaldi et al. (2006) who concluded that H_2S accelerates the resolution of the inflammation process via inhibition of polymorphonucelar (PMN) apoptosis. In another *in vitro* study, H_2S at normal colonic concentration lowered the proliferation of different colonic cancerous and normal cells and induced autophagy through the AMP-activated protein kinase (AMPK) pathway (Wu et al., 2012). At higher concentrations, H_2S acts as a genotoxic and/or cytotoxic transmitter to the colonocytes by affecting genes responsible for cell cycle progression, DNA repair and inflammatory responses (Attene-Ramos et al., 2010). The main cytotoxic effect of H_2S is the inhibition of cytochrome c oxidase activity, which is the terminal oxidase of mitochondrial respiration (Roediger et al., 1993; Leschelle et al., 2005). This leads to the prevention of the oxidation of essential metabolites such as n-butyrate, L-glutamine and acetate, which eventually decreases the bioenergetic performance of the cell (Roediger et al., 1993; Leschelle et al., 2005). Sulfide has also been shown to inhibit

butyrate oxidation in rat colonocytes through inhibition of short chain acyl dehydrogenation of activated fatty acids (Moore et al., 1997). The antagonistic effect of H_2S on butyrate may induce hyperproliferation of the colonic mucosa (Christl et al., 1996). An indirect cytotoxicity of H_2S was reported, where increased sulfide production induces the conversion of nitrite to nitric oxide, which had a damaging effect on colonocytes (Vermeiren et al., 2012).

To keep the local concentration of H_2S at a harmless level, the colonic mucosa expresses a special H_2S oxidation system that degrades H_2S to sulfate and thiosulfate (Furne et al., 2001). This oxidation system consists of sulfide quinone reductase (SQR), dioxygenase ethylmalonic encephalopathy protein 1 (ETHE1), and thiosulfate sulfur transferase (TST that is also known as rhodanese) (Mimoun et al., 2012). This mitochondrial oxidation of H_2S is not essential for cell respiration, but instead, its main purpose is the detoxification of excess H_2S (Mimoun et al., 2012). In contrast, the respiratory capacity of the cell is an important parameter that affects the efficiency of H_2S detoxification independent of the mitochondrial oxidation system (Mimoun et al., 2012).

A higher abundance of H_2S generated by gut microbiota is considered one of the strongest models associated with the pathogenesis of IBD. Higher luminal H_2S concentration in IBD could arise from either increased abundance of H_2S-producing bacteria or a deficient H_2S-detoxification pathway. The association between H_2S and IBD was first reported in UC. In Roediger et al. (1997) reviewed the role of H_2S in the pathogenesis of UC. They stated that the level of colonic sulfide and the relative abundance of sulfate reducing bacteria was higher in UC patients compared to healthy subjects (Roediger et al., 1997). In addition, the bacteria isolated from UC patients showed higher generation of H_2S than those separated from control cases (Roediger et al., 1997). Treatment of those patients with 5-aminosalicylic acid containing drugs lowered the production of H_2S as indicated by the stool sulfide level and this was proposed to contribute to the therapeutic activities of these drugs (Edmond et al., 2003). Sulfate reducing bacteria were exclusive to patients with UC, where they were isolated from 80% of UC pouches but not form patients with familial adenomatous polyposis (Duffy et al., 2002). Other indirect evidences for the role of H_2S in the pathogenesis of UC are also available. The first comes from studies of diet consumption, where high protein intake is associated with a higher risk of IBD (Ng et al., 2013). Higher protein means increased sulfur containing amino acids and subsequently, elevated H_2S levels. The second is the higher activity of fecal mucin sulphatase in UC patients (Tsai et al., 1995). Mucin sulphatase releases sulfate from the mucosal sulfomucin and this endogenous sulfate provides the source for H_2S biosynthesis by SRB (Tsai et al., 1995). Regarding CD, few reports have linked H_2S production with disease activity. Jia et al. (2012) have reported no difference in the general abundance of SRB between CD and healthy subjects. Mottawea et al. (2016) have illustrated the increased abundance of H2S producing bacteria along with downregulation of mitochondrial proteins implicated in H2S detoxification in children with CD. Some indirect links are also available. For example, increased metabolism of sulfur containing amino acids such as methionine and cysteine associated with a decrease in the metabolism of sulfur lacking amino acids such as lysine and glutamine are characteristics of ileal CD (Morgan et al., 2012). Also, the same study reported an over representation of sulfate transport genes in CD patients. Both sulfate and sulfur containing amino acids are the main precursors for H_2S biosynthesis as mentioned above. The second factor that contributes to higher colonic sulfide concentration is the dysfunctionality of H_2S detoxification. The dysfunctionality of H_2S detoxification genes in IBD is still up for debate. Pitcher et al. (1998) showed that the activity of thiol-methyl transferase (TMT) is higher in the peripheral blood of UC patients. This was then confirmed in 2007, when the activity of TMT and rhodanese were found to be higher in the erythrocytes of UC patients than controls (Picton et al., 2007). However, no difference in TMT and rhodanese activity was detected in the rectal biopsies of the same individuals. In patients with CD neither the erythrocytes nor the rectal biopsies of patients showed a change in that enzymatic activity (Picton et al., 2007). In 2009, the role of H_2S detoxification in IBD emerged again when the activity and the expression of rhodanese were shown to decrease in parallel to the development of dextran sodium sulfate-induced colitis in mice (Taniguchi et al., 2009). In concordance with formerly mentioned studies, this loss of activity is followed by an increase in its activity in red blood cells (Taniguchi et al., 2009). Impaired detoxification of H_2S has been confirmed for UC patients via the assessment of TST expression level and activity in colonic mucosal biopsies (De Preter et al., 2012). As well, it has been confirmed for CD where metaproteomic and expression analyses reported the decreased abundance of H2S detoxification proteins and transcripts in children with new onset CD and UC (Mottawea et al., 2016). These includes the sulfur dioxygenase (*ETHE1*), the thiosulfate sulfurtransferase (*TST*) and the components of complexes III and IV of the mitochondrial respiratory chain, and *tst*, cytochrome *c* oxidase subunit IV (*hcox41*) and the sulfide dehydrogenase genes (*SQRDL*) transcripts (Mottawea et al., 2016). All in all, the association between higher H_2S generation and IBD is well established. Additionally, the impaired intestinal H_2S detoxification in IBD is evident.

Short Chain Fatty Acids Metabolism

Butyrate is known as the salt or ester of butanoic (butyric) acid, which is a weak acid with a pKa of 4.8. By considering the pH of the intestine, which is approximately neutral, most of the butyrate in the intestine will be in the anionic form rather than the free acid form. Butyrate biosynthesis by the gut microbiota starts by condensation of two molecules of acetyl coA to generate one molecule of butyrylcoA. Next, butyrate are generated from butyrylcoA via two main pathways; the enzymes butyrate kinase and phosphotransbutyrylase or butyryl-CoA: acetate-CoA-transferase (Louis and Flint, 2009). The second pathway has been shown to be predominant among butyrate producing microbiota in human colon (Duncan et al., 2002). The major butyrate producing bacteria that inhabit the human gut are related to Clostridium clusters XIVa and IV with few percentage of Clostridium clusters I, XV, and XVI (Louis and Flint, 2009),

while acetate and propionate are mainly produced by *Bacteroides* (Martens et al., 2011). It was indicated that the terminal ileum and proximal colon are the main sites of butyrate production, while acetate and propionate are generated in the distal colon, where *Bacteroides*is the predominant bacteria (Walker et al., 2005). Particularly, the role of butyrate in preventing IBD is illustrated in several studies as mentioned below.

A large body of evidence has revealed the association between IBD, perturbations of butyrate metabolism and/or depleted butyrate producing bacteria in the gut. Initially, a raised luminal butyrate level, as a result of impaired oxidation by colonocytes, was correlated with the severity of mucosal inflammation and was considered as a biomarker in UC patients (Roediger, 1980; Roediger et al., 1982). On the other hand, butyrate intake was shown to have a protective effect against development of colitis independent of restored butyrate oxidation in UC patients (De Preter et al., 2011). For example, administration of butyrate either orally or via local enema to mice or rats with chemically induced colitis alleviated the mucosal inflammation (Butzner et al., 1996; Vieira et al., 2012). Indeed, butyrate exhibits several anti-inflammatory activities. First, butyrate inhibits NFκB activation, which results in suppression of proinflammatory cytokines in UC patients (Luhrs et al., 2002) and rats with trinitrobenzene sulphonic acid (TNBS) induced colitis (Segain et al., 2000). This inhibitory action is impaired in IBD individuals as revealed by assessment of butyrate effect on cytokines production by peripheral blood mononuclear cells (PBMC) isolated from IBD and healthy subjects in response to TLR-2 activation (Kovarik et al., 2011). Secondly, butyrate was reported to induce Fas-mediated apoptosis of T cells via inhibition of HDAC-1 in mice (Zimmerman et al., 2012). This in turn inhibits IFN-γ-induced STAT1 activation, which results in reduced colonocyte expression of inflammatory mediators such as nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX2) (Zimmerman et al., 2012). Finally, Butyrate is known to reduce the inflammation through contributions to intestinal barrier integrity. For example, intra-rectal inoculation of *C. tyrobutyricum*, the potent butyrate producer, has restored MUC-2 secretion, upregulated expression of (ZO)-1 tight junction protein and reduced cytokine release in DSS-treated mice (Hudcovic et al., 2012). Recently, Magnusson et al. (2020) compared impacts of butyrate on the intestinal immune profile of UC patients with active disease and non-inflamed controls. They found that butyrate exhibits different impact on gene regulation and more strongly leads to down-regulation of +expressed genes of inflammatory pathways in non-inflamed controls than in inflamed tissue of UC patients (Magnusson et al., 2020). According to the authors, such discrepancies can partly elucidate why expected anti-inflammatory impacts of local butyrate stimulation or supplementation are not always obtained.

Regarding butyrate producing bacteria, screening of fecal microbiota of 6 healthy and 6 CD individuals via *16S rDNA* clone sequencing and FISH analysis revealed a depletion of *C. leptum* cluster in CD cases compared to the healthy group (Manichanh et al., 2006). In agreement with this, Sokol et al. (2008b) illustrated that *F. prausnitzii* was depleted in CD patients mucosal microbiota and this reduction in abundance was associated with a higher risk of recurrence of ileal CD. Though, they have shown that the anti-inflammatory activity of this bacterium is independent of butyrate production using *in vitro* cellular models and *in vivo* colitis model of mice. Recently, depletion of *F. prausnitzii*and other butyrate producers was confirmed in the fecal microbiota of adults with CD compared to their unaffected relatives and other healthy controls via denaturing gradient gel electrophoresis and real time PCR (Joossens et al., 2011). This is not the case in pediatric CD, where some studies reported that *F. prausnitzii* had higher abundance in CD in comparison to controls (Hansen et al., 2012; Mottawea et al., 2016). An important difference in the pediatric studies is that the samples were collected at the time of diagnosis by colonoscopy, in contrast to Sokol et al. (2008b) where specimens were taken at surgical resection. This indicates the importance of sample collection time for these kinds of microbiota studies. The age groups screened by different studies were also different confirming the different phenotypes of the disease at different ages.

Geirnaert et al. (2017) reported the therapeutic effect of butyrate-producing bacteria in CD patients in addition to its role in enhanced intestinal epithelial barrier integrity. The four-strain probiotic supplement (containing *Lactobacillus acidophilus* NCIMB 30175, *Lactobacillus plantarum* NCIMB 30173, *Lactobacillus rhamnosus* NCIMB 30174, and *Enterococcus faecium* NCIMB 30176) was found to positively influence the immune response via colonic butyrate production *in vitro* and facilitating modulation of the gut microbiota composition and metabolic enhancing (Bjarnason et al., 2019; Moens et al., 2019).

Bile Acid Metabolism

Bile acids are self signaling molecules that regulate their own biosynthesis through a negative feedback mechanism (Sayin et al., 2013). Primary bile acids are the product of cholesterol breakdown in the liver, while secondary bile acids are the products of gut microbiota metabolism. primary and secondary bile acids have been found to act as signaling players on a group of cell membrane and nuclear receptors together named "bile acid-activated receptors." These receptors are highly expressed all over the gastrointestinal tract and control the bilateral communications of the intestinal microbiota with the host immune system (Fiorucci et al., 2021). Bile acid absorption is affected in models of inflammatory bowel disease (Fitzpatrick and Jenabzadeh, 2020). The expression of bile acid transporter apical sodium dependent bile acid transporter was inhibited in rats with colitis in addition to murine, canine and rabbit models of intestinal inflammation (Fitzpatrick and Jenabzadeh, 2020).

Bile acids mainly act as digestive aids to facilitate digestion of cholesterol, fat-soluble vitamins and triglycerides into water soluble products so they can be absorbed from the small intestine (Russell, 2003). They are also nutrient regulatory molecules that mediate some endocrine functions (Houten et al., 2006; Hylemon et al., 2009). They activate some nuclear receptors and cell signaling pathways to regulate lipid and glucose metabolism, energy expenditure and triglyceride homeostasis (Watanabe et al., 2006; Hylemon et al., 2009; Trauner et al., 2010). In addition, they have been shown to exert antimicrobial activity either directly through their detergent characteristics on bacterial

membranes or via indirect physiological function (Hofmann and Eckmann, 2006; Duboc et al., 2013). Their indirect antimicrobial activity mainly occurs in the distal small intestine, where they promote the activation of nuclear farnesoid X receptor (FXR) (Inagaki et al., 2006). Activation of FXR induces the expression of several host genes involved in mucosal defense including genes involved in oxidative stress, and antibacterial peptide biosynthesis (Bernstein et al., 1999; Hofmann and Eckmann, 2006; Inagaki et al., 2006). The expression and function of bile acid-activated receptors including FXR in addition to other receptors; G-Protein bile acid-activated receptor, pregnane-X-receptor, vitamin D receptor, and related orphan receptor gamma are strongly linked to the composition of the intestinal microbiota and negatively regulated by intestinal inflammation (Fiorucci et al., 2021).

The enterohepatic circulation of bile acids starts at the liver, where 14 enzymes are required to synthesize the two primary forms of bile acids namely cholic acid and chenodeoxycholic acid from cholesterol (Russell, 2003). Thereafter, they are conjugated to glycine or taurine to form bile salts (also termed conjugated bile acids), which are stored in the gallbladder during interdigestive periods. After meals, bile salts are released into the duodenum via the biliary duct to facilitate lipid digestion and absorption (Ridlon et al., 2006). In the gut lumen, the microbiota induce biotransformation of the primary forms of bile acids into secondary bile acids products via deconjugation, oxidation of hydroxyl groups and dehydoroxylation (Ridlon et al., 2006). This increases the hydrophobicity of bile acids so that they can passively diffuse through the small intestine and proximal colon in addition to their active transport from the small intestine to the blood stream and back to the liver (Ridlon et al., 2006). Approximately 95% of bile acids are recycled and transported through the circulation to return to the liver while 5% (400–600 mg) enter the colon, where they are dehydroxylated by colonic bacteria into secondary bile acid products [Deoxycholic acid (DCA) and Lithocholic acid (LCA); Ridlon and Hylemon, 2012].

Gut microbiota start the metabolism of bile salts via the deconjugation step through bile salt hydrolases (BSH) that generate free primary bile acids and amino acids (Ridlon et al., 2006). The distribution of BSH activity among gut microbiota was estimated via metagenomic analysis of 89,856 fecal clones (Jones et al., 2008). Firmicutes were the most predominant BSH-expressing bacteria (30%) followed by Bacteroidetes (14.4%) and Actinobacteria (8.9%) (Jones et al., 2008). Firmicutes with BSH activity include the genera *Eubacterium*, *Coprococcus*, *Clostridium*, *Ruminococcus*, *Dorea*, *Lactobacillus*, *Enterococcus*, *Listeria*, and *Lactococcus* while *Bifidobacterium* and *Collinsella* are the two Actinobacteria genera that express BSH (Jones et al., 2008). The same study revealed that Firmicutes and Actinobacteria are capable of deconjugating both glyco- and tauro-conjugated bile acids while Bacteroidetes are only able to hydrolyse tauro-conjugated bile acids. Also 9 BSH open reading frames have been identified among the gut microbiota community, one of them shares 56% homology with a protein encoded by the gut, archeon *Methanobrevibacter smithii*, which has been confirmed to have BSH activity against both glyco- and tauro-conjugated bile acids (Jones et al., 2008). The second step of bile acid metabolism by intestinal bacteria is the oxidation of the hydroxyl group at carbon 3, 7, or 12 of deconjugated bile acids via hydroxysteroid dehydrogenases to generate the oxo-derivative of bile acids (Ridlon et al., 2006). Gut microbiota expressing hydroxysteroid dehydrogenases include, but are not limited to *Clostridium* spp., *Eggerthella lentum*, *Ruminococcus* spp., *Bacteroides* spp., and *E. coli* (Ridlon et al., 2006; Fukiya et al., 2009). Simultaneously, some microbes promote 7α-/β-dehydroxylation of free cholic acid and chenodeoxycholic acid to generate deoxycholic acid (DCA) and lithocholic acid (LCA), respectively (Ridlon and Hylemon, 2012; Kakiyama et al., 2013). This dehydroxylation activity is common among the order Clostridiales of gut bacteria including *Ruminococcaceae*, *Lachnospiraceae,* and *Blautia* (Kakiyama et al., 2013). Heinken et al. (2019) performed an interesting study that included a systematic workflow to computationally model bile acid metabolism by gut microbes and microbial communities. The authors found that, each microbe could produce maximally 6 secondary bile acids *in silico*, while microbial pairs could produce up to 12 bile acids, suggesting bile acid biotransformation being a microbial community task (Heinken et al., 2019). Das et al. (2019) performed a shotgun metagenomic analysis of the bile salt biotransformation genes and their distribution at the phyla level. They reported that IBD patients harbored a significantly lower abundance of these genes in comparison with healthy individuals; many of these genes originated from Firmicutes (Das et al., 2019).

Bile acids metabolism by gut microbes has a controversial contribution to both the host and the microbe. The deconjugation step has been suggested to benefit gut microbiota colonization by increasing their resistance to bile salts, where the antimicrobial effect of bile acids benefit the host via alleviating the bacterial overgrowth (Desmet et al., 1995; Ridlon et al., 2006; Jones et al., 2008). Also, bile acid modification results in reducing cholesterol level and controls lipid metabolism, which is considered a protective factor against metabolic disorders such as obesity, cardiac diseases and diabetes (Tonack et al., 2013; Wu et al., 2013). Another link between bile acids and metabolic diseases arises from its effect on gut microbiota composition. Using *16S rDNA* clones sequencing of rats' cecal microbiota has revealed that cholic acid is a host modifier of the gut microbiota structure that is equivalent to high fat diet (Islam et al., 2011). One more benefit to the host from bile acid metabolism is the protection from pathogen colonization such as vegetative *C. difficile* (Sorg and Sonenshein, 2008). However, high levels of secondary bile acids are associated with some diseases such as gastrointestinal cancer and gallstones (Berr et al., 1996; Bernstein et al., 2005).

Four decades of research have established the association between IBD and bile acid dysmetabolism (Andersson et al., 1978; Heuman et al., 1983; Nyhlin et al., 1994; Duboc et al., 2013; Iwamoto et al., 2013). Different studies have linked the dysmetabolism of bile acids to gut microbiota dysbiosis in IBD (Wohlgemuth et al., 2011; Duboc et al., 2013). It is clear now that cholic acid or a high fat diet act as a regulatory host factor that selects for gut microbes that have the machinery to

detoxify bile acid metabolites or utilize their conjugates, glycine and taurine as metabolic substrates (De Filippo et al., 2010; Islam et al., 2011; Devkota et al., 2012). Devkota et al. (2012) reported that diets high in saturated fat induce the expansion of the H_2S producing pathobiont, *Bilophila wadsworthia*, which in turn promotes colitis in $IL10^{-/-}$ mice. This proinflammatory effect of high saturated fat diet was attributed to taurocholic acid (Devkota et al., 2012). Taurine, the conjugate in taurocholic acid, is known to have a sulfonic acid moiety that could be dissimilated by gut microbiota into H_2S as a microbial by-product (Laue et al., 2001). Diets high in fat or meat lead to more conjugation of taurine to bile acids and consequently more H_2S production, which is considered as a potential risk factor for IBD (Magee et al., 2000; Devkota et al., 2012). Alternatively, microbial dysbiosis associated with IBD may result in bile acid dysmetabolism and this in turn may affect the anti-inflammatory characteristics of bile acids (Duboc et al., 2013). Duboc et al. (2013) has reported the dysfunctionality of bacterial metabolism of bile acids because of microbial dysbiosis. Moreover, they illustrated that this intestinal dysmetabolism disturbed the intestinal bile acid pool, which in turn impacted the anti-inflammatory characteristics of bile acids (Duboc et al., 2013). Indeed, bile acids are known as anti-inflammatory mediators that inhibit NFκB activation and consequently reduce cytokine production by macrophages (Wang et al., 2011). This anti-inflammatory effect is characteristic of secondary bile acids but not the conjugated forms, which stresses the importance of deconjugation by gut microbiota (Duboc et al., 2013). Another anti-inflammatory effect of bile acids may be attributed to the activation of FXR, the bile acid receptors, which has been revealed as a protective factor against chemically induced colitis in mice (Gadaleta et al., 2011). Mice lacking FXR receptors have developed compromised intestinal barrier and antimicrobial defense in small intestine (Inagaki et al., 2006). The role of bile acids in protection against inflammation has been confirmed recently after identifying the association between a genetic variation of NR1H4, the gene encoding FXR receptor, and IBD (Attinkara et al., 2012). A pilot study of fecal bile acid and microbiota profiles in inflammatory bowel disease demonstrated that bile acid profiles were in general alike among patients with IBD and healthy controls (Vaughn et al., 2019). Oral administration of secondary bile acids in mice was reported to reduce the severity of colitis and ameliorate colitis-associated fecal dysbiosis at the phylum level (Van den Bossche et al., 2017). In accordance with the last study, dysbiosis of gut microbiota was found to induce deficiency in secondary bile acids in inflammatory-prone UC patients, which in turn leads to pro-inflammatory status in the intestine that may be treated via restoring secondary bile acids (Sinha et al., 2020). Like bile acid dysmetabolism, bile acid malabsorption was reported to be common reason of diarrhea in CD and colitis patients (Hou et al., 2018; Mena Bares et al., 2019).

Oxidative Stress

Oxidative stress is thought to be one of the key players in the tissue damage associated with IBD. Oxidative stress is defined as an imbalance between reactive oxygen species and intracellular antioxidants. Hence, oxidative stress arises from either a higher production of oxidative free radicals (Keshavarzian et al., 2003) and/or deficient antioxidant machinery (Koutroubakis et al., 2004). The inflammatory cascade starts by the infiltration of proinflammatory cells to the intestinal mucosa, which release reactive oxygen species (ROS) and/or reactive nitrogen metabolites. For example, chemiluminescence analysis of ROS has elucidated a higher release of these free radicals by monocytes and polymorphonuclear cells extracted from both CD and UC biopsies (Kitahora et al., 1988; Keshavarzian et al., 1992). These free radicals induce more infiltration of proinflammatory cells and so this cycle is sustained and eventually causes tissue damage. The disruption of the intestinal epithelium exposes the immune system to the gut microbiota or other antigenic luminal components, which exacerbate the inflammation resulting in the active phenotype of the disease (Keshavarzian et al., 2003). For the cells to protect themselves against oxidative stress, they have to induce the production of antioxidant metabolites, which is not the case in IBD. It has been reported that IBD is associated with a depleted total antioxidant capacity of the cells or individual antioxidants. For example, IBD patients are characterized by a depletion of copper/zinc containing protein (superoxide dismutase and metallothionein), lower glutathione transferase activity with higher glutathione peroxidase in UC, and lower colonic ascorbate (Koutroubakis et al., 2004). Indeed, it is well reported that increased oxidative stress level in IBD patients and the detection of oxidative stress index rate could be used as predictors for the pathogenesis of IBD (Yuksel et al., 2017; Luceri et al., 2019). Bourgonje et al. (2019) showed that plasma free thiols are reduced in patients with CD, reflection of systemic oxidative stress, in clinical remission. The authors recommended that systemic oxidative stress and plasma free thiols may be a relevant therapeutic target and biomarker to monitor disease activity in CD (Bourgonje et al., 2019). Oral administration of probiotics in IBD patients were reported to effectiveness via the decrease of oxidative stress values (Ballini et al., 2019).

For the gut microbiota to maintain their homeostasis at this oxidative stress, they must develop oxidative stress resistance machinery. Morgan et al. (2012) demonstrated a shift of IBD microbiota toward microbes that possess the glutathione generation and reduction capability, which enables them to compensate for the oxidative stress. This machinery includes higher cysteine biosynthesis, which is a precursor of glutathione, riboflavin and NADPH, which are cofactors of glutathione reduction reaction and glutathione transfer gene (Morgan et al., 2012). Glutathione is a tripeptide (γ-glutamylcysteinylglycine) thiol that is produced by the majority of living cells (Anderson, 1998). The reduced form of glutathione protects the cells from toxic oxygen metabolites and other electrophiles via keeping the cell in a reduced state (Anderson, 1998). It also has other protective functions via regulation of gene expression, cell apoptosis and transport of organic solutes (Hammond et al., 2001). With regards to gut microbiota, glutathione has been reported to be biosynthesized by Proteobacteria members and a limited number of gram positive bacteria such as some Streptococcus spp. and *Staphylococcus aureus,* but not by *Clostridium*, *Bacillus*

or *Micrococcus* members (Fahey et al., 1978). Also, glutathione sulfur transferase encoding genes that possess peroxidase activity are expressed by Proteobacteria members (Bartels et al., 1999). It is well documented now that IBD microbiota is dominated by Proteobacteria, which is known to produce some proinflammatory metabolites such as enterotoxins or LPS. This might generate a testable hypothesis that the inflammation associated stresses in the gut, such as oxidative stress, might constitute a selective pressure that induces a microbial shift toward the stress-resistant microbes. Furthermore, this shift might develop a colitogenic microbiota that could maintain the active chronic phenotype of the inflammation.

EFFECT OF IBD MEDICATIONS ON GUT MICROBIOTA

Medications for IBD can be classified into five classes: aminosalicylates (sulfasalazine, olsalazine, and mesalamine), Corticosteroids (cortisone, prednisone, prednisolone, hydrocortisone, methylprednisolone, beclometasone, and budesonide), immunosuppressive agents (6-Mercaptopurine, azathioprine, methotrexate, and tacrolimus), antibody agents (Anti-TNF agents (infliximab, adalimumab, certolizumab pegol), and antibiotics (metronidazole, ciprofloxacin and rifaximin) (Gade et al., 2020; Targownik et al., 2020). The choice of the treatment strategy depends on the severity of the disease and the response to previous therapy. The effect of some of the above-mentioned drugs on gut microbiota are summarized in **Table 2**. Interestingly some drugs that are used for treatment of IBD requires metabolic activation via the gut microbiota e.g., sulfasalazine, balsalazide (mesalamine prodrug), olsalazine, and methotrexates (Crouwel et al., 2020).

It should be mentioned that some IBD medications are reported to affect either the metabolism of gut microbiota or the metabolic status of intestinal cells by altering the intestinal biota. Sulfasalazine was reported to enhance carbohydrate metabolism, citrate cycle and decrease the oxidative stress (riboflavin, sulfur, cysteine) (Zheng H. et al., 2017). Dahl et al. (2017) showed that, mesalamine was able to decrease polyphosphate levels in bacteria, including members of the human gut microbiota. This reduction leads to bacterial sensitization to oxidative stress and decreases bacterial colonization (Arthur et al., 2012). Effenberger et al. (2021) performed an *in-silico* metabolic prediction analysis by including azathioprine or anti-TNF antibodies-treated IBD groups and assessed the effect of gut microbiota function on remission status. They found that the predicted butyrate synthesis was significantly enriched in patients achieving clinical remission. The use of oral steroids in IBD patients was demonstrated to affect two biosynthetic pathways of methanogenesis and one pathway in the biosynthesis of vitamin B2 and nucleosides (Vich Vila et al., 2020). The oral administration of metronidazole was found to reduce basal oxidative stress in colonic tissue of healthy rat (Pelissier et al., 2007) and increase the thickness of colonic mucosal layer by about twofolds (Pelissier et al., 2010). Moreover, the metronidazole-treated microbiota in murine fecal donors retained its ability to control inflammation co-occurring with enrichment of *Lactobacillus* and innate immune responses including invariant natural killer T cells in experimental colitis (Pelissier et al., 2010).

THERAPEUTIC STRATEGIES OF IBD TARGETING GUT MICROBIOME

Probiotics, Prebiotics, and Postbiotics

Probiotics are living organisms that when are given in appropriate amount to the host result in health benefit (Hill et al., 2014). Prebiotics are substances, which are utilized by probiotics leading to enhancing the health (Gibson et al., 2017). Postbiotics are non-living microorganisms with or without their cell components and metabolites that confer health benefits (Salminen et al., 2021). Although probiotics have the ability to modulate microbiome composition resulting in enhancing the growth of good species and inhibiting the growth of pathogenic ones, their use in IBD treatment is recommended only in the context of clinical trials in adults and children (Su et al., 2020). Probiotics have anti-inflammatory impact and enhance intestinal barrier functions (Abraham and Quigley, 2017). In adults and children with pouchitis, the use of eight-strain combination of *Lactobacillus plantarum*, *Lactobacillus paracasei* subsp*paracasei*, *Lactobacillus delbrueckii* subsp *bulgaricus*, *Lactobacillus acidophilus*, *Bifidobacterium breve*, *Bifidobacterium longum* subsp. *longum*, *Bifidobacterium longum* subsp. *infantis*, and *Streptococcus salivarius* subsp. *thermophilus* is recommended (Su et al., 2020). It is well documented that the probiotic cocktail VSL#3, which is composed of 3 Bifidobacteria strains, 4 lactobacilli strains, and 1 *Streptococcus* strain, is promising for treatment of patients suffering from IBD (Bibiloni et al., 2005; Miele et al., 2009; Fedorak et al., 2015). Also, the probiotic *Lactobacillus reuteri* ATCC 55730 was speculated to be helpful during the treatment of IBD cases (Oliva et al., 2012). In addition, the probiotic cocktail composed of *Lactobacillus acidophilus* NCIMB 30175, *Lactobacillus plantarum* NCIMB 30173, *Lactobacillus rhamnosus* NCIMB 30174, and *Enterococcus faecium* NCIMB 30176 has a well-documented effect in the treatment of IBD patients especially UC (Bjarnason et al., 2019; Moens et al., 2019). More recently, next generation probiotics such as *A. muciniphila* and *F. prausnitzii* and their supernatants (postbiotics) are reported to exhibit beneficial effects during IBD treatment (Sokol et al., 2008b; O'Toole et al., 2017). Fermented foods are good sources of probiotics including miso, tempeh, kefir, kimchi, pickled vegetables, yogurt with live active cultures, kombucha tea and sauerkraut (Sultan et al., 2020).

Also, prebiotics such as inulin, resistant starch, gums, pectins, and fructo-oligosaccharides are reported to be beneficial in the treatment of IBD patients through enhancing functions of the intestinal barrier and protecting vs. invasion and translocation of pathogens (Akram et al., 2019). Although there are many commercial products of these prebiotic fibers, healthy diets are considered their main source such as bananas, beans, onions, raw version of leeks, oats, dandelion greens, wheat, garlic,

TABLE 2 | The effect of medications used for treatment of IBD on gut microbiota.

Drug	Reported effects on gut microbiota diversity and composition	Model/Disease subtype
Sulfasalazine	• Restores gut diversity • Increases SCFAs- producers and lactic acid-producers • Decreases Proteobacteria (Zheng H. et al., 2017).	Rats/Colitis
5- Aminosalicylates	• Positively alters the diversity, composition, and bacterial interaction patterns. • Decreases *Escherichia–Shigella* (Xu et al., 2018).	Human mucosa /UC
Mesalamine	• Partially restores gut microbiota diversity and composition • Significant alteration of 129 correlated metabolites (Dai et al., 2020). • Increase the abundance of *Erysipelotrichaceae* species (Vich Vila et al., 2020).	Human/ UC
Oral steroids	• Increases the abundance of *Methanobrevibacter smithii* (Vich Vila et al., 2020).	Human/IBD
Azathioprine or mercaptopurine	• Inhibits the growth of *Campylobacter concisus* and other enteric microbes that are associated with IBD (Liu et al., 2017).	
Azathioprine or anti-TNF antibodies	• Restoration of intestinal microbial • Decrease in Proteobacteria • Increase of Bacteroidetes (Effenberger et al., 2021).	Human/CD
Metronidazole	• Increases the abundance of Bifidobacteria (Particularly Bifidobacterium pseudolongum) and enterobacteria (Pelissier et al., 2010). • Allowed the retention of a beneficial microbiota that reduced the severity of colitis upon fecal microbiota transplantation in an experimental colitis model (Strati et al., 2021).	Rats/Colitis
Ciprofloxacin	• Increased relative abundance of *Bacteroides* and Firmicutes genera *Blautia*, *Eubacterium* and *Roseburia* (Stewardson et al., 2015). • Reduced *Bifidobacterium*, *Alistipes* and 4 genera from the phylum Firmicutes (*Faecalibacterium*, *Oscillospira*, *Ruminococcus* and *Dialister*) (Stewardson et al., 2015).	Human/urinary tract infection.
Rifaximin	• Inducing the growth of bacteria useful to the host without changing its general composition (Ponziani et al., 2017).	

asparagus, artichokes, barley, seaweed, and other fruits and vegetables that are rich in fibers and indigestible carbohydrates (Sultan et al., 2020). Such prebiotics aid the growth of normal gut microbiota and the production of SCFAs, which results in enhancing the activity of immune cells, maintaining the levels of glucose and cholesterol as well as decreasing the pH of the colon, which results in enhancing the condition (Akram et al., 2019; Sultan et al., 2020).

Phage Therapy

Phages are the most ubiquitous organisms worldwide and they are characterized by their selectivity and specificity to bind their target host (Abd El-Aziz et al., 2019). Phages can bind and lyse specific bacterial strains within certain species (El-Mowafy et al., 2021). This capability gives the phage the advantage to be safer during the treatment of bacterial infections than commonly used antibiotics and to have limited effect on microbiota of the host (Abd El-Aziz et al., 2019; El-Mowafy et al., 2021).

The use of Russian coliphage or oral T4-like coliphages in children with bacterial infection-induced diarrhea does not induce any side effect, however, it was unsuccessful to enhance the conditions (Sarker et al., 2016). Recently, it is recommended to use phages against AIEC in patients suffering from IBD (Galtier et al., 2017). AIEC is an abnormal pathogen, which is commonly found in the ileal mucosa of IBD patients (Barnich et al., 2007). The use of bacteriophages to lyse AIEC significantly reduced the symptoms of DSS-induced colitis in transgenic mice expressing the human receptor for AIEC named CEACAM6 (Galtier et al., 2017). Therefore, phages targeting AIEC may be a promising therapeutic approach for the treatment of IBD patients.

Fecal Microbiota Transplantation

FMT is defined as the transplantation of healthy donor fecal microbiota into the gut of a patient as a trial to reverse the dysbiosis, restore homeostasis of the gut microbiota and improve the condition (Burrello et al., 2018; Hvas et al., 2019). This approach was reported to be successful during the treatment of recurrent *C. difficile* infection (CDI) (Khoruts and Sadowsky, 2016). The successful treatment of IBD patients using FMT depends on three main factors including; early treatment, content of fecal matter of the donor and usage of multiple FMTs (Moayyedi et al., 2015). Treatment of IBD patients using FMT is based on the idea that regaining the gut microbiota homeostasis will alter the growth of pathogens like *C. difficile* that are responsible for the disease (Khoruts et al., 2016). This is achieved through competition between the regained microbiota and pathogens for nutrients and colonization, production of antimicrobials by regained microbiota that directly affect pathogens, and vegetative growth and spore germination inhibition through bile acid mediated mechanism (Khoruts and Sadowsky, 2016). However, it is noteworthy that the fungal and viral content of the stool of donors may affect the outcome of IBD treatment using FMT (Draper et al., 2018; Zuo et al., 2018). It is reported that using stool rich in *C. albicans* significantly decreases the treatment efficacy (Zuo et al., 2018). Moreover, it was depicted that following FMT, different patients who received fecal matter from the same donor exhibited extremely individualized virus colonization patterns (Draper et al., 2018).

Costello et al. (2019) showed that 1 week treatment with anaerobically prepared fecal material, in mild to moderate UC patients, led to a higher probability of remission after

8 weeks. Another interesting work followed a randomized controlled study to investigate the role of FMT to maintain remission in CD patients (Sokol et al., 2020). The authors found a low similarity index between donor and recipient microbiota in some patients suggesting that a single FMT might not be enough to stimulate significant changes in these patients. An interesting study aimed to investigate the optimum timing of FMT for maintaining the long-term clinical benefits in UC (Li et al., 2020). The authors demonstrated that UC patients could take the 2nd course of FMT within 4 months after the initial course of FMT, which will allow clinicians to consider sequential FMTs as a long-term treatment strategy for UC. Moreover, they showed that the relative abundance of *Eubacterium*, *Ruminococcus*, *Eggerthella*, and *Lactobacillus* in UC patients can be used as predictors for the long-term efficacy of FMT (Li et al., 2020). Similarly, the species *Eubacterium hallii, Ruminococcus bromii, and Roseburia inulivorans* were recommended to predict the success of FMT therapy in UC patients (Paramsothy et al., 2019). However, the application of FMT has many disadvantages. First, different samples represent different bacterial ensembles which means variable efficacy and the outcome will rely on the

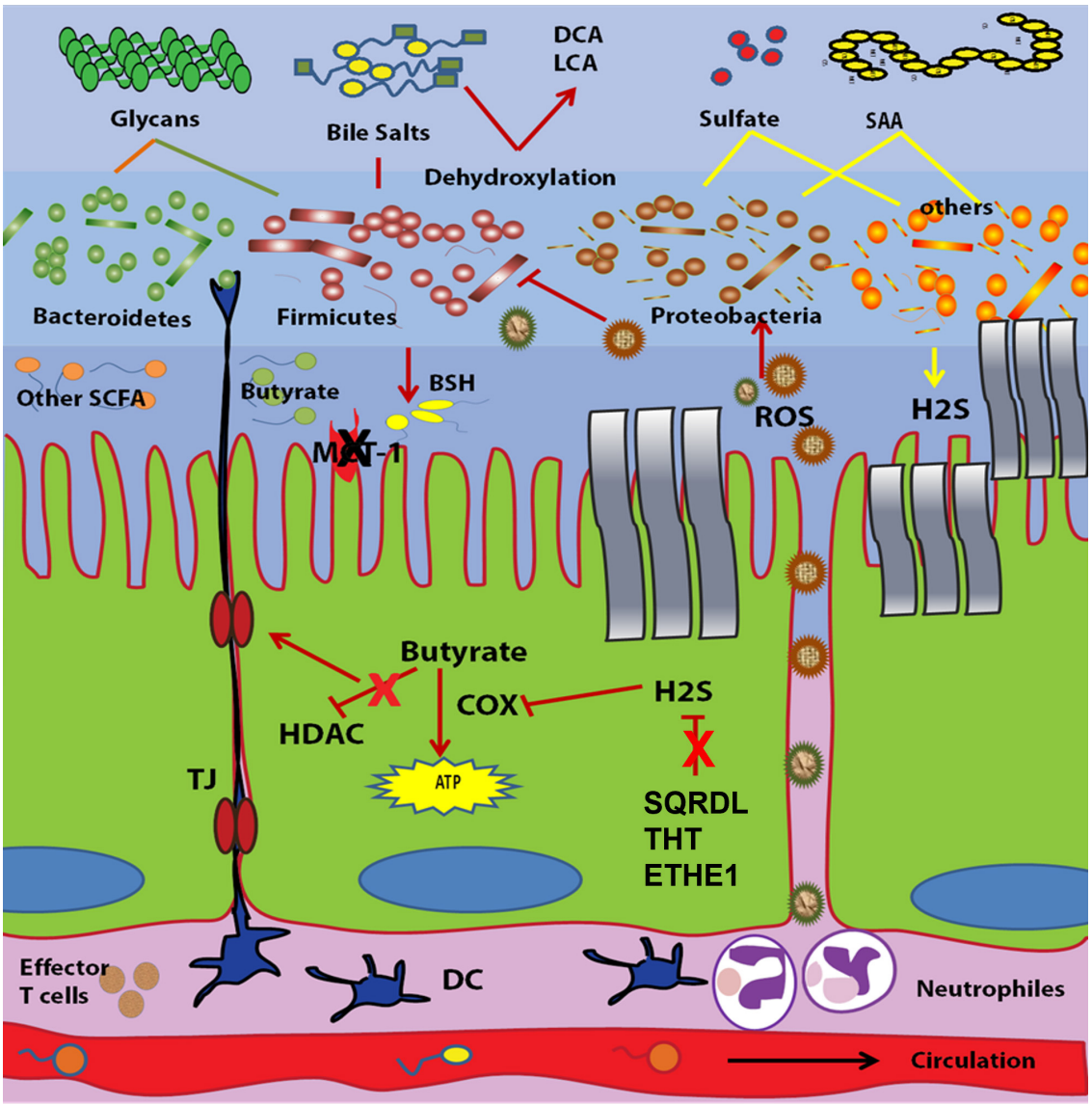

FIGURE 2 | Proposed Host-microbe metabolic dysfunctionalities in inflammatory bowel disease. The oxidative stress environment created by inflammation acts as a selective pressure. This pressure favors the microbes that are able to resist that stress such as Proteobacteria members but not Firmicutes members. This in turn leads to less short chain fatty acids (SCFAs) production, less bile salts hydrolases (BSHs) in association with higher H_2S release. From the host side, impaired butyrate transport and oxidation, less mucin secretion, low tight junction (TJ) expression, and defective H_2S detoxification are the major host metabolic perturbations associated with IBD.

sample (Choi and Cho, 2016). Additionally, safety risks may arise from the presence of undesirable strain/functionality within the donated stool sample such as transferable antibiotic resistance elements, virulence factors or pathogenic strains enteropathogenic *E. coli* (Nataro and Kaper, 1998; Choi and Cho, 2016).

Dietary Interventions to Modulate the Gut Microbiota in Inflammatory Bowel Diseases Patients

There is a tight bond between diet, gut microbiota, colonocytes, and immune cells during both the intestinal healthy conditions (homeostasis) and inflammation (Celiberto et al., 2018). Healthy diet patterns including diets rich in fruits, vegetables, probiotics, fermentable food, fibers, prebiotics, and adequate concentrations of vitamin D are good for intestinal health and homeostasis (Rinninella et al., 2019). Healthy diets generally result in more microbial diversity and an increase of good bacteria including *F. prausnitzii*, *Bifidobacterium* spp., and *Lactobacillus* spp. that result in increased production of SCFAs, especially butyrate (Celiberto et al., 2018; Rinninella et al., 2019). Butyrate has strong anti-inflammatory characters as it improves the function of intestinal barrier and endorses the proliferation of regulatory T cells (Treg) (Kespohl et al., 2017). Moreover, butyrate promotes the dendritic cells (DC) within the lamina propria and commensal microbiota antigens within the intestinal lumen. Dendritic cells mainly trigger Treg cells to produce transforming growth factor-β (TGF-β) and interleukin-10 (IL-10) through the production of TGF-β resulting in tolerant immune response and intestinal homeostasis (Zheng L. et al., 2017). These modulations result in more thick mucus layer, which is an important property of intestinal homeostasis, giving a protective shield between epithelial cells and luminal bacteria (Butzner et al., 1996; Segain et al., 2000; Geirnaert et al., 2017). Moreover, healthy diets represent a good source of aryl hydrocarbon receptor (AhR) which induce the production of IL-22, via the innate lymphoid cell 3 (ILC3) that maintains intestinal barrier function (Song et al., 2020).

Healthy diets harboring adequate concentrations of vitamin D as well as vitamin D supplements are reported to be helpful during IBD cases (Celiberto et al., 2018). It is reported that vitamin D is crucial for maintaining the composition of gut normal microbiota, where vitamin D receptor (VDR) deficient mice exhibited increased abundance of Bacteroidetes and Proteobacteria as well as diminished *Lactobacillus* spp. resembling the dysbiotic cases of IBD patients (Winter et al., 2017; Windsor and Kaplan, 2019). Vitamin D enhances the epithelial barrier function and promotes the production of antimicrobial peptides (Celiberto et al., 2018). It also promotes the proliferation of dendritic cells, which in turn promotes the production of the immunosuppressive IL-10 through provoking Treg cells (Sokol et al., 2006b; Jin et al., 2015). Vitamin D also decreases the production of pro-inflammatory cytokines including TNF-γ, IL-17, and IL-21 through its inhibitory effect on IL-12 and IL-23 that are responsible for the responses of Th-1 and Th-17, respectively (Santos-Antunes et al., 2016; Winter et al., 2017).

On the other hand, unhealthy diets such as Western dietary patterns those are low in fruits, vegetables, probiotics, fermentable food, fibers, prebiotics, and vitamin D are directly related to dysbiosis of the microbiota and diminished microbial diversity, decreasing SCFAs production, reducing good bacteria and increasing the pathobionts such as *E. coli* and *Clostridium difficile* (Sugihara and Kamada, 2021). This results in more patchy and thinner mucus layer, which as a result provides less protection between colonocytes and luminal bacteria (Sugihara and Kamada, 2021). In addition, the compromised intestinal barrier function, due to decreased expression of cellular tight junctions, leads to escape of harmful bacterial products including lipopolysaccharides (LPS) from the intestinal lumen to the lamina propria (Ghosh et al., 2020). LPS trigger the macrophages via binding to toll-like receptors (TLR) leading to the production of TNF-α. TNF-α endorses the proliferation of T helper cell type 1 (Th1) that produce the proinflammatory cytokines including TNF-α and TNF-γ resulting in inflammation and dysfunctionality of the intestinal barrier (Grassin-Delyle et al., 2020). Moreover, the condition may worsen due to decreasing Treg cells responsible for the production of IL-10, which mitigates the intestinal inflammation (Iyer and Cheng, 2012).

CONCLUSION

From what has been discussed above, gut microbiota exerts many beneficial roles to human health, where any permanent disturbance of this ensemble could metabolically lead to a disease state. Gut microbiota of IBD patients is characterized by depletion of Firmicutes and Bacteroidetes with increased abundance of Proteobacteria and Actinobacteria. These changes in microbial composition result in perturbations of microbial functions that could be summarized in less short chain fatty acid production, less bile acid hydrolysis, higher redox potential and increased H_2S production (**Figure 2**). This pattern of microbial metabolic perturbations is associated with defectiveness of some human cellular pathways such as defective H_2S detoxification, SCFAs transport and oxidation, and high luminal redox potential. The interaction between both sides of dysfunctionality is considered pathogenic to the host and might modulate the chronicity of the disease. However, the knowledge of the cause/effect relationship between these metabolic perturbations and the inflammation has so far been limited. In addition, the actual molecular mechanisms that regulate the interaction between both sides of the equation still need to be identified. Still, different strategies are being developed to manipulate gut microbiota to reduce the intestinal inflammation and improve the disease outcomes. These include live biotherapeutics such as FMT and probiotics. Taking in account the disadvantages of FMT and enrichment-based approaches in general, new approaches may rely on bottom-up rational to design identified bacterial consortia that are metabolically interdependent and exert a variety of protective functions to the host and the gut microbiota. These functions

include generation of beneficial metabolites such as SCFAs and indole, deconjugation and conversion of bile acids, competition for critical nutrients and synthesis of antimicrobial molecules against opportunistic pathogens.

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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The Microbiome in Autoimmune Liver Diseases: Metagenomic and Metabolomic Changes

Yanping Zheng[1†], Ying Ran[1†], Hongxia Zhang[1], Bangmao Wang[1] and Lu Zhou[1,2*]*

[1] Department of Gastroenterology and Hepatology, Tianjin Medical University General Hospital, Tianjin, China, [2] Department of Gastroenterology and Hepatology, Hotan People's Hospital, Xinjiang, China

Recent studies have identified the critical role of microbiota in the pathophysiology of autoimmune liver diseases (AILDs), including autoimmune hepatitis (AIH), primary biliary cholangitis (PBC), and primary sclerosing cholangitis (PSC). Metagenomic studies reveal significant decrease of gut bacterial diversity in AILDs. Although profiles of metagenomic vary widely, *Veillonella* is commonly enriched in AIH, PBC, and PSC. Apart from gut microbiome, the oral and bile microbiome seem to be associated with these diseases as well. The functional analysis of metagenomics suggests that metabolic pathways changed in the gut microbiome of the patients. Microbial metabolites, including short-chain fatty acids (SCFAs) and microbial bile acid metabolites, have been shown to modulate innate immunity, adaptive immunity, and inflammation. Taken together, the evidence of host–microbiome interactions and in-depth mechanistic studies needs further accumulation, which will offer more possibilities to clarify the mechanisms of AILDs and provide potential molecular targets for the prevention and treatment in the future.

Keywords: autoimmune liver diseases, microbiome, metagenomic, metabolomic, bile acids

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***Correspondence:**
Lu Zhou
lzhou01@tmu.edu.cn
Bangmao Wang
mwang02@tmu.edu.cn

[†] These authors have contributed equally to this work

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INTRODUCTION

Autoimmune liver diseases (AILDs) are chronic inflammatory conditions of the liver, including autoimmune hepatitis (AIH), primary biliary cholangitis (PBC), and primary sclerosing cholangitis (PSC) (Biewenga et al., 2020). Poor understanding of etiology makes the diagnosis and treatment of patients with AILDs challenging. In recent years, increasing studies have been focusing on microbiota-host interactions. Imbalanced microbial communities have been suggested to be related with aberrant immune response (Shi et al., 2017). Relationships have been established between the microbiome and autoimmune diseases, such as systemic lupus erythematosus (Katz-Agranov and Zandman-Goddard, 2017), inflammatory bowel disease (Franzosa et al., 2019), and rheumatoid arthritis (Bergot et al., 2019). Particularly, intestinal microbiome and liver could communicate through the biliary tract, portal vein, and systemic circulation, given the special anatomic and physiological relationships of liver and gut. Studies have discovered that liver diseases are intimately linked to the microbial communities of the human gut (Seki and Schnabl, 2012; Miyake and Yamamoto, 2013; LaRusso et al., 2017). Besides bacteria, the involvement of fungus and chlamydia has also been demonstrated in AILDs (Abdulkarim et al., 2004; Wang et al., 2017). Moreover, various metabolites of gut microbiome have been shown to participate in immune development and regulation (Levy et al., 2017).

Since the microbiota plays an important role in the development of the innate and adaptive immune system (Zheng et al., 2020), further study on the interaction between the diseases and microbiota may provide new insights on the etiology and management of AILDs. With the development of high throughput DNA sequencing, the diversity of the human microbiome has been greatly appreciated. The meta-omics approach, which consists of metagenomic, metatranscriptomic, and metabolomic analysis, has allowed for a more comprehensive characterization of the human microbiome (Zhang et al., 2019). Metagenome, a DNA sequencing method, aims to catalog all the genes from the samples (Wang et al., 2015). Metagenome reveals only the microbial composition of the community. The metatranscriptome could record expressed transcripts of the active members under a set of environmental conditions (Shakya et al., 2019). Changes in the composition or function of the gut microbiota lead to metabolite alterations. Through metabolomics, specific bacterial metabolic pathways and metabolites can be defined (Medina-Cleghorn and Nomura, 2014). Apparently, the microbiome is a rising star in the exploration for the prevention, diagnosis, and treatment of AILDs. Thus, we summarize a review of microbiome associated with AILDs from metagenomics and metabolomics, which may be the key for further understanding of the etiology and management of AILDs.

The Microbiome in Autoimmune Liver Diseases

Metagenomics is a powerful tool that is helpful for the analysis of microbial heterogenicity. It mainly includes two sequencing strategies: amplicon sequencing, most often amplifying portions of the hypervariable regions of 16S rRNA; or shotgun sequencing, which sequences all given genomic DNA from a sample (Rausch et al., 2019). The shotgun metagenomics sequencing can achieve species-level and potentially strain-level of microorganisms (Walsh et al., 2018). In contrast, relative abundances of bacterial taxa derived from the general 16S rRNA is usually defined at the genus level (Walsh et al., 2018).

Studies of the microbiota often focus on the bacterial diversity in the feces of the patients. Collectively, alpha diversity of fecal microbial showed a downward trend in most AILDs patients, which was shown in **Table 1** (Sabino et al., 2016; Tang et al., 2018; Wei et al., 2020). However, a German cohort study reported that the alpha-diversity of patients with PSC was similar to controls (Ruhlemann et al., 2019). The microbial communities of human gut were mainly composed of Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria at the phylum level (Dekaboruah et al., 2020). Firmicutes and Proteobacteria were increased in both AIH and PBC, while Bacteroidetes showed no significant difference in AIH and PBC when compared to healthy control (HC) (Lv et al., 2020). Remarkably, PSC was characterized by abundant Bacteroidetes and Proteobacteria, whereas Firmicutes were underrepresented (Sabino et al., 2016; Ruhlemann et al., 2019). However, different results can be observed in salivary microbiome and bile microbiome. Statistically significant differences in the phylum level in Firmicutes, Bacteroidetes, and Proteobacteria of the salivary microbiota among the PBC, AIH, and HC groups were not detected (Abe et al., 2018), although the abundance of Bacteroidetes, Firmicutes, and Fusobacteria was also lower in bile microbiome of PSC patients. The abundance of Proteobacteria was higher when compared with patients from the control group (Tyc et al., 2020). These findings suggest that both gut and oral microbiome may be involved in AILDs pathogenesis.

The Microbiome in Autoimmune Hepatitis

Microbiome studies are relatively rare in AIH. We only found 6 related articles in PubMed, which are shown in **Table 2**. Wei et al. (2020) indicated increased abundance in *Veillonella, Klebsiella, Streptococcus,* and *Lactobacillus* in AIH compared to HC. Moreover, they created a model including *Veillonella*, *Lactobacillus*, *Oscillospira,* and *Clostridiales* to distinguish AIH from controls (Wei et al., 2020). *Lachnospiraceae*, *Veillonella*, *Bacteroides*, *Roseburia*, and *Ruminococcaceae* were selected as the AIH microbial biomarkers in another study (Lou et al., 2020). They also reported a higher relative abundance of *Streptococcus* in patients. In Africa, Elsherbiny et al. (2020) reported that *Faecalibacterium, Blautia, Streptococcus, Haemophilus, Bacteroides, Veillonella, Eubacterium, Lachnospiraceae,* and *Butyricicoccus* were enriched in AIH. Given together, most studies confirmed an overrepresentation of *Veillonella* in the gut microbiota of AIH patients. Microbes enriched in gut may aggravate the disease. However, no data support a causal relationship between *Veillonella* and AIH. The actual strains will need to be identified in future studies by shotgun metagenomic sequencing.

Probiotics were believed to restore the composition of the gut microbiome (Hemarajata and Versalovic, 2013). It can also participate in regulating the immune system (Liu et al., 2018). *Bifidobacteria*-based probiotics have been shown to confer health benefits on the host by regulating gut microbiota (O'Callaghan and van Sinderen, 2016). There is a depletion of *Bifidobacterium* in AIH (Lin et al., 2015; Lv et al., 2020). Furthermore, patients with lower *Bifidobacterium* failed to achieve remission (Lv et al., 2020). Zhang et al. (2020) reported that *Bifidobacterium lactis 420* have beneficial functions in alleviating experimental autoimmune hepatitis. It suggested probiotics supplements may help to treat AIH in the future.

Except for gut microbiota, it is increasingly recognized that the oral cavity microbiota could also affect the host health (Arweiler and Netuschil, 2016). Dysbiosis of the oral microbiota has been found to be related to the pathogenesis of autoimmune diseases, such as inflammatory bowel diseases and systemic lupus erythematosus (van der Meulen et al., 2019; Elmaghrawy et al., 2020). There is a significant increase in *Veillonella* in the oral microbiota of AIH patients when compared with the HC, whereas *Streptococcus* is decreased (Abe et al., 2018). However, *Veillonella* is closely related to oral infectious diseases (Luo et al., 2020), which may influence the result. More research is needed. Notably, the change of *Veillonella* in oral bacterial community is consistent with that of fecal in AIH patients, although the relationship between the gut microbiota and the oral microbiota is still

TABLE 1 | Changes of gut microbiome in autoimmune liver diseases (AILDs).

Disease	Alpha diversity	Composition
AIH	↓	Firmicutes↓ Proteobacteria↑
PBC	↓	Firmicutes↓ Proteobacteria↑
PSC	controversial	Bacteroidetes and Proteobacteria↑ Firmicutes↓

unknown. We can look forward to seeing that *Veillonella* strain may become a microbial marker in AIH.

The Microbiome in Primary Biliary Cholangitis

Primary biliary cholangitis is a chronic occult disease which can progress to cirrhosis, and ultimately to liver failure and even death (Hirschfield et al., 2018). Lammert et al. (2021) found that fecal microbiota were related to the fibrosis and cirrhosis of PBC. Therefore, the analysis of microbiota composition in patients with PBC is meaningful. A study containing 60 UDCA treatment naive PBC patients and 80 healthy controls found 12 bacteria (**Table 3**) whose abundance changed in PBC compared with HC in China. *Haemophilus*, *Veillonella*, *Clostridium*, *Lactobacillus*, *Streptococcus*, *Pseudomonas*, *Klebsiella*, and an unknown genus in the family of *Enterobacteriaceae* were increased, and Bacteroidetes spp., *Sutterella*, *Oscillospira*, *Faecalibacterium* were decreased in PBC (Tang et al., 2018). These altered genera can help to discriminate PBC with HC (Tang et al., 2018). The relative abundance of *Streptococcus* was reported to be positively correlated with AST in alcoholic liver disease (Zhong et al., 2021). Regrettably, its role in PBC needs to be explored. Further, later study has reported that a decrease abundance of *Faecalibacterium* was associated with treatment non-responders, suggesting that *Faecalibacterium* may be beneficial for treatment response in patients with PBC (Furukawa et al., 2020). Another study from China also indicated more abundant genera including *Haemophilus*, *Veillonella*, *Lactobacillus*, *Streptococcus*, and *Klebsiella* in PBC (Lv et al., 2016). Besides *Streptococcus* and *Lactobacillus*, *Bifidobacterium* was proved to be more abundant in the PBC group compared with healthy control in Japanese (Furukawa et al., 2020). In addition, this study observed a significant reduction in the diversity of *Clostridiales*, which included amounts of butyric acid-producing symbiotic bacteria (Furukawa et al., 2020), although the findings above showed gut microbiota was strongly related to PBC. Evidence above also revealed that the change of microbial abundance is not just limited to one specie. This makes it more difficult to understand the potential mechanism between microbiome and disease. Present studies of metagenomics of gut microbiota are all from Asian patients and thus have certain limitations. In the future, more metagenomics studies of PBC are needed to identify the possible pathogens.

Gut microbiome is the hotspot in PBC research, whereas the investigation of microbiology at other body sites is also going on. As we all know, the biliary tract is traditionally considered sterile or has few bacteria. Previous study has reported that there are bacteria in PBC patients' bile, mainly including *Staphylococcus aureus, Enterococcus faecium,* and *Streptococcus pneumoniae* (Hiramatsu et al., 2000). Additionally, it is worth noting that *Propionibacterium acnes* 16S rRNA gene has been detected in epithelioid granuloma of PBC livers, but less in adjacent hepatic parenchyma (Harada et al., 2001). *Propionibacterium acnes* has been suggested as a most likely infectious pathogen of sarcoidosis, a kind of autoimmune disease (Yamaguchi et al., 2021). This study indicated that *Propionibacterium acnes* may also play a role in PBC which required further investigation. Dysbiosis of the oral microbiome has also been defined in PBC. It was characterized by increased relative abundances in *Eubacterium* and *Veillonella* as well as decreased abundances in *Fusobacterium* (Abe et al., 2018). Above all, *Veillonella* is consistently overrepresented in both stool and saliva of patients with PBC, indicating that *Veillonella* is closely associated with PBC. Further studies are warranted to investigate how *Veillonella* interact with PBC.

The Microbiome in Primary Sclerosing Cholangitis

The 16S rRNA gene analysis has also been a major method of bacterial analysis in PSC. Studies of the changes of microbiome in PSC were shown in **Table 4**. At present, there was only one study that has measured genetic diversity of fecal microbiota

TABLE 2 | Study of microbiome in autoimmune hepatitis (AIH).

Study	Country	Sample	Groups	AIH-enriched taxa	Controls-enriched taxa
Lou et al., 2020	China	Stool	AIH(37) vs. HC(48)	15 genera such as *Veillonella*, *Faecalibacterium*, *Klebsiella*, *Akkermansia*, *Enterobacteriaceae_unclassified*, *Megasphaera,* and so on	19 genera such as *Pseudobutyrivibrio*, *Blautia*, *Lachnospira*, *Erysipelotrichaceae_incertae_sedis*, *Ruminococcaceae_incertae_sedis*, *Phascolarctobacterium*, and *Alistipes* and so on
Wei et al., 2020	China	Stool	AIH(91) vs. HC(98)	*Veillonella*, *Klebsiella*, *Streptococcus,* and *Lactobacillus*	*Clostridiales, RF39, Ruminococcaceae, Rikenellaceae, Oscillospira, Parabacteroides,* and *Coprococcus*
Lin et al., 2015	China	Stool	AIH (24) vs. HC(8)	/	*Bifidobacterium, Lactobacillus*
Elsherbiny et al., 2020	Egypt	Stool	AIH(15) vs. HC(10)	*Faecalibacterium, Blautia, Streptococcus, Haemophilus, Bacteroides, Veillonella, Eubacterium, Lachnospiraceae,* and *Butyricicoccus*	*Prevotella, Parabacteroides,* and *Dilaster*
Abe et al., 2018	Japan	Stool	AIH(17) vs. HC(15)	*Lactobacillales*	*Clostridium subcluster XIVa*
Lv et al., 2020	Germany	Stool	AIH(72) vs. HC(95)	*Veillonella,* facultative anaerobic genera *Streptococcus* and *Lactobacillus*	*Bifidobacterium, Faecalibacterium*
Abe et al., 2018	Japan	Saliva	AIH(17) vs. HC(15)	*Veillonella*	*Streptococcus, Fusobacterium*

TABLE 3 | Study of the microbiome in primary biliary cholangitis (PBC).

Study	Country	Sample	Groups	PBC-enriched taxa	HC-enriched taxa
Tang et al., 2018	China	Stool	PBC(60) vs. HC(80)	*Haemophilus, Veillonella, Clostridium, Lactobacillus, Streptococcus, Pseudomonas, Klebsiella, Enterobacteriaceae*	*Bacteroidetes* spp., *Sutterella, Oscillospira, Faecalibacterium*
Furukawa et al., 2020	Japan	Stool	PBC(76) vs. HC(23)	*Bifidobacterium, Streptococcus, Lactobacillus, Enterococcus*	*Lachnospiraceae*, *Ruminococcaceae* of class *Clostridia*
Abe et al., 2018	Japan	Stool	PBC(39) vs. HC(15)	*Lactobacillales*	*Clostridium subcluster XIVa*
Lv et al., 2016	China	Stool	PBC(42) vs. HC(30)	*γ-Proteobacteria, Enterobacteriaceae, Neisseriaceae, Spirochaetaceae, Veillonella, Streptococcus, Klebsiella, Actinobacillus pleuropneumoniae, Anaeroglobus geminatus, Enterobacter asburiae, Haemophilus parainfluenzae, Megasphaera micronuciformis, Paraprevotella clara*	*Acidobacteria, Lachnobacterium* sp., *Bacteroides eggerthii, Ruminococcus bromii*
Abe et al., 2018	Japan	saliva	PBC(39) vs. HC(15)	*Veillonella, Eubacterium*	*Fusobacterium*

by shotgun metagenomic sequencing. It demonstrated the microbial gene richness reduced markedly in patients with PSC compared with HC (Kummen et al., 2020). Nine species showed an increased prevalence and 5 species were less prevalent in PSC compared to HC (Kummen et al., 2020). This study suggested that *Veillonella atypica*, *Veillonella parvula*, and an unclassified *Veillonella* species were more prevalent in PSC patients (Kummen et al., 2020). There is no study illustrating the effect of specific *Veillonella* strain in PSC. The relative abundance of *Veillonella* genera were also increased in children and teenagers with PSC (Cortez et al., 2020). Intriguingly, it has been proved that the abundance of *Veillonella* decreased after effective treatment of UDCA (Kummen and Hov, 2019). Evidence above suggested that the abundance of *Veillonella* was closely related to PSC, but it is not sufficient to distinguish PSC and controls (Ruhlemann et al., 2017). Then Ruhlemann et al. (2017) established a diagnosis model consisting of *Veillonella*, *Clostridiales*, *Lachnospiraceae,* and *Coprococcus* to help to differentiate PSC from HC.

To our knowledge, 70% of PSC patients have underlying inflammatory bowel disease (IBD) (Weismuller et al., 2017). Thus, it is necessary to distinguish the microbial profile of PSC with or without IBD. Published data suggested that the fecal microbiota of patients with PSC was significantly different from both HC and patients with IBD (Ruhlemann et al., 2019). At the genus level, *Rothia*, *Lactobacillus*, *Streptococcus,* and *Veillonella* were observed overrepresented specifically in PSC patients (Bajer et al., 2017). *Coprobacillus*, *Escherichia*, *Corynebacterium,* and *Lactobacillus* genera were related to PSC-IBD, but not PSC without IBD (Bajer et al., 2017). However, *Rothia*, *Streptococcus*, *Enterococcus*, *Veillonella*, *Clostridium*, and *Haemophilus* were more abundant in all subgroups of PSC (Bajer et al., 2017). Until now, studies have shown that all the treatments can't change the natural history of PSC. Evaluating the difference of microbiota between PSC with or without IBD may help to find specific treatments for different subgroups of PSC. A randomized placebo-controlled crossover study including 14 PSC patients suggested that probiotics supplement didn't alter the symptoms, liver biochemistry, or liver function in PSC (Vleggaar et al., 2008). However, evidence showed that fecal microbiota transplantation could increase bacterial diversity and was related with decreased alkaline phosphatase in patients with PSC (Allegretti et al., 2019).

Currently, composition of the bile microbiome in PSC is gradually emerging. In previous studies of bile microbiome in PSC, an over-representation of *Enterococcus* spp., *Prevotella* spp., *Staphylococcus* spp., *Lawsonella* spp., *Veillonella dispar,* and *Cutibacterium* was observed (Liwinski et al., 2020). *Klebsiella* spp. was also found in bile fluid of PSC patient (Liwinski et al., 2020). It has been reported that *Klebsiella pneumoniae* is associated with intestinal barrier dysfunction (Nakamoto et al., 2019). Nevertheless, the relationship between *Klebsiella* and PSC could not be confirmed in a cohort including 62 patients (Ruhlemann et al., 2019). On the contrary, Pereira et al. (2017) found similar bacterial communities of bile in non-PSC subjects and early stage PSC patients, which indicated that the initiation of PSC may not be associated with alteration in bile microbial communities.

The characteristic of oral microbiota has also been defined in PSC. Alpha-diversity of the salivary microbiome was not changed when comparing PSC with HC, but there is an overrepresentation of *Streptococcus salivarius, Prevotella histicola, Rothia mucilaginosa, V. parvula, Actinomyces, Campylobacter concisus, Bifidobacterium stellenboschense,* and Bacteroidales genus *Phocaeicola* (Lapidot et al., 2021). Furthermore, *S. salivarius, V. parvula, Actinomyces,* and *Bifidobacterium* were both significantly enriched in both the saliva and the fecal samples in patients with PSC compared with HC (Lapidot et al., 2021). Combining the analyses of fecal and oral microbiota studies may help to find out the specific bacterium which participates in the pathogenesis of disease.

Specific Microbiome in Autoimmune Liver Diseases

As we all know, Helicobacter mainly colonizes in stomach. Helicobacter species may also partly participate in the pathogenesis of AILDs. In a previous study, Helicobacter species have been detected in livers from adults suffering from AIH, PSC, and PBC using PCR or DNA sequencing (Nilsson et al., 2000; Casswall et al., 2010). However, this study could not decide whether the Helicobacter specie play a pathogenetic

TABLE 4 | Study of the microbiome in primary sclerosing cholangitis (PSC).

Study	Country	Sample	Groups	PSC-enriched taxa	HC-enriched taxa
Lapidot et al., 2021	Israel	Stool	PSC(35) vs. HC(30)	32 species including *Clostridium XlVa, Clostridium symbiosum, Clostridium perfringens, Streptococcus salivarius, Veillonella dispar, Ruminococcus gnavus, Bacteroides fragilis, Enterobacteriaceae, Lactobacillus,* and *Blautia*	261 species including *Bacteroides thetaiotaomicron* and *Faecalibacterium prausnitzii*
Kummen and Hov, 2019	Norway	Stool	PSC(85) vs. HC(263)	*Veillonella*	12 genera including *ML615J-28,Succinivibrion, Desulfovibrio, RF32, Phascolarctobacterium, Coprococcus,* and so on
Cortez et al., 2020	Brazil	Stool	PSC(11) vs. HC(23)	*Streptococcus, Veillonella*	/
Vaughn et al., 2019	United States	Stool	PSC/IBD(7) vs. HC(8)	*Megamonas*	*Clostridium XlVa, Faecalibacterium*
Ruhlemann et al., 2019	Germany and Norway	Stool	PSC(73) vs. HC(98)	p.Proteobacteria, *g.Parabacteroides*, *bacteroides* spp., c.Gammaproteobacteria, *g.Streptococcus, c.Bacilli, o.Lactobacillales, g.Veillonella*	*Coprococcus* spp.
Sabino et al., 2016	Belgium	Stool	PSC&PSC-IBD(52) vs. HC(52)	*Enterococcus, Streptococcus, Lactobacillus* and *Fusobacterium*	/
Kummen et al., 2020	Germany and Norway	Stool	PSC(136) vs. HC(158)	*Clostridium clostridioforme, Clostridiales bacterium17 47FAA, Clostridium bolteae, Bifidobacterium bifidum, Clostridium symbiosum, Eggerthella lenta, Escherichia unclassified, Eggerthella unclassified, Clostridium citroniae,Veillonella atypica, Veillonella parvula,* and *an unclassified Veillonella species*	*Coprobacter fastidiosus, Alistipes senegalensis, Eubacterium ramulus, Eubacterium hallii, Lachnospiraceae bacterium 71 58FAA*
Bajer et al., 2017	Czechia	Stool	PSC(43) vs. HC(31)	*Rothia, Enterococcus, Streptococcus, Clostridium, Veillonella,* and *Haemophilus*	*Coprococcus*
Liwinski et al., 2020	Germany	Bile	PSC(43) vs. HC(22)	*Enterococcus faecalis, Staphylococcus epidermidis, Streptococcus sanguinis, Enhydrobacter aerosaccus, Prevotella pallens, Veillonella dispar*	*Gemella sanguinis, Streptococcus gordonii*
Pereira et al., 2017	Finland	Bile	PSC(80) vs. HC(46)	/	An unclassified *Enterobacteriaceae*, *Neisseria*, *Campylobacter*, an unclassified *Neisseriaceae*
Lapidot et al., 2021	Israel	Saliva	PSC(35) vs. HC(30)	*Streptococcus salivarius, Prevotella histicola, Rothia mucilaginosa, Veillonella parvula, Actinomyces, Campylobacter concisus, Bifidobacterium stellenboschense,* Bacteroidales genus *Phocaeicola*	/

role in AILDs because of the lack of healthy controls. Boomkens et al. (2005) in the Netherlands demonstrated that Helicobacter species do not play a causal role in the pathogenesis of the PBC and PSC, by comparing Helicobacter species-specific DNA in liver tissue of patients with PBC/PSC and a control group. But another study involving 25 patients with end-stage PSC and 31 controls suggests a contributory role of *Helicobacter pylori* in the pathogenesis of PSC (Krasinskas et al., 2007). *H. pylori* gene can be detected in liver tissue samples of patients with PBC (Krasinskas et al., 2007), whereas there is no correlation between *H. pylori* antibody and PBC (Durazzo et al., 2004). Cross-reaction can be observed between *H. pylori* and mitochondria antibody of the bile duct cells, although there is no evidence so far of a common epitope between *H. pylori* and bile duct cell (Bogdanos et al., 2004). In general, the correlation between *H. pylori* antibody and AILDs is still controversial.

In addition to bacteria, the role of chlamydia in AILDs has also been studied. Although Chlamydia pneumoniae specific 16S rRNA gene and antigen can be found in PBC liver (Leung et al., 2003). No difference of Chlamydia pneumoniae IgG was seen in PBC patients compared to post-hepatitis cirrhosis patients, suggesting that infection with chlamydia may not be the triggering agent of PBC (Liu et al., 2005). Recently, Lemoinne et al. (2020) found that the fungal microbiota

of patients with PSC displayed an increased biodiversity. Moreover, their study suggested that PSC was associated with the increase of *Exophiala* genus and *Sordariomycetes* class, with a decrease of the *Saccharomycetales* order, *Saccharomycetes* class, *Saccharomycetaceae* family, and *S. cerevisiae* species (Lemoinne et al., 2020). Investigation of other microbiome such as chlamydia and fungi may provide a new direction for microbiome study in the future.

It is urgent to find related bacteria of AILDs, because of difficult diagnosis at the early stage in disease. Cumulative evidences show a linkage between microbiome and AILDs. The genera of *Veillonella* is predominant in the gut of AIH, PSC, and PBC patients, showing a vital role in AILDs. However, little is known about the potential mechanism of it in AILDs. The present result showed *Veillonella* may be enriched by suppression of bile acid synthesis (Loomba et al., 2021). Exploring the potential mechanism of microbiome in AILDs could be an opportunity for disease diagnosing and treating.

Functional Analyses of the Microbiome in Autoimmune Liver Diseases

Exploring the response of microbiome may help to find the induction factor of AILDs. Metagenomics is limited to reveal the functional activities of microorganisms, while metatranscriptomics is applied to explore the rapid response and expressed biological signatures of microorganisms to the external stimuli (Franzosa et al., 2014; Ram-Mohan and Meyer, 2020). However, there is still no metatranscriptomics research in AILDs. By PICRUSt, a tool used to infer the functional profile of microbial community, the microbial function is shown in **Figure 1**. Elsherbiny et al. (2020) demonstrated that butyrate, tryptophan, branched-chain fatty acids, pantothenate, and coenzyme A metabolisms were improved in microbial communities of AIH. However, the metabolism associated with proline and arginine was reduced (Elsherbiny et al., 2020). Changes in the metabolites have been verified in metabolomics, which will be illustrated later. Furthermore, bacterial invasion of epithelial cells, peroxisome proliferator-activated receptors (PPAR) signaling pathway, and caprolactam degradation pathways were enriched in PBC (Tang et al., 2018). The selective destruction of biliary epithelial cells is the key step in the pathogenesis of PBC (Selmi et al., 2010). PPAR agonists is suggested to regulate bile acid pool, and reduce inflammation and fibrosis of liver (Gerussi et al., 2020). Intriguingly, UDCA use not only influences the relative abundance of microbial species in feces but also alters the metabolic pathways of microbiota. The metabolic pathway predicted by 16s rRNA sequencing data showed elevation in taurine and hypotaurine metabolism in PBC after UDCA treatment, whereas glycine metabolism pathway had no difference with that of UDCA-naive PBC (Chen et al., 2020). The change of bile acids in PBC has been confirmed by metabolomic study (Yang and Duan, 2016). As for PSC patients, evidence showed that inferred microbiome functions were significantly different between PSC and healthy controls. There were an increase of 'biofilm formation by *Escherichia coli*,' 'lipopolysaccharide biosynthesis,' 'shigellosis,' 'Salmonella infection,' 'pathogenic *E. coli* infection' and 'bacterial invasion of epithelial cells,' and a decrease of 'tryptophan metabolism,' 'biosynthesis of amino acids' in PSC (Liwinski et al., 2020). In addition, Kummen et al. (2020) demonstrated that patients with PSC had more metabolic pathways related to vitamin B6 synthesis and branched-chain amino acid synthesis compared to healthy controls. In short, pioneering work has indicated that metatranscriptomics had their functional potential in AILDs. Further study is needed in the field.

Microbial Metabolites Associated With Autoimmune Liver Diseases

Microbial metabolites are emerging to be important effectors mediating the impact of microbiota on host immune responses and are critical for host-microbiota interactions. The gut microbial metabolites contain a wide variety of molecules ranging from short-chain fatty acids (SCFAs) and vitamins to secondary bile acids and neurotransmitters (Kummen and Hov, 2019). Colonic microbiota can transform carbohydrates into SCFAs, including acetate, propionate, and butyrate (Topping and Clifton, 2001). SCFAs have been verified to participate in regulating both innate immunity and antigen-specific adaptive immunity (Kim, 2018). It cannot only suppress the proinflammatory activation of macrophage but also promote peripheral regulatory T-cell generation (Dohmen et al., 2002; Arpaia et al., 2013). Previous study indicated that SCFAs can be uptaken by liver (Cummings et al., 1987). Recently, Zhang et al. (2020) demonstrated that the fecal butyrate was decreased in AIH patients compared to HC. The cause of butyrate reduction in AIH is still unknown. Furukawa et al. (2020) demonstrated a significant reduction in the diversity of the order *Clostridiales*, which included butyric acid-producing bacteria. It may partly explain the reduction of butyrate. In addition, SFCAs supplementation has been proved to ameliorate experimental autoimmune hepatitis (Hu et al., 2018). Furthermore, Wu et al. (2017) have shown that butyrate could ameliorate experimental autoimmune hepatitis through maintaining the integrity of small intestine via inhibiting TLR4 signaling pathway. Altogether, SCFAs may have beneficial effects on liver health through various mechanisms.

Excessive intrahepatic accumulation of bile acids can aggravate liver injury in cholestatic diseases (Schmucker et al., 1990). Bile acids not only act as a stimulator of numerous inflammatory mediators but also induct mitochondrial reactive oxygen species, which may contribute to the progression of cholestatic liver diseases (Li et al., 2017). In addition, bile acids are crucial for the immune modulation and study has demonstrated bile acids metabolites play a pivotal role to regulate the balance of TH17 and Treg cells (Hang et al., 2019). Bile acid can regulate gut microbial composition and immune response (Ramirez-Perez et al., 2017). On the contrary, the microbiome also plays a central role in bile acid homeostasis. Primary bile acids are synthesized in the liver and metabolized into secondary bile acids by microbiota in the gut (Ridlon and Bajaj, 2015). Liwinski et al. (2020) detected decreased concentrations of most bile acids in bile of PSC patients, except for the secondary bile

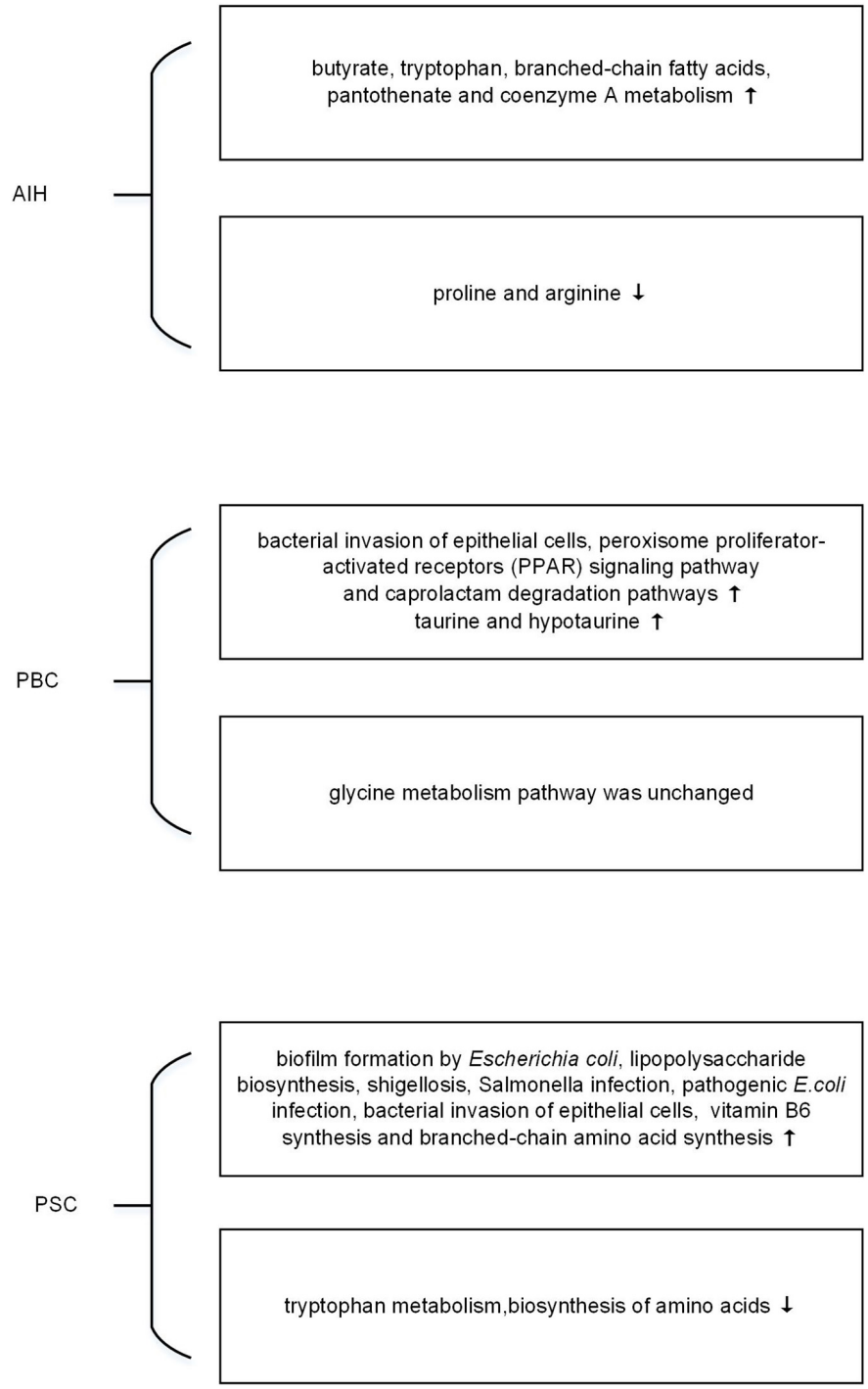

FIGURE 1 | The changes of microbial function predicted by metagenomic in autoimmune liver diseases (AILDs).

acid-taurolithocholic acid. Torres et al. (2018) also found PSC-IBD patients had a significant decrease in total stool bile acid pool compared to HC. Chen et al. (2020) identified decreased levels of lithocholic acid, glycodeoxycholic acid, and increased levels of cholic acid, taurochenodeoxycholic acid, chenodeoxycholic acid, and taurocholic acid in feces of PBC. UDCA, a kind of secondary bile acid, is just a small fraction of total bile acids. So far, it's the main therapeutic drug for PBC, which has been proved to significantly improve the outcomes of patients with PBC (Santiago et al., 2018). The production of UDCA can be modified by intestinal bacteria in gut (Tonin and Arends, 2018). UDCA was absent in germ-free mice (Tabibian et al., 2016).

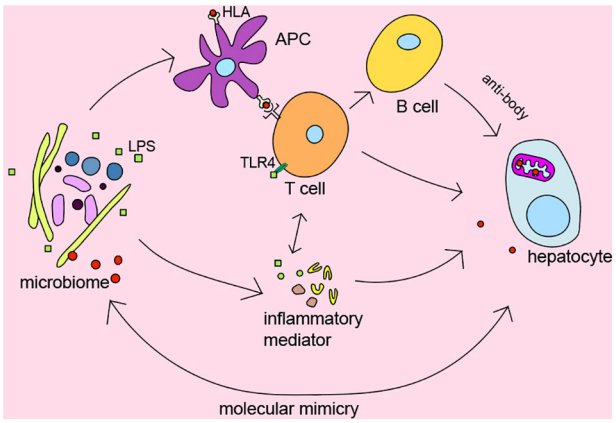

FIGURE 2 | The role of microbiome in pathogenesis of AILDs.

Farnesoid X receptor, which plays a pivotal role in regulating liver inflammation and the extent of inflammatory responses, can be activated by bile acids (Ding et al., 2015). Oral vancomycin significantly reduced the concentration of secondary fecal bile acids, emphasizing the role of microbiota in transformation of primary bile acid (Vaughn et al., 2019). It's worth noting that the abundance of *Veillonella* and *Klebsiella* have been proved to be negatively correlated with the level of secondary bile acids in serum (Chen et al., 2020).

As mentioned above, evidence showed the change of microbiota metabolomics was associated with AILDs. But there is much of the gut metabolome that remains uncharacterized. Untargeted metabolomics has great potential to identify novel molecules in AILDs in the future.

THE ROLE OF MICROBIOME IN THE PATHOGENESIS OF AUTOIMMUNE LIVER DISEASES

It's widely recognized that genetic predisposition in combination with exposure to environmental triggers and immunity dysregulation play a vital role in the pathogenesis of AILDs (Arndtz and Hirschfield, 2016). We summarized the role of intestinal microbiota in AILDs pathogenesis as follows in **Figure 2**.

Several genome-wide association studies indicated that there is significant association between HLA DR3, DR4, and AIH (de Boer et al., 2014; van Gerven et al., 2015), HLA DRB1*08, and PBC (Invernizzi et al., 2005). Moreover, early studies from Norway and the United Kingdom also identified HLA-DR3 (*DRB1*0301*) as the susceptible gene of PSC (Schrumpf et al., 1982). HLA gene has been shown to affect the microbial composition of the late infant gut in a cohort study from southeast Sweden (Russell et al., 2019). The microbiome participates in regulating inflammatory and immune responses as trigger factors. However, the detailed relationship between HLA and microbiome in AILDs still needs further exploration.

To our knowledge, T lymphocyte plays a central role in the immunopathogenesis of AILDs (Ishibashi et al., 2003; Ichiki et al., 2005; Henriksen et al., 2017). Interestingly, Clostridium has been reported to regulate the induction of T regulatory cells by providing bacterial antigens and SCFAs (Atarashi et al., 2013). Bacterial metabolites SCFAs have been demonstrated to affect the activity of T regulatory cells and reduce the levels of pro-inflammatory cytokines such as IFN-γ, IL-6, IL-1b, MIP-2, and TNF-α (Felice et al., 2015; Bhaskaran et al., 2018). In addition, bile acid metabolites, which mainly regulated by microbiota, were reported to inhibit TH17 cells by binding to the key transcription factor retinoid-related orphan receptor-γ and regulate T regulatory cells through the production of mitochondrial reactive oxygen species (Hang et al., 2019).

It is currently believed that chronic bacterial infection might play a part in the initiation or development of the autoimmune status in patients with AILDs (Haruta et al., 2010). Many studies suggest the alteration of microbes in AILDs, but the causality of this relationship is unclear. In fact, additional intestinal barrier dysfunction has been proved to exacerbate liver injury in mice (Zhang et al., 2021). Bacteria and endotoxin would enter the systemic circulation and trigger immune response because of intestinal barrier dysfunction (Krentz and Allen, 2017). Recently, it has been reported that translocation of *Enterococcus gallinarum* to the liver in germ-free C57BL/6 mice could trigger autoimmune responses (Manfredo Vieira et al., 2018). Zhang et al. (2020) found that *B. lactis 420* mitigated experimental autoimmune hepatitis through regulating intestinal barrier and liver immune cells (macrophage and Th17 cells). Specifically, the NOD.c3c4, which could spontaneously develop biliary inflammation, is a model of the human biliary disease primary biliary cirrhosis (Koarada et al., 2004). Biliary inflammation is ameliorated in antibiotic-treatment and germ-free NOD.c3c4 mice (Schrumpf et al., 2017). Nakamoto et al. (2019) demonstrated that gnotobiotic mice inoculated with PSC-derived microbiota was more susceptible to hepatobiliary injuries. Previous evidence shown that microbiome may be the promoting factors of AILDs. Antibacterial treatment may be effective options to suppress the development of the disease.

Molecular mimicry between immunodominant epitopes of the pathogen and self-peptides has been hypothesized to be the key event leading to the disease. It may provide clues to explain the relationship between microbes and disease. Investigations have detected that PDC-E2 of *E. coli* is molecularly similar to human PDC-E2, the immunodominant target of AMAs in PBC (Shimoda et al., 1995). Meanwhile, PDC-E2-like proteins of *Novosphingobium aromaticivorans* were more similar with human PDC-E2 than that of *E. coli* (Kaplan, 2004). Although *N. aromaticivorans* hasn't been detected in the liver of patients with PBC (Tanaka et al., 1999), a study has reported that *N. aromaticivorans* is present in approximately 25% of fecal samples from patients and controls (Selmi et al., 2003). *Lactobacillus delbrueckii* and *Mycobacterium gordonae* might also induce loss of tolerance to human mitochondrial proteins in genetically susceptible individuals due to molecular mimicry and immunological cross-reactivity (Vilagut et al., 1994, 1997; Bogdanos et al., 2008). Recent findings also suggested that

antibodies against *Yersinia enterocolitica* were present in PBC patients (Yamaguchi et al., 1994). Interestingly, Roesler et al. (2003) identified β-subunit of bacterial RNA-polymerase, a non-species-specific bacterial protein, as the target of antibodies in PBC. Besides bacterium, mycoplasma has also been suggested as a causative factor in the etiopathogenesis of PBC via 'molecular mimicry' (Berg et al., 2009). It is worth noting that PDC-E2 is highly conserved from prokaryotes to advanced organisms, which may be the reason why multiple microorganisms are related to AILDs. These results confirmed the hypothesis that autoimmunity in AILDs may be triggered by proteins specific for bacteria through 'molecular mimicry,' which provide a hopeful direction for further study on the pathogenesis of AILDs. In short, the relationship between the organisms and AILDs needs to be explored.

CONCLUSION

Increasing evidence has highlighted the crucial role of microbiome in AILDs. Research showed various changes of microbiome in metagenomic and metabolomic analyses. Although the studies so far didn't clearly demonstrate the causation between microbiome and AILDs, they found the associated microbiome and even possible role in the pathogenesis providing direction for further study in the pathogenesis of AILDs. Moreover, mutual authentication among metagenomic and metabolomic of the microbiome could help us to understand the detailed and correct role of microbiota in AILDs. Nevertheless, the integrated analysis of gut microbiome and metabolite is lacking because of limited data of metagenomics and metabolomics in AILDs. With the application of metagenomics and metabolomics, it is possible to identify new microbial diagnostic markers in the early diagnosis and novel treatments of AILDs. The role of the microbiome, not only bacteria, in the mechanisms of AILDs needs more comprehensive and in-depth research to explore in the future.

AUTHOR CONTRIBUTIONS

BW and LZ guided the outline and carried out manuscript editing. YZ and YR collected data and drafted the manuscript. HZ performed manuscript review. Each of the co-authors has approved the final draft submitted. All authors contributed to the article and approved the submitted version.

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REVIEW
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Pathogenesis of Children's Allergic Diseases: Refocusing the Role of the Gut Microbiota

Tingting Hu, Yinmiao Dong, Chenghao Yang, Mingyi Zhao and Qingnan He**

Department of Pediatrics, The Third Xiangya Hospital, Central South University, Changsha, China

Allergic diseases comprise a genetically heterogeneous cluster of immunologically mediated diseases, including asthma, food allergy (FA), allergic rhinitis (AR) and eczema, that have become major worldwide health problems. Over the past few decades, the spread of allergic diseases has displayed an increasing trend, and it has been reported that 22% of 1.39 billion people in 30 countries have a type of allergic disease. Undoubtedly, allergic diseases, which can be chronic, with significant morbidity, mortality and dynamic progression, impose major economic burdens on society and families; thus, exploring the cause of allergic diseases and reducing their prevalence is a top priority. Recently, it has been reported that the gastrointestinal (GI) microbiota can provide vital signals for the development, function, and regulation of the immune system, and the above-mentioned contributions make the GI microbiota a key player in allergic diseases. Notably, the GI microbiota is highly influenced by the mode of delivery, infant diet, environment, antibiotic use and so on. Specifically, changes in the environment can result in the dysbiosis of the GI microbiota. The proper function of the GI microbiota depends on a stable cellular composition which in the case of the human microbiota consists mainly of bacteria. Large shifts in the ratio between these phyla or the expansion of new bacterial groups lead to a disease-promoting imbalance, which is often referred to as dysbiosis. And the dysbiosis can lead to alterations of the composition of the microbiota and subsequent changes in metabolism. Further, the GI microbiota can affect the physiological characteristics of the human host and modulate the immune response of the host. The objectives of this review are to evaluate the development of the GI microbiota, the main drivers of the colonization of the GI tract, and the potential role of the GI microbiota in allergic diseases and provide a theoretical basis as well as molecular strategies for clinical practice.

Keywords: children's allergic diseases, gastrointestinal (GI) microbiota, asthma, allergic rhinitis, food allergy (FA)

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****Correspondence:***
Mingyi Zhao
zhao_mingyi@csu.edu.cn
Qingnan He
heqn2629@csu.edu.cn

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INTRODUCTION

The World Health Organization (WHO) has identified allergic diseases as one of the three major diseases that need focal prevention and a cure; moreover, allergic diseases are considered a current serious global problem (Nwaru and Virtanen, 2017). Allergic diseases, mainly asthma, food allergy (FA), allergic rhinitis (AR), eczema and so on, are traditionally referred to as immediate or type 1 hypersensitivity reactions, with IgE as a critical factor (Han et al., 2020; Justiz Vaillant et al., 2021).

This kind of anaphylactic reaction is mediated by IgE antibodies that are produced by the immune system in response to environmental proteins which are termed allergens including pollens, animal danders, or dust mites. These IgE antibodies bind to mast cells and basophils, which include histamine particles released during the reaction, and eventually cause inflammation (Crosson et al., 2021; Justiz Vaillant et al., 2021; Shamji et al., 2021). According to the World Allergy Organization (WAO)[1], an epidemiological survey on allergic diseases showed that 22% of the population in 30 countries has a type of allergic disease. A study in 2003 indicated that the morbidity of asthma among children under 4 years old in the United States has increased by 160% since the 1980s and 1990s (Eichenfield et al., 2003). According to a cross-sectional study of the whole population in six regions of Inner Mongolia, northern China, the report said that 4,441 of the respondents (18.0%) self-reported food allergies, and the incidence rate of children was higher than that of adults (38.7 vs. 11.9%, $P = 0.335$, respectively) (Wang et al., 2018). The incidence of major allergic diseases, i.e., asthma, allergic rhinitis and eczema, in the urban centers of Peking, Chongqing, and Guangzhou are summarized in **Table 1** (Zhao et al., 2011). As shown, the incidence rate of asthma in Peking, Chongqing, and Guangzhou were 3.15, 7.45, and 2.09%, respectively; the incidence of allergic rhinitis was 14.46, 20.42, and 7.22%, respectively; and the self-reported in of eczema was 20.64, 10.02, and 7.22%, respectively (Zhao et al., 2011). According to a cross-sectional study aimed at children aged 0–14 years in Hong Kong, China, 352 (4.8%; 95% CI 4.3–5.3%) of 7,393 children were reported to suffer from FA (Ho et al., 2012). In addition, the above-mentioned tables all show that the morbidity of allergic diseases was higher in males than in females. In general, the incidence rate of allergic diseases continues to rise, particularly in Asia (Hong et al., 2004; Anandan et al., 2010; Wong et al., 2013). Therefore, exploring the cause of allergic diseases and reducing the incidence of allergic diseases are of vital importance.

The "hygiene hypothesis" proposed by Strachan in the late 1980s is currently considered the main theory targeted to explain the peculiar generational rise in immune dysregulation (Strachan, 1989), this hypothesis argues that a lack of exposure to infectious sources, parasites and symbiotic microorganisms such as the GI microbiota limits the normal development of the immune system, ultimately resulting in the increasing incidence of allergic diseases (Hong et al., 2004; Anandan et al., 2010; Wong et al., 2013). However, research over the past decade has provided evidence which linked the commensal and symbiotic microbes (GI microbiota) and parasitic worms to immune development, extending the hygiene hypothesis to the "microflora" and "old friends" hypotheses, respectively (**Figure 1**; Rook et al., 2004; Noverr and Huffnagle, 2005). Rook et al. (2004) proposed the "old friends" hypotheses. This hypotheses argues that microorganisms and macroorganisms such as parasitic helminths co-evolved with the development of the human immune system, and it also notes that these organisms are necessary for normal immune system development, which are similar to the "hygiene hypothesis" (Rook et al., 2004; Stiemsma et al., 2015). In 2005, the "microflora" hypothesis is proposed. In fact, the "microflora" hypothesis is considered as an another modern extension of the "hygiene hypothesis," which indicates that early life disturbance (by antibiotic use, infection, or diet) to the bacteria which resides in the human intestine (the GI microbiota) destroy the normal microbial mediated mechanisms promoting immunological tolerance, and finally makes the immune system toward a state of promoting allergic disease (Noverr and Huffnagle, 2005; Bae, 2018).

Now, we have a much clearer understanding of the interactions between the GI microbiota and immune system with the emergence of next-generation sequencing (NGS) and personal genome sequencing. This review aims to summarize the research progress on the relationship between the GI microbiota and children's allergic diseases, such as asthma, allergic rhinitis, eczema, and FA; in addition, it provides novel thoughts for preventive strategies and the treatment of allergic diseases.

THE DEVELOPMENT OF THE GUT MICROBIOTA

The Composition of the Gut Microbiota

In healthy individuals, there is a diverse microbial community present in the gut with numerous bacterial species, defined as the microbiota (Harmsen and de Goffau, 2016). Research has shown that the human gut microbiota likely contains 1,000–1,500 bacterial species; nevertheless, each person has only approximately 160 bacterial species (Lee and Mazmanian, 2010; Shi et al., 2017). Tierney et al. (2019) made an effort to sequence the bacterial genomes of the microbiota, indicating that our gut microbiota contains about 22.2 million genes, which is over 700 times the length of the human genome. The GI microbiota of healthy individuals was primarily composed of four phyla of bacteria: *Firmicutes, Bacteroidetes, Proteobacteria*, and *Actinobacteria* (Wright et al., 2015). Furthermore, most of the microbiota was composed of the phyla *Firmicutes* and *Bacteroidetes*, and the class *Clostridia* dominated the *Firmicutes* sequences (Cai et al., 2021). Of note, the microbiome includes not only bacteria, but also viruses, phage, fungi, archaea and so on (Gong et al., 2021).

Origins of Fetal Microbiota and Maternal Gut Microbiota

In the past decades, numerous studies on the GI microbiota have been restricted to the point of view that fetuses, considered microbiologically sterile, would not be exposed to bacteria until they come into contact with the vaginal and intestinal microbiota of the mother or with other environmental microorganisms at birth (Satokari et al., 2009). Nevertheless, this traditional view has been challenged by a new view that microbial colonization of the healthy new-born intestinal tract may begin *in utero* rather than during or after birth (Milani et al., 2017). Studies have found that the placenta harbors a unique microbiota composed of non-pathogenic commensal microbiota, which is similar to the oral microbiota of the mother, indicating that the placental microbiota may be established by the hematogenous spread

[1]https://www.world allergy.org/

TABLE 1 | Incidence of allergic diseases in children of different cities and genders (Zhao et al., 2011).

	Asthma			Allergic rhinitis			Eczema		
	Overall	Male	Female	Overall	Male	Female	Overall	Male	Female
Peking	3.15%	4.09%	2.05%	14.46%	16.44%	12.26%	20.64%	21.13%	20.09%
Chongqing	7.45%	8.22%	6.06%	20.42%	22.08%	18.53%	10.02%	11.34%	10.66%
Guangzhou	2.55%	3.87%	1.60%	7.22%	8.93%	6.55%	7.22%	7.52%	6.87%

Data are n (%) unless otherwise stated. The data are from reference (Zhao et al., 2011).

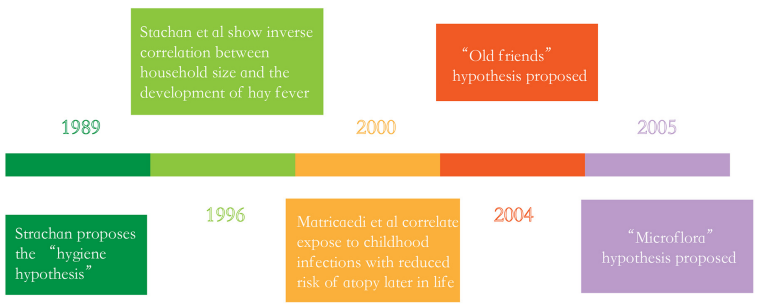

FIGURE 1 | The history of the "hygiene hypothesis".

of oral microbiota (Aagaard, 2014; Prince et al., 2016; Theis et al., 2019). Jimenez et al. (2008) detected microbial DNA in meconium (Mshvildadze et al., 2010). An additional study has found a high degree of similarity between microbes in meconium and those in amniotic fluid; presumably, this is due to the high likelihood that fetuses swallow plentiful amounts of amniotic fluid during the later stages of pregnancy, and meconium microbes are derived from the amniotic fluid that is swallowed (Nanthakumar et al., 2000; Ardissone et al., 2014; Cereta et al., 2021). Taken together, these finding show that microbial colonization of the healthy new-born GI tract may begin *in utero*.

While there is good evidence that prenatal exposure to maternal gut microbes may occur, the mechanisms by which microbes may be transported from the maternal GI to the developing fetus have yet to be identified. At present, the most likely hypothesis is that microbes are transferred from the intestinal epithelium to the bloodstream and then transported to the placenta. Normally, an epithelial barrier prevents bacteria from entering the bloodstream; however, dendritic cells (DCs) have been shown to actively penetrate intestinal epithelial cells and absorb bacteria from the intestinal lumen. Studies have shown that DCs can isolate living symbiotic bacteria here for a few days. Once attached to dendritic cells and transferred to the lymphatic system, the bacteria can spread to other parts of the body (Laubereau et al., 2004). These bacteria-loaded dendritic cells can be transported to mesenteric lymph nodes through GI lymphatic vessels (Jimenez et al., 2008). GI microbiota are also found in breast milk, and the same transport mechanism has been theorized (Forsberg et al., 2013).

However, it is of importance to be mindful that the above-mentioned source may not be the only one, that may vary between pregnancies and that fetal seeding may be a dynamic, gestational age-dependent process. And by determining the origins of the fetal microbiome, we might find chances for pre- and post-natal health interventions to prevent dysbiosis and minimize the incidence and impact of non-communicable diseases.

Neonates demonstrate a complex microbiota in the gut within the week immediately following birth, with dynamic fluctuations in the composition of the gut microbiota until a stable adult microbiota composition is reached at the age of 2–2.5 years. Research suggests that facultative and aerotolerant bacteria such as lactic acid bacteria, *Enterobacteria*, and streptococci dominate the initial flora in the neonatal gut, followed by an increasing number of strict anaerobes, including *Bifidobacterium, Clostridium, Bacteroides* (Bottacini et al., 2017; Milani et al., 2017; Brosseau et al., 2021).

MAIN DRIVERS OF COLONIZATION AND AFFECTING FACTORS OF THE INFANT GASTROINTESTINAL TRACT

The most important affecting factors of the GI tract composition in infants are divided into those occurring before birth, during/at birth and after birth, with the main factors being the mode of delivery, infant diet (i.e., the type of infant feeding) environment, antibiotic use by the infant and so forth (**Figure 2**; Azad et al., 2016; Cryan et al., 2019).

Mode of Delivery

The delivery mode is thought to be a vital driver of the early GI tract composition in full-term infants (Dominguez-Bello et al., 2010). A large number of studies have reported differences in full-term infants gut microbiota

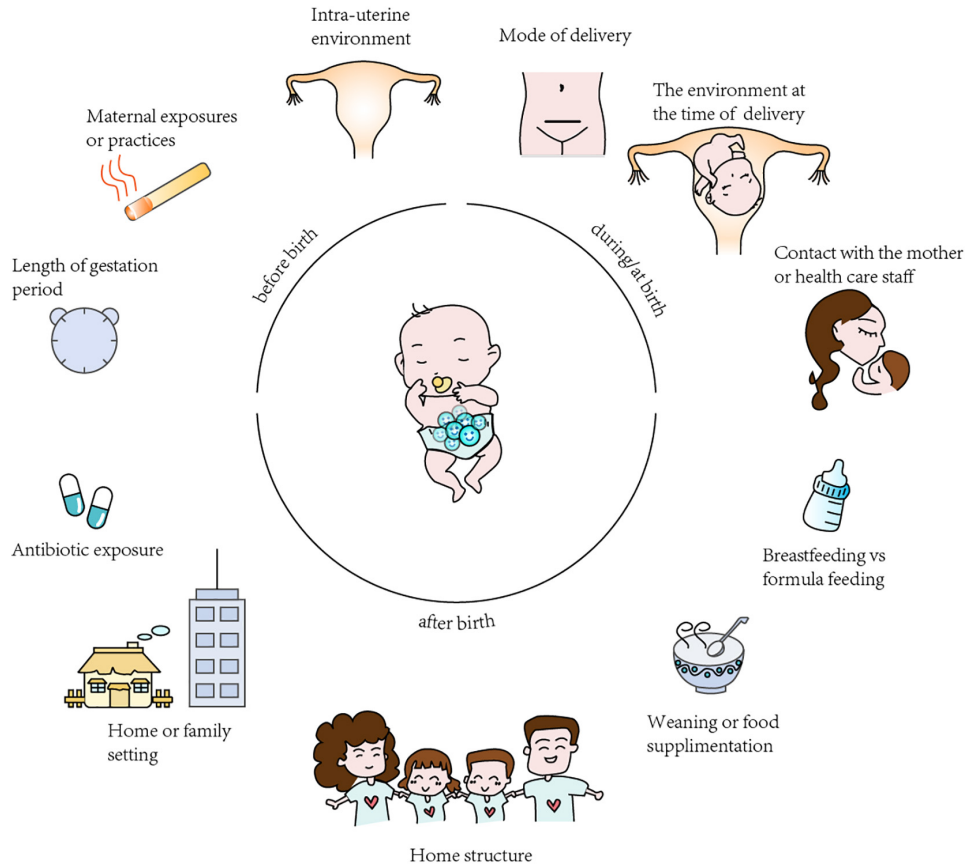

FIGURE 2 | Summary of the factors affecting gut microbiota colonization in infants. The "before birth", "during birth", "after birth" factors affecting analysis and results in microbiota studies.

diversity and/or composition between caesarean delivery (CD) and vaginal delivery (VD) delivered infants. And consistent among many of these researches are findings of lower overall microbial diversity and decreased abundance of *Bifidobacterium*, *Bacteroides*, and *Lactobacillus* in infants born by CD compared to those VD (Dominguez-Bello et al., 2010; Jakobsson et al., 2014; Brumbaugh et al., 2016; Francino, 2018). For example, Lee et al. (2016) collected fecal samples and performed 16S rRNA gene analysis via 454 pyrosequencing of the V1–V3 regions. Unsurprisingly, they found that during the neonatal period, infants born by caesarean delivery (CD) showed a lower richness and diversity of the GI microbiota than those born by vaginal delivery (VD; Lee et al., 2016).

Differences in microbial colonization patterns are of importance, not only because of their potential impact on the eventual composition of the microbiota, but also because they affect the concomitant development of the infant's immune system. The maturation of intestinal mucosa and its associated lymphoid tissue depends on intestinal colonization. The GI-associated lymphoid tissue Peyer's patches, mesenteric lymph nodes, and isolated lymphoid follicles require signals from the GI microbiota to fully develop and/or recruit mature immune cell complement (Maynard et al., 2012). In addition, microbiota shape the immune phenotype in early infancy by binding to molecular receptors in various intestinal immune cells and initiating cascades of signals that indicate cell differentiation and control inflammatory states (Romagnani, 2006). In conclusion, under the influence of the GI microbiota, the immune cell pool established during this critical period will influence the host's lifetime immunity and susceptibility to disease (Maynard et al., 2012; Francino, 2014; Brugman et al., 2015). Thus, the disruption of colonization caused by CD may be reflected in altered immune system development with potential long-term consequences. Several studies have addressed the impact of CD on T-cell response development and production of different cytokines. The study by Jakobsson et al. (2014) identified limited evidence that CD is thought to be linked with lowered cyclic dynamic levels of Th1-associated chemokines in infancy (Jakobsson et al., 2014). And the study by Jakobsson et al. (2014) showed that *Bacteroides* can exert strong effects on the immune system. In the case of *Bacteroides fragilis*, a surface polysaccharide is recognized by toll-like receptor 2 (TLR2) in Treg cells and induces production of IL-10 and other cytokines that affect the Th1/Th2 balance and promote immune tolerance (Round and Mazmanian, 2010). So the absence of *Bacteroides* may be related to the reduction of Th1 responses detected in CD infants. And

poor immune system can result in remarkably lower levels of Th1-associated chemokines, such as CXL10 and CXL11, which are associated with immune responses (Munyaka et al., 2014). Here, it is conjectured that birth via CD is related to adverse effects on immune development and allergies.

Infant Diet

The type of infant feeding is another profound element affecting the composition and function of the GI microbiota (Cerdo et al., 2019). Human milk oligosaccharides (HMOs) are important components of breast milk and can regulate the proliferation and maturity of GI cells and benefit the growth and development of babies. In addition, HMOs tend to provide nutrients, probiotics, and IgAs, which protect infants from exogenous infections and makes the immune system work more efficiently. Research on the transcription of enterocytes by Praveen et al. (2015) suggests that the infant diet also has a great influence on the expression of host genes and that breastfeeding can lead to the promotion of gene transcription related to immunity and metabolism (Praveen et al., 2015). Furthermore, it is well documented that the GI microbiota of formula-fed infants shows higher diversity than that of breastfed babies (Bezirtzoglou et al., 2011). The GI microbiota of breastfed infants tends to be dominated by *Bifidobacteria* during the first week of life, with members of the *Enterobacteriaceae* family concomitantly decreasing (Cerdo et al., 2019). Therefore, for the first 6 months of life, babies can benefit from exclusively being fed breast milk.

Antibiotic Use

Antibiotics are the most frequently used drugs in pediatric therapy, nonetheless, excess antimicrobial use and long-term use of antibiotics can not only clear pathogenic microorganisms from the host but can also kill beneficial gut bacteria, induce the alteration of intestinal flora and affect gene expression in the intestine via immunoreactions (Chai et al., 2012; Cox et al., 2014; McArdle et al., 2021). Gasparrini et al. (2019) used 16S rDNA community analysis to study the GI microbiota of 437 infant stools that had taken antibiotics and concluded that these newborns, including 41 preterm infants, had a greater abundance of ***Enterobacter***, ***Escherichia***, and ***Enterococcus***, and a lower relative abundance of ***Bifidobacterium*** during the course of antibiotic use. Furthermore, animal models have demonstrated that antibiotic use can destroy the GI microbiota and subsequently slow the growth of the immune system, which results in airway hyper-responsiveness in susceptible children and thereby increases the risk of allergic diseases such as asthma and allergic rhinitis (Korpela et al., 2016). As mentioned above, even short-term use, especially during the first 2 years of childhood, may lead to long-term changes in the GI microbiota that alter host interactions (Vangay et al., 2015). Gut dysbiosis promotes the horizontal transfer of resistance genes and fuels the evolution of drug-resistant pathogens and the spread of antibiotic resistance (Stecher et al., 2013).

Environment

Environment, which mostly includes family lifestyle, family members, close siblings, family size and structure, birth order and geographical location, has also been considered a correlative factor that may affect the infant GI microbiota. Some recent studies defined the "sibling effect," namely, that the anaerobe-to-facultative anaerobe ratio and the abundance of *Bifidobacteria*, in infants with older siblings are higher than those in infants without siblings (Rodriguez et al., 2015). An analysis comparing American children in an upper-middle-class community and Bangladeshi children living in an urban slum of the same age living in poorer conditions indicated that the American children had a greater abundance of *Bacteroides* and were depleted in *Prevotella* than the Bangladeshi children (Lin et al., 2013). These results suggest that differing environmental or genetic factors may shape the microbiota of healthy children in the two countries. Another analysis comparing children from southeastern Africa and northern Europe showed differences in microbiota composition and structure, with the microbiota of African children being enriched in *Bifidobacterium*, *Bacteroides*, and *Prevotella* compared to the microbiota of European children (Lin et al., 2013).

THE GASTROINTESTINAL MICROBIOTA AND ALLERGIC DISEASES

Allergic Asthma

Allergic asthma is a complicated chronic respiratory disease (CRD) characterized by airway hyper-responsiveness (AHR) and airway remodeling. Allergic asthma is a global health issue that has affected over 300 million patients of all age groups and all regions worldwide; over the last few decades, the morbidity of allergic asthma has been increasing (Bousquet et al., 2010; McKenzie et al., 2017). Currently, asthma is commonly treated with inhaled corticosteroids (ICSs) and beta-2 adrenergic receptor agonist (i.e., Salbutamol), which can help control symptoms; unfortunately, this treatment is not a cure. Therefore, there is priority in exploring novel, effective therapeutic methods to combat allergic asthma, and the microbiota and its associated metabolites have been considered as a therapeutic target.

In fact, the colonization of microorganisms in human lung is closely related to its anatomical and physiological functions. Respiratory microbes that enter the mouth travel to the lungs and are suspended in the air or on particles. The upper respiratory tract is layered with cylindrical respiratory epithelium covered by mucous membrane. The constant fluctuation of mucus and airflow determines the balance between microbial migration and elimination. Mucociliary micromotility and coughing support microbial clearance, all of which are influenced by host immune status (Dickson et al., 2014). It has been proposed that the main source of lower respiratory colonization is the resident upper respiratory microbiome. It also seems plausible that bacteria may reach the lower respiratory tract through micro-inhalation of oropharyngeal secretions and, to a lesser extent, direct inhalation (Segal et al., 2013). And in healthy individuals, low density and continuous renewal of the lung microbiome and low bacterial replication rates have been observed. Conditions conducive to the replication and persistence of certain bacterial species may lead to imbalances or disorders in the lung microbiome, which

may lead to the development of asthma (Teo et al., 2015). Colonization of the upper respiratory tract began very early, as tracheal aspirations of newborns only a few hours after birth showed that *Firmicutes* and *Proteobacteria* were the dominant phyla, in addition to *Actinomycetes* and *Bacteroides*. Interestingly, the development of resident respiratory microbiota is largely dependent on exposure in the first few hours, including mode of delivery, and the environment over the next 4–5 months. Many human clinical studies found a relation between alterations in the lung microbiota and asthma. The potential of airway microbes to regulate asthma is readily recognized due to their proximity to sites of allergic inflammation, while the GI microbiota, although anatomically isolated, are now recognized to play a vital role in asthma. The roles and dysfunction of the GI microbiota are considered to have indirect physiological effects on distal anatomical sites, including the lungs. The communication between the colonization of GI tract and the mechanism of asthma, termed the gut-lung axis, is highly complex, which stresses the interaction between the GI tract and the lung. The gut-lung axis, which in asthma mainly involves changes in the immune differentiation of cells and partial production of metabolites impacted by the mechanisms of the GI microbiota, has been confirmed. Namely, a disrupted connection within the axis or a disruption to the composition of the gut microbiota can lead to negative effects on the immunological equilibrium mechanism; in turn, this may result in allergic reactions (Durack et al., 2018; Chatenoud et al., 2020). In fact, we do not fully understand the mechanisms underlying this axis, but the GI microbiota is considered to play a crucial role in the gut-lung axis, and several contributing pathways have been confirmed (see **Figure 3**). Based on the existing mechanisms, we summarized the important cells in these pathways and stated their effects in **Table 2** (Choy et al., 2015; Kumar et al., 2017; Cait et al., 2018).

New models for the diagnosis, phenotyping and prediction of asthma treatment responses are the result of our growing understanding of the role of symbiotic microbiota in asthma; as a result, microbial ecology can lead to new therapeutic approaches to prevent and treat asthma. Some clinical studies have shown that the main mechanism in treating allergic asthma with probiotics involves regulating the composition of the GI microbiota, binding to the receptor competitively, preventing bacterial invasion and producing bacteriocins to prevent the growth of pathogens. A meta-analysis by Lin et al. (2013) suggested that probiotics can significantly reduce episodes of asthma; nevertheless, no significant differences were obtained regarding FEV1 (i.e., forced expiratory volume in the first second), Childhood Asthma Control Test (CACT) scores, PEF (i.e., peak expiratory flow) and quality of life (Zuccotti et al., 2015). Of note, the severity of the airflow limitation, as measured by percent predicted FEV1; PEF can evaluate the degree of airway blockage and make an effective judgment on the diagnosis or recovery of asthma and the severity of the disease; and the CACT is a test questionnaire to assess asthma control in children aged 4–11 years and can be used at home for long-term monitoring of the condition. As underlined in two recent reviews, the specific effects of probiotics in children who suffer from asthma need to be further confirmed based randomized-controlled trials (RCTs)

TABLE 2 | Important cells in these pathways and stated their effects.

Cells	Primary functions	Note	References
Treg cells	Play a crucial role immune homeostasis, particularly in allergy	Tregs modulate production of the major mucosal antibody, IgA, through the production of TGFβ	Bousquet et al., 2010
iNKT cells	Fill an important niche, bridging both innate and adaptive immune functions	The potential of gut microbiota to direct differentiation and function of immune populations in the lungs	Durack et al., 2018
Th17 cells	Maintain barrier function and clearing pathogens at mucosal surfaces	A potential therapeutic target in severe asthma	Chatenoud et al., 2020

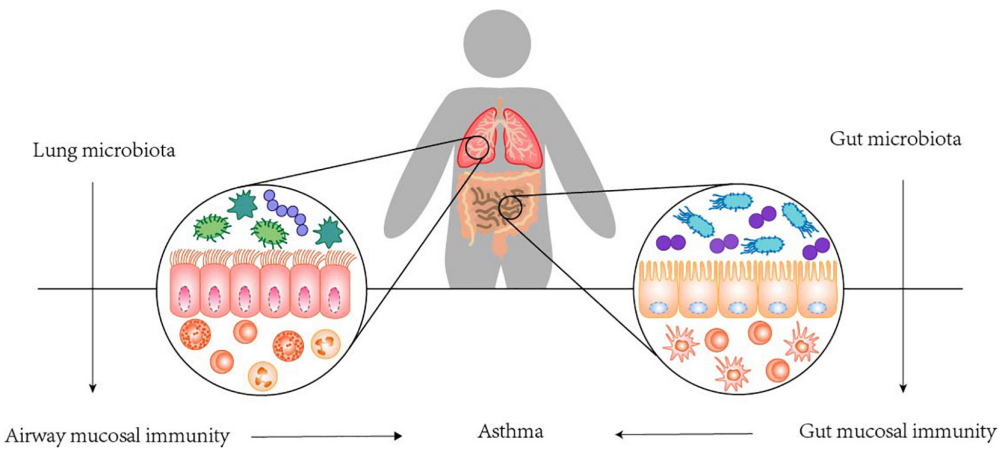

FIGURE 3 | The gut-lung axis.

with larger sample sizes (Zuccotti et al., 2015; Huang et al., 2019). In our opinion, pharmacodynamic alteration of the GI microbiota to change its composition or to target specific host pathways impacted by the GI microbiota in asthma patients remain promising therapeutic options.

Food Allergy

Food allergy, a severe health issue, has aroused public concern, especially in developed countries. About 5% of children in the United States who are 5 years old or younger suffer from FA, and evidence shows that SCFAs produced by the GI microbiota, dietary elements and the GI microbiota seriously affect immune tolerance (Kim et al., 2016).

This first studies to use germiculture showed that babies who were allergic to milk had a higher total bacterial and anoxybiotic microbe count. In addition, there is evidence that the dysbiosis of the GI microbiota prior to FA subsequently impacts the progression of FA. An investigation by Goldberg et al. (2020) discovered that babies with food allergies showed a lower abundance of *Prevotella copri* than babies without food allergies, with the concomitant increase in SCFAs, which may destroy intestinal homeostasis and barrier function. Currently, microbiota-directed therapy has been a new focus for the treatment of food allergies, and studies with larger sample sizes are needed to clarify the specific effects (Schuijs et al., 2015).

Eczema

Eczema is a common chronic disease of the skin that commonly begins in infancy (Biagini Myers and Khurana Hershey, 2010). Reduced diversity of the GI microbiota has been proven by several prospective studies to be related to the morbidity of eczema. In 2012, a prospective study by Abrahamsson et al. (2012) discovered that babies who suffered from eczema at the age of one-and-a-half had a lower diversity of the GI microbiota than healthy babies. Additionally, a lower abundance of *Lactobacillus, Bifidobacterium* and SCFAs was detected in children with eczema at the age of one than in healthy babies (Song et al., 2016). Interestingly, several probiotics that mainly contain the genera *Lactobacillus* and *Bifidobacterium* can be used as therapy for eczema because they decrease IgE levels (Toh et al., 2012; Abrahamsson et al., 2014). Nevertheless, the literature on the effectiveness of probiotic use for treating eczema is limited and hampered because studies fail to address important confounding factors. In fact, more rigorous experiments and long-term follow-up are needed to prove the efficacy of this type of treatment.

Allergic Rhinitis

Compared to other allergic diseases, there is markedly less literature specifically discussing the effect of the GI microbiota on the development of allergic rhinitis, and many of the studies that do exist are based on mouse models. A study by Flach and Diefenbach (2015). found a fixed relation between lifetime antibiotic administration and the eventual development of allergic rhinitis (OR 1.06; 95% CI 1.04–1.09) (Flach and Diefenbach, 2015). For allergic rhinitis, the GI microbiota is considered a new target for early intervention, but its mechanism and therapeutic effect need to be further studied. While there are few significant experimental results and studies correlated with allergic diseases, it is well documented that a correlation can be drawn between the GI microbiota and asthma (Bousquet et al., 2010; Suzuki et al., 2010; Shi et al., 2017; van den Elsen et al., 2017), which is frequently associated with allergic rhinitis. Thus, further research in this direction is needed.

CONCLUSION AND OUTLOOK

The recent development of NGS, genomic analysis, metabolomics and proteomics has facilitated a clearer understanding of the important role of the GI microbiota in allergic diseases, and more emphasis has been placed on the significance of maintaining intestinal microbial communities. Overwhelming evidence shows that the composition of the intestinal flora of children who suffer from allergic diseases is significantly different from that of healthy children. In addition, the composition of the GI microbiota is mainly influenced by the mode of delivery, infant diet, environment, antibiotic use by the infant, etc., and the abnormal structure of the GI microbiota has been closely related to the incidence rate of allergic diseases, which provided us with a new idea that intestinal flora disorder in infancy may be considered an important predictor of allergic diseases later in life. Taken together, these findings show that an understanding of these interactions can contribute to the development of valid prevention and therapeutic strategies for allergic diseases. Probiotics can considerably reduce episodes of allergic diseases, but additional RCTs with larger sample sizes need to be conducted to evaluate the curative effect of these strategies.

AUTHOR CONTRIBUTIONS

TH drafted the manuscript. YD generated the figure. MZ performed the background research. MZ and QH edited the manuscript. All authors have read and approved the content of the manuscript.

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The Intratumor Microbiota Signatures Associate With Subtype, Tumor Stage, and Survival Status of Esophageal Carcinoma

Yangyang Wang[1], *Hua Guo*[2], *Xiaoguang Gao*[1] *and Jihan Wang*[3]*

[1] School of Electronics and Information, Northwestern Polytechnical University, Xi'an, China, [2] Department of Nursing, Shaanxi Provincial People's Hospital, Xi'an, China, [3] Institute of Medical Research, Northwestern Polytechnical University, Xi'an, China

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Phillipp Hartmann, University of California, San Diego, United States

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***Correspondence:**
Jihan Wang
jihanwang@nwpu.edu.cn

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Altered human microbiome characteristic has been linked with esophageal carcinoma (ESCA), analysis of microbial profiling directly derived from ESCA tumor tissue is beneficial for studying the microbial functions in tumorigenesis and development of ESCA. In this study, we identified the intratumor microbiome signature and investigated the correlation between microbes and clinical characteristics of patients with ESCA, on the basis of data and information obtained from The Cancer Microbiome Atlas (TCMA) and The Cancer Genome Atlas (TCGA) databases. A total of 82 samples were analyzed for microbial composition at various taxonomic levels, including 40 tumor samples of esophageal squamous cell carcinoma (ESCC), 20 tumor samples of esophageal adenocarcinoma (EAD), and 22 adjacent normal samples. The results showed that the relative abundance of several microbes changed in tumors compared to their paired normal tissues, such as *Firmicutes* increased significantly while *Proteobacteria* decreased in tumor samples. We also identified a microbial signature composed of ten microbes that may help in the classification of ESCC and EAD, the two subtypes of ESCA. Correlation analysis demonstrated that compositions of microbes *Fusobacteria*/*Fusobacteriia*/*Fusobacteriales*, *Lactobacillales*/*Lactobacillaceae*/*Lactobacillus*, *Clostridia*/*Clostridiales*, *Proteobacteria*, and *Negativicutes* were correlated with the clinical characteristics of ESCA patients. In summary, this study supports the feasibility of detecting intratumor microbial composition derived from tumor sequencing data, and it provides novel insights into the roles of microbiota in tumors. Ultimately, as the second genome of human body, microbiome signature analysis may help to add more information to the blueprint of human biology.

Keywords: esophageal carcinoma, intratumor microbiota, The Cancer Microbiome Atlas, The Cancer Genome Atlas, microbial biomarker

INTRODUCTION

Esophageal carcinoma (ESCA) is a common type of cancer and one of the leading causes of mortality associated with the gastrointestinal tract. There are two main histological subtypes of ESCA, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAD). The two subtypes differ significantly in incidence, geographic distribution, and etiology. ESCC accounts for almost 90% of the ESCA incidence each year, and the geographic distribution of ESCC varies greatly, with the highest incidence rates occurring in Asia, especially China. Approximately half of all ESCC cases worldwide is reported in China, and these high rates are mainly due to China's large population (1). In the West, EAD represents the main histological subtype and its incidence has increased rapidly over the past 30 years (2). Although the prognosis of EAD has slightly improved over the last few decades, it is still worse than that of most other cancer types. Moreover, since most patients are diagnosed at late stages, the motility of esophageal carcinoma remains high; in most countries, approximately only 15%-25% of patients survive 5 years.

The etiology of ESCA is multifactorial and includes cigarette smoking, alcohol consumption, and low fruit/vegetable intake for ESCC and gastroesophageal reflux disease (GERD), Barrett's esophagus (BE), obesity, low fruit/vegetable intake, and cigarette smoking for EAD. The current understanding of these risk factors cannot fully explain the etiology of ESCC and EAD. Microbiota have recently emerged as novel tumorigenesis regulators and biomarkers in disease and multiple types of cancer, including ESCA (3–6). Microbial dysbiosis contributes to cancer susceptibility through complex mechanisms, including inducing inflammation and immune disfunction and interfering with anticancer drug pharmacodynamics. Dysbiosis of the gut microbiota (GM) has been studied in ESCA patients (3, 7). In addition, investigation of the esophageal microbiota is a relatively new approach in the field of ESCA (8). Several studies have indicated alterations in the esophageal microbiota in esophagitis, BE, EAD, and ESCC (7, 9, 10). There is evidence that the microbial composition of the esophagus is diverse, with gram-positive bacteria dominating in healthy conditions, while gram-negative bacteria predominating in disease status including GERD and BE (8). Exploring esophageal microbiota changes will help us better understand the tumor pathophysiology and provide potential diagnostic and/or therapeutic approaches for ESCA.

Recently, The Cancer Microbiome Atlas (TCMA) revealed a pan-cancer analysis identifying tissue-resident microbiota (11). The sample types that were analyzed for microbial prevalence were derived from The Cancer Genome Atlas (TCGA) program, and over 20,000 primary cancer and matched normal samples spanning 33 cancer types were molecularly characterized. Until now, the TCMA has been a resource for exploring the tissue-resident microbiota prevalence in several cancer types, including tissues of the oropharynx, esophagus, stomach, and colorectum. Previously, we explored the microbiota signature in four major types of gastrointestinal cancer, and the results demonstrated that the microbial profile is highly site-specific and notably differed between upper and lower gastrointestinal tumors (12). Several other studies have also investigated the intratumor microbiota derived from TCGA sequences of different cancer types (13, 14). In the study of a TCGA breast cancer cohort, the results indicated an increased *Proteobacteria* presence in tumor tissues, while the composition of *Actinobacteria* was elevated in the adjacent normal tissues (15). Rodriguez et al. detected the global microbial composition in tumor and adjacent normal tissues across 9 TCGA cancer cohorts (16). Microbiome analysis from tumor tissues as well as human blood samples will also reveal a new class of microbial-based cancer diagnostics (17). Overall, exploring the intratumor microbial signature will help improve our knowledge of the host-microbiota interaction, which is important to understand the linkage of dysbiosis with chronic inflammation and processes that influence tumorigenesis.

Understanding the relationship between clinical phenotype information and multiomics data such as the genome or microbiome is critical for human biological and medical research. To the best of our knowledge, no studies have been conducted to investigate the comprehensive microbial signature or its relationship with the clinicopathological characterization of ESCA. Here, we profiled the microbiome of patients with ESCA from the TCMA, and also analyzed the clinical phenotype and survival data of the corresponding samples from the TCGA. The global microbial composition at the phylum, class, order, family, and genus levels of tumor and noncancerous adjacent normal tissues was calculated to analyze the differential microbes. We further evaluated the correlation between the microbes and the clinical variables of the tumors. Specifically, we identified the microbial signatures related to cancer subtype, tumor stage, and survival status. We believe that the intratumor microbial study will provide a better understanding of dysbiosis and establish a new foundation for studying host-microbiota interactions and the role of microbiota in the tumorigenesis of esophageal carcinoma.

MATERIALS AND METHODS

Sample Acquisition and Information Collection

In this study, the microbiota profiles of samples from 82 cases (including 40 ESCC tumors, 20 EAD tumors, and 22 noncancerous adjacent tissues used as normal samples) at the phylum, class, order, family, and genus levels were obtained from the TCMA database (https://tcma.pratt.duke.edu/); the corresponding clinical phenotype information and survival data for the 82 patients were obtained from the TCGA program (for phenotype information, https://gdc-hub.s3.us-east-1.amazonaws.com/download/TCGA-ESCA.GDC_phenotype.tsv.gz; for survival data, https://gdc-hub.s3.us-east-1.amazonaws.com/download/TCGA-ESCA.survival.tsv). **Figure 1A** displays an overview of the study design, and the clinical information about the patient samples is summarized in **Table 1**.

Microbiota Abundance Analysis of Tumor and Normal Tissues of Esophageal Carcinoma

The relative abundance of the microbiota composition at the phylum, class, order, family, and genus levels of each sample was

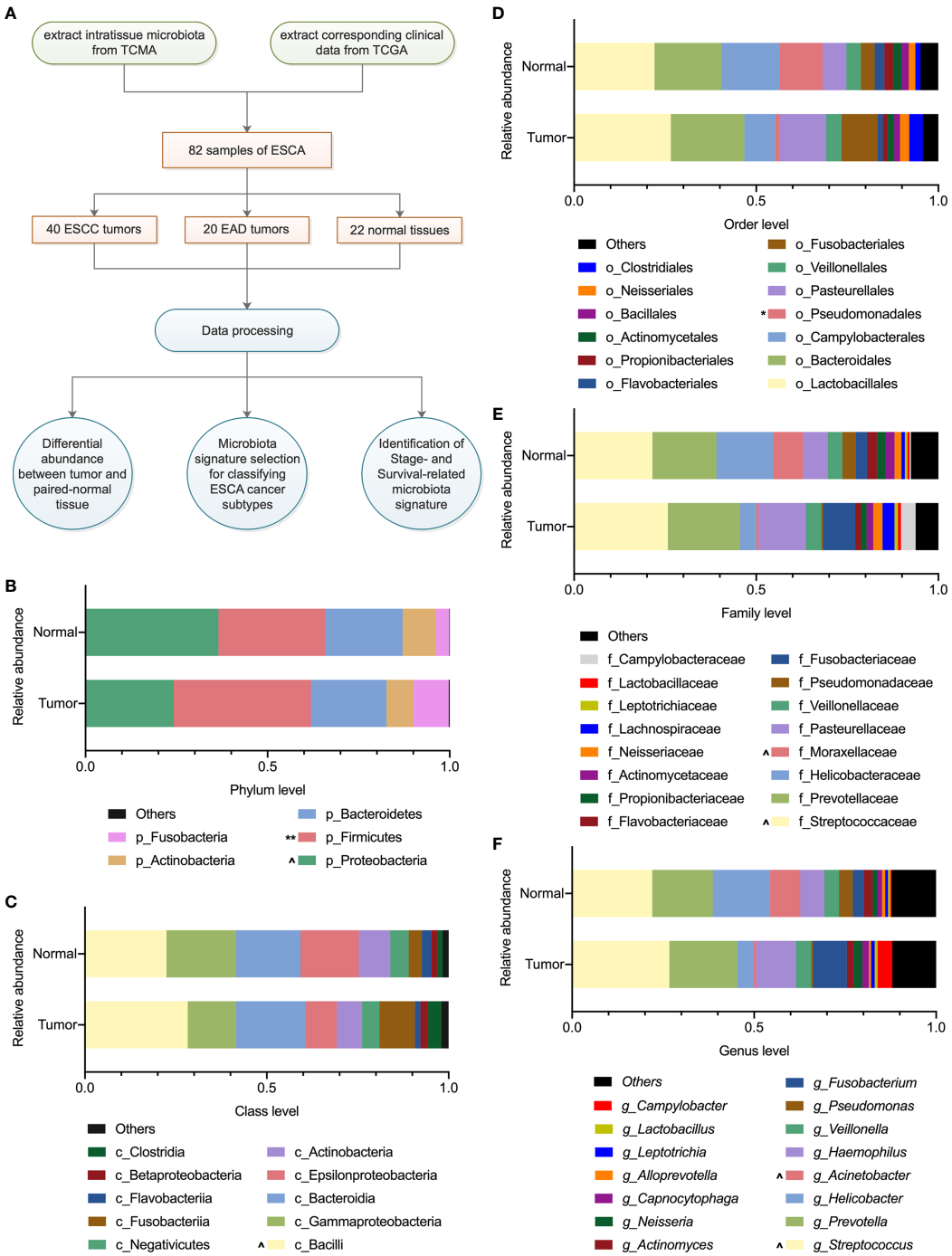

FIGURE 1 | Overview of the study design and microbiota profiling. **(A)** Schematic overview of the study design. TCMA, the cancer microbiome atlas; TCGA, the cancer genome atlas; ESCA, esophageal carcinoma; ESCC, esophageal squamous cell carcinoma; EAD, esophageal adenocarcinoma. **(B–F)** The composition of microbiota with an average abundance > 1% in tumor and paired normal tissues at the phylum, class, order, family, and genus levels, respectively. The relative microbial abundance in tumors compared with normal samples, ^0.1 < P < 0.01, *P < 0.05, **P < 0.01 in paired-samples t-test.

calculated, and the microbial composition with an average relative abundance > 1% was selected for further analysis. In this study, there were 22 pairs of strictly matched tumor-adjacent normal samples of ESCA. The paired-samples t-test was used to analyze the differential microbial composition in tumors and their normal tissues, with a false discovery rate (FDR)-adjusted P-value < 0.05 considered significant. In addition, linear discriminant analysis effect size (LEfSe) analysis was performed

TABLE 1 | Clinical characteristics of cases in this study (information derived from the TCGA database).

Clinical characteristics		ESCA tumor tissue		Normal tissue
		ESCC	EAD	
Age (year)		36-90	47-86	51-90
[range (mean ± SD)]		(59.45 ± 10.60)	(72.75 ± 10.75)	(73.45 ± 10.98)
Gender	Male	36	15	15
	Female	4	5	7
Pathologic T-stage	T1	2	8	–
	T2	10	2	–
	T3	24	9	–
	T4	2	0	–
	Not reported	2	1	–
Pathologic N-stage	N0	27	6	–
	N1	11	8	–
	N2	0	3	–
	N3	0	2	–
	Not reported	2	1	–
Pathologic M-stage	M0	35	9	–
	M1	1	1	–
	MX	1	6	–
	Not reported	3	4	–
Overall stage	Stage I	2	6	–
	Stage II	26	4	–
	Stage III	9	6	–
	Stage IV	1	1	–
	Not reported	2	3	–
Overall survival status	Alive	28	14	–
	Dead	11	6	–
	Not reported	1	0	–
Overall survival time (day)		96-1688	9-1458	–
[range (mean ± SD)]		(551.90 ± 435.05)	(405.28 ± 284.02)	
Total number		**40**	**20**	**22**

ESCA, esophageal carcinoma; EAD, esophageal adenocarcinoma; ESCC, esophageal squamous cell carcinoma. SD, standard deviation.

by using OECloud tools at https://cloud.oebiotech.cn. Specifically, the nonparametric factorial Kruskal-Wallis (KW) sum-rank test and Wilcoxon rank-sum test were used to identify taxa biomarkers for tumor and normal samples, and linear discriminant analysis (LDA) was further performed to evaluate the microbial effects for each group. The microbes with LDA values > 2 and $P < 0.1$ were considered significantly enriched in that group.

Microbiota Signature Selection for Classifying Subtypes of Esophageal Carcinoma

There were 40 ESCC and 20 EAD tumor samples in the esophageal carcinoma group. We investigated whether these two cancer subtypes could be classified based on the tumor microbiota profile. The SHapley Additive exPlanations (SHAP) (18, 19) theoretic approach was performed for microbial feature selection to identify the more important microbial profile, which may predict the classification of the two different cancer subtypes. The global microbiota (as the variables to distinguish the two cancer subtypes) importance scores were evaluated and visualized by SHAP, and we then selected the top ten most important microbial features for further analysis. Principal component analysis (PCA) and partial least squares discrimination analysis (PLS-DA) were performed by using the packages "FactoMineR" and "mixOmics" in R version 4.0.2, respectively.

Identification of Stage- and Survival-related Microbiota Signatures for Esophageal Carcinoma

The microbiota profile of ESCA at the phylum, class, order, family, and genus levels derived from the TCMA database and the corresponding clinical information of all the samples obtained from the TCGA database were integrated for correlation analysis. Specifically, the Pearson cor.test () function in R version 4.0.2 was performed to analyze the correlation between the relative abundance of specific microbiota and tumor stage. Kaplan-Meier survival analysis was performed to assess the survival-related microbiota. The "survival" and "survminer" packages in R version 4.0.2 were used for survival analysis and curve visualization based on the microbial composition.

RESULTS

Differential Microbiota Signatures in Tumor and Normal Tissues of Esophageal Carcinoma

There were 82 samples of esophageal carcinoma (**Table 1**) in the TCMA database. A total of 11, 22, 38, 67, and 221 taxa were obtained for each sample at the phylum, class, order, family, and

genus levels, respectively. **Table S1** summarizes the global microbial profiling at each taxonomic level. We then explored the differential microbial compositions between tumor and normal tissues. Overall, there were 5, 10, 13, 16, and 15 microbial taxa with an average relative abundance > 1% at the phylum, class, order, family, and genus levels, respectively (**Figures 1B–F**). At the phylum level, the intratissue microbiota was dominated by *Proteobacteria* (36.5% for normal tissue, 24.2% for tumor tissue) and *Firmicutes* (29.3% for normal tissue, 37.7% for tumor tissue), followed by *Bacteroidetes* (21.2% for normal tissue, 20.7% for tumor tissue), *Actinobacteria* (9.2% for normal tissue, 7.4% for tumor tissue), and *Fusobacteria* (3.6% for normal tissue, 9.7% for tumor tissue). The microbial composition of *Firmicutes* increased significantly ($P < 0.01$), while that of *Proteobacteria* decreased ($0.1 < P < 0.05$) in tumor samples compared with their paired normal tissues (**Figure 1B**). As **Figures 1C–F** show, the difference in microbial composition profiling in tumors was not obviously significant compared with that in normal tissues, except that the relative abundance of *Pseudomonadales* was less abundant in tumors than in normal samples at the order level ($P < 0.05$, **Figure 1D**).

LEfSe analysis helps to identify specific enriched microbial biomarkers for different groups. As **Figure 2** shows, the compositional abundances of *Fusobacteria*/*Fusobacteriia*/*Fusobacteriales* were higher in tumor tissues, while compositional abundances of *Proteobacteria*, *Moraxellaceae*, *Acinetobacter*, and *Flavobacteriia*/*Flavobacteriales*/*Flavobacteriaceae* were enriched in normal tissues. Specifically, the compositional positive ratio of order *Fusobacteriales* was 63.6% (14 of 22) for tumor and 68.2% (15 of 22) for normal tissue, while the average abundance of *Fusobacteriales* for the positive samples was higher ($0.1 < P < 0.05$) in tumor (14 positive samples with an average abundance of 15.4%) than that in normal tissues (15 positive samples with an average abundance of 5.6%). In contrast, a higher compositional positive ratio of *Flavobacteriaceae* (5 of 22 for tumor vs. 12 of 22 for normal, $P < 0.05$) and *Moraxellaceae* (2 of 22 for tumor vs. 6 of 22 for normal, $P < 0.01$) at the family level as well as *Acinetobacter* (2 of 22 for tumor vs. 6 of 22 for normal, $P < 0.01$) at the genus level was detected more in normal tissues than in tumors.

Identification of Intratumor Microbiota Signatures Associated With Cancer Subtypes of Esophageal Carcinoma

As the ESCA tumor samples were histologically subdivided into ESCC and EAD subtypes, we further investigated whether the intratumor microbial signature was somehow subtype-correlated in esophageal carcinoma. The SHAP (Shapley additive explanations) approach was applied to select the most valuable features in predicting the different groups, as it provided reference information about feature ranking and feature selection. Previously, a total of 59 microbial taxa were identified with an average relative composition > 1% at different taxonomic levels. To prioritize microbes of the 59 taxa, we relied on feature importance (contribution) obtained from SHAP to evaluate the whole importance value of each individual microbe in predicting cancer subtypes. A signature containing the top 10 microbial features was identified from the 59 microbial taxa as predictable factors, including *Actinobacteria*, *Fusobacteria*, *Bacilli*, *Epsilonproteobacteria*, *Negativicutes*, *Bacillales*, *Pasteurellales*, *Fusobacteriaceae*, *Lactobacillaceae*, and *Streptococcaceae* (**Figure 3A**). PCA (**Figure S1A**) and PLS-DA (**Figure S1B**) of all 59 microbial profiles were performed before feature ranking and selection. A relatively improved separate model was observed when performing PCA (**Figure 3B**) and PLS-DA (**Figure 3C**) based on the ten microbial features obtained from SHAP after feature ranking and selection.

Abundances of Specific Microbes in Relation to Patients' Clinical Characteristics

The TCGA database contains comprehensive clinical characterization of multiple types of cancer. **Table 1** summarizes the clinicopathological information of the 82 cases analysed in this study. We next investigated whether there were specific microbes associated with the clinicopathological variables of ESCA patients. The results showed that the composition of *Fusobacteria*/*Fusobacteriales* was positively correlated ($P < 0.01$), while the relative abundance of *Lactobacillales* was negatively correlated ($0.05 < P < 0.1$) with the tumor stage status of ESCA (**Figure 4**). The survival analysis indicated that the enrichment of several microbes could reflect the overall survival probability of patients (**Figure 5**). Detailed information about the survival status of tumor patients and the microbial composition were summarized in **Table S2**. High abundances of *Proteobacteria*, *Negativicutes*, and *Lactobacillaceae*/*Lactobacillus* were associated with better prognosis ($P < 0.05$), while a high composition of *Clostridia*/*Clostridiales* and *Fusobacteriia*/*Fusobacteriales* reflected poorer prognosis ($P < 0.05$). The eight microbes were then applied in a multivariate Cox regression analysis. Four microbes were identified as independent prognostic factors of ESCA patients ($P < 0.05$), as shown in **Table 2**.

It is essential to assess clinical-pathological prognostic factors such as lymph nodes, esophageal wall size and infiltration, and metastasis in relation to the microbiota under investigation. The information about lymph nodes in the current study was relatively incomplete (**Table S3**). As a result, we did not conduct the lymph node microbiota correlation analysis, which will require more attention in future research.

DISCUSSION

Studies on the bacterial or viral composition of human tumors using sequencing data from databases such as the TCGA have recently emerged (14, 20). Investigation of the intratumor microbiota will provide valuable information for better understanding the occurrence and progression of tumors. In addition to recent studies about microbiota changes in esophageal disease, our research performed a more comprehensive investigation of microbial characteristics in ESCA.

After microbial detection and calculation at different taxonomic levels, we found differential microbial abundance in

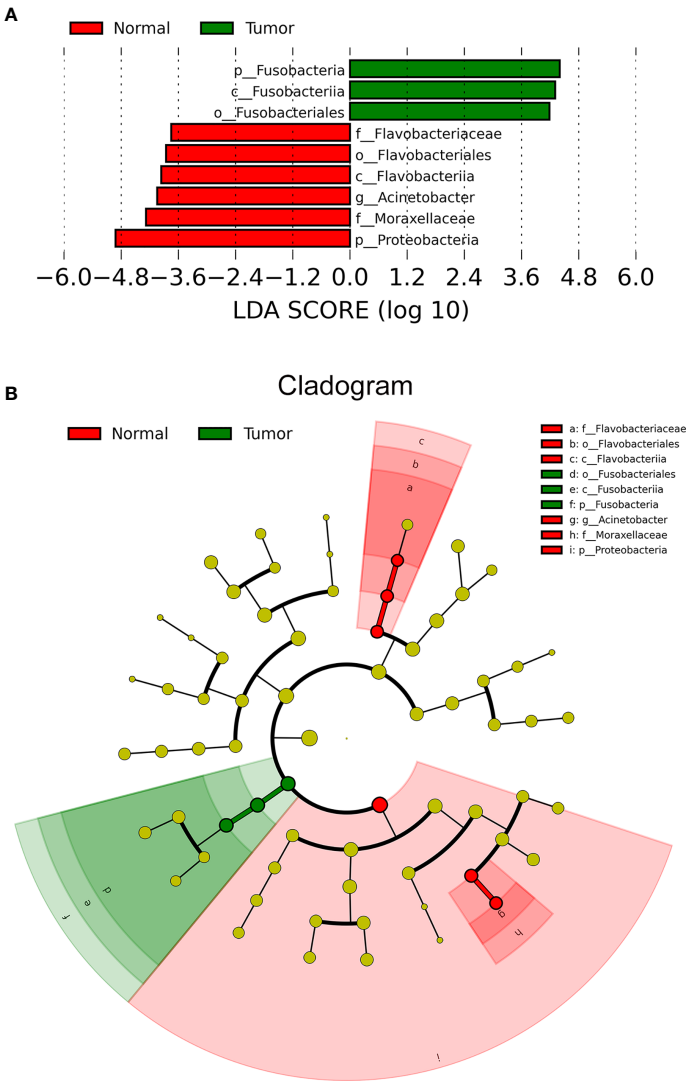

FIGURE 2 | LEfSe analysis identifying tumor- and normal-enriched microbiota. **(A)** The LDA score of specific microbial taxa in the tumor group and normal group. Threshold LDA score is 2. **(B)** Cladogram showing tumor- and normal-enriched microbial taxa. LEfSe, linear discriminant analysis effect size; LDA, linear discriminant analysis.

tumor and normal samples of ESCA. Consistent with other studies, the relative abundance of *Firmicutes* and *Fusobacteria* increased, while that of *Proteobacteria* decreased in esophageal tumors compared with normal tissues (21–23). In another study, the enriched composition of *Firmicutes* and the unenriched composition of *Proteobacteria* were reported to be associated with BE (24). The LEfSe analysis in this study also indicated *Fusobacteria*/*Fusobacteriia*/*Fusobacteriales* as tumor-enriched microbes, suggesting that it might be a potential biomarker for the tumorigenesis and development of ESCA. In general, the alteration of microbial abundance at the class, order, family, and genus levels in tumors compared with adjacent normal tissue was not significant.

The human genome has been referred to as the blueprint of human biology (25). It is well established that cancer genome signature analysis helps to predict different cancer systems and their subtypes and contributes to precision medicine (26–28). The microbiome, as the second genome of the human body, plays crucial roles in health and disease (25, 29). A study reported that the intracellular microbiome of human tumors is tumor type-specific across multiple types of tumors, and intratumor bacteria or their predicted functions correlate with tumor types, subtypes, patient smoking status, and the response to immunotherapy (30). The microbiome could also be a potential biomarker/rule for subgrouping different cancer subtypes and used as a factor for exploring the complicated microenvironment components associated with tumorigenesis (31). In our research, we further identified a signature containing 10 microbial features that was somehow predictive of ESCC and EAD, the two subtypes of ESCA, by applying the SHAP

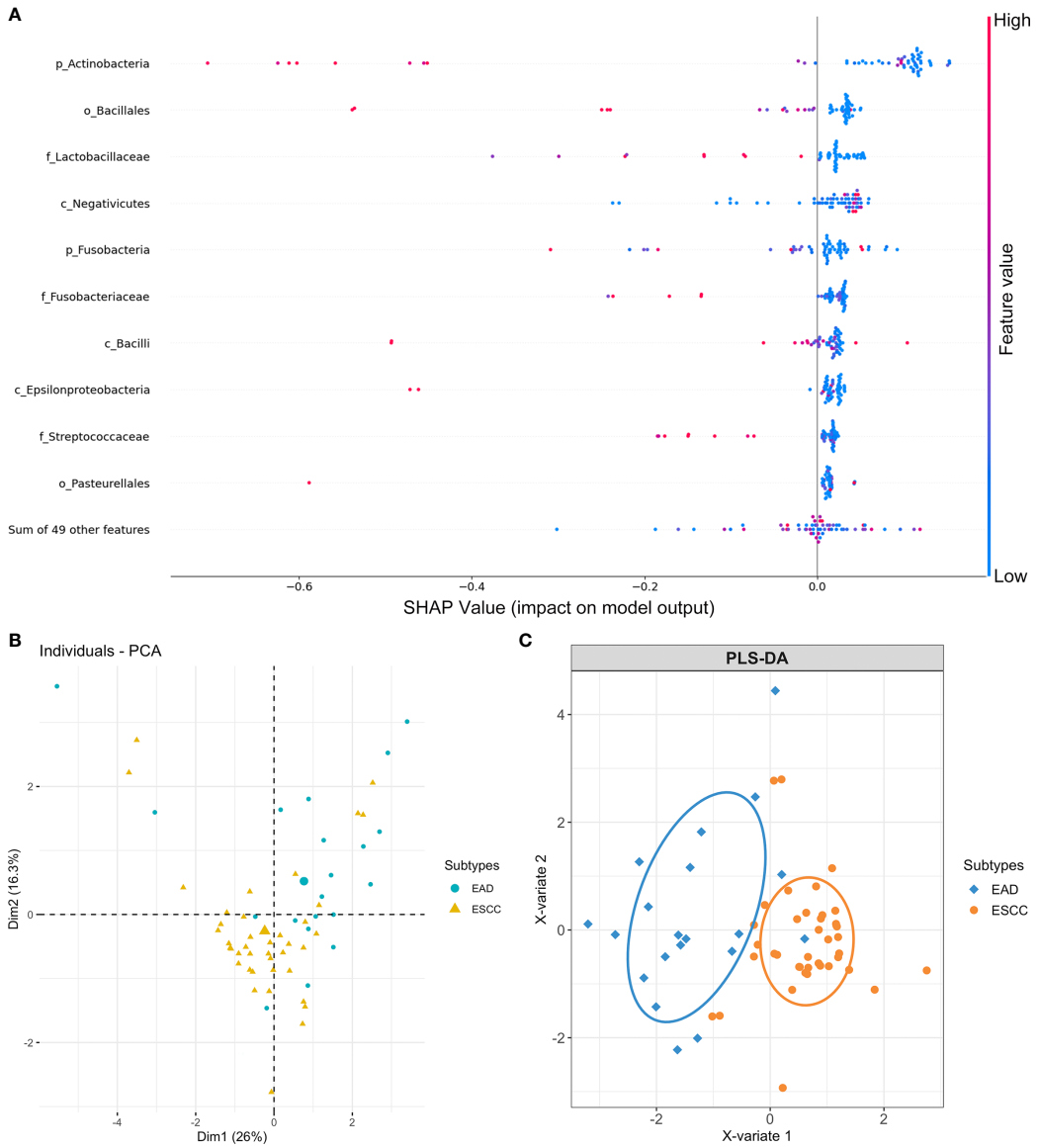

FIGURE 3 | Microbial feature selection in predicting different cancer subtypes of esophageal carcinoma. **(A)** Summary plots of the importance values of the top 10 predictable microbes. **(B)** PCA plots and **(C)** PLS-DA plots based on the top 10 microbial features display the EAD and ESCC subtypes of esophageal carcinoma.

approach. The human oral cavity harbours the second most abundant microbiota after the gut microbiota in the gastrointestinal tract (32). Microorganisms that exist in the oral cavity and its contiguous extensions (stopping at the distal esophagus) are all considered the oral microbiome and are altered within different oral structures and tissues (33). Here, we provide evidence that the microbial signature could be cancer subtype-related.

We then examined the relationship between specific microbes and the clinical index of ESCA patients by integrating the TCMA microbiome profile with clinical data from the TCGA. There were few links between tumor stage and microbial abundance, except that the composition of tumor-enriched *Fusobacteria*/*Fusobacteriales* was found to be positively correlated with tumor stage. *Fusobacteria* contribute to the formation of a proinflammatory microenvironment that promotes the colorectal neoplasia progression by recruiting tumor-infiltrating immune cells (34). In addition to its role in in colorectal cancer, *Fusobacteria* has been reported to be enriched in various cancer types, including oral, stomach, and breast cancer (35, 36). Studies have demonstrated that breast cancer colonized by *Fusobacterium nucleatum* accelerates tumor growth and metastasis (36). Furthermore, the high relative abundance of *Fusobacteriia*/*Fusobacteriales* correlated with a poorer prognosis in ESCA patients in our study, indicating that targeting *Fusobacteria* may be beneficial for the treatment of not only colorectal cancer but also other types of cancers. Thus, exploring

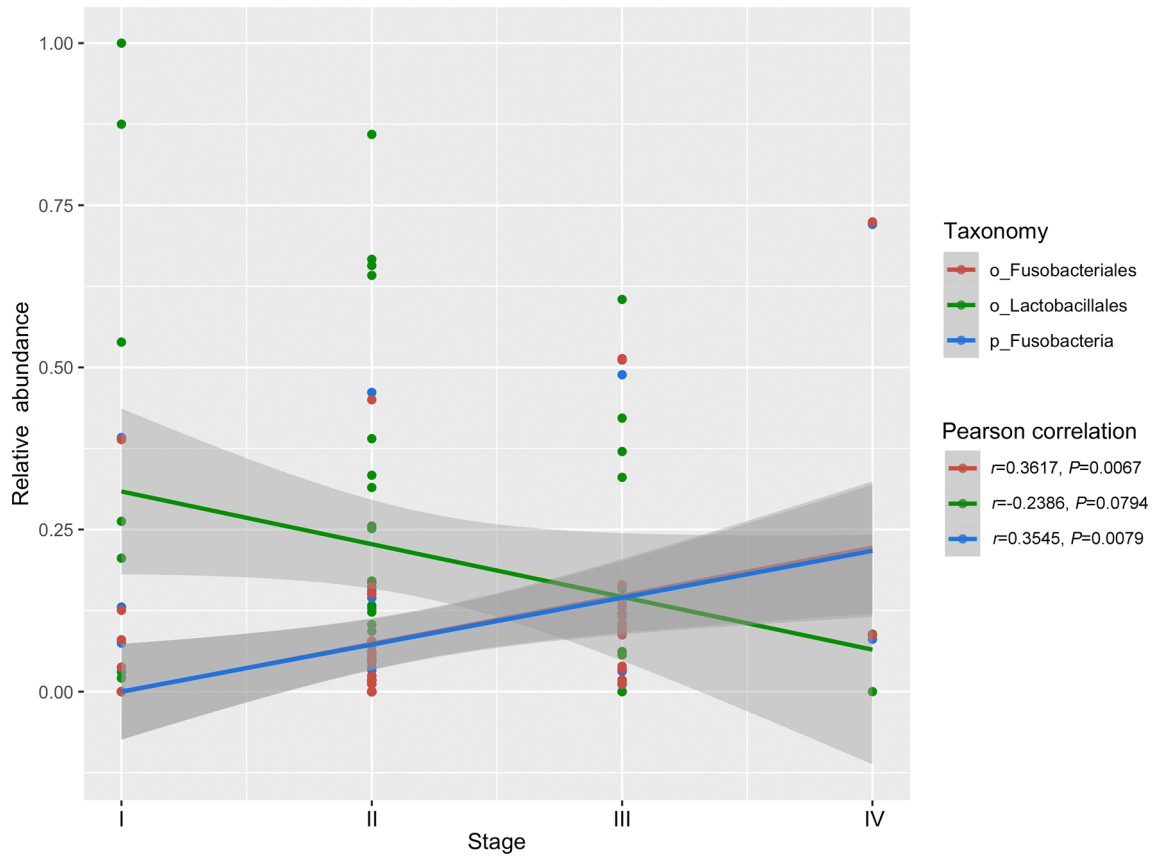

FIGURE 4 | Correlation of specific microbes with the tumor stage status of ESCA. The analysis was performed using Pearson correlation in R.

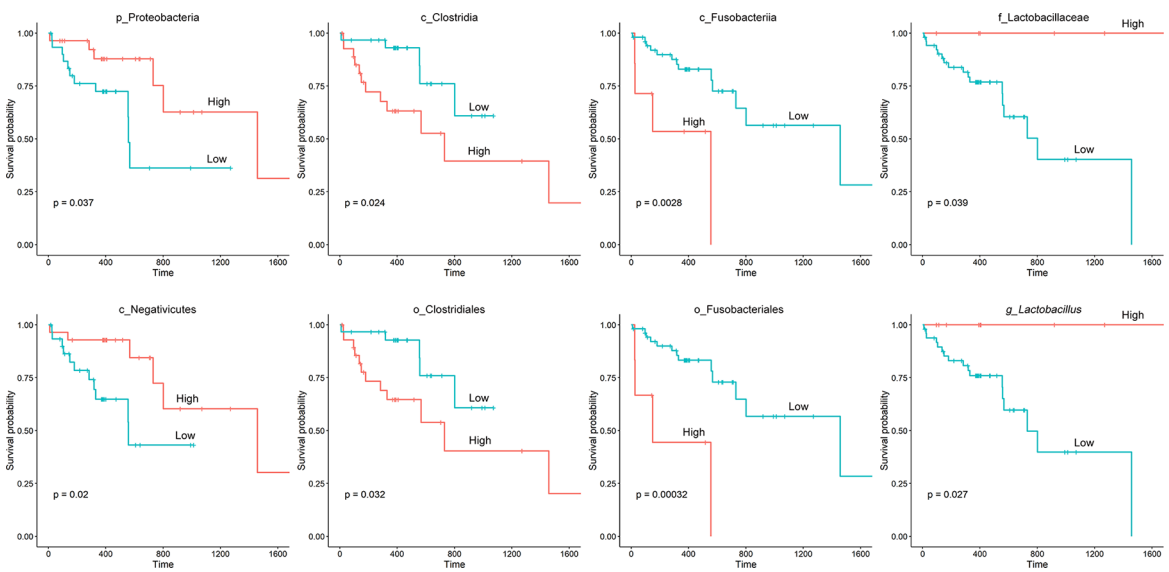

FIGURE 5 | Kaplan-Meier survival curves based on microbial composition. The "survival" and "survminer" packages in R were used for survival analysis. The process included determining the optimal cut-off point of variables, categorizing variables into "high" and "low" subgroups based on microbial abundance, fitting survival curves and visualization.

TABLE 2 | Multivariate Cox regression analysis of the independent significance of eight microbes as prognostic factors.

Microbes		HR	HR (0.95 lower)	HR (0.95 upper)	*P*-value
Phylum	*Proteobacteria*	5.61E-02	1.27E-03	2.48E+00	0.136
Class	*Clostridia*	Inf	Inf	Inf	**<2E-16**
	Fusobacteriia	3.94E-86	3.47E-87	4.48E-85	**<2E-16**
	Negativicutes	7.34E-03	7.57E-07	7.11E+01	0.294
Order	*Clostridiales*	0.00E+00	0.00E+00	0.00E+00	**<2E-16**
	Fusobacteriales	1.11E+86	9.81E+84	1.27E+87	**<2E-16**
Family	*Lactobacillaceae*	0.00E+00	0.00E+00	Inf	0.988
Genus	*Lactobacillus*	1.60E+298	0.00E+00	Inf	0.994

HR, hazard ratio. The results were analyzed by multivariate Cox regression model based on "survival" package in R. There were four microbes with P < 0.05 in the multivariate Cox regression analysis, as showed in bold values.

the intratumor microbiome signature will facilitate the discovery of novel microbial biomarkers for cancer research.

CONCLUSION

In this study, we conducted a comprehensive analysis of the intratumor microbiome in ESCA samples. Taken together, there are differences in the abundance of several microbial taxa between the tumor and adjacent normal tissues, and the potential functions of these microbes in ESCA merit further study. We also identified the intratumor microbiota signatures that were correlated with the subtype, tumor stage, and survival status of ESCA. We expect that our research will facilitate a better understanding of the intratumor microbiome of ESCA and identify potential biomarkers for the disease, as well as provide a novel perspective on the role of the microbiome in tumors, since studies of genome variation and disease risk will necessitate the integration of human and microbial genomic data.

Our study has limitations; the number of tumor and paired normal tissue samples in the subgroups was relatively small and did not allow us to make any generalizable conclusions. Large-scale and mechanistic studies are needed to further confirm the results of this study.

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.

AUTHOR CONTRIBUTIONS

YW and JW designed, performed the study, and co-wrote the manuscript. HG and XG contributed to data and statistical analysis, and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.754788/full#supplementary-material

Supplementary Figure 1 | PCA and PLS-DA plots based on the 59 microbial taxa displaying EAD and ESCC subtypes of esophageal carcinoma. **(A)** PCA plots. **(B)** PLS-DA plots.

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Gut Microbial Dysbiosis and Changes in Fecal Metabolic Phenotype in Precancerous Lesions of Gastric Cancer Induced With N-Methyl-N′-Nitro-N-Nitrosoguanidine, Sodium Salicylate, Ranitidine, and Irregular Diet

Fuhao Chu[1,2,3†], Yicong Li[4†], Xiangmei Meng[2,3†], Yuan Li[2,3], Tao Li[3,5], Mengyin Zhai[2,3], Haocheng Zheng[2,3], Tianxi Xin[2,3], Zeqi Su[3,6], Jie Lin[2,3], Ping Zhang[7]* and Xia Ding[2,3]**

[1] Institute of Regulatory Science for Traditional Chinese Medicine, Beijing University of Chinese Medicine, Beijing, China, [2] School of Traditional Chinese Medicine, Beijing University of Chinese Medicine, Beijing, China, [3] Research Center for Spleen and Stomach Diseases of Traditional Chinese Medicine, Beijing University of Chinese Medicine, Beijing, China, [4] Dongfang Hospital, Beijing University of Chinese Medicine, Beijing, China, [5] Dongzhimen Hospital, Beijing University of Chinese Medicine, Beijing, China, [6] Beijing Research Institute of Chinese Medicine, Beijing University of Chinese Medicine, Beijing, China, [7] Wangjing Hospital, China Academy of Chinese Medical Sciences, Beijing, China

Background and Aims: Precancerous lesions of gastric cancer (PLGC) are the most important pathological phase with increased risk of gastric cancer (GC) and encompass the key stage in which the occurrence of GC can be prevented. In this study, we found that the gut microbiome changed significantly during the process of malignant transformation from chronic gastritis to GC in N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) multiple factors-induced rat model. Accumulating evidence has shown that alterations in gut microbiota and metabolism are potentially linked to chronic inflammation and cancer of the gastrointestinal tract. However, the correlation of gut microbiota and metabolites, inflammatory factors, and the potential mechanism in the formation of PLGC have not yet been revealed.

Methods: In this study, multiple factors including MNNG, sodium salicylate drinking, ranitidine feed, and irregular diet were used to establish a PLGC rat model. The pathological state of the gastric mucosa of rats was identified through HE staining and the main inflammatory cytokine levels in the serum were detected by the Luminex liquid suspension chip (Wayen Biotechnologies, Shanghai, China). The microbial composition and metabolites in the stool samples were tested by using *16S ribosomal RNA* (*rRNA*) gene sequencing and non-targeted metabolomics. The correlation analysis of gut microbiota and inflammatory cytokines in the serum and gut microbiota and differential metabolites in feces was performed to clarify their biological function.

Results: The results showed that compared to the control group, the gastric mucosa of the model rats had obvious morphological and pathological malignant changes and the serum levels of inflammatory cytokines including interleukin-1β

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Phillipp Hartmann, University of California, San Diego, United States

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Haoming Xu, Guangzhou First People's Hospital, China
Min Gu, Louisiana State University, United States
Jiaojiao Xie, Zhejiang Chinese Medical University, China

***Correspondence:**
Jie Lin
linjie@bucm.edu.cn
Ping Zhang
pinglele@sina.comand
Xia Ding
dingx@bucm.edu.cn

† These authors have contributed equally to this work

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(IL-1β), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10), interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and macrophage colony-stimulating factor (M-CSF) increased significantly, while the level of chemokine (C-X-C motif) ligand 1 (CXCL1) in serum reduced significantly. There were significant differences in the composition of the gut microbiota and fecal metabolic profiles between the model and control rats. Among them, *Lactobacillus* and *Bifidobacterium* increased significantly, while *Turicibacter*, *Romboutsia*, *Ruminococcaceae_UCG-014*, *Ruminococcaceae_UCG-005*, and *Ruminococcus_1* reduced significantly in the model rats compared to the control rats. The metabolites related to the lipid metabolism and peroxisome proliferator-activated receptor (PPAR) signaling pathway have also undergone significant changes. In addition, there was a significant correlation between the changes of the differential inflammatory cytokines in the serum, fecal metabolic phenotypes, and gut microbial dysbiosis in model rats.

Conclusion: The activation of the inflammatory response, disturbance of the gut microbiota, and changes in the fecal metabolic phenotype could be closely related to the occurrence of PLGC. This study provides a new idea to reveal the mechanism of risk factors of chronic gastritis and GC from the perspective of inflammation-immune homeostasis, gut microbiota, and metabolic function balance.

Keywords: precancerous lesions of gastric cancer (PLGC), gut microbial and metabolic dysbiosis, MNNG, *16S rRNA* genes sequencing, untargeted metabolomics analysis

INTRODUCTION

Gastric carcinoma (GC) is the fifth most frequently diagnosed cancer and the third leading cause of cancer-related death worldwide (Bray et al., 2018), and approximately half of the world GC cases and deaths occur in China (Nie et al., 2017). Evidence from the pathology and epidemiology studies indicated that the human model of gastric carcinogenesis evolved from the following sequential stages known as Correa cascade: chronic non-atrophic gastritis (CNAG), followed by precancerous lesions of gastric cancer (PLGC), including chronic atrophic gastritis (CAG), intestinal metaplasia (IM), and dysplasia (DYS) (Correa et al., 1975; Correa, 1992; Park and Kim, 2015), eventually worsened into gastric adenocarcinoma. PLGC is the most important pathological phase with increasing the risk of gastric cancer (Correa et al., 1990, 2010; You et al., 1993; de Vries et al., 2008; Song H. et al., 2015; Li et al., 2016). Exploring the pathogenesis of the PLGC stage could provide new strategies for preventing the occurrence of GC.

The occurrence of GC is closely related to *Helicobacter pylori* (*H. pylori*) infection, dietary habits, gastroesophageal reflux, long-term stomach inflammation, and proton pump inhibitors (PPIs) abuse (Engel et al., 2003; Carrasco and Corvalan, 2013; Palmer, 2017; Laterza et al., 2019). Long-term exposure to *H. pylori* infection is one of the main etiologic factors for the progression of PLGC and GC (Díaz et al., 2018). *Helicobacter pylori* infection could release a variety of bacterial virulence factors (VacA, CagA, etc.), which could promote gastric cell death, induce genetic and epigenetic changes in the gastric epithelial cells and the initial inflammatory response, and eventually resulting in primary tissue lesions (Valenzuela et al., 2015). More notably, as the food additives in processed meats, consumption of nitrates was related to a decreased risk of GC, while high intake of nitrites and N-nitrosodimethylamine (NDMA) resulted in an increased risk of GC (Song P. et al., 2015). In addition, drug abuse could also cause inflammation and damage to the gastric mucosa, which, in turn, increased the risk of GC. Among them, PPI abuse could inhibit the production of gastric acid and then contribute to gastric cancer pathogenesis, which could be related to the growth of excessive pathogenic microorganisms in a low-acid state in the stomach (Palmer, 2017; Laterza et al., 2019). The non-steroidal anti-inflammatory drugs (NSAIDs) are well-known cyclooxygenase (COX) inhibitors for the treatment and prevention of cancer due to the relationship between chronic inflammation and cancer (Wong, 2019). However, there are conflicting findings on the role of NSAIDs in the prevention and treatment of cancer. Long-term NSAID use is often associated with serious gastrointestinal (GI), cardiovascular, renal, and other side effects (Harirforoosh et al., 2013) and increased risk or mortality in certain types of cancer (Brasky et al., 2011; Choueiri et al., 2014). Although the report about the dietary habits and drugs abuse increasing the risk of gastritis and gastric cancer is mostly epidemiologic, the mechanisms underlying the increased risk are less well delineated.

Increasing evidence has shown that alterations in gut microbiota are potentially linked to inflammation and cancer (Schwabe and Jobin, 2013; Abreu and Peek, 2014; Garrett, 2015; Vogtmann and Goedert, 2016; Bhatt et al., 2017). The gut microbiota consists of a huge number of bacteria, which participate in the absorption of nutrients, energy metabolism, maturation of the intestinal immune system, and protection of GI mucosa from the infection of pathogens (Lozupone et al., 2012;

Nicholson et al., 2012). The gut microbiota is easily influenced by a variety of factors including diet, age, antibiotics, and environmental and psychological factors (Osadchiy et al., 2019). The gut microbial dysbiosis resulted in immunological dysregulation has been associated with the pathogenesis of *H. pylori* infection, chronic inflammation, and cancer (Round and Mazmanian, 2009; Lam et al., 2017; Zitvogel et al., 2017; Wong et al., 2019). The increased risk of gastritis and GC caused by the unhealthy diet and drug abuse is related to the imbalance of gut microbiota that has not been elucidated.

N-Methyl-N′-nitro-N-nitrosoguanidine (MNNG)-induced mouse model, one of the chemical carcinogenesis models, has been widely used for the GC and development of gastric precancerous lesions (Cai et al., 2018, 2019; Xu et al., 2018; Zhang et al., 2018; Zhao et al., 2019). In this study, we modified the MNNG-induced model to combined with sodium salicylate drinking, ranitidine feed, and irregular diet (multifactor induction) that were used to simulate the main factors leading to chronic gastritis and GC such as irregular diet, gastric mucosal inflammation damage, and PPI abuse to establish PLGC rat model regarding our previous report (Yu et al., 2020).

To explore the mechanisms of the interaction of gastric inflammation, gut microbiota, and metabolic in PLGC, we used the multifactor-induced rat PLGC model and performed the serum cytokines detection, gut microbiota 16S ribosomal RNA (rRNA) sequence, and fecal non-targeted metabolomics assay.

MATERIALS AND METHODS

Materials

N-Methyl-N′-nitro-N-nitrosoguanidine was purchased from the TCI (Shanghai) Huacheng Industrial Development Corporation Ltd. (Shanghai, China). Sodium salicylate was purchased from the Sinopharm Chemical Reagent Corporation Ltd. (Beijing, China) and prepared 2% solution with the SPF-grade animal drinking water every day. Granulated SPF-grade rat fodder containing 0.05% ranitidine was purchased from the Beijing Keao Xieli Feed Corporation Ltd. (Beijing, China). All the other chemicals used in ultra performance liquid chromatography-quadrupole-time-of-flight mass spectrometry (UPLC-Q-TOF/MS) were of MS grade and purchased from the Thermo Fisher Scientific Incorporation (Waltham, MA, United States).

Animal Study

A total of 18 SPF-grade Wistar rats (male, 100–130 g, 4-week-old) were purchased from the Beijing Vital River Laboratory Animal Technology Corporation Ltd. (Beijing, China). All the animal experiments were approved by the Ethics Committee of Beijing University of Chinese Medicine. Animals were received food and water under the SPF conditions with a standard 12 h light/dark cycle, a temperature of 22 ± 2°C, and relative humidity of 60 ± 5%. After 1 week of acclimatization, all the 18 rats were randomly divided into the control group and model group. The rats of the model group were free to drink 120 μg/ml of MNNG aqueous solution and feed with granular SPF-grade fodder containing 0.05% ranitidine and the rats of the control group were given with normal chow and water. Besides, on every Tuesday and Friday, abrosia and gavage of 0.05 mL/kg 2% sodium salicylate solution were given to rats of the model group and normal feeding and gavage of 0.05 ml/kg water were given to rats of the control group (**Figure 1**). After the 32nd week, the feces of all the rats were collected in separate cryotubes and stored at −80°C for the detection of gut microbiota and metabolites. Rats were anesthetized by intraperitoneal injection with 0.2 ml/100 g 2% pentobarbital sodium solution after fasting for 18 h. The whole stomach was removed and cut along the stomach bend. After removed the contents and washed with physiological saline, the stomach tissue was unfolded on the filter paper to observe whether mucosal damage or not takes place.

Histopathological Analysis

The histopathological analysis of gastric mucosa tissues was performed on all the rats. The samples were embedded in paraffin and sectioned to 4 μm slides. The sections were stained by using HE dying and then observed by using light microscopy. The HE scores were evaluated by the professional pathology researchers in our experiment according to the Gastritis and Gastric Cancer Scale. Scoring criteria were as follows: (1) Multifocal aggregates of mononuclear ± polymorphonuclear leukocytes; (2) Coalescing aggregates of the inflammatory cells in submucosa ± mucosa; (3) Organizing nodules of the lymphocytes and other inflammatory cells in submucosa ± mucosa; and (4) Follicles or sheets of the inflammatory cells extending into or through muscularis ± adventitia (Rogers, 2012).

High-Throughput Detection of Inflammatory Cytokines in Serum

The levels of inflammatory cytokines in the serum of rats in each group were detected by the Luminex liquid suspension chip (Wayen Biotechnologies, Shanghai, China). The Bio-Plex Pro Rat Cytokine 23-Plex Group I Panel System (Hercules, CA, United States) was used following the instructions of the manufacturer. Rat serum was incubated in 96-well plates embedded with microbeads for 1 h and then incubated with detection antibody for 30 min. Subsequently, streptavidin-phycoerythrin (PE) was added to each well for 10 min and values were read by using the Bio-Plex MAGPIX System (Bio-Plex) (Hercules, CA, United States).

Gut Microbiota Analysis

Microbial DNA was extracted from the fecal samples of rats by using the E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek Incorporation, Norcross, GA, United States) according to the protocols of the manufacturer. The final DNA concentration and purity were determined by the NanoDrop™ 2000c UV-Visible Spectrophotometer (Thermo Scientific, Wilmington, DE, United States) and DNA quality was checked by 1% agarose gel electrophoresis. The V3–V4 hypervariable regions of the bacteria *16S rRNA* gene were amplified with primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) by thermocycler PCR system (GeneAmp 9700 PCR System 9700, ABI, CA,

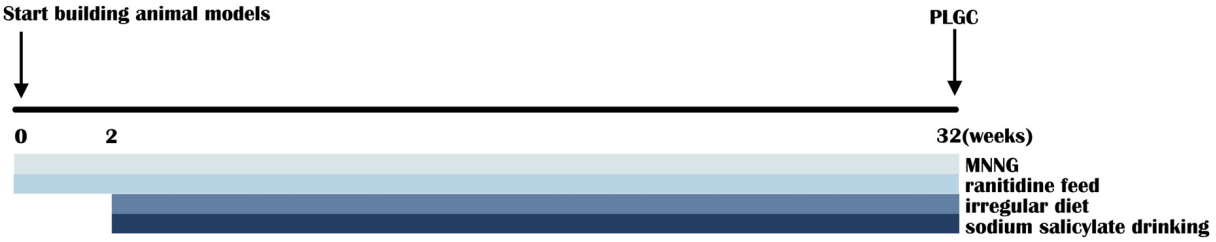

FIGURE 1 | Schematic view of the establishment of multifactor-induced precancerous lesions of gastric cancer (PLGC) rat model.

United States). PCR reactions were performed in triplicate 20 μl mixture containing 4 μl of 5 × FastPfu Buffer, 2 μl of 2.5 mM dNTPs, 0.8 μl of each primer (5 μM), 0.4 μl of FastPfu DNA Polymerase, and 10 ng of template DNA. The PCR reactions were conducted three repetitions independently by using the following program: 3 min of denaturation at 95°C, 27 cycles of 30 s at 95°C, 30 s for annealing at 55°C, 45 s for elongation at 72°C, and a final extension at 72°C for 10 min. The resulted PCR products were extracted from a 2% agarose gel and further purified by using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) and quantified by using the QuantiFluor^{TM}-ST (Promega Corporation, United States) according to the protocol of the manufacturer. The amplified complementary DNA (cDNA) was built by the following steps: first, the fragment was connected to the "Y" glyph connector, the self-connected fragment of the connector was removed by magnetic bead screening, then the library template was enriched by PCR amplification, and finally, sodium hydroxide degeneration was used to produce a single-stranded DNA fragment. Purified amplicons were pooled in equimolar and paired-end sequencing (2 × 300) on the Illumina MiSeq Platform (Illumina, San Diego, CA, United States) according to the standard protocols by the Majorbio Bio-Pharm Technology Corporation Ltd. (Shanghai, China). Raw FastQ files were quality filtered by trimmomatic and merged by Fast Length Adjustment of SHort reads (FLASH) with the following criteria: (i) The reads were truncated at any site receiving an average quality score < 20 over a 50-bp sliding window, (ii) Sequences whose overlap being longer than 10 bp were merged according to their overlap with mismatch no more than 2 bp, (iii) Sequences of each sample were separated according to the barcodes (exactly matching), and (iv) Primers (allowing two nucleotide mismatching) and reads containing ambiguous bases were removed. Operational taxonomic units (OTUs) were clustered with 97% similarity cutoff by using the UPARSE (version 7.1)[1] with a novel "greedy" algorithm that performs the chimera filtering and OTU clustering simultaneously. The taxonomy of each *16S rRNA* gene sequence was analyzed by the ribosomal database project (RDP) Classifier Algorithm[2] against the Silva (SSU123) 16S rRNA database by using a confidence threshold of 70%.

The α-diversity of gut microbiota was described separately by the Ace index and the Shannon index. The β-diversity of gut microbiota was analyzed by the principal coordinate analysis (PCoA) and hierarchical clustering analysis (HCA). The compositional similarity of microbiota in the two groups was reflected by the Venn analysis at the OTU level. The difference in microbial composition between the groups was more intuitive and visualized through the community Bar plot analysis. The significant differences in the phylum and genus levels were tested according to the relative abundance of the two groups of samples. The correlation values of the gut microbiota and inflammatory factors in serum are visually evaluated by using the correlation heatmap graphs.

Untargeted Metabolomics Analysis

Fecal samples (50 mg) were suspended in a 400 μl extraction solution (methanol: water = 4:1), crushed at −20°C with the high-throughput tissue disrupter (60 Hz), vortexed, and extracted by ultrasonic on ice for 10 min three times (40 Hz, 300 W). After standing at −20°C for 30 min, the extracted samples were centrifuged at 13,000 *g* for 15 min at 4°C, removed the supernatant, and transferred to a 200 L vial for UPLC-Q-TOF/MS analysis. The pooled quality control (QC) sample was prepared by mixing equal volumes of all the samples. The QC samples were disposed of and tested in the same manner as the analytic samples, which would be injected every six samples in order to monitor the stability of the analysis. Chromatographic separation of the metabolites was performed on the ExionLC AD System (AB SCIEX, United States) equipped with the ACQUITY UPLC BEH C18 Column (100 mm × 2.1 mm in diameter, 1.7 μm; Waters, Milford, MA, United States). The mobile phases consisted of 0.1% formic acid in water with formic acid (0.1%) (solvent A) and 0.1% formic acid in acetonitrile:isopropanol (1:1, v/v) (solvent B). The solvent gradient changed according to the following conditions: from 0 to 3 min, 95% (A): 5% (B) to 80% (A): 20% (B); from 3 to 9 min, 80% (A): 20% (B) to 5% (A): 95% (B); from 9 to 13 min, 5% (A): 95% (B) to 5% (A): 95% (B); from 13 to 13.1 min, 5% (A): 95% (B) to 95% (A): 5% (B); from 13.1 to 16 min, 95% (A): 5% (B) to 95% (A): 5% (B) for equilibrating the systems. The sample injection volume was 20 μl and the flow rate was set to 0.4 ml/min. The column temperature was maintained at 40°C. During the period of analysis, all these samples were stored at 4°C. The UPLC system was coupled to a quadrupole-time-of-flight mass spectrometer (Triple TOFTM 5600+, AB SCIEX, United States) equipped with an electrospray ionization (ESI) source operating

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

[2]https://sourceforge.net/projects/rdp-classifier/

in the positive mode and negative mode. The optimal conditions were set as follows: source temperature, 500°C; curtain gas (CUR), 30 psi; both ion source GS1 and GS2, 50 psi; ion spray voltage floating (ISVF), −4,000 V in the negative mode and 5,000 V in positive mode, respectively; declustering potential, 80 V; collision energy (CE), 20–60 V rolling for MS/MS. Data acquisition was performed with the data-dependent acquisition (DDA) mode. The detection was carried out over a mass range of 50–1,000 m/z. The detailed methods were provided in the **Supplementary Methods**. Differential metabolites analysis, the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis, and correlation analysis with the bacterial flora and differential metabolites were completed on the free online Majorbio I-Sanger Cloud Platform.[3] The differential metabolites between the groups were analyzed by the orthogonal partial least squares discriminant analysis (OPLS-DA) method [variable importance in projection (VIP) > 1, $p < 0.05$]. The fold changes of the metabolite ratio of the model group (numerator) to the control group (denominator) and the expression levels of differential metabolites between the normal group and the model group are listed in the **Supplementary Materials**.

Statistical Analysis

Data are expressed as the mean ± SEM. The analysis methods of cytokine data were the Mann–Whitney test for interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) and the unpaired *t*-test for interleukin-4 (IL-4), interleukin-10 (IL-10), interferon-γ (IFN-γ), macrophage colony-stimulating factor (M-CSF), and chemokine (C-X-C motif) ligand 1 (CXCL1). The analysis of significant differences between groups in the phylum and genus levels was tested by the Wilcoxon rank-sum test. The significance of differences between the two groups was evaluated for $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

RESULTS

Histopathological Observation of Gastric Mucosa and Detection of Serum Inflammatory Cytokines

The histopathological images of the gastric mucosa of the control group and model group are shown in **Figure 2A**. The results showed that with respect to the control group, there was obvious gastric inflammation in the rats of the model group, there was a significant polymorphonuclear cells infiltration in the mucosa, submucosa, and muscularis. In addition, the bodyweight of rats at 32 weeks decreased significantly compared with the control group **(Figure 2B)**. It indicated that multifactor induction could cause precancerous lesions of rat gastric mucosa, which was consistent with our previous report (Yu et al., 2020).

To further detect the systematic inflammatory state of the rats, the levels of serum inflammatory cytokines were detected by the Bio-Plex Pro Rat Cytokine 23-Plex Group I Panel System (Bio-Plex). As shown in **Figure 2C**, after multifactor stimulation, the levels of inflammatory cytokines in the serum in the model group were significantly different from the control group. Among them, the levels of IL-1β, IL-4, IL-6, IL-10, IFN-γ, TNF-α, and M-CSF in serum of the rats in the model group were significantly increased, while the level of CXCL1 in serum of the rats in the model group was significantly reduced compared with the control group. It indicated that the differential role of CXCL1 takes place in the formation of PLGC.

Alteration in the Composition and Function of Gut Microbiota After Multifactor Induction

In total, 8,99,441 valid *16S rRNA* gene sequences were obtained from 18 rat stool samples. The analysis was performed at a level of similarity higher than 97% and a total of 644 OTUs were obtained. According to the Shannon index (control vs. model: 4.144 ± 0.256 vs. 2.954 ± 0.321, $p < 0.001$) and the Ace index (control vs. model: 492.01 ± 14.50 vs. 415.51 ± 44.01, $p < 0.001$), there was a significant difference in microbiota diversity and richness between the two groups. It indicated that the Shannon index and the Ace index of the microbiota in the model group reduced significantly compared with the control group (**Figures 3A,B**). As shown in **Figure 3C**, the β-diversity analyzed by PCoA indicated that there was a significant difference between the two groups on the OTU level and good similarity within the group. The Venn analysis showed that the total number of microbiotas in both groups was 555 and the number of unique microbiotas in the control group was 74, while that in the model group was 15 (**Figure 3D**).

The microbial compositions between the groups were significantly different on the phylum and genus levels (**Figures 3E,G**). The results revealed that the predominant phyla in both the groups were Firmicutes (76.83% in the control group and 80.85% in the model group) and Bacteroidetes (19.81% in the control group and 16.31% in the model group) on the phylum level, while there was significantly reduced proportion of *Proteobacteria*, *Tenericutes*, and *Saccharibacteria* in the model group compared with the control group. On the genus level, 67 genera were significantly different ($p \leq 0.05$) between the two groups. Compared to those in the control group, *Lactobacillus* and *Bifidobacterium*, two major probiotics with anti-inflammatory activity, increased significantly, while *Turicibacter*, *Romboutsia*, *Ruminococcaceae_UCG-014*, *Ruminococcaceae_UCG-005*, and *Ruminococcus_1* reduced significantly in the model group (**Figures 3F,H**).

Inflammatory Cytokines Increasing Are Correlated With Differential Bacteria

The imbalance of the intestinal flora is associated with the inflammatory response of the host. To assess the correlation of the differential systemic inflammation and microbiota changes between the control group and model group, Spearman's rank correlation coefficient was used for the differential microbiota ($p < 0.05$) and the inflammatory cytokines. The heatmap is ordered by the correlation level and significance (*p*-value) within the top 20 floras shown in **Figure 4** and 17 floras are significantly

[3]www.i-sanger.com

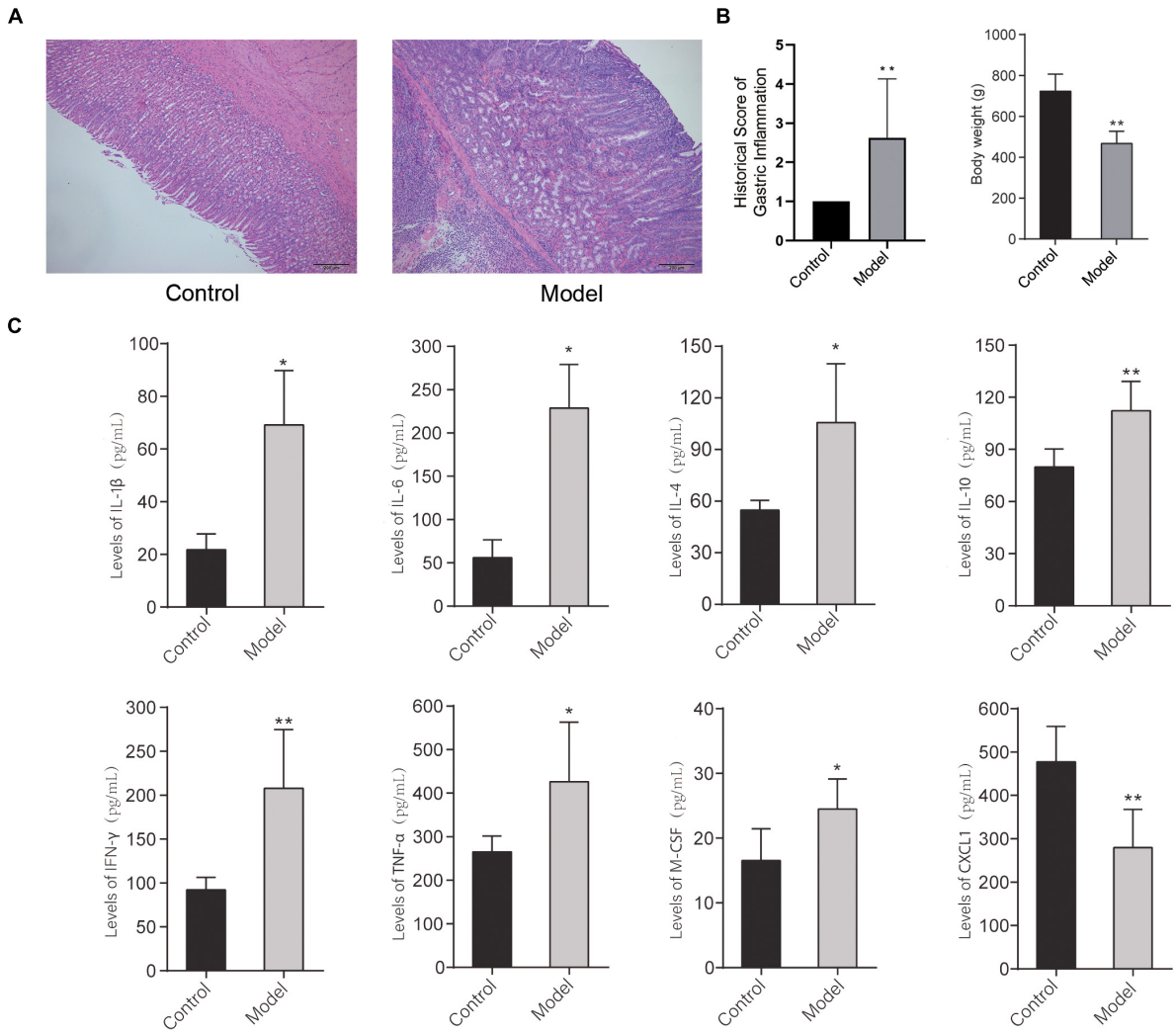

FIGURE 2 | (A) Histopathological image and inflammation score of the gastric mucosa of the control group and model group stained with H&E dying. **(B)** Bodyweight of rats in the control group and model group at 32 weeks. **(C)** The levels of inflammatory cytokines in the serum of the rats in the control group and model group (*$p < 0.05$, **$p < 0.01$). $n = 9$.

correlated with the serum cytokines level alternation. Among them, there was a significant positive correlation between IL-4, IL-10, M-CSF, IFN-γ, IL-1β, TNF-α, and IL-6 with *Bifidobacterium*, *Lactobacillus*, *Allobaculum* and a negative correlation with *Ruminococcus_1*, *Romboutsia*, *Ruminococcaceae*, *Ruminiclostridium_6*, and *Turicibacter*. On the contrary, CXCL1 showed a converse trend of correlation compared with other cytokines, which mainly attributed to the opposite change profile of CXCL1 in the model group (**Figure 2C**). In addition, the *Ruminococcaceae* family, *Bifidobacterium*, and *Lactobacillus* are most relative to cytokines change among all the differential flora at the genus level. It indicated that intestinal floras might regulate the inflammatory cytokines in the serum of model rats and the *Ruminococcaceae* family, *Bifidobacterium*, and *Lactobacillus* are most potentially involved in the intestinal homeostasis disorder and PLGC formation.

Analysis of Differential Metabolites in the Stool Samples

To further explore the interaction mechanism between microbiota and the systematic inflammatory response, total ion chromatograms of the stool sample were detected by using UPLC-Q-TOF/MS in the positive and negative ion modes. Samples were analyzed by the OPLS-DA and the OPLS-DA score plots presented a distinct clustering of metabolites in the stool samples between the control group and model group in both the positive (**Figures 5A,B**) and negative (**Figures 5C,D**) ion modes, which suggested that the fecal metabolic profiles had been changed after intervened by the multifactor stimulation.

Next, a total of 337 significantly differentially expressed metabolites with annotated names were detected under the screening conditions of $p < 0.05$ and VIP > 1

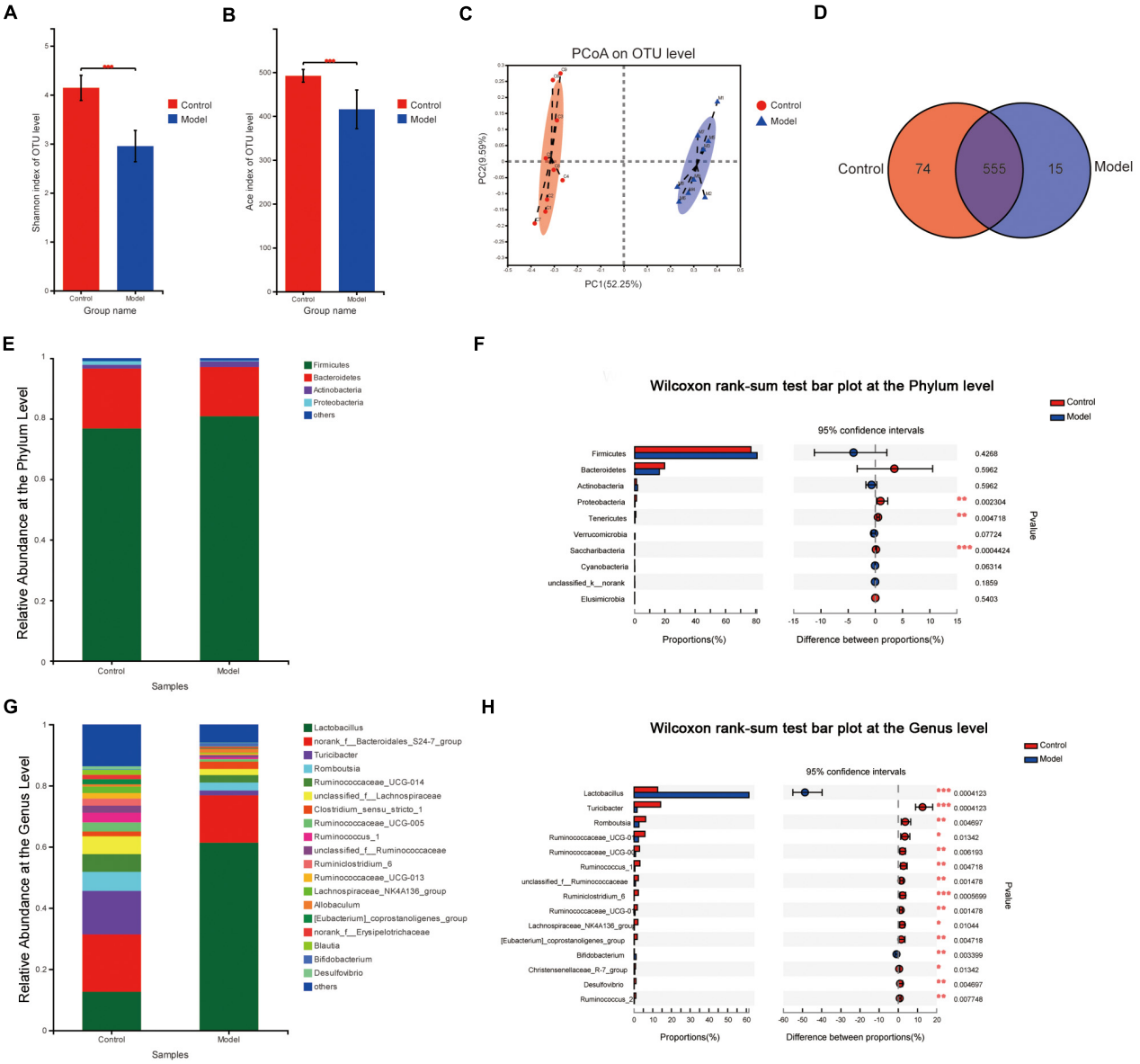

FIGURE 3 | Microbiota change after multifactor stimulation. **(A)** The Shannon index. **(B)** The Ace index. **(C)** Principal coordinate analysis (PCoA) analysis of β-diversity. **(D)** Venn analysis of gut microbiota. **(E,G)** The relative abundance at the phylum and genus levels. **(F,H)** Statistical results of the Wilcoxon rank-sum test in the phylum and genus levels (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). $n = 9$.

(**Supplementary Table 1**). The expression profile and VIP of the top 30 differential metabolites such as N-arachidonoyl tyrosine, Pro-Ser-Thr-Lys, cucurbitacin D, 1α,25-dihydroxy-26,27-dimethyl-17,20,21,22,22,23,23-hexadehydrovitamin D3, tryptophylhydroxyproline, and phenylalanylvaline were showed as a heatmap in **Figure 5G** and the full chemical information of the metabolites of the top 30 differential metabolites was shown in **Table 1**. Besides, the KEGG functional pathway and enrichment analysis of the differential metabolites found that the differential metabolites are mainly related to metabolism, peroxisome proliferator-activated receptor (PPAR) signaling pathway, insect hormone biosynthesis, (alpha-) linoleic acid metabolism, steroid biosynthesis, secondary bile acid biosynthesis, and steroid hormone biosynthesis (**Figures 5E,F**).

Metabolites Are Correlated With Intestinal Differential Microbes

To further explore the relationship between the intestinal microbial changes and differences in metabolites, the correlation between the differential metabolites ($p < 0.05$) and intestinal microbes was analyzed by correlation heatmap analysis to calculate the Spearman's rank correlation coefficient. As shown in **Figure 6**, there is a good clustering relationship in the differential

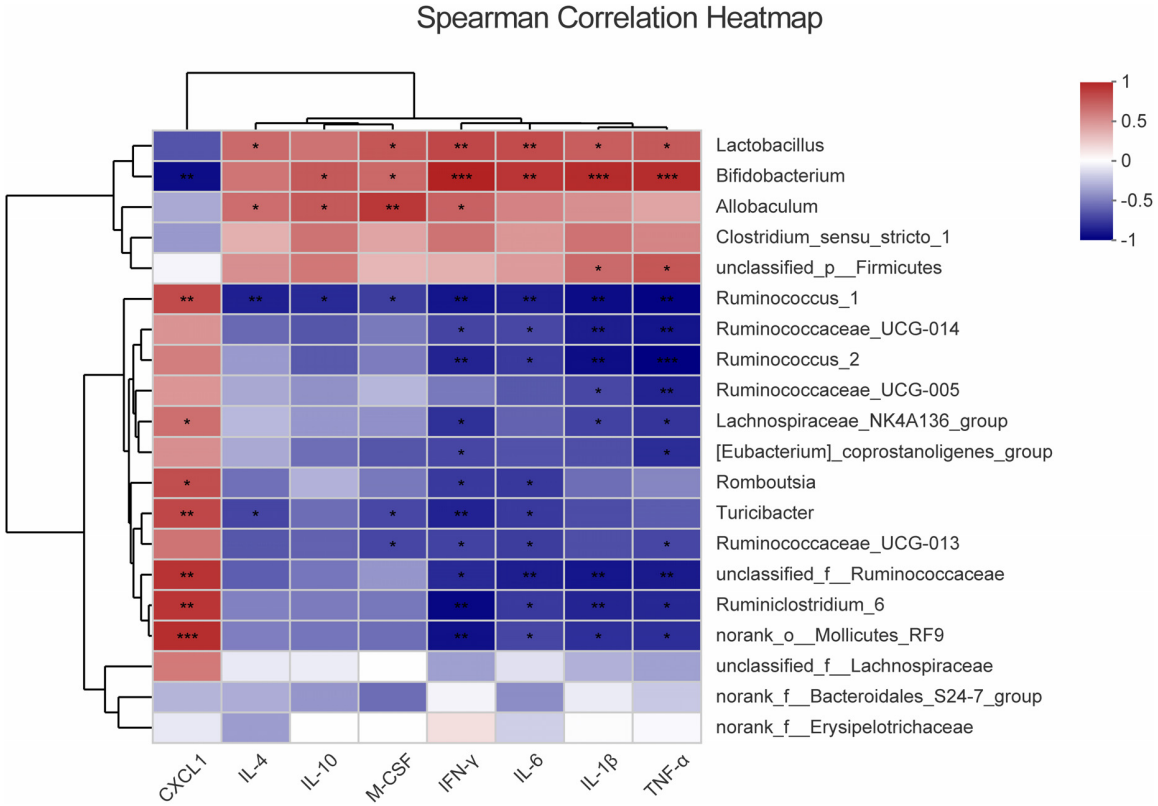

FIGURE 4 | The correlation between the different cytokines and differential intestinal microbes, respectively. The color represents the correlation coefficient. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

metabolites and intestinal microbes, respectively, which may indicate that they have similarities in structural and biological function. The *Bifidobacterium* and *Lactobacillus*, which is the main genus related to the alternation of the cytokines (**Figure 4**), shared the same correlation profile of metabolic (**Figure 5**). On the contrary, the genus within the *Ruminococcaceae* family does not have the same correlation profile. Among all the metabolic, N-arachidonoyl tyrosine, cucurbitacin D, PI [P-18:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z)], Phosphatidyl inositols (PI) [17:1(9Z)/22:2(13Z,16Z)], diacyl glycerol (DG) [15:0/16:1(9Z)/0:0], Pro-Ser-Thr-Lys, tryptophylhydroxyproline, 26,26,26-trifluoro-25-hydroxyvitamin D3, 1α,25-dihydroxy-26,27-dimethyl-17,20,21,22,22,23,23-hexadehydrovitamin D3, 13(S)-HODE, and 10-epoxy-12-octadecenoic acid were mainly positively associated with the genus *Lactobacillus* and *Bifidobacterium* and ursodeoxycholic acid, β-amyrin, phenylalanylvaline, and 1α,25-dihydroxy-3α-methyl-3-deoxyvitamin D3 were mainly negatively associated with the genus *Lactobacillus* and *Bifidobacterium*.

DISCUSSION

Gastric cancer is an important health problem with the fifth most common cancer and the third leading cause of cancer-related death worldwide. There are about 1.2 million new cases of GC worldwide each year and about 40% of them occur in China. The risk factors of GC development have been identified to some extent such as *H. pylori* infection, dietary habits, nitrite intake, and PPI abuse. But, some of the mechanisms between risk factors remain unclear. Gut microbial dysbiosis are linked to the aberrant immune responses accompanied by abnormal production of inflammatory cytokines. Validation of the predicted host–microbial interactions reveals that TNF-α and IFN-γ production are associated with specific microbial metabolic pathways such as palmitoleic acid metabolism and tryptophan degradation to tryptophol (Schirmer et al., 2016). This study aims to explore how multirisk factors influence the gut microbiota and gastric inflammation and the underlying mechanisms. We reported that the gut microbial dysbiosis and changes in fecal metabolic phenotype in PLGC rats could stimulate the immune responses to regulate the levels of a variety of inflammatory cytokines, thereby accelerating the formation of PLGC.

The malignant transformation of chronic inflammation is a common process in the development of most cancers. The persistence of chronic inflammation plays an important role in initiating, maintaining, and promoting the growth of GC. The possibility of abnormal expansion of cancer cell DNA was promoted by increasing the infiltration of chemokines on the gastric mucosal epithelial cells. Inflammatory cytokines

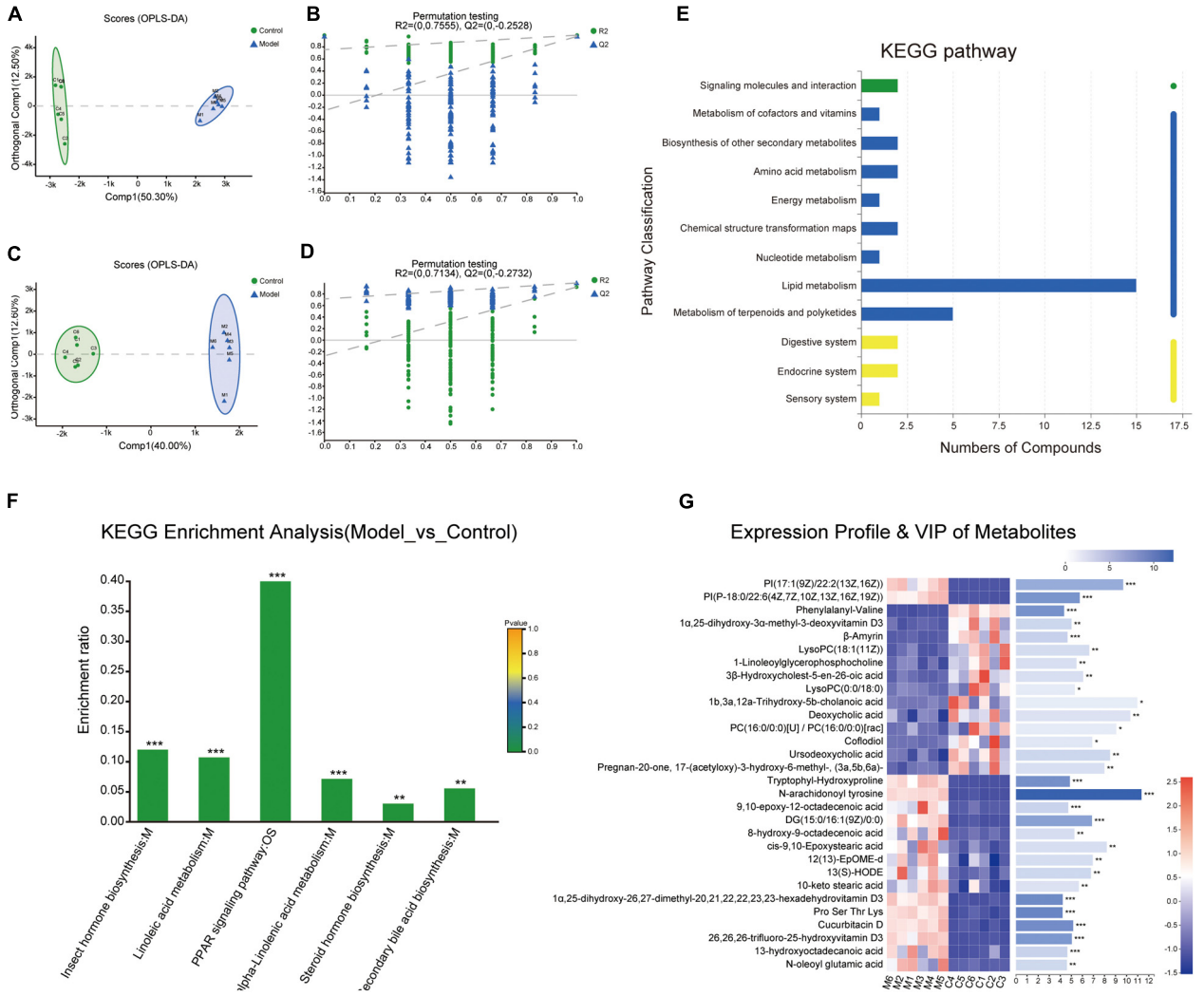

FIGURE 5 | Metabolites analysis between the control and model groups. **(A–D)** Orthogonal partial least squares discriminant analysis (OPLS-DA) score plots of fecal metabolites in comparisons of the control group and model group in the positive and negative ion modes. **(E)** The Kyoto Encyclopedia of Genes and Genomes (KEGG) functional pathway analysis. The abscissa is the number of metabolites annotated to this pathway and the ordinate is the name of the KEGG metabolic pathway. **(F)** KEGG enrichment analysis. **(G)** Expression profile and variable importance in projection (VIP) of the top 30 differential metabolites analyzed through heatmap analysis methods. (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$). $n = 6$.

such as IL-1β, IL-6, and TNF-α are often used to assess the severity of gastric mucosal injury (Michalkiewicz et al., 2015). IL-1β is a typical proinflammatory cytokine. Inflammatory signals can activate the specific immune responses by activating inflammatory corpuscles IL-1β, stimulating secreted IL-1β with a small amount of expression to produce the appropriate inflammatory responses (Apte and Voronov, 2002). A large number of IL-1β can cause a wide range of inflammatory reactions, leading to inflammatory damage. IL-6 is regarded as a proinflammatory cytokine that can participate in the tumor development and development process through the mechanisms of promoting tumor vascular generation, regulating the genes related to the cell cycle, accelerating the speed of tumor stem cell occurrence and self-renewal, and regulating the local inflammatory environment of the body. Gastric mucosal inflammatory lesions lead to an abnormal rise in IL-6 serum cytokines, which have important reference value in detecting the inflammation and pathological degree (Hodge et al., 2005; Nilsson et al., 2005; Landskron et al., 2014). IL-4 can mediate the activation of T cells and B cells and cause excessive immune inflammatory injury in tissues (Fang et al., 2020). The cytokine IL-10 is a key anti-inflammatory cytokine to protect the host from overexuberant responses to microbiota and pathogens and functions as a negative regulator of immune responses to microbial antigens while playing important roles in maintaining the intestinal microbe–immune homeostasis, sterile wound healing, autoimmunity, cancer, and homeostasis (Neumann et al., 2019; Saraiva et al., 2020). IFN-γ is the uppermost cytokine implicated in antitumor immunity with cytostatic, proapoptotic, and immune-provoking

TABLE 1 | The chemical information of the top 30 differential metabolites.

Metabolite	Mode	M/Z	RT (min)	VIP value	P value	Fold changes (Control/Model)	HMDB Subclass
N-arachidonoyl tyrosine	pos	468.306	3.9072	11.3507	5.982E-13	6.1857	Amino acids, peptides, and analogs
Pro Ser Thr Lys	pos	432.2408	4.6296	4.2109	1.139E-09	3.8443	Amino acids, peptides, and analogs
1α,25-dihydroxy-26,27-dimethyl-20,21,22,22,23,23-hexadehydrovitamin D3	pos	439.3152	4.3854	4.2169	3.083E-09	3.3114	Vitamin D and derivatives
Cucurbitacin D	pos	517.3244	4.8337	5.1645	3.993E-09	2.6176	Cucurbitacins
Tryptophyl-Hydroxyproline	pos	300.1373	1.2872	4.8653	5.607E-09	14.2487	Amino acids, peptides, and analogs
Phenylalanyl-Valine	pos	297.1799	2.1592	4.3459	7.864E-09	0.1724	Amino acids, peptides, and analogs
PI[P-18:0/22:6(4Z, 7Z, 10Z, 13Z, 16Z, 19Z)]	pos	895.5791	8.5257	5.7623	1.241E-08	119.4175	Amino acids, peptides, and analogs
26,26,26-Trifluoro-25-hydroxyvitamin D3	pos	455.3099	4.2291	5.0392	1.924E-08	3.5673	Vitamin D and derivatives
DG[15:0/16:1(9Z)/0:0]	pos	575.4659	9.4817	6.8613	1.057E-07	6.8347	Diradylglycerols
PI[17:1(9Z)/22:2(13Z,16Z)]	pos	903.5846	8.3	9.6808	7.928E-07	2976.4356	Glycerophosphoinositols
β-Amyrin	pos	409.3816	10.2151	4.6561	0.000575	0.1848	Triterpenoids
13-hydroxyoctadecanoic acid	pos	283.262	7.8558	4.642	0.0006128	4.395	Fatty acids and conjugates
9,10-epoxy-12-octadecenoic acid	pos	297.2413	6.4444	4.7207	0.0008167	6.3232	Lineolic acids and derivatives
1α,25-dihydroxy-3α-methyl-3-deoxyvitamin D3	pos	415.3556	9.3034	5.0118	0.001137	0.2422	Vitamin D and derivatives
Ursodeoxycholic acid	pos	357.2779	7.0011	8.5	0.001209	0.6283	Bile acids, alcohols and derivatives
13(S)-HODE	pos	297.2413	7.6725	6.7751	0.001627	3.2121	Lineolic acids and derivatives
N-oleoyl glutamic acid	pos	412.3041	4.9695	4.5913	0.001791	4.2917	Amino acids, peptides, and analogs
LysoPC[18:1(11Z)]	pos	522.3538	8.3284	6.6158	0.003252	0.3125	Glycerophosphocholines
8-hydroxy-9-octadecenoic acid	pos	299.2571	7.8453	5.2733	0.004067	8.2188	Lineolic acids and derivatives
3β-Hydroxycholest-5-en-26-oic acid	pos	480.3438	8.4665	6.0719	0.005779	0.1319	Bile acids, alcohols and derivatives
(3a,5b,6a)-3-hydroxy-6-methyl-17-(acetyloxy)-Pregnan-20-one	pos	391.2833	6.2504	7.9849	0.005887	0.3124	Pregnane steroids
1-Linoleoylglycerophosphocholine	pos	520.3382	7.8047	5.4651	0.006243	0.4019	Glycerophosphocholines
Coflodiol	pos	425.3763	9.2733	6.8958	0.01892	0.1551	Triterpenoids
PC(16:0/0:0)[U]/PC(16:0/0:0)[rac]	pos	496.3384	8.2299	9.0627	0.03449	0.4909	Glycerophosphocholines
LysoPC(0:0/18:0)	pos	524.3696	8.8922	5.3144	0.03774	0.451	Glycerophosphocholines
1b,3a,12a-Trihydroxy-5b-cholanoic acid	pos	817.5801	5.3651	10.9706	0.04306	0.0896	Bile acids, alcohols, and derivatives

effects. Many clinical trials and immunotherapy approaches have been designed to reinforce IFN-γ-mediated immunity for the different types of cancer (Kursunel and Esendagli, 2016). TNF-α is a typical proinflammatory factor produced during the initiation of inflammation to maintain chronic inflammation, promote the expression of other inflammatory cytokines, aggravate inflammation, and play an important role in the inflammation and tumor genesis. It had been reported that the level of TNF-α increased in *H. pylori*-positive PLGC, through *H. pylori* secreted TNF-α-inducing protein (Tipα) (Suganuma et al., 2012; Landskron et al., 2014). M-CSF is related to monocytes and macrophages proliferation. Elevated M-CSF is correlated with invasion, metastasis, and poor survival of patients with tumors. M-CSF, combined with IL-34, tumor associated macrophages (TAMs) were the novel biological markers for GC, which may provide new insight for both the diagnosis and cellular therapy of GC (Liu et al., 2020). CXCL1 can promote and enhance the killing of macrophages to tumor cells and microorganisms, regulate the release of cytokines and other inflammatory regulators by macrophages, and stimulate cell phagocytosis. CXCL1, one of the CXCR2 ligands secreted by macrophages in the GC microenvironment, could promote migration of GC cells through activating the CXCR2/STAT3 feed-forward loop. TNF-α secreted by GC cells could induce the release of CXCL1 by macrophages (Zhou et al., 2019). Recently, with the constitutional understanding of the tumor microenvironment, the role of several cytokines needs to be revalued. The anti-inflammatory cytokines such as IL-4 and IL-10, which were considered protected cytokines in the past, become a double-edged sword in the protection of cancer. On one side, anti-inflammatory cytokines are important to alleviate inflammation, a huge risk factor of cancerization. On the other side, most of the anti-inflammatory cytokines could suppress the immune response, which is important for mutation surveillance and elimination (Salazar-Onfray et al., 2007; Li et al., 2009; Musolino et al., 2017). This study showed that both the proinflammatory and anti-inflammatory cytokines are increased in the model group, except CXCL1, which indicated the systematic activation of the immune system. The CXCL1 is often cosecreted with inflammatory cytokines such as IL-1, IL-6, and TNF-α by infiltrating macrophage or cancer cells. The decrease of CXCL1 of the model group described that the upstream of multifactor-induced systematic immune activation is different from the normal activation pathway. The multifactor

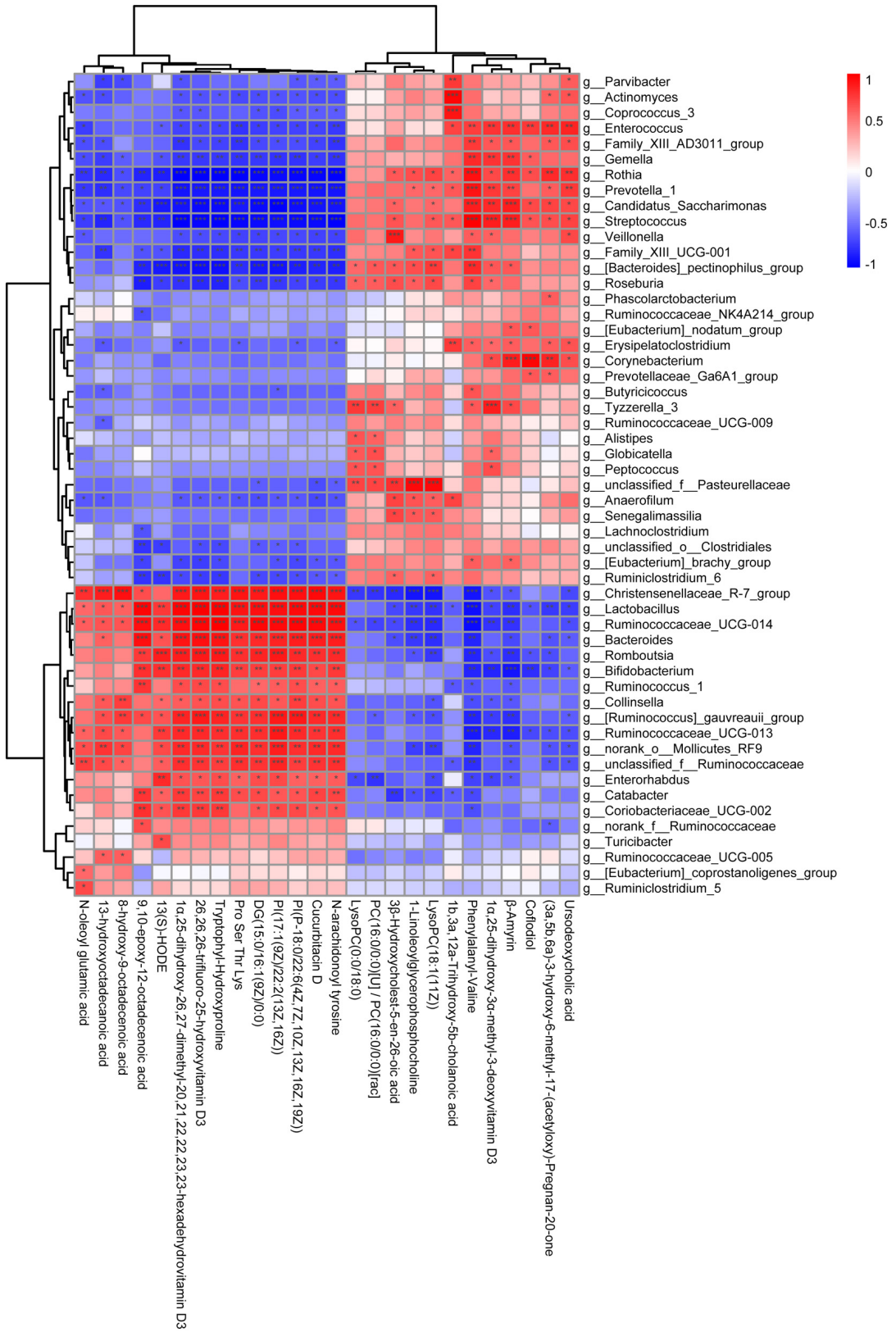

FIGURE 6 | The correlation analysis of the differential metabolites and intestinal microbes in the genus level. The ordinate represents the differential metabolites and the abscissa represents the intestinal microbes in the genus level. The color represents the correlation coefficient (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).

induction has a wide effect on both the digestion system and other organs in rats, the underlying mechanism of it still needed further study.

In addition, we try to link systematic inflammation with intestinal microbiota alternation. This study showed that *Lactobacillus* and *Bifidobacterium* are increased significantly in the model group, which makes us reconsider their role in cancer. In this study, unlike in other diseases, the *Lactobacillus* and *Bifidobacterium* are potentially contributed to the development of cancer (Dicksved et al., 2009; Aviles-Jimenez et al., 2014; Castano-Rodriguez et al., 2017). Some researchers attribute the abnormal role of *Bifidobacterium* and *Lactobacillus* in cancer to the unusual cancer metabolic environment (Vinasco et al., 2019). This study revealed a strong positive correlation between *Lactobacillus* and *Bifidobacterium* with serum cytokines arising in PLGC rats and some of the flora shared a similar clustering profile of metabolic including *Bifidobacterium* and *Lactobacillus*. All of these pointed out the vital role of *Lactobacillus* and *Bifidobacterium* in precancerous lesions and the potential to study the metabolic environment of these two floras.

The microbiota is associated with host systemic metabolites, most of the metabolic released by floras are shared across the subjects (Visconti et al., 2019). In our healthy GI tract, there are over 90% of the bacteria belong to the phyla of *Firmicutes* and *Bacteroidetes*, followed by *Actinobacteria*, *Proteobacteria*, *Verrucomicrobia*, and *Fusobacteria*, which are in a relatively stable balance (Bäckhed et al., 2005; Qin et al., 2010). Gut microbiota could produce several metabolites and bioproducts necessary to protect the health and gastrointestinal homeostasis of the host. However, the constitution and metabolic function of gut microbiota are easily influenced by a variety of factors including genetics, diet, age, antibiotics, mode of delivery, stress, and environmental and psychological factors (Osadchiy et al., 2019). The disorders in the constitution and metabolic function of gut microbiota could be increasingly linked to various diseases including inflammation, cancers, obesity, metabolic diseases, allergies, depression, and disorders in the immune system (Turnbaugh et al., 2007; Shanahan, 2013). The increasing evidence has confirmed that the disturbance of the composition and function of the GI tract microorganism is closely related to the occurrence of GI inflammation and cancers, likely due to altered mucosal immunity and proinflammatory immune microenvironment (Aviles-Jimenez et al., 2014; Brawner et al., 2014; Coker et al., 2018; Thakkar et al., 2018; Kong and Cai, 2019). Ranitidine, a potent histamine H2 receptor antagonist inhibiting gastric acid secretion, could cause an imbalance of gut microbiota, including the excessive proliferation of *H. pylori* and other microorganisms in the GI tract (Zeldis et al., 1983).

In addition to the gut microbiota involved in human health and disease, microbial metabolites or cometabolites from both the host and microbes can contribute to inflammatory and regulation of the immune, endocrine, and nervous system. The disorder of gut microbes also leads to the production of harmful metabolites such as acetaldehyde, secondary bile acids, and glucuronic acid to induce DNA damage and contribute to carcinogenesis (Kong and Cai, 2019). We next proved that the metabolic change of microbiota is highly associated with host metabolism homeostasis. On this basis, we exerted the metabolomics methods to bridge the gut microbiota with precancerous inflammation. The results showed that the control group and model group are significantly different, especially on the lipid metabolism. It also partly revealed the potential mechanism of how alternation of gut microbiota contributes to the PLGC formation. The molecule correlated with differential microbiota deserves future exploration and more convincing evidence is still needed.

This study provides a comprehensive view of gut microbiota, inflammation, and gut metabolism in the PLGC stage in the rat. Besides, we brought new evidence of the precancerous role of *Lactobacillus* and *Bifidobacterium*, which is of interest to clinical microbiologists and physicians. However, the crosstalk of specific floras, metabolic, and cytokines needs further exploration and validation.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA752833.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Ethics Committee of Beijing University of Chinese Medicine, China (No. BUCM-4-2017120101-4038).

AUTHOR CONTRIBUTIONS

FC, ZS, and XD conceived and designed the experiments. YuL, HZ, XM, and TL analyzed and interpreted the data. YiL, MZ, JL, and TX carried out animal experiments. PZ carried out a pathology experiments and analysis. FC wrote the manuscript. All the authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.733979/full#supplementary-material

Supplementary Table 1 | The significantly differential expressed metabolites in the stool samples of the control group and model group rats.

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Dysbiosis in the Human Microbiome of Cholangiocarcinoma

Benchen Rao[1,2†], Tong Ren[3†], Xuemei Wang[1,2†], Haiyu Wang[1,2], Yawen Zou[1,2], Ying Sun[1,2], Shanshuo Liu[1,2], Zhigang Ren[1,2] and Zujiang Yu[1,2*]*

[1] Department of Infectious Diseases, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [2] Gene Hospital of Henan Province, Precision Medicine Center, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [3] Department of Breast Surgery, Affiliated Cancer Hospital of Zhengzhou University, Zhengzhou, China

Cholangiocarcinoma (CCA) is the most common malignant tumor of the biliary system with a very poor prognosis. The human microbiome, which is the sum of the genetic information of human microorganisms, plays an important role in regulating the digestion, absorption, immune response, and metabolism of the host. Increasing evidence indicates a close relationship between CCA and the human microbiome. Specific alterations occur in the human microbiome of patients with CCA. Therefore, in this review, we aimed to summarize the recent evidence on dysbiosis in the human microbiome of CCA. Then, we generalized the effect of *Helicobacter pylori* on CCA. Additionally, the potential mechanism of human microbial dysbiosis promoted the progress of CCA, and its precancerous disease was also explored. Furthermore, the possibility of the human microbiome as a diagnostic and therapeutic target of CCA was discussed.

Keywords: cholangiocarcinoma (CCA), human microbiome, gut microbiome, bile microbiome, microbial dysbiosis

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****Correspondence:***
Zhigang Ren
fccrenzg@zzu.edu.cn
Zujiang Yu
johnyuem@zzu.edu.cn

† These authors have contributed equally to this work

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INTRODUCTION

Cholangiocarcinoma (CCA) is the most common malignant tumor of the biliary system and the second most common primary hepatobiliary malignancy, accounting for 15% of hepatobiliary malignancies (Banales et al., 2020; Siegel et al., 2020). According to the anatomical location, CCA is classified into intrahepatic cholangiocarcinoma (iCCA), perihilar cholangiocarcinoma, and distal cholangiocarcinoma (Razumilava and Gores, 2014). There are no obvious clinical symptoms in the early stage of CCA, and approximately 70% of patients are in the late stage at the time of diagnosis (Nathan et al., 2009). In 2017, it was expected to have 40,710 new cases of hepatobiliary malignancies, of which approximately 28,920 will eventually die from hepatobiliary malignancies in the United States (Islami et al., 2017).

Cholestasis and chronic inflammation lead to malignant transformation of bile duct cells, which is a common way for the tumorigenesis and development of CCA. Conjugated bile acid stimulates the production and secretion of growth factors and inhibition of apoptosis. A variety of proto-oncogenes are activated, leading to the destruction of the intracellular pathways controlled by the proto-oncogenes and cell canceration (Papoutsoglou et al., 2019; Jin et al., 2020). The independent risk factors recognized by CCA mainly include primary sclerosing cholangitis (PSC), hepatobiliary parasites, bile duct stones, choledochal cysts, and carcinogens (i.e., asbestos, dioxins, and nitrosamines). The potential risk factors include liver cirrhosis, hepatitis B virus infection, hepatitis C virus infection, diabetes, obesity, chronic consumption of alcohol, smoking, biliary,

and disorders of enteric circulation (Razumilava and Gores, 2014; Blechacz, 2017). However, most patients with CCA were not exposed to relevant risk factors before the tumorigenesis. Therefore, the etiology and pathogenesis of CCA are still unknown. Increasing evidence showed that CCA developed through the accumulation of genetic and epigenetic changes, which were affected by the human microbiome (Jusakul et al., 2017; McKenzie et al., 2017; Banales et al., 2020).

The human microbiome, which is the sum of the genetic information of human microorganisms, mainly includes the microbiome in the intestinal tract, bile duct, oral cavity, vagina, nasal cavity, and skin (Turnbaugh et al., 2007; Human Microbiome Project Consortium, 2012). The human intestine is colonized with a large number (about 10^{14}) and a complex structure (over 1,000 species of bacteria) of microbial community (about 1.5 kg) (Hayes et al., 2010). In the process of symbiosis and co-evolution of the intestinal flora and the host, the gut microbiome plays an important role in regulating the digestion, absorption, immune response, and metabolism of the host (Backhed et al., 2005). As for the bile microbiome, the alterations of the bile microbiome will lead to the occurrence and progression of a variety of hepatobiliary and pancreatic and intestinal diseases (Liwinski et al., 2020). At the same time, the metabolism of bile acids and the characteristics of the gut microbiome will be affected by it (Molinero et al., 2019; Serra et al., 2021). Studies on oral (Hayes et al., 2018), vagina (Nene et al., 2019), nasal (Mika et al., 2016), and skin microbiome (Chen et al., 2018) have also made great progress.

In this article, we reviewed and focused on the alterations in the human microbiome of patients with CCA and its precancerous diseases and analyzed the potential mechanism of the development and progress of CCA caused by the dysbiosis of the human microbiome. In addition, we evaluated the potential of the human microbiome as a diagnostic biomarker and therapeutic target for CCA.

The Close Relation Between Gut Microbiome and Hepatobiliary Diseases

The gut–liver axis and the biliary-enteric circulation are closely related to the function of the hepatobiliary system and gut. The hepatobiliary system has a close relationship with the gut based on the anatomical position and physiological function (Schwabe and Greten, 2020). About 70% of the blood in the hepatobiliary system comes from the portal vein. Nutrients and toxins from the intestine enter the liver through the portal vein, and then they are transported to the whole body after the detoxification process in the liver. At the same time, the hepatobiliary system can secrete bile acids and other substances into the intestine to participate in the regulation of hormone levels and immune response, and affect intestinal homeostasis. Then, the gut microbiome can convert the primary bile acids that are excreted into the intestine from the hepatobiliary system into secondary bile acids. Moreover, 95% of the bile acids can be reabsorbed by the intestinal wall, and then enter the hepatobiliary system again through the portal vein (Tripathi et al., 2018; Jiang et al., 2019; Chopyk and Grakoui, 2020). The liver defends against intestinal toxins such as lipopolysaccharides (LPS) and products of the gut microbiome by its innate immune system. Moreover, the liver regulates metabolism and immune response and affects intestinal function through bile secretion and enterohepatic circulation. Dysbiosis of the gut microbiome and increased permeability of the intestinal wall are closely related to hepatobiliary disease through immune responses. Similarly, hepatobiliary insufficiency is also positively correlated with dysbiosis of the gut microbiome (**Figure 1**).

Human Microbial Dysbiosis in the Precancerous Diseases of Cholangiocarcinoma

It is well known that patients with bile duct stones, PSC, liver fluke infection, and bile duct obstruction have a higher risk of CCA tumorigenesis. Patients with these precancerous diseases of CCA have human microbial dysbiosis. In turn, human microbial dysbiosis will promote the progression of precancerous diseases to CCA (Clements et al., 2020).

Human Microbial Dysbiosis in Gallstones

Studies have shown that abnormal secretion and metabolism of cholesterol and bile acids are important reasons for the formation of gallstones (Wang et al., 2009). The gut microbiome can regulate the metabolism of bile acids to influence the formation of gallstones to vary degrees. On the contrary, bile duct stones can cause alterations in the secretion of bile and inflammation response, resulting in dysbiosis of the gut microbiome (Sayin et al., 2013).

Keren et al. (2015) analyzed the difference between patients with gallstones and healthy controls in the gut microbiome. They found that the diversity of gut microbiome was significantly decreased in patients with gallstones. In addition, *Oscillospira* was enriched, while *Roseburia* was decreased when compared with healthy controls. Then, the increased level of bile acids in feces was detected; at the same time, the abundance of *Oscillospira* was negative with primary bile acids and correlated positively with secondary bile acids. In contrast, the correlation between *Bacteroides* and bile acids displayed the opposite trend to *Oscillospira* (**Table 1**). In another study, feces, bile, and gallstones from patients were collected and induced to 16S gene sequences. Proteobacteria was significantly increased in the gut microbiome compared with the healthy controls, while *Faecalibacterium*, *Lachnospira*, and *Roseburia* were decreased. Meanwhile, they reported that the bacteria diversity of the gut microbiome was significantly lower than that of the bile (Wu et al., 2013). The presence of dysbiosis in gut microbiome may be a key factor in the formation of gallstones. A large number of bacteria also exist in the bile and gallstones (Hazrah et al., 2004; Peng et al., 2015; Molinero et al., 2019). In addition, there was a difference in the bile microbiome between different types of gallstones (Abeysuriya et al., 2008). It was reported that the species of bile microbiome were largely consistent with that of the gut microbiome at the phylum level (Wu et al., 2013). There was no difference in the α and β diversity of the bile microbiome between patients with gallstones and healthy controls (Kim et al., 2021).

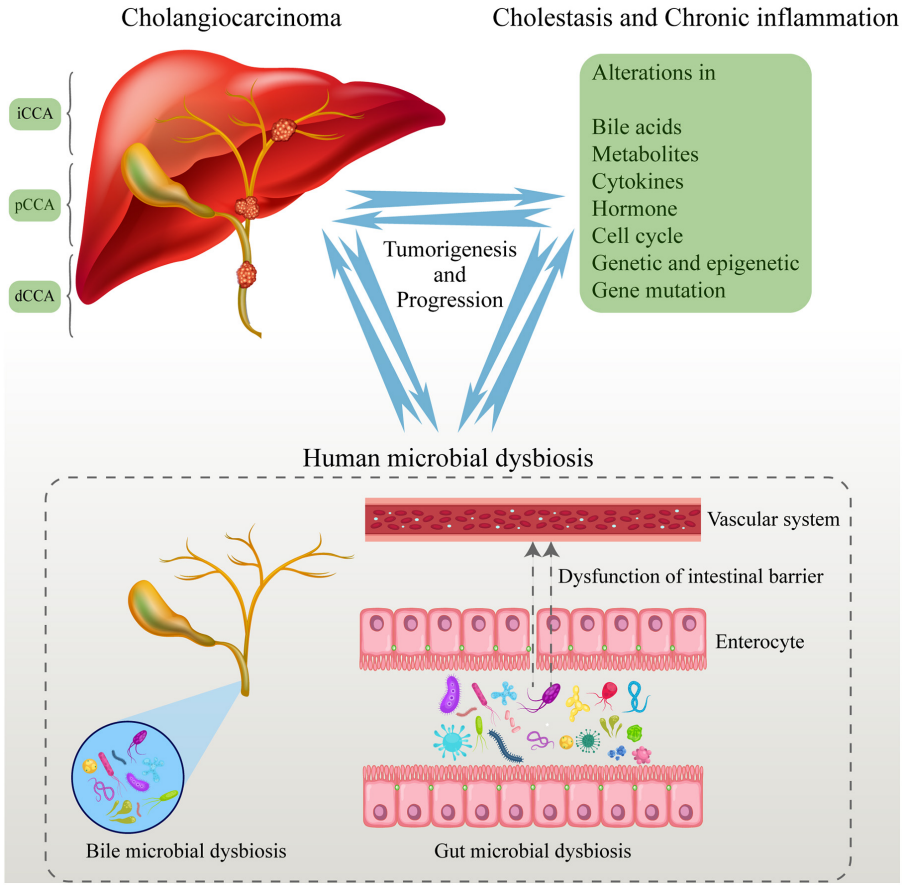

FIGURE 1 | Human microbial dysbiosis promotes the tumorigenesis and progression of CCA. The human microbiome dysbiosis occurred in the process of CCA tumorigenesis. The entry of abnormal microbiome and their metabolites into the blood promotes the progression of CCA. At the same time, CCA aggravates the dysbiosis of the human microbiome by many ways, leading to a vicious circle. CCA, cholangiocarcinoma.

Then, *Pseudomonas*, *Bacillus*, *Enterococcus*, and *Acinetobacter* were found as the dominant genera in the bile (Peng et al., 2015). Moreover, the level of *Enterococcus* was significantly higher in patients with gallstones than that in other causes of biliary obstruction (Kim et al., 2021).

Surprisingly, the microbiome in the gallstones has been detected. Many species of *E. coli* and *Pseudomonas* were found in the cholesterol gallstones (Lee et al., 1999). Alterations in the microbiome of gallstones in patients with cholesterol gallstones were identified over time (Swidsinski et al., 1998). These findings help to figure out the development mechanism of cholesterol stones. In sum, the human microbial dysbiosis was involved in the process of the occurrence, progression, and deterioration of gallstones.

Human Microbial Dysbiosis in Primary Sclerosing Cholangitis

Primary sclerosing cholangitis is a chronic cholestatic liver disease. Approximately 70% of patients also suffer from inflammatory bowel disease. In addition, the inflammation of the colon causes the loss of the intestinal mucosal barrier, leading intestinal bacteria to enter the biliary tract *via* the portal vein, which contributes to infection of the biliary tract and progression of PSC. Studies have shown that the risk of CCA in patients with PSC is hundreds of times that of patients without PSC (Dyson et al., 2018).

A total of 543 stool samples, which included patients with PSC and healthy controls, were collected and induced to the 16S rRNA gene sequencing by Kummen et al. (2017). They identified the characteristics of the gut microbiome in patients with PSC. The diversity of gut microbiome in patients with PSC was significantly reduced compared with healthy controls, but there was no difference in the diversity of gut microbiome of in patients with PSC and with and without IBD. Comparing the relative abundance of the gut microbiome at the genus level, the data showed that *Veillonella* increased significantly in PSC (Kummen et al., 2017). And the abundance of the *Veillonella* genus was positively correlated with the Mayo risk score, which indicates the severity of the disease. Interestingly, an operational taxonomic unit (OUT), which belonged to the *Enterococcus* genus, was positively related to the serum level of alkaline phosphatase, which was regarded as a biomarker of disease severity (Sabino et al., 2016). Metagenomic shotgun sequencing showed that the bacteria genes that regulated the

TABLE 1 | Dysbiosis in the human microbiome of cholangiocarcinoma and its precancerous diseases.

Hepatobiliary disease	Type of the human microbiome	Dysbiosis in the microbiome	Clinical significance	Reference
Gallstones	Gut	*Oscillospira* ↑ *Roseburia*↓ *Proteobacteria* ↑ *Faecalibacterium* ↓ *Lachnospira* ↓ *Roseburia* ↓	Human microbial dysbiosis happened in patients with different types of gallstones	Wu et al., 2013; Keren et al., 2015
	Bile	*Pseudomonas* ↑ *Bacillus* ↑ *Enterococcus* ↑ *Acinetobacter* ↑		Peng et al., 2015; Kim et al., 2021
	Gallstones	*E. coli* ↑ *Pseudomonas* ↑		Lee et al., 1999
Primary sclerosing cholangitis	Gut	*Veillonella* ↑ *Exophiala* ↑ *Saccharomyces cerevisiae*↓ *Oscillospira* ↑ *Anaeroplasma* ↑ *Ruminococcus* ↑ *Akkermansia* ↑ *Allobaculum*↓ *Mucispirillum*↓ *Anaerostipesa*↓ *Coprococcus*↓	Human microbial dysbiosis may influence disease progression. The human microbiome served as a non-invasive diagnostic biomarker for PSC	Sabino et al., 2016; Kummen et al., 2017, 2021; Schrumpf et al., 2017; Lemoinne et al., 2020
	Bile	*Proteobacteria* ↑ *Enterococcus* ↑ *Staphylococcus* ↑ *Neisseria* ↑		Liwinski et al., 2020
Liver fluke infection	Bile	*Lactobacillus spp.* ↑ *Aggregatibacter spp.* ↑ *Klebsiella spp.* ↑ *Treponema spp.* ↑ *Staphylococcus equorum* ↑ *Treponema socranskii*↓ *Streptomyces sp.*↓ *Xanthobacter sp.*↓ *Flectobacillus sp*↓	Liver fluke infection was associated with human microbial dysbiosis and promoted diseases progression	Saltykova et al., 2016
	Bile duct tissues	*Bifidobacteriaceae* ↑ *Enterobacteriaceae* ↑ *Enterococcaceae* ↑		Chng et al., 2016
Choledochal cysts	Bile	*Escherichia coli* ↑ *Klebsiella species* ↑	Choledochal cysts contributed to the development of CCA by inducing human microbial dysbiosis	Kaneko et al., 2005
Cholangiocarcinoma	Gut	*Alloscardovia*↑ *Peptostreptococcaceae* ↑ *Actinomyces* ↑ *Lactobacillus* ↑ *Ruminococcus*↓ *Leuconostocaceae*↓	The increased lipopolysaccharide-producing genera and cholestasis promoted tumor progression. The human microbiome served as a non-invasive diagnostic biomarker for CCA	Jia et al., 2019
	Bile	*Nitrospirae* ↑ *Gemmatimonadetes* ↑ *Geobacillus* ↑ *Bacteroides* ↑ *Enterococcus faecalis* ↑ *Enterococcus faecium* ↑ *Enterobacter cloacae* ↑ *Escherichia coli* ↑		Chen et al., 2019; Saab et al., 2021
	Bile duct tissues	*Dietziaceae* ↑ *Pseudomonadaceae* ↑ *Oxalobacteraceae* ↑ *Bifidobacteriaceae* ↑ *Enterobacteriaceae* ↑ *Enterococcaceae* ↑		Chng et al., 2016
	Blood	*Cyanobacteria* ↑ *Bacteroidetes* ↑ *Actinobacteria* ↑ *Firmicutes* ↑ *Proteobacteria* ↑		Lee et al., 2020

synthesis of vitamin B6 and branched-chain amino acids were significantly decreased in patients with PSC, and this was validated in the serum metabolomics (Kummen et al., 2021). Notably, in addition to bacteria, fungi were also involved in the dysbiosis of the gut microbiome in patients with PSC. Lemoinne et al. (2020) identified a relative increase in the diversity of fungal species. They found that the relative abundance of *Exophiala* increased significantly, and that of *Saccharomyces cerevisiae* decreased significantly (Lemoinne et al., 2020). Schrumpf et al. (2017) constructed the mouse model of spontaneous bile duct inflammation and reported that at the genus level, *Oscillospira*, *Anaeroplasma*, *Ruminococcus,* and *Akkermansia* were significantly enriched compared with control mice. In contrast, *Allobaculum*, *Mucispirillum*, *Anaerostipes*, and *Coprococcus* were decreased (Schrumpf et al., 2017).

Meanwhile, microbial dysbiosis also existed in the bile of patients with PSC. Bile samples were collected using endoscopic retrograde cholangiography from patients with PSC and controls. Analysis results showed a decrease in diversity of the species of bile microbiome in patients with PSC vs. controls. And an enrichment of *Enterococcus faecalis* was found in patients with PSC. Moreover, the relative abundance of *E. faecalis* was positively related to the level of the harmful taurolithocholic acid (Liwinski et al., 2020). Dysbiosis in the biliary was associated with increased concentrations of the proinflammatory and potential carcinogen taurocholic acid. This may provide new insights for the treatment of PSC. The above research indicated that there was a close relationship between human microbial dysbiosis and the occurrence, progression, and deterioration of PSC.

Human Microbial Dysbiosis in Liver Fluke Infection

Liver fluke infection, which is defined as the group 1 carcinogen, will greatly increase the risk of CCA. Liver flukes are located in the biliary tree after entering the human body. And chronic infection of liver flukes will lead to tumorigenesis. In addition, chronic infection causes alterations in the human microbiome (Saltykova et al., 2018).

Saltykova et al. (2016) collected 56 bile samples from gallstone patients with or without liver fluke infection and conducted the 16S rRNA sequence. They claimed that *Lactobacillus* spp., *Aggregatibacter* spp., *Klebsiella* spp., *Treponema* spp., and *Staphylococcus equorum* were enriched in the bile from infected patients compared with non-infected patients, whereas *Treponema socranskii*, *Streptomyces* sp., *Xanthobacter* sp., and *Flectobacillus* sp. were increased in the uninfected group (Saltykova et al., 2016). Another study showed that the Shannon diversity index was significantly increased in the normal bile duct tissues because of the infection of liver fluke (Chng et al., 2016). Surprisingly, they found that Bifidobacteriaceae, Enterobacteriaceae, and Enterococcaceae were enriched in the liver fluke-associated bile duct tissues. These indicated that the Bifidobacteriaceae in the bile duct tissue may be introduced by liver flukes. In addition, the increased potential for producing bile acids and ammonia was associated with the alterations in the microbiome. In conclusion, the human microbiome was related to the process of the occurrence, progression, and deterioration of liver fluke infection.

Human Microbial Dysbiosis in Choledochal Cysts

Choledochal cysts (CCs) are cystic dilatation of the biliary tract which often occurs in children. CC is also known as a rare precancerous lesion of CCA (Soares et al., 2015). Kaneko et al. (2005) collected 122 bile specimens from patients with CCs and carried out bacterial culture. The results showed that gram-negative enterobacteria, such as *Escherichia coli* and *Klebsiella* species, and non-enteric bacteria were enriched in the bile. Chronic bile infection is important for stone formation and tumorigenesis. The detailed mechanism needs further research.

Human Microbial Dysbiosis in Cholangiocarcinoma

The human microbiome, including gut, bile, bile duct tissue, and blood microbiome, was closely related to the tumorigenesis and progress of CCA. Jia et al. (2019) recruited 84 volunteers, including 28 patients with iCCA, 28 patients with hepatocellular carcinoma (HCC), 16 patients with liver cirrhosis, and 12 healthy controls. They found that Firmicutes, Bacteroidetes, Actinobacteria, and Verrucomicrobia were the most dominant gut microbiome at the phylum level. Additionally, the gut microbiome of the patients with ICC had the highest diversity (both α-diversity and β-diversity). Compared with other groups, at the genus level, *Alloscardovia*, Peptostreptococcaceae, *Actinomyces*, and *Lactobacillus* were significantly enriched in the patients with ICC. Also, *Ruminococcus* and Leuconostocaceae were enriched in the healthy controls. Furthermore, they identified the association between specific bacterial characteristics and clinical characteristics. The genera *Lactobacillus* and *Alloscardovia* were positively correlated with tauroursodeoxycholic acid. Plasma tauroursodeoxycholic acid was negatively correlated with the genus *Pseudoramibacter* and with survival time but positively correlated with vascular invasion. There were positive correlations among plasma tricarboxylic acid, IL-4, and vascular invasion.

When it comes to the bile microbiome of CCA, the bile samples from patients with PCC and gallstones were collected and compared. The analysis results showed that *Pyramidobacter*, *Klebsiella*, *Bacteroides*, and *Enterococcus* were the most dominant bacteria in the bile of patients with PCC. Then, compared with patients with gallstones, the phylum Nitrospirae and Gemmatimonadetes and the genus *Geobacillus* and *Bacteroides* were enriched in patients with PCC (Chen et al., 2019; Saab et al., 2021). Meanwhile, Jan Bednarsch et al. (2021) studied the bile microbiome of patients with PCC. They identified a large number of bacterial colonization in the bile of patients with PCC using bacterial culture. Notably, *E. faecalis, Enterococcus faecium, Enterobacter cloacae*, and *E. coli* were the most common bacterial species.

The microbiomes of the bile duct tissue in patients with CCA were characterized using 16S rRNA sequencing. The result showed that Dietziaceae, Pseudomonadaceae, and Oxalobacteraceae were relatively abundant microbiomes in the CCA tissues compared with the other nearby tissues (Chng et al., 2016). In addition, the difference in the tissue microbiomes between liver fluke-associated CCA and non-liver fluke-associated CCA was identified. It was reported that

Bifidobacteriaceae, Enterobacteriaceae, and Enterococcaceae were significantly enriched in the fluke-associated CCA tissues. Furthermore, surprisingly, the gene functional analysis found that the functions of producing bile acid and ammonia were significantly enhanced in CCA tissue microbiome (Chng et al., 2016).

Surprisingly, the blood microbiome of patients with CCA was also identified by Lee et al. (2020). At the phylum level, the most dominant bacteria were Cyanobacteria, Bacteroidetes, Actinobacteria, Firmicutes, and Proteobacteria in patients with CCA (Lee et al., 2020). Then, they constructed a diagnostic model using blood microbiome for patients with CCA. But the blood microbiome is poorly understood, and more studies are needed to understand alterations and mechanisms.

The Effect of Helicobacter Pylori on Cholangiocarcinoma

Helicobacter pylori, which is a gram-negative bacteria, is closely related to peptic ulcer and gastric cancer. However, more researchers believed that *H. pylori* plays an important role in tumors other than gastric cancer (Pellicano et al., 2008; Pandey and Shukla, 2009; Suzuki et al., 2011). In CCA, studies showed that *H. pylori* was associated with the possible increase risk. The bile and bile duct tissue from 19 patients with hepatobiliary cancer and 19 controls were collected and induced to PCR (Fukuda et al., 2002). *H. pylori* was identified in 10 patients (52.6%) and 3 controls (15.7%). At the same time, the multiple regression analysis showed that there is a close correlation between the presence of *H. pylori* and the increase of the proliferating cell nuclear antigen marker index in the bile duct epithelium. Bulajic et al. (2002) have also reached a similar result that *H. pylori* in the bile is closely related to an increased risk of malignant biliary disease.

Additionally, *H. pylor*i in the blood of patients with CCA has also been detected. Murphy et al. (2014) collected the serum from 64 patients with biliary tract cancer, 122 patients with HCC, and 224 healthy controls and analyzed the seropositivity to *Helicobacter* species. Then, they reported that the prevalence of seropositivity in healthy controls was 88%, while the prevalence in ICC and PCC rose to 97% and 96%, respectively. And the odds ratios were 7.01 (95% CI: 0.79–62.33) and 10.67 (95% CI: 0.76–150.08), respectively. Their research proved that seropositivity of *H. pylori* protein had a close relationship with the increased risk of CCA.

Human Microbial Dysbiosis Promotes the Progress of Cholangiocarcinoma and Its Precancerous Diseases

The gut microbiome is involved in the process of antitumor immunity in CCA. Microbiome dysbiosis was detected in the mouse model of CCA. Additionally, the intestinal barrier function was destroyed, allowing intestinal bacteria and LPSs to enter the liver through the portal vein. Then, the TLR4-dependent mechanism and the CXCL1-CXCR2 axis led to the accumulation of polymorphonuclear myeloid–derived suppressor cells (PMN-MDSC), thereby promoting the immune escape and progression of CCA. On the contrary, healthy mice orally took neomycin to remove part of the gut microbiome and change the structure. The results showed that the reduction in the expression of CXCL1 and suppression of the aggregation of PMN-MDSC resulted in inhibiting tumor growth (Zhang et al., 2021). Thus, gut microbial dysbiosis promotes the progress of CCA.

The bile microbiome played an important role in gallstone disease. There were differences in the pathogenesis of different gallstones. The biliary microbiome promoted the formation of brown pigment gallstones. Then, LPSs, oxygen free radicals, oxysterols, and prostaglandins were involved in the formation of gallstones. In addition, the appearance of cholesterol gallstones was closely related to *H. pylori* and *Salmonella*. Furthermore, the metabolites of bile microbiome have been identified in promoting the formation of gallstones. Thus, bile microbial dysbiosis promotes the progress of gallstones (Yoshida et al., 2003; Haigh et al., 2006; Peng et al., 2015; Wang et al., 2018).

Gut microbial dysbiosis promotes the progress of PSC. In a PSC mouse model, researchers found gut microbial dysbiosis in PSC mice. Additionally, the results showed dysfunction of intestinal barrier, increased bacterial translocation, and activation of NLRP3 inflammasome. To identify the detail mechanism, they transplanted the feces of PSC mice into healthy mice. Surprisingly, the recipient healthy mice showed obvious liver damage, highlighting that the gut microbial dysbiosis promotes the progress of PSC (Liao et al., 2019; Dean et al., 2020). Thus, dysbiosis of the human microflora promotes the progress of CCA and its cancerous diseases.

Human Microbiome Serves as a Non-invasive Diagnostic Biomarker for Cholangiocarcinoma

The diagnostic value for the human microbiome has been reported in many diseases, such as type 2 diabetes (Qin et al., 2012), autoimmune hepatitis (Wei et al., 2020), liver cirrhosis (Qin et al., 2014), and colorectal cancer (Yu et al., 2017). A total of 486 fecal samples of patients with HCC and controls from Central, East, and Northwest China have been collected and induced to 16S rRNA sequencing (Ren et al., 2018). Then, the characteristics were described and the alterations of the gut microbiome in patients with HCC were figured out. Compared with cirrhosis, Actinobacteria phylum, *Gemmiger* genus, and *Parabacteroides* genus were significantly enriched in HCC. In addition, a diagnostic model was constructed using the random forest model based on the 30 optimal microbial biomarkers. A diagnostic efficiency of 80.64% was obtained between HCC and non-HCC samples in the discovery cohort. Moreover, high diagnostic efficiencies were also obtained in the validation cohort and independent diagnosis cohort. In another study, we illustrated the gut and oral microbial characteristics of patients with coronavirus disease 2019 (COVID-19) (Ren et al., 2021). In the meanwhile, the diagnostic value of human microbiomes in COVID-19 was identified and validated. Jia et al. (2019) constructed a microbial non-invasive diagnostic model for CCA by the logistic regression model. They selected two key genera

(*Lactobacillus* and *Alloscardovia*) to construct the diagnostic model, and the ACU value was achieved at 0.968, 0.965, and 0.987 to distinguish CCA from HCC, cirrhosis, and healthy controls, respectively (Jia et al., 2019). The diagnostic value of other human microbiome except the gut microbiome in CCA needs further exploration.

The Prospect of Translational Medical Research of Human Microbiome of Cholangiocarcinoma

In recent years, human microbiome and high-throughput sequencing have contributed much to the diagnosis, prognosis, and treatment of CCA (Yu and Schwabe, 2017; Tripathi et al., 2018). But there are many unknown facts about the interaction between the human microbiome and CCA. With the progress of research in microbiome diversity, studies in future should concentrate on the functions of microbiome and their metabolites, so as to further analyze the mechanism of between microbiome and diseases. At the same time, the above results need to be verified in a large number of rigorous animal experiments. Sterile animals would be a good choice, which can eliminate the influence of the original gut microbiome and the external environment. Moreover, in sterile animals, fecal microbiota transplantation is used to validate the causal relationship between microbiome and CCA.

The microbiome can serve as a potential treatment for CCA in future. We can use drugs that will interfere with the structure of the microbiome and microbiota transplantation to improve the human microbiome dysbiosis (Bajaj and Hays, 2019; Nicoletti et al., 2020; Shah et al., 2020). With the deepening of the understanding of the link between the gut microbiome and the liver, it will provide new opportunities for the development of new CCA treatment strategies.

AUTHOR CONTRIBUTIONS

ZY and ZR designed the study. BR, TR, and XW provided equal contributions to the data curation and writing of the manuscript. HW, YZ, YS, SL, ZR, and ZY revised the manuscript. All authors contributed and approved the submitted version of the manuscript.

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Going Beyond Bacteria: Uncovering the Role of Archaeome and Mycobiome in Inflammatory Bowel Disease

Yashar Houshyar[1†], Luca Massimino[2†], Luigi Antonio Lamparelli[1], Silvio Danese[2,3‡] and Federica Ungaro[2‡]*

[1] IBD Center, IRCCS Humanitas Research Hospital, Milan, Italy, [2] Department of Gastroenterology and Digestive Endoscopy, IRCCS Ospedale San Raffaele, Milan, Italy, [3] Faculty of Medicine, Università Vita-Salute San Raffaele, Milan, Italy

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University of Patras, Greece

***Correspondence:**
Federica Ungaro
ungaro.federica@hsr.it;
federicaungaro@hotmail.com

[†] These authors have contributed equally to this work and share first authorship

[‡] These authors have contributed equally to this work and share senior authorship

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Inflammatory Bowel Disease (IBD) is a multifaceted class of relapsing-remitting chronic inflammatory conditions where microbiota dysbiosis plays a key role during its onset and progression. The human microbiota is a rich community of bacteria, viruses, fungi, protists, and archaea, and is an integral part of the body influencing its overall homeostasis. Emerging evidence highlights dysbiosis of the archaeome and mycobiome to influence the overall intestinal microbiota composition in health and disease, including IBD, although they remain some of the least understood components of the gut microbiota. Nonetheless, their ability to directly impact the other commensals, or the host, reasonably makes them important contributors to either the maintenance of the mucosal tissue physiology or to chronic intestinal inflammation development. Therefore, the full understanding of the archaeome and mycobiome dysbiosis during IBD pathogenesis may pave the way to the discovery of novel mechanisms, finally providing innovative therapeutic targets that can soon implement the currently available treatments for IBD patients.

Keywords: intestinal microbiota, archaeome, mycobiome, inflammation, inflammatory bowel disease, immunity

INTRODUCTION

The human gastrointestinal (GI) tract is the host of 10^{13}-to-10^{14} microorganisms since birth (Gill et al., 2006) when the GI tract starts being colonized by microbial species, forming the gut microbiota (Barko et al., 2018). Changes in specific microbial species abundances and diversity occur until adulthood when the gut microbiota becomes more stable and in symbiosis with the host (Yatsunenko et al., 2012).

Microbial species composing the gut microbiota include bacteria, protozoa, eukaryotes, fungi, viruses, and archaea (Thursby and Juge, 2017). All these entities interact with each other and with the host, existing in a continuum of predator-prey interactions (Frank et al., 2007) which are pivotal for ensuring human health by participating in a variety of physiological functions, such as regulating host immunity, protection against pathogens, as well as host energy harvesting (Clemente et al., 2012; Zhang et al., 2015).

However, symbiotic relationships between microbiota and host may be disrupted by different factors such as antibiotic treatments, dietary habits, lifestyle, environmental triggers, and pathologic conditions leading to gut dysbiosis (Hasan and Yang, 2019), mainly consisting of a reduction of commensal bacteria and increase of enteric pathogens which may lead to disease condition (Hasan and Yang, 2019), such as Inflammatory Bowel Disease (IBD).

Inflammatory Bowel Disease (IBD) includes two main types of chronic inflammatory conditions, ulcerative colitis (UC) and Crohn's disease (CD) whose aetiopathogenesis is still unknown (Matsuoka and Kanai, 2015; Presti et al., 2019). Although CD and UC differ in disease location and symptoms (Rodrigues et al., 2020), they are both featured by a resolution-failing mucosal immune response (Strober et al., 2007; Shim, 2013), a dysfunctional gut epithelial barrier that fails to defend against pathogens (Presti et al., 2019), and an intestinal dysbiosis (Shim, 2013; Massimino et al., 2021).

Previous studies revealed that IBD patient intestinal microbiota is characterized by a reduced abundance of *Firmicutes* and *Bacteroidetes*, as compared to the healthy individuals, whereas the proportion of *Proteobacteria* and *Actinobacteria* are increased (Walker et al., 2011; Dong et al., 2019). Moreover, a lower abundance of *Faecalibacterium prausnitzii,* the main butyrate producer in the human intestine (Lopez-Siles et al., 2017), has been reported for IBD patients as compared to the healthy subjects. Indeed, the reduced levels of this commensal causes reduced levels of butyrate, known as a proinflammatory cytokine inhibitor, thus sustaining, chronic intestinal inflammation (Presti et al., 2019). Interestingly, numerous studies have been associating increased numbers of virulent *Escherichia coli* strains in IBD patients compared to healthy controls (Mirsepasi-Lauridsen et al., 2019), especially when focusing on IBD patients during disease relapses (Burke and Axon, 1988; Mirsepasi-Lauridsen et al., 2019).

Along with bacteria, the viral component of the gut microbiota, the enteric virome, has been recently characterized as a large and composite community made of both eukaryotic-targeting and prokaryotic-targeting viruses (bacteriophages) (Yue et al., 2019). While bacteriophages contribute to the maintenance of bacterial composition of the gut microbiota and changes in their abundances (virome dysbiosis) may affect bacteria diversity in the GI tract (Lin and Lin, 2019), the gut eukaryotic viruses (Taboada et al., 2021) may exert beneficial effects and directly impact the host's cells (Ungaro et al., 2019b). Gut virome dysbiosis was reported also in IBD patients and many studies profiled virome composition in the context of GI disease, as extensively reviewed elsewhere (Lin and Lin, 2019; Ungaro et al., 2019a; Massimino et al., 2021).

Despite the extensive analysis of the bacteriome and the virome, archaeome (archaeal components) and mycome (fungal and yeast component) populating the GI tract have been poorly investigated so far, mainly because of the lack of appropriate and reproducible techniques for their profiling (Ungaro et al., 2019a).

This minireview aims to compile all notions depicting these two neglected components of the intestinal microbiota as, on one hand, possible contributors to the maintenance of the intestinal homeostasis and, on the other, precipitators of IBD, eventually prompting future studies toward their better definition and comprehension as actors within the intestinal microbiota.

THE GUT ARCHAEOME AND THE MYCOBIOME: FROM TISSUE PHYSIOLOGY TO INFLAMMATORY BOWEL DISEASE PATHOGENESIS

The Gut Archaeome

Archaea constitute a domain of single-celled organisms alongside two other domains, eukarya, and bacteria (Woese et al., 1990). Although archaea share some features with both bacteria (lack of nucleus, introns, the presence of a single circular chromosome) and eukaryotes (presence of histones for chromosomal DNA packaging) (Gaci et al., 2014), they are classified as a distinct class of organisms that comprises two major kingdoms: *Euryarchaeota* encompassing the methanogens and their phenotypically diverse relatives, and *Crenarchaeota* comprising the relatively tight clustering of extremely thermophilic archaebacteria, whose general phenotype appears to resemble the ancestral phenotype of the Archaea (Woese et al., 1990).

Archaea have the distinctive feature of colonizing a broad range of habitats, because of their evolutionary advantage in using specific pathways metabolizing a versatile panel of energy sources, ranging from the sunlight to both organic and inorganic substances (Valentine, 2007). Now we know that archaea are not only extremophile species colonizing severe environments, but they can be also found in moderate climates and can populate plants and animal intestines, representing an important constituent of the gut microbiota (Janssen and Kirs, 2008; Berg et al., 2016; Raymann et al., 2017).

Previous pieces of evidence positioned the archaeal species as ranging from 0.1 to 21.3% of the microbial entities colonizing the digestive tract (Kim et al., 2020). Recently, the development of culture-independent methods (i.e., Next-Generation Sequencing) has been opening a new horizon for the study of the composition of gut microbiota, where the methanogens have been suggested as a predominant archaeal group among gut microbial entities (Matijašić et al., 2020) and their colonization rate ranges from 25 to 95% of human stools (Stewart et al., 2006; Dridi et al., 2009; Hoffmann et al., 2013). Specifically, methanogens perform anaerobic respiration generating methane as a final product of metabolism (methanogenesis). Indeed, they decrease the gas pressure in the colon by consuming H_2 and CO_2 to produce methane (Gaci et al., 2014). Therefore, archaeal methanogens are likely to compete with sulfate-reducing bacteria for H_2 production in the human colon (Conway de Macario and Macario, 2009). Consequently, unbalance between methanogens and sulfate-reducing bacteria may alter gut mucosal homeostasis, resulting in intestinal dysbiosis.

Detailed analysis of gut archaeome revealed the predominance in the intestine of *Methanobrevibacter (M.) smithii* and *Methanosphaera (M.) stadtmanae* species, belonging to the

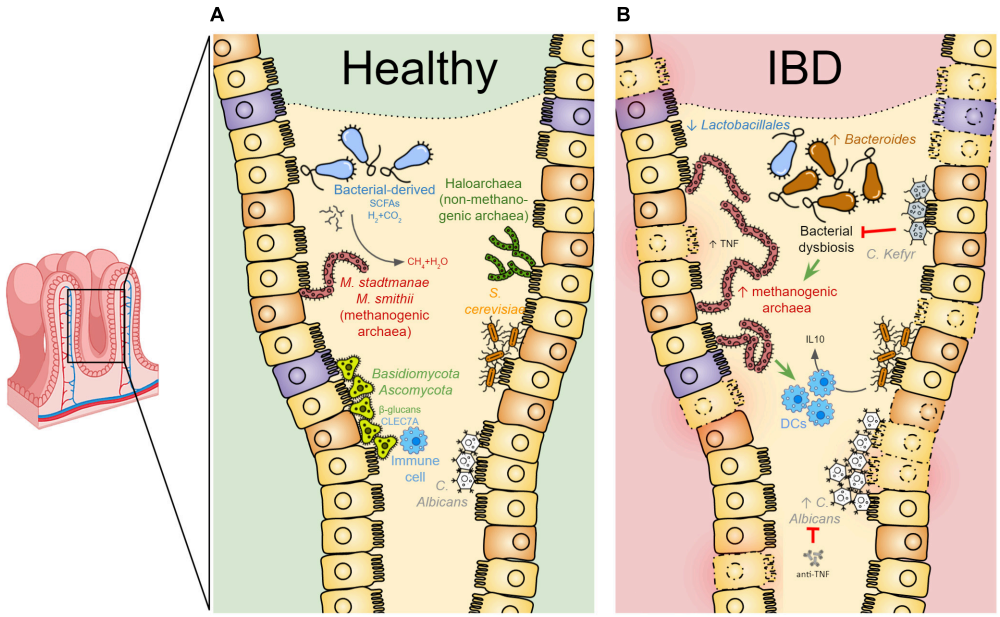

FIGURE 1 | The gut archaeome and mycobiome communities in health and disease. **(A)** In healthy conditions, the intestinal mucosa is colonized by specific archaeal and fungal species contributing, together with the other components of the microbiota, to the overall gut physiology. The archaeal methanogens [i.e., *Methanobrevibacter (M.) smithii* and *Methanosphaera (M.) stadtmanae* species] exist in a syntrophic relationship with bacterial species within the gut. Short-chain fatty acids (SCFAs) as well as hydrogen gas (H_2), produced by anaerobic bacterial fermentation, are used by methanogens to decrease the gas pressure in the colon by consuming H_2 and CO_2 to produce methane. In physiologic conditions, the human gut is also colonized by the haloarchaea. In parallel, the human gut mycobiota is predominantly characterized by *Ascomycota, Basidiomycota, Candida (C.) albicans,* and *Saccharomyces (S.) cerevisiae.* Fungi can be detected by the host immune system through CLEC7Aa C-type lectin receptor recognizing fungal wall β-glucans. **(B)** In IBD patient intestine, bacterial dysbiosis may contribute to increased methanogen species abundance, known to likely promote TNF production and activate dendritic cells (DCs), eventually contributing to the inflammatory state of the mucosa. *Candida kefyr* acts as a probiotic during intestinal inflammation by re-establishing the *Bacteroides* and *Lactobacillales* abundances. *S. cerevisiae* may exert anti-inflammatory effects by stimulating the IL10-production by DCs. This image has been designed using resources from https://www.twinkl.fr/ and https://www.flaticon.com/.

archaeal *phylum* of *Euryarchaeota* (Conway de Macario and Macario, 2009; Dridi et al., 2009; Gaci et al., 2014) and likely harboring the strong capability to establish a syntrophic association with several bacterial species (Samuel et al., 2007). Besides these two species, haloarchaea, a non-methanogenic euryarchaeota belonging to the salt-loving family of archaea (Kim et al., 2020) (**Figure 1A**), and other members of several archaeal orders have been identified as components of the gut microbiota. Among these, methanogenic members of the orders *Methanosarcinales*, *Methanobacteriales*, *Methanococcales, Methanomicrobiales*, and *Methanopyrales* were found to populate the human gut, along with the members of *Desulfurococcales, Sulfolobales, Thermoproteales, Nitrososphaerales,* and *Halobacteriales* orders, also detected in the human intestine (Gaci et al., 2014). A recent work, while confirming an increased prevalence of *M. stadtmanae* in the majority of human samples, failed to detect any non-methanogenic archaeal lineages (Raymann et al., 2017), unless the *Haloferax massiliensis* and *Haloferax assiliense*, demonstrating that halophilic archaea can inhabit the human gut (Khelaifia and Raoult, 2016; Khelaifia et al., 2018).

The contribution of the archaeome to the host physiology has not been completely understood, even if some relationship with the metabolism of dietary food has been highlighted, as for the *M. smithii*, found to trigger calories intake from the diet (Gaci et al., 2014). Moreover, methanogens exist in a syntrophic relationship with bacterial species within the gut. Indeed, short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate, as well as hydrogen gas, are produced as a result of anaerobic bacterial fermentation (Samuel and Gordon, 2006). As mentioned above, by eliminating H_2 in the colon and thus affecting bacterial energy production, methanogens optimize the energy yield of the entire human microbiota (Chaudhary et al., 2018) (**Figure 1A**). As another example of host-microbiome interaction perpetuated by archaeal commensals, *Methanomassiliicoccus luminyensis*, recently isolated from human feces (Borrel et al., 2014), was found as sensitive to human-derived antimicrobial peptides and exhibited low immunogenicity toward human immune cells *in vitro*, thus perfectly resembling a commensal gut microbe (Bang et al., 2017).

Despite the evidence depicting the archaea as commensals, their role in IBD pathogenesis has not been fully elucidated yet (Aldars-García et al., 2021). Some indications come from studies regarding the *M. stadtmanae,* found to promote *in vitro* production of TNF and to be more abundant in IBD patient stools by comparison with the controls, suggesting a possible involvement in gut inflammation (Lecours et al.,

2014) (**Figure 1B**). Importantly, *M. stadtmanae* was displayed to significantly activate human dendritic cells (Bang et al., 2014), further supporting this archaeon to participate in IBD pathogenesis (Lecours et al., 2014). Also, a recent report showed significantly lower *M. smithii* levels among IBD patients compared to healthy individuals, while normal *M. smithii* level was recovered in disease remission (Ghavami et al., 2018).

The recent IBD metatranscriptomics meta-analysis, the IBD TaMMA, analyzing a very large sample size (Massimino et al., 2021), highlighted for the first time differences in archaeome composition between colon and ileum from UC and CD patients. Indeed, such a transcriptome analysis compendium revealed colonic and ileal samples to largely differ in terms of archaeal compositions (Massimino et al., 2021). More interestingly, *Nitrosophaerales*, *Haloferacales*, *Natrialbales*, and *Thermococcales* were among the most abundant archaea orders in CD ileum, whereas the most abundant orders in UC ileum were *Methanococcales*, *Methanobacteriales*, *Methanosarcinales*, *Methanomicrobiales*, evidencing the differences between the two diseases in the ileal part, more "methanogenic" in UC. Also, *Methanomicrobiales* were found higher in UC colons, where it was the sole archaeal order to be statistically significant, while no differences were observed in colons from CD patients. From these insights, we can conclude that each intestinal tract may display differential abundances of archaea not only featuring the specific gut tract, but also the specific disease conditions. Such differences may contribute also to fostering diversified metabolic processes in the different intestinal segments, finally influencing the overall intestinal homeostasis. The archeaome composition in healthy conditions and the changes occurrgin during IBD pathogenesis are summarized in **Table 1**.

Notably, the IBD-associated bacterial dysbiosis may contribute to the archaeome composition shift, with an advantage of methylotrophic archaeal species (**Figure 1B**), particularly *M. stadtmanae*, finally increasing the inflammatory response within the human gut (Lecours et al., 2014). In this regard, a proposed leading theory explaining the possible contribution of archaea to IBD pathogenesis is that the SCFA butyric acid, produced in the colon by bacterial fermentation of dietary fibers and resistant starch, is a key regulator of syntrophism between archaea and bacteria in the gut (Matijašić et al., 2020). However, the archaeal overgrowth and the subsequent increased removal of SCFA from the biofilms are the two potential factors that cause dysbiosis, triggering bacteria to become endoparasites and enter intestinal epithelial tissues, which in turn leads to inflammatory processes in the human gut (White, 2017) (**Figure 1B**).

In conclusion, even if a causal link between archaeome dysbiosis and IBD pathogenesis has not been demonstrated yet, the evidence coming from previous studies suggested that archaea may play a major role in both health and IBD conditions. However, the way to the full definition of archaea-triggered mechanisms and functions in the gut is still long and deserves much more attention as a key player in regulating intestinal physiology. Moreover, its dysregulation may contribute, in combination with the other commensals, to sustain gut chronic inflammation, thus resulting important for the better comprehension of IBD pathogenesis and the development of new therapeutic lines of interventions aimed at the reconstitution of the archaeal composition within the intestinal microbiota.

The Gut Mycobiome

Mycobiome is a general designation used for the description of fungal communities (molds and yeasts) of the microbiome (Cui et al., 2013) and is supposed to include nearly 0.1% of the total microbes in the gut (Arumugam et al., 2011; Nash et al., 2017). Mycobiome has been detected in the GI tracts of several mammals including humans, mice, rats, pigs, and many ruminants and non-ruminants (Liggenstoffer et al., 2010; Iliev et al., 2012).

Although most of the fungi-related studies were based on culture-dependent methods (Anderson, 1917), thus hampering the understanding of the fungal community of the microbiota, the recent advance in sequencing techniques (Meyer et al., 2010) has been progressively enlarging our knowledge about the mycobiome composition within the gut (Sokol et al., 2017; Qiu et al., 2020), where a lower diversity of fungal community than the bacterial was found (Nash et al., 2017).

In the human gut, many studies reported *Ascomycota, Basidiomycota,* and *Mortierellomycota* as the most dominant *phyla* of fungi (Qiu et al., 2020), while the most abundant *genera* composing the gut mycobiome are the *Candida* (particularly *C. albicans*), *Saccharomyces* (particularly *S. cerevisiae*), *Penicillium*, *Aspergillus*, *Cryptococcus*, *Malassezia* (particularly *M. restricta*), *Cladosporium*, *Galactomyces*, *Debaryomyces*, and *Trichosporon* (Sokol et al., 2017; Qiu et al., 2020) (**Figure 1A**).

Also, mycobiome entities exist in a tight equilibrium with the host and with the other actors of the intestinal microbiota, such as bacteria, thus altogether contributing to the maintenance of the overall tissue homeostasis (Santus et al., 2021). Evidence from experimental models showed *C. Albicans* to contribute to the recolonization of the intestine by bacterial species (*Bacteroides)* after antibiotic treatment (Mason et al., 2012). Interestingly, another study suggested the overall fungal and bacterial composition of the microbiota to be impacted by dietary habits in Japanese and Indian individuals. Results from this study revealed the higher abundance in Indian participants of *Candida* and *Prevotella,* maybe resulting from a higher dietary intake of vegetables used as a growth factor by the various *Candida* species (Pareek et al., 2019).

Some species populating the gut mycobiome were demonstrated to act as probiotics, with therapeutic potential for the host. As an example, the probiotic *Saccharomyces (S.) boulardii* was found to prevent antibiotic-associated diarrhea (Szajewska and Kołodziej, 2015) by regulating the immune system and by exerting an antimicrobial activity (Kelesidis and Pothoulakis, 2012). Another probiotic species is the *Candida kefyr,* found to reduce the severity of colitis by decreasing the abundance of *Bacteroides* and increasing the *Lactobacillales* (Takata et al., 2015) (**Figure 1B**). This shift in microbiota composition was associated with a decrease in interleukin (IL) 6 production (Takata et al., 2015). Moreover, it is suggested that fungi can be detected by the host immune system through Dectin-1 (CLEC7A), a c-type lectin receptor recognizing fungal

TABLE 1 | Microbiota composition in healthy and IBD conditions.

Kingdom	Phylum	Order	Genus/Species	IBD	References
Archaea	*Crenarchaeota*	*Desulfurococcales*			Gaci et al., 2014
		Sulfolobales			Gaci et al., 2014
		Thermoproteales			Gaci et al., 2014
	Euryarchaeota	*Halobacteriales*	*Haloferax assiliense*		Gaci et al., 2014; Khelaifia and Raoult, 2016; Khelaifia et al., 2018; Kim et al., 2020
			Haloferax massiliensis		Gaci et al., 2014; Khelaifia and Raoult, 2016; Khelaifia et al., 2018; Kim et al., 2020
		Haloferacales		Increased	Massimino et al., 2021
		Methanobacteriales	*Methanobrevibacter smithii*	Decreased	Conway de Macario and Macario, 2009; Dridi et al., 2009; Gaci et al., 2014; Ghavami et al., 2018
			Methanosphaera stadtmanae	Increased	Conway de Macario and Macario, 2009; Dridi et al., 2009; Bang et al., 2014; Gaci et al., 2014; Lecours et al., 2014; Raymann et al., 2017
				Increased	Gaci et al., 2014; Massimino et al., 2021
		Methanococcales		Increased	Gaci et al., 2014; Massimino et al., 2021
		Methanomassiliicoccales	*Methanomassiliicoccus luminyensis*		Borrel et al., 2014
		Methanomicrobiales		Increased	Gaci et al., 2014; Massimino et al., 2021
		Methanopyrales			Gaci et al., 2014
		Methanosarcinales		Increased	Gaci et al., 2014; Massimino et al., 2021
		Natrialbales		Increased	Massimino et al., 2021
		Thermococcales		Increased	Massimino et al., 2021
	Thaumarchaeota	*Nitrososphaerales*		Increased	Gaci et al., 2014; Massimino et al., 2021
Fungi	*Ascomycota*	*Capnodiales*	*Cladosporium*		Qiu et al., 2020; Sokol et al., 2017
		Eurotiales	*Aspergillus clavatus*	Increased	Liguori et al., 2016; Massimino et al., 2021; Qiu et al., 2020; Sokol et al., 2017
			Penicillium	Increased	Massimino et al., 2021; Qiu et al., 2020; Sokol et al., 2017
		Glomerellales		Increased	Massimino et al., 2021
		Hypocreales	*Gibberella moniliformis*	Increased	Liguori et al., 2016; Qiu et al., 2020; Massimino et al., 2021;
		Magnaporthales		Increased	Massimino et al., 2021
		Mycosphaerellales		Increased	Massimino et al., 2021
		Pleosporales	*Alternaria brassicola*	Increased	Liguori et al., 2016; Qiu et al., 2020
		Saccharomycetales	*Candida albicans*	Increased	Mason et al., 2012; Chehoud et al., 2015; Sokol et al., 2017; Qiu et al., 2020; Massimino et al., 2021
			Candida glabrata	Increased	Hoarau et al., 2016; Liguori et al., 2016; Sokol et al., 2017; Qiu et al., 2020; Massimino et al., 2021
			Candida tropicalis	Increased	Hoarau et al., 2016; Liguori et al., 2016; Sokol et al., 2017; Qiu et al., 2020; Massimino et al., 2021
			Debaryomyces	Increased	Sokol et al., 2017; Qiu et al., 2020; Massimino et al., 2021
			Galactomyces	Increased	Sokol et al., 2017; Qiu et al., 2020; Massimino et al., 2021
			Saccharomyces cerevisiae	Decreased	Quinton et al., 1998; Chehoud et al., 2015; Sokol et al., 2017; Qiu et al., 2020; Massimino et al., 2021
		Schizosaccharomycetales		Decreased	Massimino et al., 2021
		Sordariales		Increased	Massimino et al., 2021
	Basidiomycota	*Malasseziales*	*Malassezia restricta*	Increased	Sokol et al., 2017; Qiu et al., 2020; Massimino et al., 2021
			Malassezia sympodialis	Decreased	Hoarau et al., 2016; Sokol et al., 2017; Qiu et al., 2020; Massimino et al., 2021
		Tremellales	*Cryptococcus*	Increased	Sokol et al., 2017; Qiu et al., 2020; Massimino et al., 2021
			Cystofilobasidium	Increased	Liguori et al., 2016; Qiu et al., 2020; Massimino et al., 2021;
			Trichosporon	Increased	Sokol et al., 2017; Qiu et al., 2020; Massimino et al., 2021
		Ustilaginales		Increased	Massimino et al., 2021
	Mortierellomycota				Qiu et al., 2020

Archaeome and mycobiome species composing the gut microbiota independently of inflammatory conditions are indicated in green. Archaeome and mycobiome species shifts during IBD pathogenesis are indicated in blue and red for the downregulation and upregulation of these microbial entities, respectively.

wall β-glucans, resulting in a host immune response (Iliev et al., 2012) (**Figure 1A**).

Besides this beneficial impact on the host's health, mycobiome dysbiosis was also associated with IBD. Higher levels of anti-*S. cerevisiae* antibodies raised against a component of the fungal cell wall were detected in CD patients' sera compared to controls resulting in a reliable CD biomarker and predictors of the disease course (Quinton et al., 1998).

Reduced fungal diversity and increased abundance of *Candida* (*C.*) species were found in both pediatric and adult IBD patients (**Figure 1B**). More specifically, in adult IBD patients an increased *Basidiomycota/Ascomycota ratio* was found, coupled with the enrichment of *C. albicans* and the reduction of *S. cerevisiae* (Chehoud et al., 2015; Sokol et al., 2017). Moreover, *Gibberella Moniliformis*, *Alternaria Brassicola, Aspergillus clavatus*, and the *Cystofilobasidiaceae* were found to be increased in IBD patients (Liguori et al., 2016), while *Malassezia sympodialis* was markedly decreased (Hoarau et al., 2016). In CD, *C. cropicalis* and *C. glabrata* were found augmented by comparison with the control (Hoarau et al., 2016; Liguori et al., 2016).

Fungal dysbiosis observed in IBD patients was found to be associated also with composition shifts of other microbial compartments. As an example, the reduction of *S. cerevisiae* was observed in association with the reduction of several bacterial genera, such as *Bifidobacterium*, *Blautia*, *Roseburia*, and *Ruminococcus* (Qiu et al., 2020).

From a mechanistic point of view, the intestinal mycobiome was delineated also as a contributor during the inflammatory process. For example, by treating bone marrow-derived dendritic cells with two heat-killed yeast strains of *S. cerevisiae* and *C. albicans*, Sokol and colleagues found that the IL6 levels were comparable among treatments, but the anti-inflammatory cytokine IL10 was significantly higher after the stimulation with *S. cerevisiae* by comparison with the *C. albicans* (**Figure 1B**), suggesting an anti-inflammatory effect of the former (Sokol et al., 2017).

Additionally, *C. tropicalis* together with *E. coli* and *Serratia marcescens* bacteria were found to form a biofilm functioning as a commensal niche enriched in fungal hyphae, usually increased in pathogenic conditions (Hoarau et al., 2016). Interestingly, *C. tropicalis* was shown to positively correlate with *Serratia marcescens* and *Escherichia coli* in CD, further supporting their role in sustaining chronic inflammation as a "team" in the commensal niche (Hoarau et al., 2016).

The widely accepted mechanisms through which intestinal mycobiota may interact with the host's mucosal physiology are prevalently based on the interaction between fungal species and innate immunity, involving specific receptors and signals driving the inflammatory response (Mahmoudi et al., 2021).

Among the main types of innate immune receptors that can recognize fungal Pathogen-Associated Molecular Patterns (PAMPs), Toll-Like Receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), and galectin 3 on antigen-presenting cells are well recognized and characterized (Bourgeois and Kuchler, 2012). The most investigated belonging to the CLRs include Dectin-1, recognizing the PAMP β-glucan (Taylor et al., 2007), Dectin-2, Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin receptor (DC-SIGN), Macrophage inducible Ca^{2+}-dependent lectin receptor (MINCLE), suggested to recognize the α-mannose (Yamasaki et al., 2009), the Mannose Receptor (MR). Some of CLRs were found to directly interact with TLRs to recognize fungi (Vautier et al., 2012). Within the intestine, also, the fractalkine receptor (CX3CR-1) expressed by resident mononuclear phagocytes was recently discovered to mediate the interaction between the intestinal mycobiota and host immunity in both health conditions and inflammatory state (Leonardi et al., 2018). The proposed mechanism through which fungal recognition by immune cells triggers an innate immunity cascade occurs through the activation of the spleen tyrosine kinase (SYK) or the SYK-independent RAF-1 activation, which ultimately trigger the NF-κB signaling pathway toward T helper 1 and/or T helper 17 immunophenotypes (Richard and Sokol, 2019).

Susceptibility against mycobiome-related complications in IBD patients may rely on the genetic alteration within fungal recognition receptors-encoding genes. As some examples, polymorphisms in genes encoding for *Detectin-1, TLR-1 and 3, MINCLE, Caspase recruitment domain-containing protein 9 (CARD9), Dectin-1, CD209, and CX3CR1* are the most investigated as associated with fungal dysbiosis/altered innate immunity during IBD pathogenesis (Iliev et al., 2012; Sokol et al., 2017; Leonardi et al., 2018; Limon et al., 2019).

The majority of the studies reported so far elucidated the mycobiome composition in small sample-sized IBD patient cohorts, mainly on stools and exploiting targeted DNA sequencing and PCR (Mahmoudi et al., 2021). By contrast, IBD TaMMA (Massimino et al., 2021) highlighted CD and UC ilea both featuring an increased abundance of *Glomerellales*, *Tremellales*, and *Hypocreales*, coupled with decreased abundance of *Schizosaccharomycetales*. Some orders were instead differentially abundant in the two conditions. *Saccharomycetales*, *Ustilaginales*, *Malasseziales*, *Eurotiales*, *Mycosphaerellales*, and *Magnaporthales* were found to be differentially abundant exclusively in UC ileum, while *Saccharomycetales*, *Ustilaginales*, and *Sordariales* were dysregulated only in CD ileum. Interestingly, differently from the ileal tract, very few orders were found to be differentially abundant in colons, perhaps resembling the different immune competence of the two tissues (Mann et al., 2016).

The mycobiome composition in healthy conditions and during IBD pathogenesis are summarized in **Table 1**.

Besides the long list of results coming from experimental colitis models extensively narrated by Beheshti-Maal and colleagues (Beheshti-Maal et al., 2021) and discussed later in this review, the real link between IBD pathogenesis and mycobiome dysbiosis is still missing, although some evidence lied ahead. Indeed, by combining the results from previous studies, fungal dysbiosis has been emerging to likely modulate the IBD bacteriome (Iliev and Leonardi, 2017), known to be pivotal in chronic inflammation onset and perpetuation as much as the immune system (Iliev and Leonardi, 2017). In this regard, in a study comparing samples from CD patients and their not-affected relatives, the inter- and intra-kingdom interactions between bacteriome and mycobiome were proposed to impact

the host's immune system in the setting of CD. Specifically, *C. tropicalis* interacts with potential bacterial pathogens, and that these interactions may play an important role in CD pathogenesis (Hoarau et al., 2016). Of note, the authors also indicated the microbiotas, specifically the mycobiomes, of familial samples as distinct from the non-familial (Hoarau et al., 2016). This may be partially explained by the consensus that members of a family share genetics, environment, diet, and bacterial microbiota being more similar to each other than they are to the unrelated individuals (Schloss et al., 2014).

Additionally, mycobiome entities were proposed to directly intervene in the release of IBD-specific proinflammatory cytokines (Beheshti-Maal et al., 2021), as suggested by the correlation found between specific gut mycobiome compositions with the expression levels of a series of pro-inflammatory cytokines in UC inflamed mucosa (Qiu et al., 2017). As a consequence, it is reasonable to indicate the mycobiome composition alterations, such as the increased abundances of *Candida* species often found in IBD mucosa, to be associated with inflammation and disease severity, as previously shown by Li and colleagues (Li et al., 2014). Consistently, Kowalska-Duplaga and colleagues observed that the abundance of *Candida* in CD patients decreased during the therapeutic intervention, particularly with anti-TNFα treatment (Kowalska-Duplaga et al., 2019), indicating a possible direct link between pro-inflammatory conditions and mycobiome composition. Besides the IBD-associated *Candida* species enrichment, an unbalanced *Basidiomycota/Ascomycota* ratio was also observed to correlate with flare-up/remission conditions in IBD (Sokol et al., 2017).

Overall, these pieces of evidence, although not specifically defining the mycobiome-triggered functional mechanisms underlying IBD pathogenesis, propose the mycobiome as important as the other microbiota compartments in orchestrating and contributing to the chronic gut inflammation onset and perpetuation, thus opening additional horizons for the investigation of IBD-associated microbiota diversity.

MAJOR CHALLENGES IN STUDYING MYCOBIOME AND ARCHEOME

Even if archaea resemble bacteria as we discussed above, they are so evolutionarily distant from bacteria that they retain some eukaryotic traits (i.e., molecular machinery for transmission and manipulation of genetic information) (Borrel et al., 2020). Such diversity makes challenging their detection and analysis if bacteria-centric methodologies are exploited, such as the nucleic-acid-based fluorescence *in situ* hybridization, cultivation, and molecular quantitative analyses. In the majority of commercially available kits for DNA extraction, for example, lysozyme is one of the most-used components. However, this is not suitable for archaeal DNA extraction since it cannot disrupt archaeal pseudo-peptidoglycan (Borrel et al., 2020). Indeed, more aggressive treatments may be required for archaeal cell wall break, as in the case of *Methanobacteriales* (Lee et al., 2009). No fewer difficulties are encountered while performing molecular analysis, where the so-called "universal" 16S rRNA primers fail to cover the broad archaeal diversity and to correctly annotate certain archaeal lineages (Mahnert et al., 2018). This hurdle is even huger if we consider the limited availability of well-annotated genomes in under-represented archaeal *phyla*, eventually failing to correctly assign archaeal sequences (Mahnert et al., 2018).

Similar limitations are met when fungi should be analyzed. Although some fungal entities can be cultured *in vitro* (Chevalier et al., 2018) and used to manipulate animal models as we discussed later in this review, there are significantly few complete fungal genomes yet available (Underhill and Iliev, 2014), making also their classification challenging. The approach most commonly used for fungal analysis is to amplify the fungal "internal transcribed spacer" (ITS) regions (Tang et al., 2015). Since the ITS regions are not part of the conserved transcribed regions of the structural ribosomal RNAs, they are highly divergent between fungi, allowing their classification at the species level. However, fungal ITS sequences can differ widely in size and sequence content (Santamaria et al., 2012) and there is no well-established database of ITS sequences (Tang et al., 2015).

As a matter of fact, in both mycobiome and archaeome contexts, the use of metatranscriptomics may help to better classify and annotate the fungal and archaeal composition of the gut microbiota, as we recently reported in IBD TaMMA (Massimino et al., 2021). However, much more effort in developing and improving both sequencing and cultivation approaches is required to make plausible the mycobiome and archaeome studies to unravel their physiological properties. This also represents a major limitation that renders the animal and human studies for elucidating their role during intestinal inflammation harder, as we discuss shortly.

THE ROLE OF MYCOBIOME AND ARCHAEOME DURING INTESTINAL INFLAMMATION: EVIDENCE FROM ANIMAL STUDIES

The Mycobiome in Experimental Colitis

Whether on one hand human studies are relevant from a clinical point of view, unfortunately, they cannot provide mechanistic and functional notions that may help to determine whether gut mycobiota and archaeome dysbiosis are causal for intestinal inflammation onset and progression or this is only a consequence associated with the inflammatory process. Over the years, the established models of experimental colitis have been offering the chance to depict a more comprehensive view of the entire process involving intestinal microbiota in the aetiogenesis of gut inflammation, mainly illustrating the intestinal bacteriome roles (Zhang et al., 2017) and, to a lesser extent, the mycobiome and archaeome functions. As an example, Qiu and colleagues reported how the fungal composition of the GI tract may change during Dextran sulfate sodium (DSS)-induced colitis, finally uncovering that, while fungi were higher in the ileal tract by comparison with the colon independently of the inflammation state, the fungal Shannon diversity index of the DSS-induced colitis mice was lower than

the controls in each gut segment (Qiu et al., 2015). Additionally, the inflammatory process caused a fungal translocation from the gut lumen to extra-enteric organs (such as the spleen and the mesenteric lymph node) during the experimental model of chronic colitis only in the inflamed intestinal tracts (Qiu et al., 2015), indicating that the inflammatory process itself may accelerate distal fungal invasion, likely because of the increased intestinal permeability. In line with previous evidence, the authors also showed that the fungal depletion was paralleled with a shift in mucosal bacterial composition (Qiu et al., 2015).

The direct association between fungi and bacteria during experimental intestinal inflammation was shown in another recent study highlighting *Enterobacteriaceae* to have a positive effect on fungal colonization of the gut, finally influencing the progression of gut inflammation. Indeed, *C. albicans* requires the presence of specific bacteria that trigger intestinal inflammation to increase the intensity, so that antibiotic treatment resulted as beneficial against DSS-induced colitis (Sovran et al., 2018). Also, *C. albicans* colonization of mouse intestines induced a strong Th17 response, suggesting that fungal composition infer specific immune changes in the host. Furthermore, Chiaro and colleagues in 2017 demonstrated that increased intestinal colonization with S. cerevisiae aggravated colitis by influencing purine metabolism, leading to extensive damage of the gut epithelia, reversible thanks to the inhibition of the purine pathway (Chiaro et al., 2017). These results further strengthened the concept that a tight interaction between mycobiota and the host's metabolism exists, overall influencing the host's physiology.

The Archaea in Experimental Colitis

While some studies about fungi are existing in the context of experimental colitis, fewer pieces of evidence described the functional contribution by archaea during gut inflammation *in vivo*. This is mainly due to the difficulty in isolating archaeal species (Vemuri et al., 2020), and thus the functional studies *in vivo* manipulating the archaeome composition are still missing. Nevertheless, a very recent study reported its characterization in animals. Indeed, Mohamed and colleagues found methanogens to be increased during experimental colitis, coupled with bacterial dysbiosis, thus confirming the importance of archaeome-bacteriome equilibrium also *in vivo* (Mohamed et al., 2021).

Moreover, previous evidence reported that *Methanobrevibacter smithii* contributed to digestive health by directing *Bacteroides thetaiotaomicron*-mediated fermentation of dietary fructans to acetate, and in turn *B. thetaiotaomicron*-derived formate fostered *M. smithii* for methanogenesis, thus demonstrating a link between this archaeon and bacterial utilization in balancing host's metabolism (Samuel and Gordon, 2006). Although this study did not demonstrate the effect of methanogens during intestinal inflammation, it does propose their contribution to metabolic health, suggesting their involvement in maintaining tissue physiology.

Conclusively, the paucity of studies about mycobiome and archaeome manipulation *in vivo* during intestinal inflammation strongly suggests that there is an urgent need to enlarge the knowledge about the mechanism directed by these two neglected components of the intestinal microbiota to make a step forward to the full comprehension of the entire microbiota-mediated mechanism in health and disease.

MANIPULATING THE GUT MYCOBIOME AND ARCHAEOME FOR THE TREATMENT OF INFLAMMATORY BOWEL DISEASE: EVIDENCE FROM CLINICAL TRIALS

Alteration in gut microbiota compositions during IBD pathogenesis and the evidence that specific microbial entities may result beneficial, or detrimental, have been leading over the years to the development of an enormous number of clinical trials, as those assessing the efficacy of the Fecal Microbiota Transplant (FMT) (Oka and Sartor, 2020). Reasonably, FMT exerts beneficial effects by transferring fecal microbiota from a healthy individual to an IBD patient, re-establishing the correct balance among microbial entities in the gut. Also, antibiotics are used as primary therapy for inducing or maintaining remission based on the hypothesis that certain bacteria cause IBD (Sartor and Wu, 2017). Likewise, virome modulation has been proposed as beneficial by administering CD patients with a bacteriophage cocktail parasitizing *adhesive-invasive E. coli*. Currently, this approach is under investigation in a clinical trial (NCT03808103) (Ungaro et al., 2019a).

Regarding the modulation of fungal composition, treatments may encompass some anti-fungal medications. Specifically, the NCT03476317 small pilot study has completed the recruitment of patients to determine the effect of a novel gut microbiota-targeted therapeutic regimen (bowel lavage and antibiotics with or without the antifungal fluconazole) in the management of active CD or indeterminate colitis (IBDU) that is refractory to conventional, immunosuppressive therapy.

Among future clinical trials, the NCT05049525, not recruiting yet, aims at the evaluation of the response of the combined anti-fungal itraconazole and terbinafine therapies compared to placebo in patients with CD, further strengthening the concept that targeting fungal entities in these patients may help their remission. Similarly, the NCT04966585 pilot study, not recruiting yet, will investigate whether the microbial changes induced by antifungal treatment are associated with dampened downstream immune responses in CD patients with a genetic predisposition to developing strong immune responses to *Malassezia*.

It is noteworthy that the use of probiotics, including live biotherapeutic products (LBPs) based on bacterial- and fungal-derived molecules, is establishing a new line of treatment of IBD patients (Oka and Sartor, 2020). In specific regard to fungal-derived factors, a randomized clinical trial assessing the efficacy of *Saccharomyces boulardii*, Plein and colleagues observed an improved disease activity index in a cohort of CD patients (Plein and Hotz, 1993). Similar trials have been performed later, in CD patients, highlighting improved relapse rate (Guslandi et al., 2000) and intestinal permeability (Garcia Vilela et al., 2008).

The possibility to target archaeome is more challenging. Indeed, human methanogenic archaea are highly resistant to antibiotics (Dridi et al., 2011), being susceptible only to molecules that are also effective against both bacteria and eukarya, thus hampering a possible specific therapy. Quite recently, however, statins have been elucidated as inhibitors of archaeal cell membrane biosynthesis without affecting bacterial numbers, opening the possibility of a therapeutic intervention that targets a specific aetiological factor while protecting the intestinal microbiome (Gottlieb et al., 2016). This may be the starting point also for the modulation of the archaeome in IBD patients as a therapeutic intervention, even if the route to success is still long and much more effort and attention to this aspect for IBD treatment need to be dedicated.

CONCLUDING REMARKS

Inflammatory Bowel Disease (IBD) is a complex disease where different factors, ranging from cytokines, molecules, to immune cells and microbial entities, play major roles in directing and sustaining chronic inflammation. Despite the enormous number of studies aimed and the definition of its aetiogenesis, at present, IBD is dominated by repetitive technology-based analyses of gut dysbiosis and by clinical trials based on the cyclical blockade of an endless series of cytokines, signaling molecules, and homing receptors. It is evident that the field of IBD currently lacks fresh concepts and original discoveries. Hence, more NGS-based investigations for the elucidation of the mechanisms sustained by intestinal microbiota components are urgently needed to lay down new hypotheses and raise theories delineating the IBD aetiopathogenesis. At the same time, more animal studies are required to further elucidate functions and mechanisms mediated by all components of the intestinal microbiota, including the more neglected such as fungi and archaea.

In this direction, the full comprehension of all microbiota components that may cause IBD onset and progression will help to develop novel therapeutic strategies that will finally consider IBD in its real nature, that is complexity and heterogeneity. Establishing how the diverse microbial commensals interact with each other and with the host is the basis for solving this complexity, finally leading to tailored therapies considering patient-specific characteristics within the intestinal microbiota.

AUTHOR CONTRIBUTIONS

YH, LM, LL, and FU: conceptualization and writing, review, and editing. SD and FU: supervision, review, and editing. FU: funding acquisition. All authors contributed to the article and approved the submitted version.

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Gut Microbiota Was Involved in the Process of Liver Injury During Intra-Abdominal Hypertension

*Zeyu Zhao, Zhengchang Guo, Zhengliang Yin, Yue Qiu and Bo Zhou**

Department of General Surgery, The First Affiliated Hospital of Anhui Medical University, Hefei, China

Background: Intestinal damage caused by intra-abdominal hypertension (IAH) and abdominal compartment syndrome (ACS) can lead to the ectopic gut microbiota, which can contribute to liver injury *via* portal veins. Therefore, it is speculated that gut microbiota disorder caused by IAH/ACS may result in liver injury. The relationship between gut microbiota and IAH/ACS-related liver injury was investigated in this study.

Methods: A model of IAH was established in rats, and 16S rRNA sequencing was analyzed for gut microbiota in the feces of rats. The elimination of gut microbiota was completed by antibiotics gavage, and fecal microbiota transplantation (FMT) was used to change the composition of gut microbiota in rats.

Results: In addition to the traditional cause of liver blood vessel compression, liver injury caused by IAH was also associated with gut microbiota dysbiosis. Gut microbiota clearance can relieve liver injury caused by IAH, while FMT from IAH-intervened rats can aggravate IAH-related liver injury.

Conclusion: The gut microbiota was one of the most important factors contributing to the IAH-related liver injury, and the JNK/p38 signaling pathway was activated in this process.

Keywords: intra-abdominal hypertension, abdominal compartment syndrome, fecal microbiota transplantation, 16S rRNA, gut microbiota dysbiosis

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****Correspondence:***
Bo Zhou
zhb0468@gmail.com
orcid.org/0000-0002-9591-730X

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INTRODUCTION

Intra-abdominal hypertension (IAH) and abdominal compartment syndrome (ACS) are increasingly recognized as established causes of potential complications in critically ill patients (De Laet et al., 2020). According to the consensus of the World Society for the Abdominal Compartment Syndrome (WSACS) in 2013, IAH was defined as an increased level of intra-abdominal pressure (IAP) reaching no less than 12 mmHg, and ACS was defined as a sustained

Abbreviations: IAH, intra-abdominal hypertension; ACS, abdominal compartment syndrome; MOF, multiple organ failure; IAP, intra-abdominal pressure; FMT, fecal microbiota transplantation; AST, aspartate aminotransferase; ALT, alanine aminotransferase; OTU, operational taxonomic unit; PCA, principal components analysis; PCoA, principal coordinates analysis; NMDS, non-metric multidimensional scaling; LEfSe, Linear discriminant analysis effect size; H&E, hematoxylin and eosin; SD, standard deviation; UPGMA, unweighted pair-group method with arithmetic mean; MAPK, mitogen-activated protein kinase; TLR, toll-like receptor.

elevation in IAP exceeding 20 mmHg and development of new-onset organ dysfunction (with or without an abdominal perfusion pressure under 60 mmHg) (Kirkpatrick et al., 2013). IAH/ACS influences the blood flow to various organs and affects all body systems, including circulatory disease, respiratory failure, liver failure, renal failure, and gastrointestinal dysfunction (Papavramidis et al., 2011; Reintam Blaser et al., 2017; Nazer et al., 2019).

The liver function is damaged with edema, inflammation, and necrosis when the IAP keeps elevating (Lima et al., 2017). Numerous studies have explored the reason for liver injury in IAH/ACS, and they found that elevated IAP can exert a severe impact on hepatic hemodynamics, liver perfusion, and parenchymal histology (Diebel et al., 1992; Cheatham, 2009; Inal et al., 2011; Antoniou et al., 2018; Regli et al., 2019). IAH/ACS can impair several functions of the intestinal barrier, enhance intestinal permeability, and decrease the abundance and diversity of gut microbiota (Zhao et al., 2020). Previous studies have reported the association between gut microbiota and sepsis-induced liver injury (Gong et al., 2019). However, the relationship between liver function damage and gut microbiota in IAH/ACS remains to be illustrated. Hence, we aimed to investigate the role of gut microbiota in the IAH/ACS-associated liver injury in this study.

MATERIALS AND METHODS

Experimental Animals

Sprague-Dawley male rats ($n = 36$, weight: 200 ± 20 g) of 6–8-week-old specific-pathogen-free were obtained from the Experimental Animal Center of Anhui Medical University, Hefei, China. The animals were housed in a controlled environment (25 ± 2°C, 50–60% humidity, 12 h/12 h light/dark cycle) and fed with standard rat chow and tap water *ad libitum*. One week of adaption was offered to all the animals before starting the study. The experimental protocol was reviewed and approved by the Animal Ethics Committees of Anhui Medical University (Approval number: 20170354). All efforts were taken to minimize the animal suffering during experiments.

Intra-Abdominal Hypertension Model Established

Rats fasted for 12 h while water was free to access before operation. The IAH model was established according to the method previously described (Gong et al., 2011; Leng et al., 2016). In brief, rats were anesthetized with sodium pentobarbital 40 mg/kg by intraperitoneal injection. Their body was kept warm by a thermostatic blanket. After the abdomen was shaved and sterilized, a disposable venous infusion needle with a micro-infusion pump was punctured on the abdominal cavity for nitrogen gas insufflation. The IAP was raised exceeding 20 mmHg and maintained for 4 h. The sham group was subjected to the same operation without nitrogen gas insufflation. After 4 h of IAH, rats were sacrificed by an overdose of sodium pentobarbital (160 mg/kg). Samples of whole blood, hepatic tissue, jejunum tissue, and feces in the cecum were collected.

Experimental Groups

Rats were randomly divided into six groups ($n = 6$ in each group): (1) Control group (sham operation, Con); (2) IAH 4-h group, a sustained elevation in IAP exceeding 20 mmHg for 4 h; (3) PBS group, in which the rats were administered PBS intragastrically once daily for 5 consecutive days; (4) ABX group, in which the rats were administered antibiotics intragastrically to deplete the gut microbiota; (5) FMT-C group, in which the rats received feces oral gavage from the control group rats; (6) FMT-I group, in which the rats received feces oral gavage from the IAH 4-h group rats.

Gut Microbiota Clearance and Fecal Microbiota Transplantation

Antibiotics (vancomycin, 100 mg/kg; ampicillin, 200 mg/kg; metronidazole, 200 mg/kg; and neomycin sulfate, 200 mg/kg) were administered to rats intragastrically one time daily for 5 consecutive days to deplete the gut microbiota (Gong et al., 2021). Another group of rats was administered as described above, except the antibiotics were replaced by PBS. FMT in recipient rats was performed according to the several modified methods described previously (Gong et al., 2018; Xia et al., 2021). In brief, the stool contents of the donor rats (control group rats or IAH 4-h group rats) were harvested and resuspended in sterile PBS at 0.05 g/ml and centrifuged at 1,000 rpm for 15 min. The supernatants were aliquoted and frozen at −80°C until used. Recipient rats were orally gavaged with antibiotics as mentioned above for 5 consecutive days to deplete the gut microbiota, and then, an amount of 1 ml donor fecal contents were administered to the recipient rats by gavage once daily for 3 consecutive days.

Biochemical Analysis

Blood was obtained *via* cardiac puncture before the rats were sacrificed. The blood samples were centrifuged at 3,000 × *g* for 10 min at 4°C to collect the serum. An automated analyzer (HITACHI 3100, Tokyo, Japan) was used to measure the levels of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

Gut Microbiota 16S rRNA-Sequencing Analysis

Total genome DNA from collected feces was extracted and monitored by Equalbit dsDNA HS Assay Kit. The V3–V4 hypervariable regions of the 16S rDNA sequence were selected for generating amplicons and following taxonomy. A linker with an index was added to the end of the PCR product of 16S rDNA by PCR for NGS sequencing, and the library was purified with magnetic beads. The library was quantified to 10 nM, and PE250/FE300 paired-end sequencing was performed according to the Illumina MiSeq/NovaSeq (Illumina, San Diego, CA, United States) instrument manual. A quality filtered, purified, chimeric sequenced, VSEARCH clustering sequence was used for operational taxonomic unit (OTU) clustering (the standard of sequence similarity is set to 97%). Then, the ribosomal database program classifier Bayesian

algorithm was used to analyze the representative sequences of OTU species taxonomy. The community composition of each sample was statistically analyzed under different species classification levels. Based on the OTU analysis results, the method of random sampling sample sequences was used; Chao1 index and Shannon alpha diversity index were calculated; community species abundance, diversity of rarefaction curves, and rank-abundance graph were drawn. Principal components analysis (PCA) was performed based on the sample OTU abundances table. Principal coordinates analysis (PCoA) and non-metric multidimensional scaling (NMDS) were calculated based on the distance between the Bray–Curtis matrix. Linear discriminant analysis effect size (LEfSe) was used to compare the hierarchy of evolution between-group differences of microbial community structure and species, which were shown by the species and the branching tree diagram. Metastats gap analysis was used to present the species abundance differences of microbial communities.

Hematoxylin and Eosin Staining and Liver Injury Scoring

The liver tissues of rats were fixed in 10% formaldehyde, embedded in paraffin, and sectioned into 4 μm thick layers. After staining with hematoxylin and eosin (H&E), the pathological changes of images were detected using the TissueFAXSi-plus imaging system (TissueGnostics, Vienna, Austria). The histological changes of the hepatic sections were evaluated by pathologists *via* a blind test. The histological score of the liver was graded from 0 to 4 based on the severity of the inflammatory and necrosis process. The score was calculated by accumulating all the scores of each parameter and a maximum score was 12 (Ko et al., 2020).

Immunohistochemical Staining

The expression of claudin-1, occludin, and ZO-1 in the paraffin-embedded sections was detected by immunohistochemical staining. Briefly, the sections were incubated with claudin-1 (1:200, 28674-1-AP, Proteintech, Rosemont, IL, United States), occludin (1:200, 27260-1-AP, Proteintech, Rosemont, IL, United States), and ZO-1 (1:500, ab221547, Abcam, CA, United States) antibody overnight at 4°C after receiving antigen recovery. Then, incubation with goat anti-rabbit IgM (1:200, ab2891, Abcam, CA, United States) for 1 h was carried out. Images were scanned by the TissueFAXSi-plus imaging system (TissueGnostics, Vienna, Austria).

Western Blot

The rat hepatic tissues were extracted with RIPA lysis buffer (Beyotime, Jiangsu, China) to obtain protein samples. The concentration of protein was detected by the bicinchoninic acid assay kit (Beyotime, Jiangsu, China). Equal volumes of protein samples were fractionated by SDS-PAGE and then transferred to the PVDF membranes. Later, membranes were blocked in 5% skimmed milk for 1 h at room temperature. After incubation with primary antibody ERK1/2 (1:5,000, ab184699, Abcam, CA, United States), p-ERK 1/2 (1:1,000, ab201015, Abcam, CA, United States), JNK (1:2,000, ab208035, Abcam, CA, United States), p-JNK (1:5,000, ab76572, Abcam, CA, United States), p38 (1:2,000, 66234-1-Ig, Proteintech, Rosemont, IL, United States), p-p38 (1:2,000, 28796-1-AP, Proteintech, Rosemont, IL, United States) overnight at 4°C, HRP-conjugated secondary antibodies (1:1,000, SA00001-1/SA00001-2, Proteintech, Rosemont, IL, United States) were incubated for 1 h at room temperature. Protein bands were visualized by the BeyoECL Plus assay kit (Beyotime, Jiangsu, China). The quantification of target protein was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, United States).

Statistical Analysis

All data were statistically analyzed by GraphPad Prism 8.4. The results were expressed as mean ± SD and evaluated using a two-tailed Student's *t*-test. $p < 0.05$ was considered statistically significant between groups.

RESULTS

Intra-Abdominal Hypertension Induced Liver Injury in Rats

Figure 1 shows that the levels of ALT and AST in plasma were significantly increased in the IAH 4-h group ($p < 0.05$, **Figures 1A,B**). The appearance of the liver after H&E staining implied that rats in the IAH 4-h group also presented with more severe hepatic damage (**Figures 1C–E**).

Intra-Abdominal Hypertension Induced the Gut Microbiota Dysbiosis

An amount of 1,113,391 valid reads were acquired from 12 specimens by MiSeq sequencing (Con group and IAH 4 h group, $n = 6$ each group). The sequencing for our samples was deep enough to obtain most of the OTUs due to the rarefaction curves almost reaching the saturation plateau (**Figure 2A**). The Venn diagram (**Figure 2B**) result showed that 12 unique OTUs in the control group and 2 unique OTUs in the IAH 4-h group and 274 universal OTUs in both groups were detected. The OTUs abundance and alteration of gut microbiota in both groups were revealed by the OTU rank curve and OTU heatmap (**Figures 2C,D**). The alpha diversity of microbiota presented in this study illustrated that ACE (**Figure 2E**), Chao1 (**Figure 2F**), Shannon (**Figure 2G**), and Simpson (**Figure 2H**) indices were significantly lower in the IAH 4-h group than that in the control group ($p < 0.05$), indicating that the richness and diversity of gut flora in the IAH 4-h group were lower than that in the control group. The beta diversity of microbiota in the IAH 4-h group was different from the control group based on the unweighted pair-group method with arithmetic mean (UPGMA) analysis (**Figure 3A**), NMDS analysis (**Figure 3B**), PCoA (**Figure 3C**), and PCA (**Figure 3D**).

The changes in microbial composition at the class, family, genus, order, phylum, and species levels during IAH were presented in this study (**Figures 3E,F** and

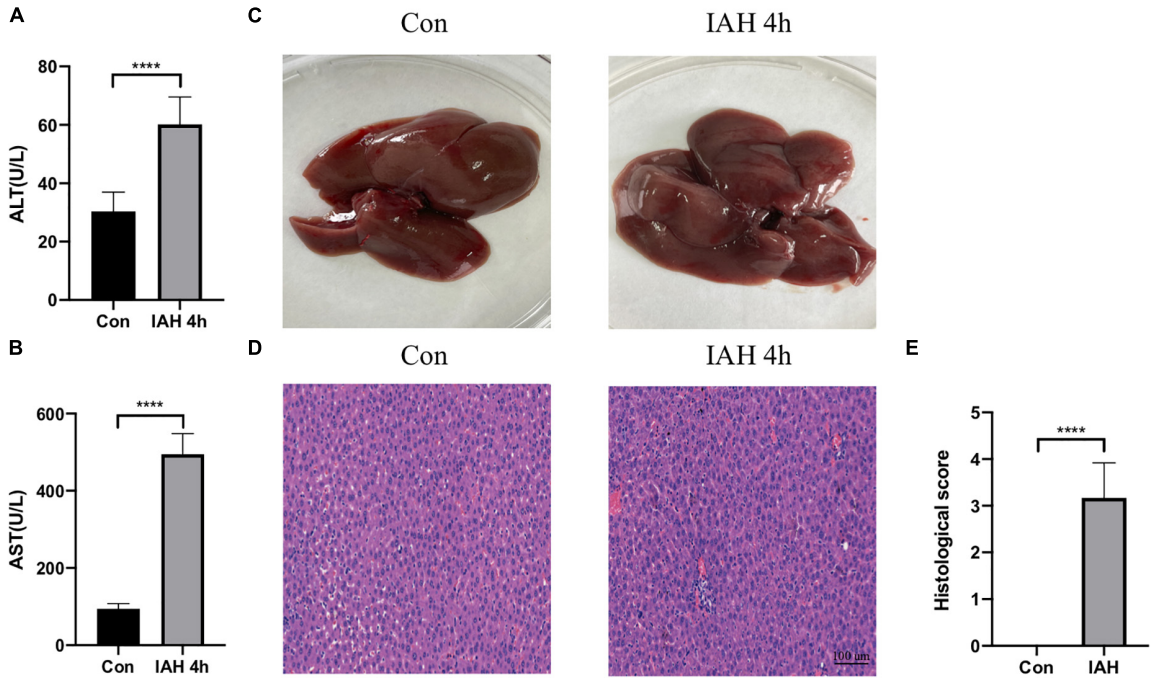

FIGURE 1 | Liver injury induced by IAH. **(A,B)** The levels of plasma ALT and AST after IAH intervention for 4 h. **(C)** Representative liver morphology. **(D,E)** HE staining and the histological score of liver injury after IAH intervention for 4 h (mean ± SD. ****$p < 0.0001$; Con group vs. IAH 4-h group; $n = 6$ per group).

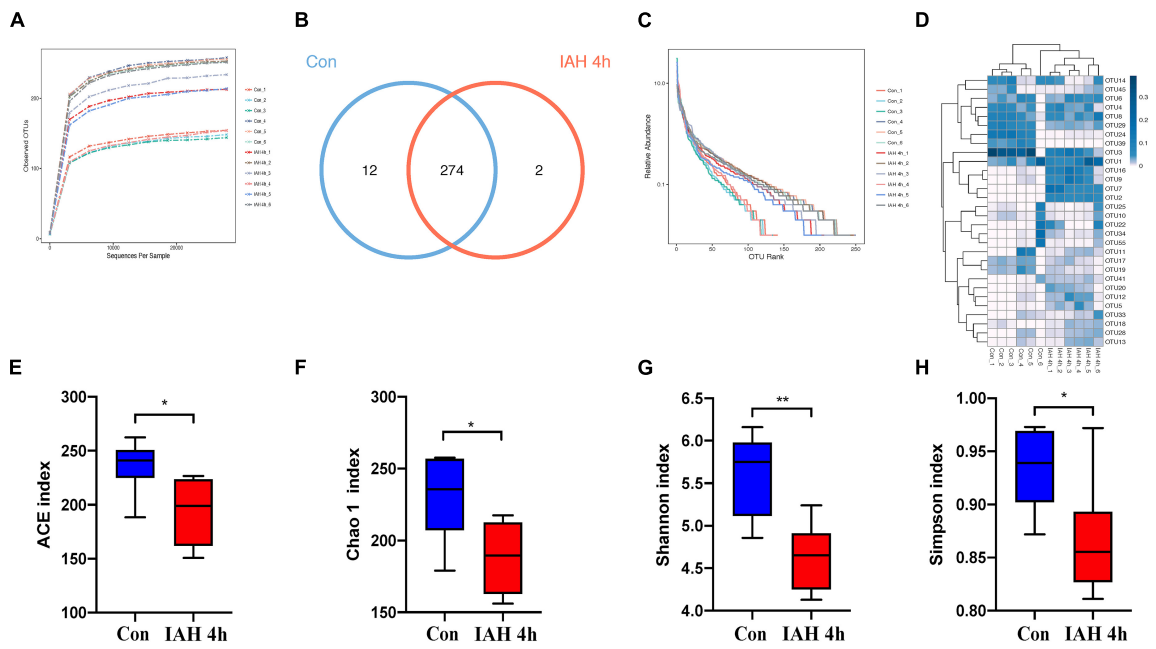

FIGURE 2 | Gut microbiota dysbiosis after IAH intervention for 4 h. Rarefaction curves **(A)**, bacteria OTUs **(B)**, OTU rank curves **(C)**, heatmap **(D)**, and composition of alpha-diversity including ACE **(E)**, Chao1 **(F)**, Shannon **(G)**, and Simpson **(H)** indices between control group with IAH 4-h group (*$p < 0.05$; **$p < 0.01$; Con group vs. IAH 4-h group; $n = 6$ per group).

Supplementary Figure 1). At the phylum level, the most dominant phyla of the control group and the IAH 4-h group were Firmicutes, Bacteroidota, and Proteobacteria species. IAH increased the relative abundance of Firmicutes and Proteobacteria species and decreased the relative abundance of Bacteroidota species. Additionally, the relative differences

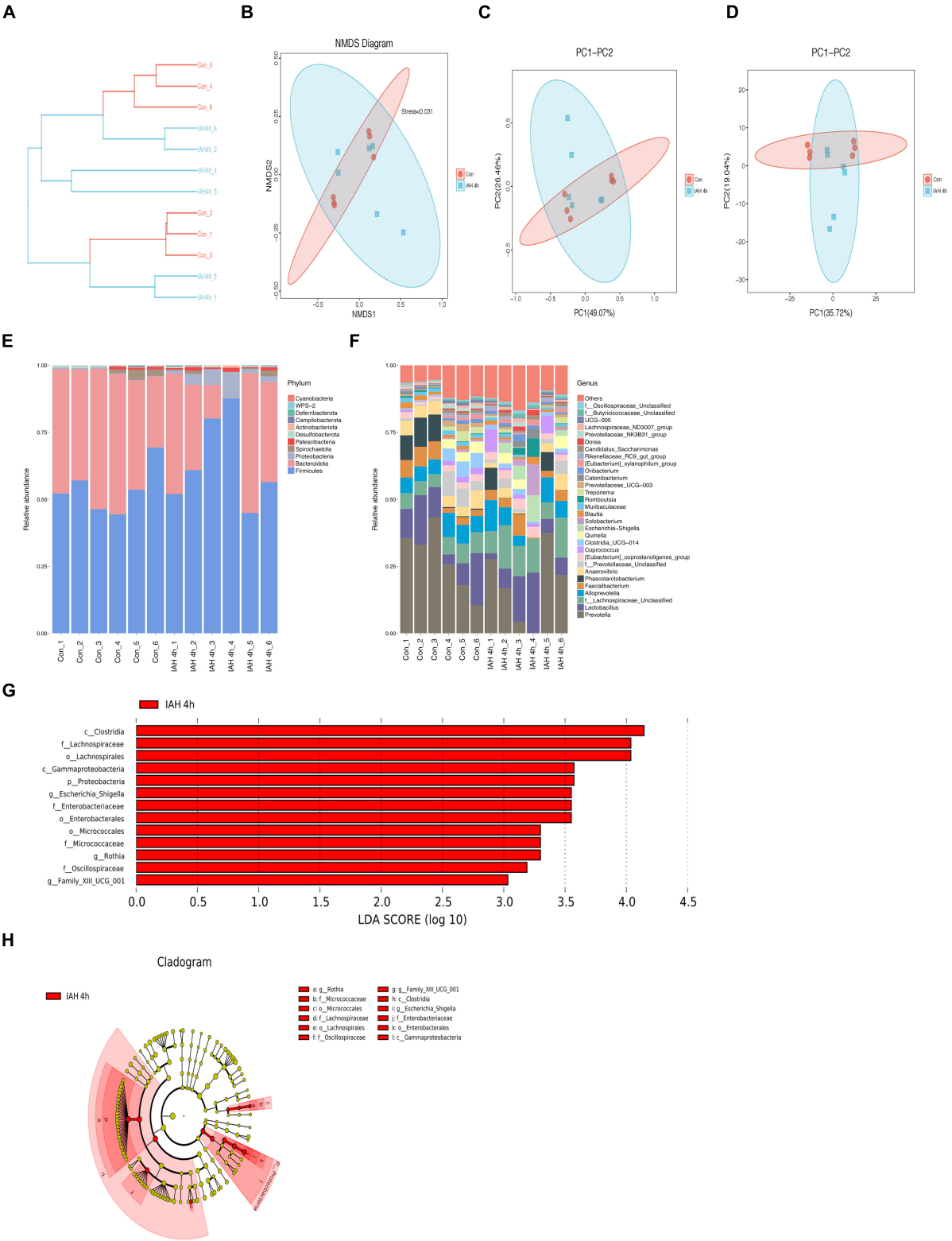

FIGURE 3 | (A) Different branches accumulated in the IAH 4 h group and control group were, respectively, presented in the UPGMA clustering tree. The composition of beta-diversity between the control group and the IAH 4-h group. Scatter plots of NMDS **(B)**, PCoA (Bray–Curtis matrix) **(C)**, and PCA (OTU abundances) **(D)** for gut microbiota. Each symbol represents one sample. The percentage of total microbiota between the control group and the IAH 4-h group was presented at phylum **(E)** and genus **(F)** levels. The enriched bacteria in the IAH 4-h group was presented by the LEfSe analysis **(G,H)**.

in the microbial composition were further revealed by the levels of other microbial compositions. The LEfSe shows that the relative abundance of Clostridia, Lachnospiraceae, Gammaproteobacteria, Proteobacteria, Escherichia_Shigella, Enterobacteriaceae, Micrococcaceae, Rothia, Oscillospiraceae, and Family_XIII_UCG_001 was significantly higher in the IAH 4-h group (LDA score > |3|, **Figures 3G,H**).

Intra-Abdominal Hypertension-Related Liver Injury Was Alleviated by ABX Pretreatment

As shown in **Figure 4C**, the IAH-induced liver injury was significantly ameliorated in the ABX group. Compared with the PBS group, the levels of plasma ALT and AST in the ABX group were decreased ($p < 0.05$, **Figures 4A,B**), and the HE scores of the liver in the ABX group were also declined ($p < 0.05$, **Figures 4D,E**).

Intra-Abdominal Hypertension-Related Liver Injury Was Affected by Fecal Microbiota Transplantation

To facilitate certification of our assumption that IAH-related gut microbiota dysbiosis promotes liver damage, an FMT operation was performed. First, the rats received antibiotics mentioned above once daily for 5 consecutive days to deplete gut microbiota, and then they received donor feces resuspended in sterile PBS from control or IAH-intervened rats for 3 days (**Figure 5A**). The rats that received feces of the IAH-intervened group exhibited more severe hepatic injury than those that received the feces of the control group after IAH intervention ($p < 0.05$, **Figures 5B–F**). Hence, we summarized that gut microbiota plays a key role in IAH-related hepatic damage.

Intra-Abdominal Hypertension Induced the Intestinal Barrier Dysfunction

The expression levels of tight junction proteins were investigated in this study, including claudin-1, occludin, and ZO-1. The immunohistochemical staining results indicate that the expression levels of claudin-1, occludin, and ZO-1 proteins were decreased after the rats intervened by IAH for 4 h and recovered after antibiotics were administered. Furthermore, the expression levels of the three intestinal tight junction proteins were lower in the FMT-I group than that in the FMT-C group (**Figure 6A**).

JNK/p38 Signaling Pathway Was Activated by the Gut Microbiota in the Intra-Abdominal Hypertension-Related Liver Injury

Figure 6 shows that the phosphorylation levels of JNK and p38 proteins were significantly higher in the IAH 4-h group than that in the control group ($p < 0.05$, **Figures 6C,D**), while no statistical difference was observed in the phosphorylation level of ERK1/2 protein between the two groups (**Figure 6B**). The phosphorylation levels of JNK and p38 proteins were lower in the ABX group compared with the PBS group and higher in

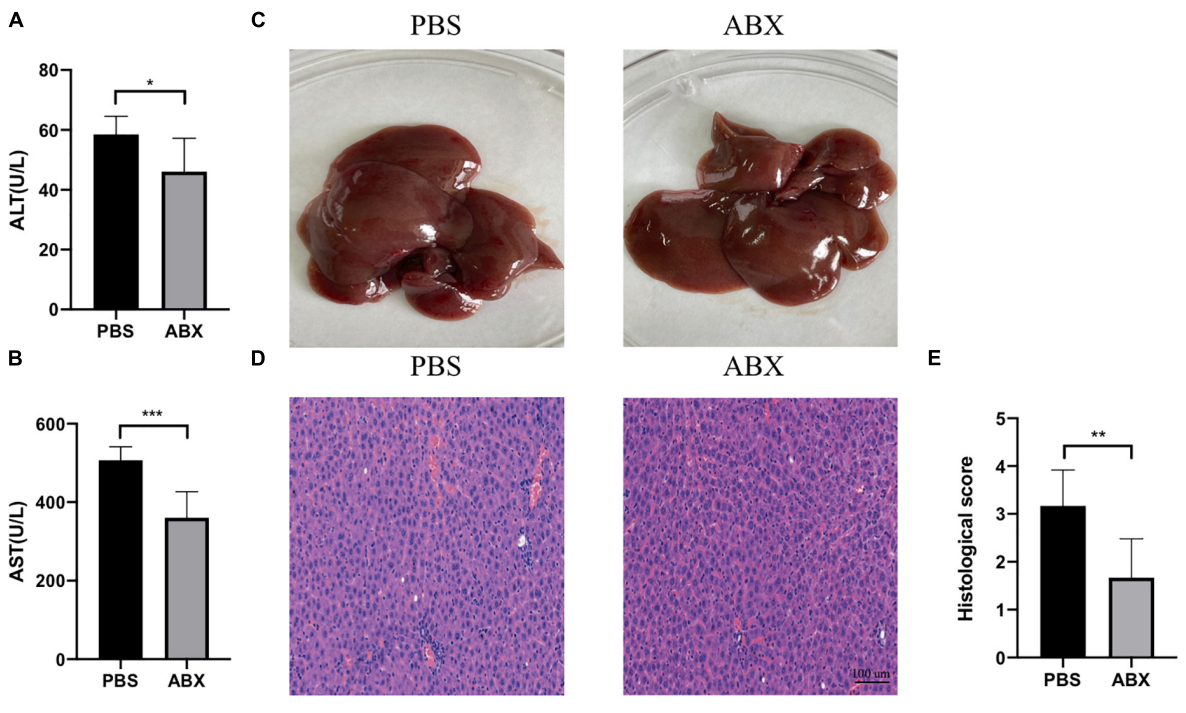

FIGURE 4 | IAH-associated liver injury was alleviated by ABX pretreatment. **(A,B)** The levels of plasma ALT and AST. **(C)** Representative liver morphology. **(D,E)** HE staining and histological score of liver injury (mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; PBS group vs. ABX group; $n = 6$ per group).

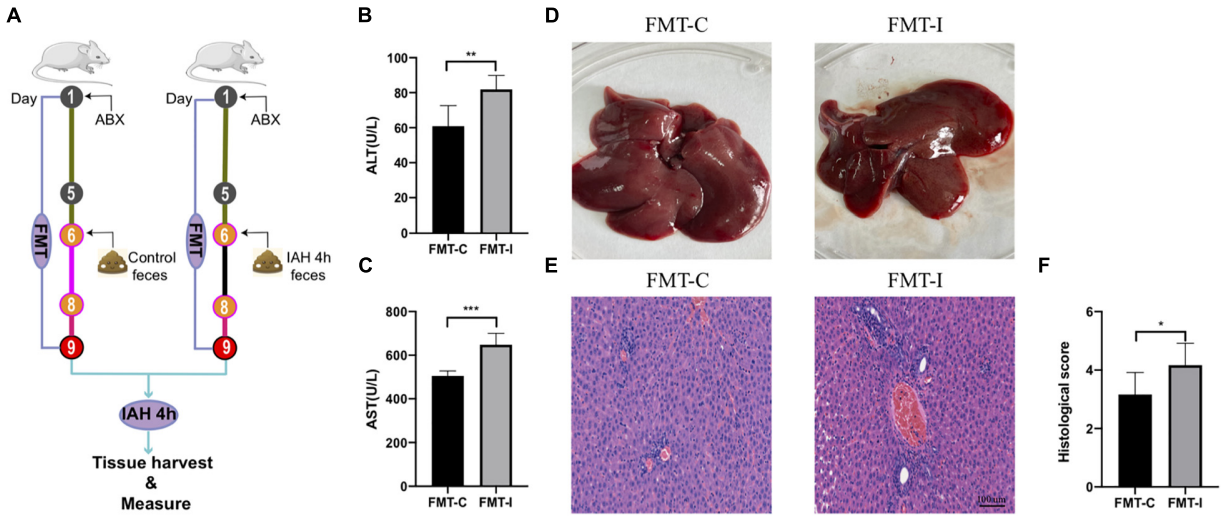

FIGURE 5 | IAH-associated liver injury was aggravated by FMT of donor feces from IAH-intervened rats. **(A)** FMT operation diagram. **(B,C)** The levels of plasma ALT and AST. **(D)** Representative liver morphology. **(E,F)** HE staining and histological score of liver injury (mean ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; FMT-C group vs. FMT-I group; $n = 6$ per group).

the FMT-I group compared with the FMT-C group ($p < 0.05$, **Figures 6C,D**). The phosphorylation levels of ERK1/2 protein were also not statistically different (**Figure 6B**).

DISCUSSION

Critical illness-induced IAH/ACS may lead to hypoperfusion of both the viscera and microbiome, exerting potentially catastrophic effects on the host (Roberts et al., 2016). It seems the gut would be one of the most sensitive organs in IAH/ACS, and the gut appears to be the initial organ that causes IAH/ACS-induced multiple organ dysfunction syndromes (Cheatham, 2009). The impact of IAH/ACS on the gut is a complex multiple cascade reaction involving decreased gut perfusion, intestinal necrosis, increased permeability of bowel wall, heightened bacterial translocation, endotoxin absorption, and released proinflammatory mediators (Kirkpatrick et al., 2020). This study experimentally indicates that the elevated IAP results in the disturbance of gut microbiota homeostasis and the decreased expressions of tight junction proteins. The intestinal mucosal barrier is the first line of host defense against both encroaching enteric pathogens and invading commensal bacteria (Turner, 2009), and its dysfunction could contribute to numerous diseases, including gastrointestinal disease (Camilleri et al., 2012), liver injury (Li et al., 2021), and septic shock (Assimakopoulos et al., 2021). These demonstrate that as the first line of gastrointestinal defense, the intestinal mucosal barrier was disturbed in IAH.

In recent years, the crosstalk between the liver and gut is increasingly recognized. The relationship between the gut and liver is established by the gut–liver axis through the vascular path of the portal vein system that transfers gut-derived metabolites directly to the liver and carries bile and antibody excreted from the liver to the intestine (Albillos et al., 2020). A variety of acute and chronic liver diseases, including pyogenic liver abscess (Zheng et al., 2021), non-alcoholic fatty liver disease (Boursier et al., 2016), alcohol-associated liver disease (Gu et al., 2021), and drug-induced liver injury (Gong et al., 2018, 2021) are closely linked to disordered gut microbiota. We speculated that the gut flora may be one important switch of the host response to IAH-related liver injury due to the reason that IAH leads to gut microbiota dysbiosis, which plays a critical role in the polymicrobial sepsis-induced liver injury (Gong et al., 2019). To decipher the mechanisms, antibiotics were used to exhaust gut microbiota before IAH intervention. We found that the liver was injured by IAH intervention, and the gut microbiota depletion could reduce this effect. Furthermore, FMT of donor feces from the IAH-intervened group rats could worsen the IAH-related liver injury compared with FMT of donor feces from the control group rats.

In the process of IAH/ACS, the liver blood flow was blocked, and the liver was damaged, while the gut microbiota was disturbed in the IAH/ACS, which may contribute to an exacerbation of liver injury. Therefore, we concluded that the intestinal flora gets out of balance when IAH occurs, and the intestinal barrier was damaged and the intestinal permeability increased due to the reason that beneficial bacteria decreased and harmful bacteria enhanced, worsening the IAH-associated liver injury.

Currently, whether the Firmicutes/Bacteroidetes ratio could be a valid marker linked with metabolic alterations or not in mice and humans is controversial (Magne et al., 2020). In our work, we observed that the Firmicutes/Bacteroidetes ratio (data not shown) and the relative abundance of Proteobacteria and Escherichia_Shigella species were increased in the IAH 4 h group rats compared with the control group rats. It

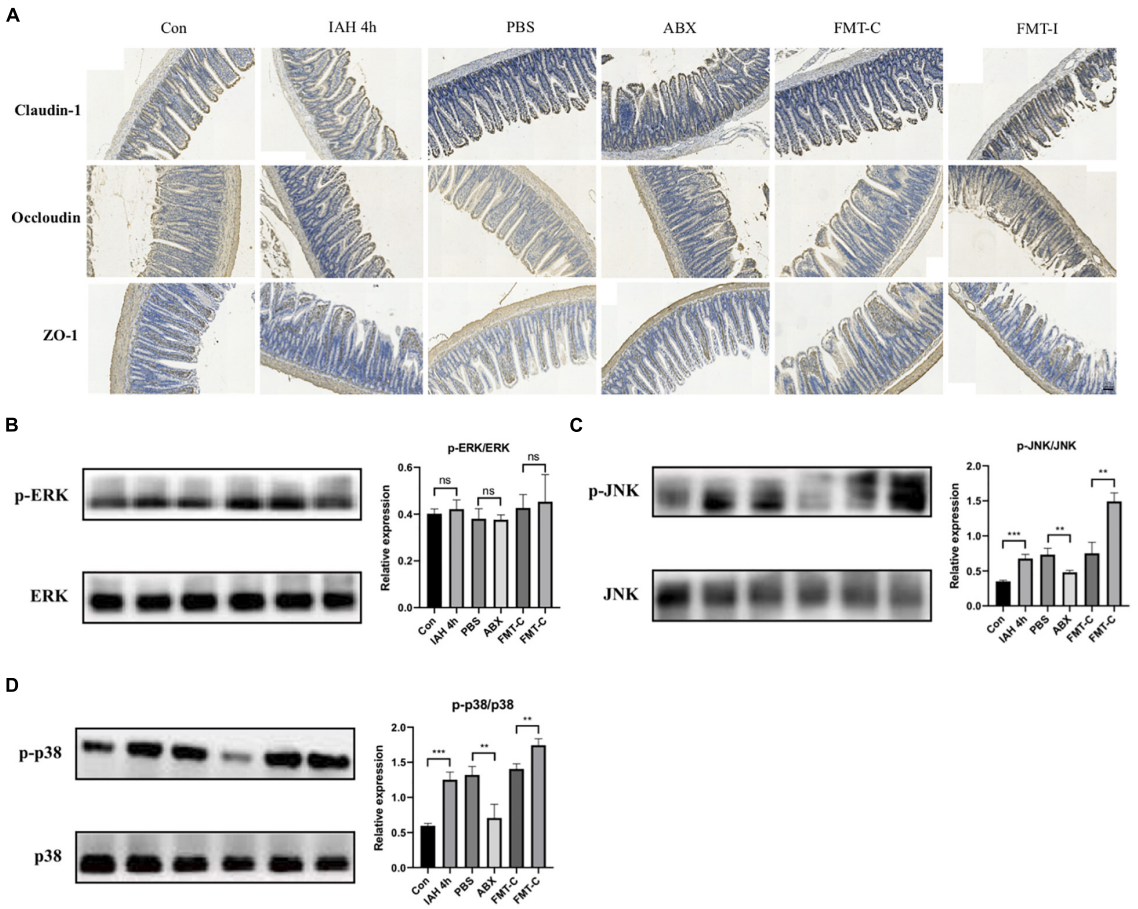

FIGURE 6 | (A) The expression of claudin-1, occludin, and ZO-1 proteins was detected by immunohistochemistry. Protein levels of p-ERK/ERK **(B)**, p-JNK/JNK **(C)**, and p-p38/p38 **(D)** in liver tissue were measured by western blot, and quantification was performed using ImageJ software (mean ± SD. $^{**}p < 0.01$; $^{***}p < 0.001$; $n = 6$ per group).

has been described that Proteobacteria is kept at a higher level in certain diseased host intestines compared with that in the healthy states, and many proliferating pathogens generate proinflammatory factors that lead to intestinal inflammation, increasing the level of Proteobacteria in the gut (Durban et al., 2013; Shin et al., 2015). Within the gut microbiota, Proteobacteria is the main source of lipopolysaccharides (LPS) synthesized (Lin et al., 2020). High LPS levels damage the intestinal barrier and increase intestinal permeability, resulting in the leakage of endotoxin into the plasma, triggering steatosis, inflammation, and apoptosis of the liver (Vasques-Monteiro et al., 2021). The Escherichia_Shigella could impair hepatic lipid metabolism (Liu, 2014), and the elevated levels of Proteobacteria and Escherichia_Shigella species in the IAH-intervened group rats may contribute to the IAH-related liver injury. Although the liver damage of rats in the ABX group was less severe than that in the PBS group, it was still more severe than that in the control group (**Figures 1**, **4**). Therefore, in addition to the role of gut microbiota, there are other factors such as the compressed liver blood vessel mentioned above contributing to the IAH-related liver injury. These factors were confirmed by previous studies; however, we did not explore them in the current work.

Several studies have highlighted that the metabolites from gut microbiota could translocate into the liver through a leaky gut and then deteriorate the host inflammatory response by binding to the toll-like receptor (TLR) 4 receptors and activating the mitogen-activated protein kinase (MAPK) signaling pathway (Boulange et al., 2016). It is well known that MAPKs contain three subfamilies, ERK1/2, JNK, and p38 (Flores et al., 2019), and the phosphorylation of MAPKs was activated in the septic rat model with liver injury (Baranova et al., 2016). MAPKs are activated in hepatic ischemia-reperfusion injury, and JNK and p38 respond to stress stimulation, whereas ERK1/2 are phosphorylated by proliferative stimulation (Jimenez-Castro et al., 2019). To explore the relationship between MAPKs signaling pathway and the gut microbiota-mediated IAH-related liver injury, the protein levels of ERK1/2, JNK, and p38 were analyzed in our study. Our results illustrate that the phosphorylation of the JNK/p38 signaling pathway was activated in the process of gut microbiota-mediated IAH-related liver injury, while the ERK1/2 signaling pathway may not be activated.

LIMITATIONS

Our study indicates that the gut microbiota has an impact on the IAH-induced liver injury, which was not reported by previous studies; however, several limitations need to be recognized. First, we did not eliminate the confounding factor that blocked portal system contributes to the IAH-induced liver injury. Second, the specific classes or metabolites of gut microbiota responsible for the IAH-induced liver injury were not investigated in this study. Further research is required to reveal the detailed mechanism of the gut microbiota associated with IAH-induced liver injury.

CONCLUSION

Our results indicate that the IAH could induce gut microbiota dysbiosis, intestinal barrier dysfunction, and liver injury. Our study suggests that the disordered gut microbiota may be one of the most important regulators of IAH-induced liver injury. These results would provide novel insights for finding a new treatment target for IAH-related liver injury.

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 the Animal Ethics Committees of Anhui Medical University.

AUTHOR CONTRIBUTIONS

BZ conceived the study idea. ZZ and ZG designed the study and completed the experiment. ZY collected the data. ZY and YQ analyzed the data and drew the figures. ZZ wrote the initial draft with all other authors providing critical feedback and edits to subsequent revisions. All authors approved the final draft of the manuscript and accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.790182/full#supplementary-material

Supplementary Figure 1 | The percentage of total microbiota between the control group and the IAH 4-h group presented at class **(A,B)**, family **(C,D)**, genus **(E)**, order **(F,G)**, phylum **(H)**, and species levels **(I,J)**.

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Human Beta Defensin 2 Ameliorated Alcohol-Associated Liver Disease in Mice

*Jeffrey B. Warner[1,2], Ida S. Larsen[3], Josiah E. Hardesty[1], Ying L. Song[1], Dennis R. Warner[1], Craig J. McClain[1,2,4,5,6], Rui Sun[7], Zhongbin Deng[4,5,7,8], Benjamin A. H. Jensen[3,9] and Irina A. Kirpich[1,2,4,5]**

[1] Division of Gastroenterology, Hepatology, and Nutrition, Department of Medicine, University of Louisville, Louisville, KY, United States, [2] Department of Pharmacology and Toxicology, University of Louisville School of Medicine, Louisville, KY, United States, [3] Québec Heart and Lung Institute (IUCPQ), Faculty of Medicine, Laval University, Québec city, QC, Canada, [4] University of Louisville Alcohol Center, University of Louisville School of Medicine, Louisville, KY, United States, [5] University of Louisville Hepatobiology and Toxicology Center, University of Louisville School of Medicine, Louisville, KY, United States, [6] Robley Rex Veterans Medical Center, Louisville, KY, United States, [7] James Graham Brown Cancer Center, University of Louisville, Louisville, KY, United States, [8] Department of Surgery, University of Louisville, Louisville, KY, United States, [9] Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

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Edited by:
Phillipp Hartmann, University of California, San Diego, United States

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Richard Hu, University of California, Los Angeles, United States

***Correspondence:**
Irina A. Kirpich
Irina.Kirpich@Louisville.edu

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Alcohol-associated liver disease (ALD) is a prevalent liver disorder and significant global healthcare burden with limited effective therapeutic options. The gut-liver axis is a critical factor contributing to susceptibility to liver injury due to alcohol consumption. In the current study, we tested whether human beta defensin-2 (hBD-2), a small anti-microbial peptide, attenuates experimental chronic ALD. Male C57Bl/6J mice were fed an ethanol (EtOH)-containing diet for 6 weeks with daily administration of hBD-2 (1.2 mg/kg) by oral gavage during the final week. Two independent cohorts of mice with distinct baseline gut microbiota were used. Oral hBD-2 administration attenuated liver injury in both cohorts as determined by decreased plasma ALT activity. Notably, the degree of hBD-2-mediated reduction of EtOH-associated liver steatosis, hepatocellular death, and inflammation was different between cohorts, suggesting microbiota-specific mechanisms underlying the beneficial effects of hBD-2. Indeed, we observed differential mechanisms of hBD-2 between cohorts, which included an induction of hepatic and small intestinal IL-17A and IL-22, as well as an increase in T regulatory cell abundance in the gut and mesenteric lymph nodes. Lastly, hBD-2 modulated the gut microbiota composition in EtOH-fed mice in both cohorts, with significant decreases in multiple genera including *Barnesiella*, *Parabacteroides*, *Akkermansia*, and *Alistipes*, as well as altered abundance of several bacteria within the family Ruminococcaceae. Collectively, our results demonstrated a protective effect of hBD-2 in experimental ALD associated with immunomodulation and microbiota alteration. These data suggest that while the beneficial effects of hBD-2 on liver injury are uniform, the specific mechanisms of action are associated with baseline microbiota.

Keywords: alcohol, defensin, gut-liver axis, intestine, ALD, immunomodulation, microbiota

INTRODUCTION

Alcohol consumption is a major global health burden and key etiological factor in the development of alcohol-associated liver disease (ALD), which is responsible for nearly half of liver cirrhosis deaths (Seitz et al., 2018). ALD is a spectrum of liver pathologies ranging from hepatic steatosis to inflammation, fibrosis, and hepatocellular carcinoma (Seitz et al., 2018). In addition to the liver, alcohol also induces alterations in other organs, such as the intestine. In both the liver and the gut, alcohol consumption has marked immunomodulatory effects on innate and adaptive immunity, including alterations in the anti-inflammatory response, increased pro-inflammatory cytokine production, and impaired antigen presentation and T cell responses (Szabo and Saha, 2015). Additionally, alcohol exposure can impair gut barrier function (Kirpich et al., 2013; Bishehsari et al., 2017; Warner et al., 2019), alter intestinal anti-microbial defense (Szabo and Saha, 2015), and induce changes in the gut microbiota composition and function, thus contributing to alcohol-induced liver pathology *via* the gut-liver axis (Engen et al., 2015; Sarin et al., 2019). Importantly, the host gut microbiota is not only a target of alcohol toxicity but also may modulate susceptibility to alcohol-associated liver and intestinal damage (Llopis et al., 2016; Ferrere et al., 2017).

Despite the high prevalence of ALD, effective therapeutic options for this disease are limited. One group of compounds that may be beneficial for alcohol-associated liver and gut injury is anti-microbial peptides (AMPs), molecules which serve as a first line of innate defense against invading pathogens, primarily on mucosal surfaces (Zhao and Lu, 2014; Hendrikx and Schnabl, 2019). Several previous reports support favorable effects of various AMPs, such as regenerating islet-derived lectins (e.g., Reg3β and Reg3γ) (Wang et al., 2016) and cathelicidin-related AMP (CRAMP) (Li et al., 2020), which ameliorate ALD by mechanisms including reduction of bacterial colonization of mucosal surfaces, prevention of bacteria/bacterial product translocation through the gut, and abrogation of hepatic inflammasome activation, among others. Similarly, a recent study demonstrated that a functional knockout of human alpha defensins exacerbated bacterial product translocation and subsequent liver injury (Zhong et al., 2020). Therapeutically, alpha defensin 5 administration ameliorated the above-mentioned effects, suggesting a beneficial role for this molecule in treating ALD (Zhong et al., 2020). Of particular interest in the current study are the structurally similar human beta defensins (hBDs), a subclass of AMPs consisting of six identified isoforms (numbered hBD-1–6) (Yamaguchi et al., 2002) that are secreted by leukocytes and epithelial cells in the intestine, skin, lungs, liver, and other organs (Harada et al., 2004; Pazgier et al., 2006). While some human beta defensins are constitutively expressed, others are induced following exposure to microbial pathogens (e.g., hBD-2–4). Of the induced hBDs, evidence suggests hBD-2 is a highly up-regulated isoform in diseases associated with inflammation, such as inflammatory bowel disease (Wehkamp et al., 2002) and colitis (Rahman et al., 2011), where inflammatory signals and bacterial products induce its expression. hBD-2 exerted favorable effects in various disease models through its dual anti-microbial and immunomodulatory functions which are mediated by either direct binding to bacteria through lipopolysaccharide and pore complex formation, or *via* host cell surface receptors such as CCR2, CCR6, and TLR4 (Semple and Dorin, 2012). For example, hBD-2 has been shown to attenuate experimental colitis by a CCR2-mediated immunomodulatory mechanism, highlighting its potential therapeutic benefits (Koeninger et al., 2020). Still, the effects of hBD-2 in ALD remain largely unknown.

In the current study, we investigated whether hBD-2 is beneficial in experimental ALD due to chronic EtOH consumption. The study was performed using two independent cohorts of mice with distinct gut microbiota to determine the impact of the microbiome on the effects of hBD-2. Our data demonstrated that hBD-2 ameliorated liver injury in both mouse cohorts. Mechanistically, hBD-2 exerted favorable, but differential effects in these cohorts including immunomodulatory changes in the liver and the gut, suggesting that the mechanisms contributing to the beneficial effects of hBD-2 are broad and gut microbiota-dependent.

MATERIALS AND METHODS

Animal Model and Human Beta Defensin 2 Administration

All animal studies were approved by and executed within the guidelines of the University of Louisville (UofL) Institutional Animal Care and Use Committee and the NIH Office of Laboratory Animal Welfare Guidelines. The study was performed with two independent cohorts of mice, Cohort 1 (referred to as "Jackson Labs Cohort," C57BL/6J mice directly purchased from Jackson Laboratories, Bar Harbor, ME) and Cohort 2 (referred to as "UofL Cohort," second to third generation C57BL/6J offspring generated at the UofL Animal facility from breeder pairs purchased from Jackson Laboratories). Mice were maintained in micro-isolator cages on a 12-h light/dark cycle. 8- to 10-week-old male mice were subjected to a chronic ethanol (EtOH) feeding protocol. In this feeding paradigm, mice were first adjusted to a control liquid diet during a one-week acclimation phase, then ramped up to a 5% (w/v) EtOH diet as follows: 2 days on 1%, 2 days on 2%, one week on 4%, then 5% EtOH for the remainder of the study, for a total of 6–8 weeks of EtOH feeding. The diets, F1258 [EtOH] and F1259 [control] were purchased from Bio-Serv (Flemington, NJ). hBD-2 (a generous gift from Defensin Therapeutics, Copenhagen, Denmark) was administered by oral gavage once daily for the 7 days prior to sacrifice at a dose of 1.2 mg/kg. This dose was chosen based on its therapeutic efficacy and low toxicity in mice with different pathologies, such as experimental colitis (Koeninger et al., 2020) and asthma (Borchers et al., 2021; Pinkerton et al., 2021). As per the manufacturer, the hBD-2 solution had a purity of 98.6% determined by Ultra Performance Liquid Chromatography. The crystal structure of hBD-2 has been previously reported (Hoover et al., 2000; Sawai et al., 2001). Mice that did not receive hBD-2 were administered an equivalent volume of phosphate-buffered saline by oral gavage

as vehicle control. Cohort 1 included PF, EtOH + vehicle, and EtOH + hBD-2 experimental groups. To confirm the effects of hBD-2 on EtOH-mediated changes, Cohort 2 included only EtOH + vehicle and EtOH + hBD-2 experimental groups. At the conclusion of the experiment, animals were euthanized by deep anesthesia with ketamine/xylazine prior to blood draw and tissue/organ harvest. A scheme of the experimental design is presented in the **Figure 1A**.

Plasma Alanine Aminotransferase Measurement

Plasma alanine aminotransferase (ALT) levels were measured as a biomarker of liver injury using the ALT/GPT reagent as per manufacturer's instructions (Thermo Fisher, Waltham, MA).

Hepatic Triglyceride Analysis

Liver triglyceride levels were measured as previously described (Kirpich et al., 2011) using Thermo Fisher reagents.

Blood Alcohol Concentration Measurement

Blood alcohol concentration was measured in undiluted plasma samples with the EnzyChrom Ethanol Assay Kit as per the manufacturer's instructions (BioAssay Systems, Hayward, CA).

Histopathological and Immunohistochemical Analysis of Liver Tissue

Formalin-fixed, paraffin-embedded samples of liver tissue were cut to 5 μm thickness, then stained with either hematoxylin & eosin (H&E staining) to evaluate gross hepatic pathology, Oil Red O to evaluate hepatic steatosis (ORO, Electron Microscopy Sciences, Hatfield, PA), chloroacetate esterase (CAE, Sigma Aldrich, St. Louis, MO) or myeloperoxidase (MPO, R&D Systems, Minneapolis, MN) for analysis of neutrophil infiltration. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) was used for analysis of hepatocellular death (ApopTag Peroxidase *in situ* Apoptosis Detection Kit, Millipore, Burlington, MA). Quantification of CAE, MPO, and TUNEL staining was performed *via* light microscopy by blindly quantifying positive cells in 10–20 random digital images (200 × magnification) per liver section (n = 5–10 sections per group) by two independent investigators. Positive cells were then averaged between images to obtain an average per mouse. Quantification of ORO staining was performed in a similar manner, except using the color threshold feature of ImageJ software to determine percentage of the image positive for ORO staining.

RNA Isolation and Real-Time Quantitative PCR

Total RNA was isolated from the liver and ileum tissue using Trizol (Thermo Fisher). Genomic DNA contamination was removed with the TURBO DNA-free kit (Thermo Fisher). cDNA was synthesized from 1 μg of total RNA with qScript cDNA Supermix (Quanta Biosciences, Beverly, MA). 10 ng cDNA was then used for each reverse transcription qPCR reaction using PerfeCTa SYBR Green Fast Mix (Quanta Biosciences) in a BioRad CFX384 qPCR instrument (Hercules, CA). qPCR data was analyzed by the ΔΔCt method (Livak and Schmittgen, 2001). Primer sequences are presented in **Table 1**.

Immunoassay of Cytokines in Liver and Ileum Tissues

Liver and ileum tissue samples were homogenized in 250 μL PBS plus 0.05% Tween-20, protease and phosphatase inhibitors (Halt, Thermo Fisher), 0.5 M EDTA, 0.5 M EGTA, and 0.25 M sucrose using 2.8 mm ceramic beads (Biotage, Charlotte, NC) by shaking at 50 cycles/second for 1 min with a Qiagen TissueLyser LT instrument (Qiagen, Germantown, MD). Insoluble material was removed by centrifugation at 19,000 × g for 10 min. Protein concentrations were determined by the bicinchoninic acid assay (BCA) (Pierce BCA protein assay kit, Thermo Fisher), and then 300 μg protein for ileum samples or 600 μg protein for liver samples was analyzed using the MesoScale Discovery (MesoScale Discovery, Rockville, MD) V-PLEX TH17 Panel 1 kit (catalog number K15085D). Data were collected using the MESO Sector S 600 instrument and reduced with Discovery Workbench (v 4.0) software (MesoScale Discovery).

Intestine and Mesenteric Lymph Node Immune Cell Isolation and Flow Cytometry Analysis

Immune cell isolation from small intestines (intraepithelial lymphocytes [IELs] and lamina propria leukocytes [LPLs]), large intestines (IELs and LPLs), liver, and mesenteric lymph nodes (MLNs) was performed by a previously described procedure (Chu et al., 2020). Briefly, to isolate small intestine and large intestine IELs and LPLs, the intestine was cut into 0.5 cm pieces and incubated in HBSS with 5 mM EDTA for 30 min at 37°C with 180 RPM shaking. IELs were recovered in the supernatant. Large intestine pieces were then incubated in an additional HBSS solution with 0.5 mg/mL DNase I (Roche, Indianapolis, IN) and 1 mg/mL collagenase type IV (Worthington, Lakewood, NJ), then passed through a 100 μm strainer. Lamina propria lymphocytes were recovered at the interface between 40 and 72% Percoll layer (GE Healthcare, Chicago, IL). For liver and MLNs, tissue was mechanically homogenized with a rubber-tipped syringe and passed through a 70 μm strainer. Immune cells were labeled using the FOXP3/Transcription Factor Staining Buffer Set as per manufacturer's instructions (Thermo Fisher). 1/100 dilutions of APC-labeled anti-CD4 (RM4-5) and either FITC-labeled FOXP3 (FJK-16s), PE-labeled anti-IFN gamma (XMG1.2) or PerCP-Cyanine5.5-labeled anti-IL17 (eBio17B7) were used to label T regulatory cells, Th1 cells, and Th17 cells, respectively. The same dilution of APC-labeled anti-B220 (RA3-6B2) and either FITC-labeled anti-IgM (II/41) or PE-labeled anti-IgA (mA-6E1) were used to label B cells. All antibodies were obtained from Thermo Fisher. Data were collected using a BD FACSCanto II flow cytometer and analyzed with FlowJo software v10.7 (Becton Dickinson, Franklin Lake, NJ).

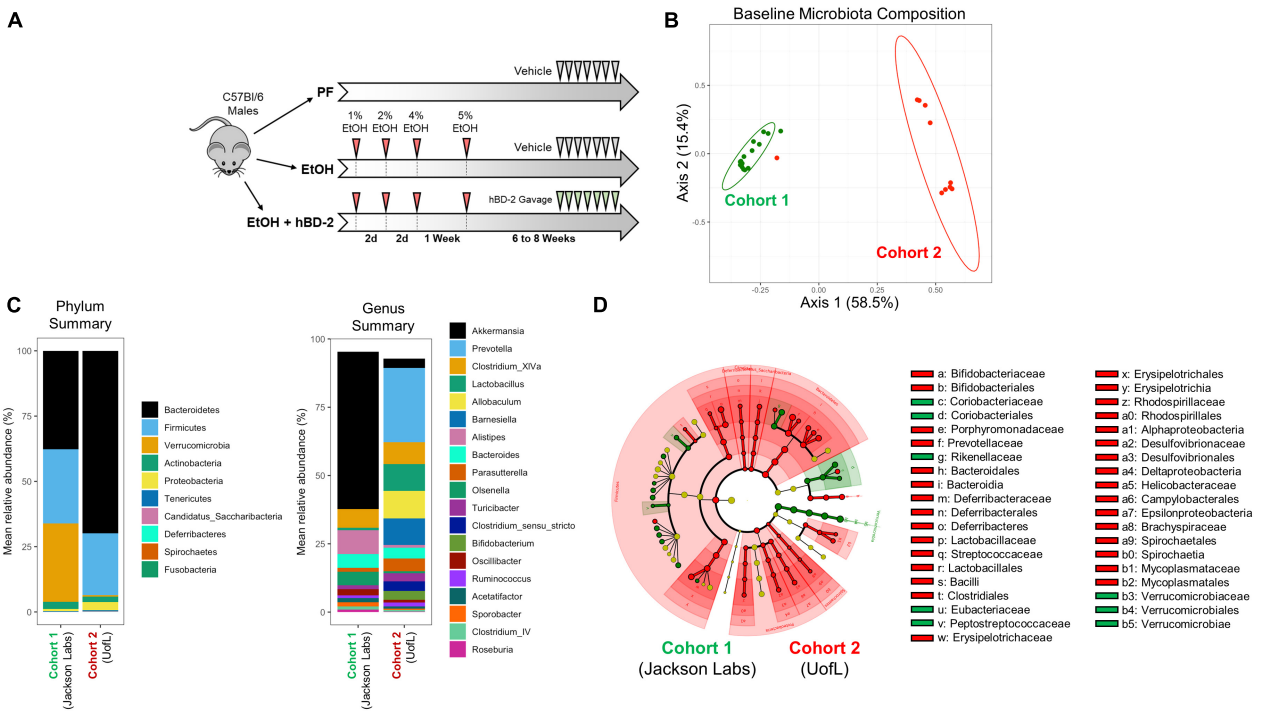

FIGURE 1 | Experimental design and gut microbiota differences between cohorts at baseline. **(A)** Schematic representation of study design. Two cohorts of male C57BL/6J mice were subjected to chronic EtOH feeding for 6–8 weeks in three experimental groups: pair-fed (PF), EtOH-fed + vehicle, EtOH-fed + hBD-2. hBD-2 was administered daily by oral gavage on the final 7 days of the EtOH feeding. **(B)** Principal coordinate analysis using Bray-Curtis distance from fecal 16S sequencing data showing independent microbiota between the two cohorts at baseline, before EtOH feeding began. Individual data points are shown as points with indicated cohort 50% confidence interval. PERMANOVA $p = 0.001$ between the cohorts. **(C)** Phylum- and genus-level summary of mean relative bacterial abundance between the cohorts at baseline. **(D)** LEfSe cladogram generated from fecal 16S sequencing data showing significant differences in taxonomical groups of bacteria at baseline associated with Cohort 1 (green) or Cohort 2 (red).

TABLE 1 | qPCR primer sequences (5′–3′).

Target	Forward	Reverse
18S	CTCAACACGGGAAACCTCAC	CGCTCCACCAACTAAGAACG
Il-17a	GGAGAGCTTCATCTGTGTCTCTGA	GAAGTCCTTGGCCTCAGTGTT
Il-22	ATCAGCTCAGCTCCTGTCACAT	TCCAGTTCCCCAATCGCCTT
Cxcl1	GGAAGTGTGATGACTCAGGTTTGC	GATGGTTCCTTCCGGTGGTTTCTTC
Mcp1	GGCTCAGCCAGATGCAGT	TGAGCTTGGTGACAAAAACTACAG
Mip2α	GCGCCCAGACAGAAGTCATA	TCCAGGTCAGTTAGCCTTGC
Zo-1	TGGGAACAGCACACAGTGAC	GCTGGCCCTCCTTTTAACAC
Zo-2	CGCTGATGGCTTGCTTCA	AACCTTCCGGGGTCTCTTG
Occludin	ACCCGAAGAAAGATGGATCG	CATAGTCAGATGGGGGTGGA

Fecal Microbiota Composition Analysis and Bioinformatics

Changes in fecal microbiota were analyzed by 16S rRNA gene amplicon sequencing. Fresh feces were collected at baseline prior to EtOH feeding as well as at the termination of the study, and DNA was extracted using the NucleoSpin 96 soil kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. Baseline samples were processed as previously described (Jensen et al., 2021). Briefly, 30 ng DNA and 16S rRNA fusion primers were added for PCR followed by purification with Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, IN). Library size and concentration was measured by Agilent 2100 Bioanalyzer and sequenced on the HiSeq platform (Illumina, San Diego, CA) according to insert size. Raw sequences were filtered to obtain the high-quality clean data, then overlapping sequences were merged to tags and further clustered to operational taxonomic units. Taxonomic classifications of operational taxonomical units were annotated using the Ribosomal Database Project database. The 16S rRNA genes of endpoint samples were amplified using primers for the V3-V4 region with Illumina adaptors (S-D-Bact-0341-b-S-17: *5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGG*

GNGGCWGCAG-3′ and S-D-Bact-0785-a-A-21: *5′-GTCTCGT GGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTA TCTAATCC-3′)* (Klindworth et al., 2013). Indices were added in a second PCR using the Nextera XT Index Kit V2 (Illumina) followed by purification with Agencourt AMPure XP beads (Beckman Coulter). The library was sequenced using a MiSeq desktop sequencer (Illumina). Raw sequences were filtered, and amplicon sequencing variants (ASVs) were generated with usearch and taxonomic classification of ASVs were annotated using the Silva database (Quast et al., 2013).

Analysis of microbiota composition was carried out in R and R Studio using the phyloseq package (McMurdie and Holmes, 2013). Permutational analysis of variance (PERMANOVA) on principal coordinate analyses (PCoAs) was carried out using the vegan package (Oksanen et al., 2020). Linear discriminant analysis Effect Size (LEfSe) was carried out using the public Galaxy server (Afgan et al., 2018). Linear mixed-effects modeling was performed using the lme4 package (Bates et al., 2015) at ASV level using a cutoff of ASVs with an overall relative abundance of minimum 0.1%. The ggplot2 package was used for visualization of the data.

Statistical Analysis

Data are reported as the mean ± standard error of the mean (SEM). For data that fit a normal distribution, differences between two groups were evaluated using the unpaired two-tailed Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test for three groups. For data that did not fit a normal distribution (as determined by the Shapiro-Wilk normality test), differences between two groups were analyzed by Mann-Whitney *U* test or Kruskal Wallis *H* test followed by Dunn's multiple comparisons test for three or more groups. Differences were considered statistically significant at a p value of less than 0.05. Statistical analyses were carried out using GraphPad Prism 8.1 software (GraphPad, La Jolla, CA).

RESULTS

Human Beta Defensin 2 Ameliorated Experimental Alcohol-Associated Liver Disease in Two Independent Cohorts of Mice

To determine the effects of hBD-2 in experimental chronic ALD, we used a chronic EtOH feeding animal model with hBD-2 administered by oral gavage in a treatment paradigm (**Figure 1A**). This model recapitulates many features of human ALD including mild liver injury and steatosis (Ghosh Dastidar et al., 2018). The study was performed with two independent cohorts of mice: Cohort 1 (the Jackson Laboratories cohort) and Cohort 2 (the UofL cohort) with distinct baseline gut microbiota compositions (**Figures 1B–D**). Specifically, at the phylum level, Cohort 1 had greater abundance of Verrucomicrobia and lower abundance of Spirochaetia than Cohort 2, and at the genus level, Cohort 1 had greater abundance of *Akkermansia* and *Alistipes*, whereas Cohort 2 had greater abundance of *Prevotella* and *Lactobacillus*, among numerous other differences (**Figures 1C,D**). Over the course of the feeding protocol, there were no significant differences in food consumption or body weights (**Table 2**). Similarly, at the conclusion of the experiment, there were no significant changes in liver/body weight or fat/body weight ratios between groups in each cohort or between cohorts (**Table 2**).

Human beta defensin 2 administration significantly attenuated EtOH-induced liver injury in Cohort 1 as shown by reduced ALT levels in EtOH + hBD-2-treated mice compared to EtOH-fed mice (**Figure 2A**) with a slight decrease in liver steatosis as reflected in H&E staining and as assessed by ORO staining (**Figures 2B,C**). hBD-2 also reduced EtOH-induced hepatocyte cell death (**Figure 2D**) and modestly decreased hepatic neutrophil infiltration (**Figure 2E**). As in Cohort 1, ALT levels were significantly decreased in Cohort 2 EtOH + hBD-2 mice compared to EtOH-fed mice (**Figure 3A**). In contrast to Cohort 1, Cohort 2 mice had significant hBD-2-mediated attenuation of hepatic steatosis as shown by H&E staining and assessed by ORO staining (**Figures 3B,C**), as well as significantly decreased hepatocyte cell death (**Figure 3D**), and liver neutrophil infiltration (**Figure 3E**). Collectively, our data demonstrated that hBD-2 afforded a significant protection against experimental ALD in two independent cohorts of mice with distinct initial gut microbiota. When comparing the two Cohorts, hepatic steatosis and inflammation were attenuated to a greater extent in Cohort 2 mice.

Intestinal Barrier Permeability Was Not Affected by Human Beta Defensin 2

Human beta defensin 2 had little impact on gut barrier permeability, as demonstrated by similar plasma LPS levels between groups in each cohort and between cohorts (**Table 2**). Similarly, mRNA expression of small intestine tight junction proteins *Zo1*, *Zo2*, and *Occludin* was unchanged between experimental groups in both cohorts (**Table 3**).

Human Beta Defensin 2 Led to Favorable Immunomodulatory Changes in a Cohort-Dependent Manner

To determine the potential mechanisms underlying the beneficial effects of hBD-2 on EtOH-associated liver injury, we first analyzed the immunomodulatory effects of hBD-2, specifically focusing on well-known cytokines that play an important role in ALD pathogenesis and innate immunity. We measured the expression of liver and small intestine IL-22, a hepatoprotective cytokine which also exerts beneficial effects in the gut (Wang et al., 2014), and IL-17A, a cytokine with pleiotropic effects including gut microbial defense (Hong et al., 2017; Jensen et al., 2021). In Cohort 1, liver and intestinal IL-22 and IL-17A protein levels were unchanged following either EtOH feeding or hBD-2 administration (**Figures 4A,B**). However, Cohort 2 EtOH + hBD-2-treated mice, compared to EtOH alone, had a significant increase in both IL-22 and IL-17A levels in the liver and small intestine (**Figures 4C,D**). At the mRNA level, hBD-2 had limited effects on *Il22* and *Il17a* expression, as well as the

TABLE 2 | Metabolic characteristics of the experimental mice.

Characteristic	Cohort 1			Cohort 2	
	PF	EtOH	EtOH + hBD-2	EtOH	EtOH + hBD-2
	n = 6	*n* = 6	*n* = 6	*n* = 8	*n* = 10
Initial BW (g)	26.15 ± 0.31	26.05 ± 0.33	26.16 ± 0.45	26.69 ± 1.10	25.98 ± 0.41
Final BW (g)	33.79 ± 0.92	32.06 ± 0.33	33.75 ± 1.75	27.51 ± 1.19	28.44 ± 0.85
Liver/BW Ratio (%)	3.894 ± 0.21	4.364 ± 0.14	4.487 ± 0.13	3.755 ± 0.07	3.793 ± 0.07
Fat/BW Ratio (%)	2.861 ± 0.19	2.844 ± 0.20	2.722 ± 0.28	2.358 ± 0.24	2.842 ± 0.28
Food consumption (g per day per mouse)	*	13.21 ± 0.25	12.84 ± 0.28	13.27 ± 0.29	13.50 ± 0.28
Biochemical measurements					
Liver triglycerides (mg per g liver)	45.57 ± 6.416	35.91 ± 2.717	41.03 ± 10.470	71.53 ± 8.389	54.49 ± 4.720
Plasma LPS (EU/mL)	0.475 ± 0.048	0.393 ± 0.028	0.478 ± 0.042	0.300 ± 0.025	0.660 ± 0.217
Blood alcohol concentration (mM)	**	1.458 ± 0.172	1.673 ± 0.100	2.026 ± 0.425	1.595 ± 0.157

Values are expressed as mean ± SEM.
**PF mice consume the same amount of food as EtOH-fed mice (pair-feeding paradigm).*
***Blood alcohol concentration was not measured in PF mice.*

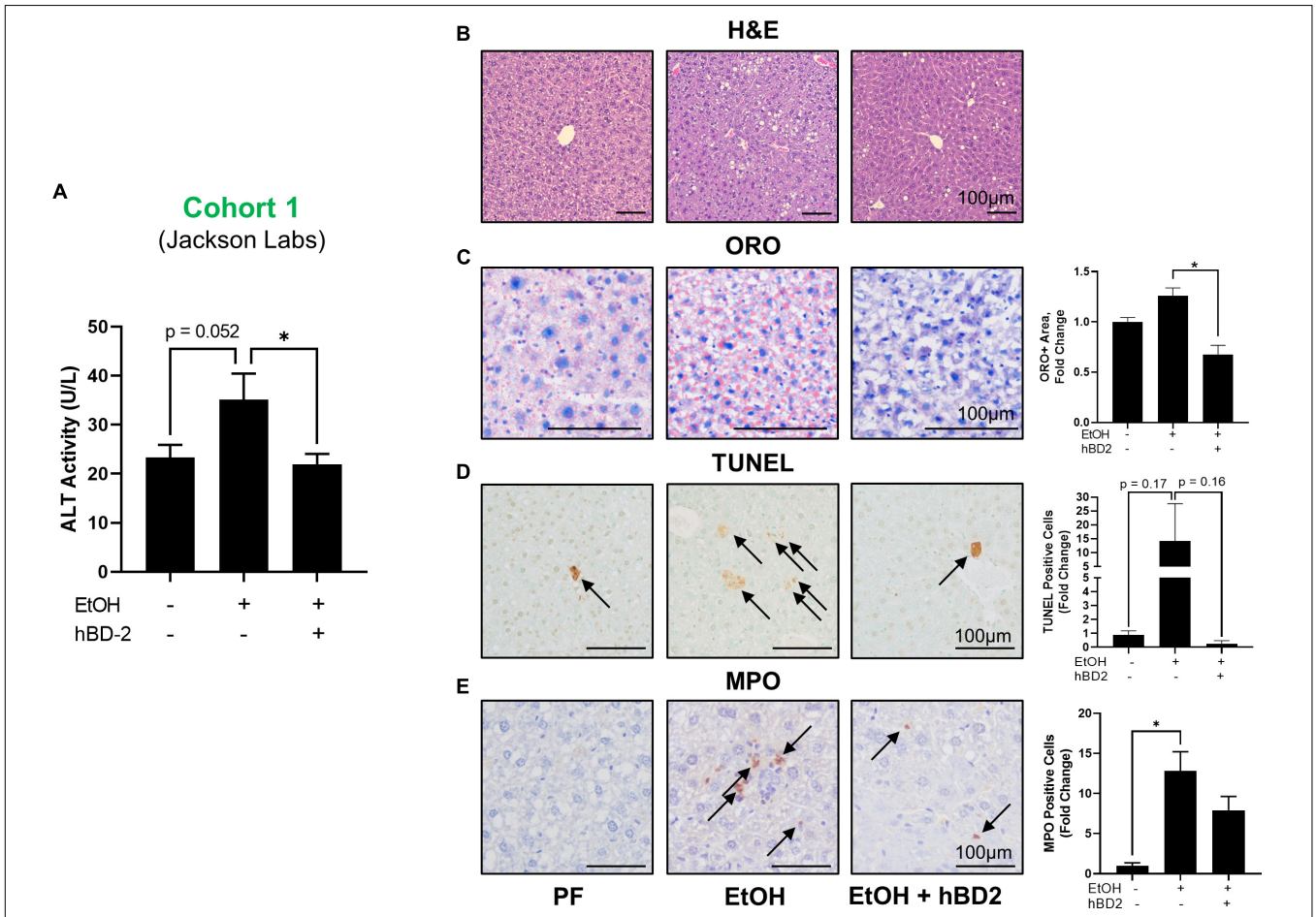

FIGURE 2 | Human beta defensin 2 ameliorated liver injury in Cohort 1. **(A)** ALT activity levels. **(B–E)** Representative digital micrographs of H&E-stained liver sections, ORO-stained liver sections, TUNEL-stained liver sections, and MPO immunohistochemistry with quantitation of ORO+ area, TUNEL+ cells, and MPO+ neutrophils shown to the right. H&E images were captured at 100 × magnification; all other images were captured at 200 × magnification. Scale bar represents 100 μm in each panel. Data are reported as mean ± standard error of the mean. *$p < 0.05$.

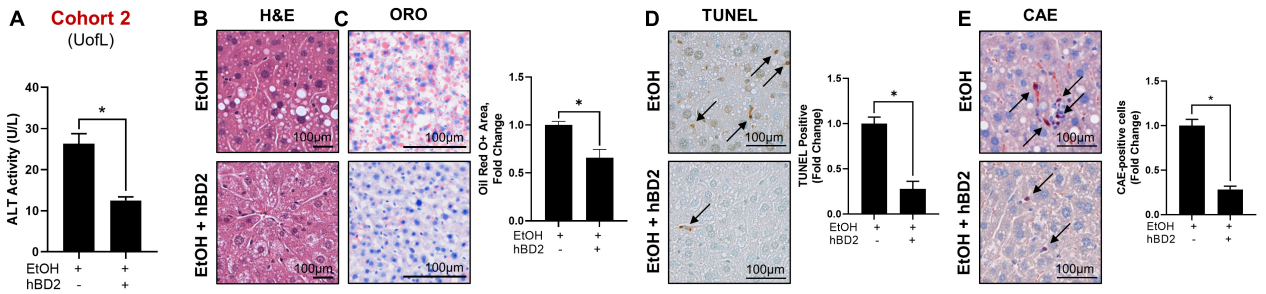

FIGURE 3 | Human beta defensin 2 ameliorated liver injury in Cohort 2. **(A)** ALT activity levels. **(B–E)** Representative digital micrographs of H&E-stained liver sections, ORO-stained liver sections, TUNEL-stained liver sections, and CAE-stained liver sections with quantitation of ORO+ area, TUNEL+ cells, and CAE+ neutrophils shown to the right of each set of images, respectively. H&E images were captured at 100 × magnification; all other images were captured at 200 × magnification. Scale bar represents 100 μm in each panel. Data are reported as mean ± standard error of the mean. *$p < 0.05$.

TABLE 3 | Small intestine mRNA expression of tight junction proteins in PF or EtOH-fed control or hBD-2-treated mice.

	Cohort 1			Cohort 2	
	PF	**EtOH**	**EtOH + hBD-2**	**EtOH**	**EtOH + hBD-2**
	***n* = 6**	***n* = 6**	***n* = 6**	***n* = 8**	***n* = 10**
Zo-1	1.040 ± 0.119	1.137 ± 0.090	1.367 ± 0.124	1.223 ± 0.279	0.840 ± 0.119
Zo-2	1.055 ± 0.136	1.162 ± 0.125	1.218 ± 0.159	1.509 ± 0.348	1.065 ± 0.180
Occludin	1.246 ± 0.342	1.295 ± 0.348	1.416 ± 0.372	1.553 ± 0.444	1.081 ± 0.225

Values are expressed as mean Fold Change (vs. PF for Cohort 1 or EtOH for Cohort 2) ± SEM.

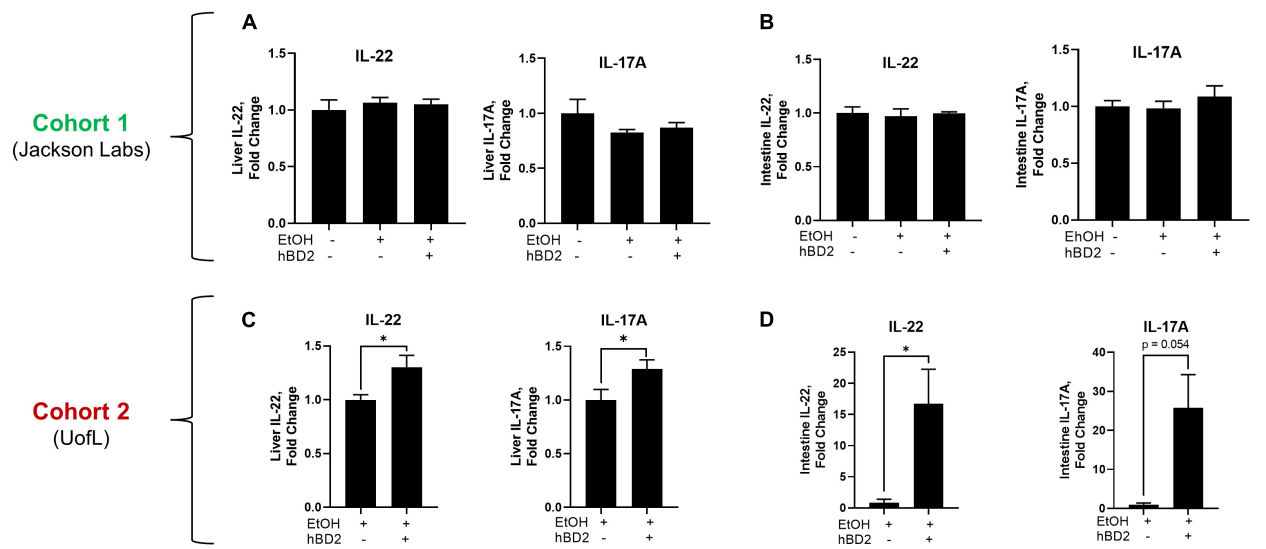

FIGURE 4 | Human beta defensin 2 altered liver and small intestine immunomodulatory cytokines. Protein levels of IL-22 and IL-17A in the liver **(A,C)** and small intestine tissue **(B,D)** of Cohort 1 and Cohort 2 mice, respectively. Data are reported as mean ± standard error of the mean. *$p < 0.05$.

expression of other pro-inflammatory cytokines, including *Cxcl1*, *Mcp1*, and *Mip2a* in both cohorts (**Table 4**).

Taken together, the differences in IL-22 and IL-17A expression between cohorts (each were elevated in Cohort 2 and unchanged in Cohort 1) support cohort-specific mechanisms contributing to hBD-2-mediated attenuation of EtOH-associated liver injury.

Human Beta Defensin 2 Increased Intestine and Liver T Regulatory Cell Populations

Considering the immune regulatory potential of hBD-2 by dendritic cell engagement (Koeninger et al., 2020), we next investigated whether immune cells which interact with dendritic

TABLE 4 | Liver and small intestine mRNA expression of immunomodulatory or pro-inflammatory cytokines in PF or EtOH-fed control or hBD-2-treated mice.

Gene	Cohort 1			Cohort 2	
	PF	EtOH	EtOH + hBD-2	EtOH	EtOH + hBD-2
	n = 6	*n* = 6	*n* = 6	*n* = 8	*n* = 10
Immunomodulatory cytokines					
Liver *Il-17a*	1.095 ± 0.176	0.890 ± 0.150	0.813 ± 0.107	1.242 ± 0.241	1.860 ± 0.731
Liver *Il-22*	1.854 ± 0.786	1.672 ± 0.549	1.454 ± 0.781	1.491 ± 0.925	1.370 ± 0.528
Small intestine *Il-17a*	1.064 ± 0.085	0.999 ± 0.225	1.417 ± 0.141	1.102 ± 0.181	1.245 ± 0.209
Small intestine *Il-22*	1.118 ± 0.217	1.244 ± 0.421	0.559 ± 0.128	2.728 ± 1.369	13.440 ± 4.409
Pro-inflammatory cytokines					
Liver *Cxcl1*	1.185 ± 0.255	4.393 ± 1.173	3.504 ± 0.481	0.792 ± 0.105	0.740 ± 0.163
Liver *Mcp1*	1.638 ± 0.398	2.307 ± 0.357	2.068 ± 0.391	0.959 ± 0.211	0.719 ± 0.079
Liver *Mip2α*	1.273 ± 0.340	0.652 ± 0.151	0.422 ± 0.065	1.121 ± 0.224	0.690 ± 0.063

Values are expressed as mean Fold Change (vs. PF for Cohort 1 or EtOH for Cohort 2) ± SEM.

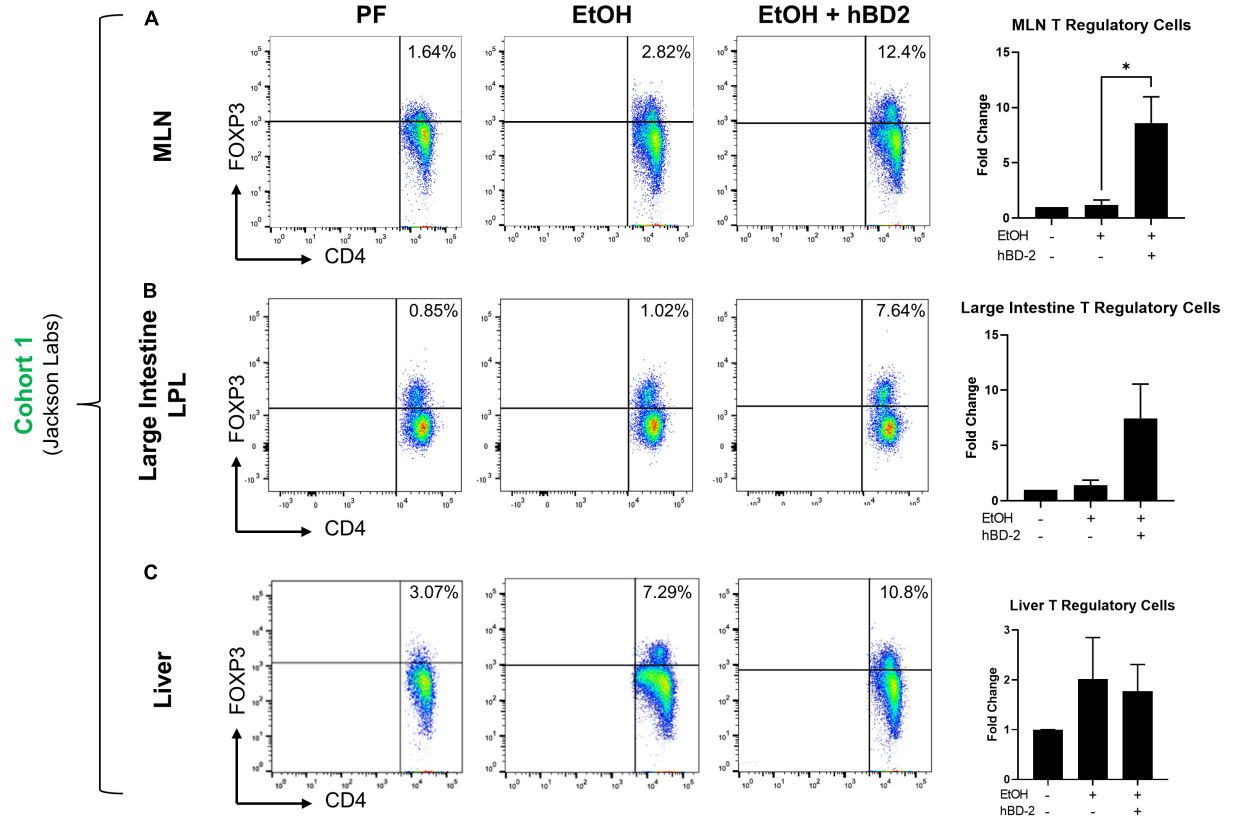

FIGURE 5 | Gut immunomodulatory effects of hBD-2 on Treg populations. Relative abundance of Tregs in EtOH-fed mice relative to pair-fed mice was determined in the mesenteric lymph nodes **(A)**, large intestine lamina propria **(B)**, and liver **(C)** in Cohort 1 animals. For each tissue, representative dot plots for each group are shown on the left (as determined by distance from mean), with percentages of FOXP3-positive cells displayed in the upper-right-hand quartile, and overall quantitation for the whole group shown on the right. Data are reported as mean ± standard error of the mean. *$p < 0.05$.

cells were altered in hBD-2 treated mice, including $CD4^+$ $IFN\gamma^+$ Th1 and $CD4^+$ $IL17^+$ Th17 T helper cells, $CD4^+$ $FOXP3^+$ T regulatory cells (Tregs), and $B220^+$ IgA^+ and $B220^+$ IgM^+ B cells. We analyzed these cell populations in the mesenteric lymph nodes (MLN), lamina propria, intraepithelial large and small intestine compartments, and liver of Cohort 1 mice (workflow and gating strategy is described in **Supplementary Figure 1**). We found that in the MLNs, hBD-2 caused a significant, ∼8.5-fold increase in Treg abundance relative to EtOH-fed mice (**Figure 5A**), indicating a distinct shift toward an immune-resolving profile. In the large intestine lamina propria, hBD-2 also caused an increase in the Treg population (**Figure 5B**), although

this trend did not reach statistical significance. In the liver, hBD-2 did not increase Treg abundance (**Figure 5C**), indicating a gut-specific effect. In addition, there were no significant effects of hBD-2 on the abundance of Th1/Th17 cells in the MLN, large intestine lamina propria, or liver, and IgA^+ and IgM^+ B cells in the MLN and small intestine lamina propria, despite some nominal trends (**Supplementary Figures 2A,B**, respectively).

Human Beta Defensin 2 Shifted the Composition of the Gut Microbiota Toward a Pair Fed-Like Phenotype

Since microbial imprinting is a major mechanism of AMPs, we next investigated whether hBD-2 administration altered the luminal gut microbiota in our two cohorts. Chronic EtOH feeding alone led to significant changes in gut microbiota, as apparent when comparing pair-fed (PF) and EtOH-fed mice in Cohort 1, which separated into distinct clusters by PCoA (**Figure 6A**). hBD-2, however, shifted the microbial composition in EtOH-fed mice toward a PF-like phenotype resulting in a clear separation of EtOH + hBD-2-treated mice from EtOH alone-treated mice in Cohort 1 (**Figure 6A**), as well as in Cohort 2 (**Figure 6B**), indicating a strong effect of hBD-2 on the EtOH-induced perturbation of the gut microbiota in both cohorts. Analysis of major phyla and genera revealed that in both study cohorts, Firmicutes, Bacteroides, and Verrucomicrobia constituted the most abundant phyla regardless of EtOH or hBD-2 treatment (**Figure 6C**). The ratio between Firmicutes and Bacteroides, an indicator of pathological changes in the gut microbiota (Stojanov et al., 2020), was increased by EtOH in Cohort 1 (**Figure 6D**), primarily due to an increase in Firmicutes. This increase was attenuated by hBD-2. Interestingly, in Cohort 2, the Firmicutes/Bacteroides ratio was relatively unchanged by hBD-2. At the genus level, in both cohorts, *Ruminococcaceae*, *Akkermansia*, and *Bacteroides* constituted the most abundant genera, whereas *Dubosiella* and *Parabacteroides* were prominent genera in Cohort 2 only (**Figure 6E**).

Next, we sought out specific taxonomical groups that were enriched by EtOH or hBD-2 administration (cladograms, **Figure 6F**). Taxonomical groups that were enriched by EtOH feeding included orders Betaproteobacteriales, Verrucomicrobiales, and Clostridiales, as well as several families including *Tannerellaceae*, *Burkholderiaceae*, *Akkermansiaceae*, *Rikenellaceae*, *Clostridiaceae*, *Ruminococcaceae*, and *Christensenellaceae*. In Cohort 1, relative to EtOH-fed mice, PF mice were enriched for orders Bacteroidales and Enterobacteriales, as well as family *Enterobacteriaceae*. In this cohort, hBD-2 enriched order Verrucomicrobiales and genus *Akkermansia*. hBD-2 also enriched several taxonomical groups in Cohort 2 including orders Desulfovibrionales and Bifidobacteriales as well as families *Peptococcaceae*, *Leuconostocaceae*, *Muribaculaceae*, and *Clostridialesvadin BB60*. When adjusting for differences in microbiota between the two cohorts *via* linear mixed-effects modeling, we further identified that numerous amplicon sequence variants (ASVs) within the family *Ruminococcaceae* were altered by hBD-2, indicating strong effects within this group (**Table 5**). Despite these varied EtOH and hBD-2 effects, neither condition changed overall alpha diversity by multiple indices (**Supplementary Figures 3A–C**).

TABLE 5 | Linear mixed-effects modeling identified bacterial genera which were altered by hBD-2 in both cohorts adjusted for cohort-specific differences.

Genus	*P* value
g__*Bacteroides*	0.0443
g__*Desulfovibrio*	0.0250
g__*Dubosiella*	0.0245
g__*Dubosiella*	0.0400
g__*Dubosiella*	0.0425
g__*Faecalibaculum*	0.0497
g__*Parabacteroides*	0.0099
g__*Parabacteroides*	0.0299
g__*Rikenellaceae RC9 gut group*	0.0325
g__*Ruminiclostridium 9*	0.0148
g__*Ruminococcaceae UCG-009*	0.0453
g__*Ruminococcaceae UCG-013*	0.0201
g__*Ruminococcaceae UCG-013*	0.0257
g__*Ruminococcaceae UCG-013*	0.0312
g__*Ruminococcaceae UCG –013*	0.0320
g__*Ruminococcaceae UCG-013*	0.0394
g__*Ruminococcaceae UCG-013*	0.0403
g__*Ruminococcaceae UCG-013*	0.0417
g__*Ruminococcaceae UCG-013*	0.0433
g__*Ruminococcaceae UCG-013*	0.0446
g__*Ruminococcaceae UCG-013*	0.0474
g__*Ruminococcaceae UCG-013*	0.0489

Lastly, we analyzed changes in abundance of several major genera with well characterized roles in ALD (**Figure 7**). hBD-2 led to a significant reduction in abundance of *Barnesiella*, *Parabacteroides*, and *Akkermansia* in our Cohort 2 mice (**Figures 7A–C**, respectively). Similarly, genus *Alistipes* was significantly decreased by hBD-2 in our Cohort 1 mice (**Figure 7D**). Another genus, *Roseburia*, was unaffected by EtOH and hBD-2 in both cohorts (**Figure 7E**). Additionally, these data revealed several differences between cohorts, specifically, an absence of *Barnesiella* and *Parabacteroides* in Cohort 1 (**Figures 7A,B**). The abundance of *Oscillobacter* and *Lactobacillus* was not affected in any group in both cohorts (**Figures 7F,G**).

DISCUSSION

In the current study, we demonstrated the beneficial effects of hBD-2 in an experimental model of chronic ALD (as summarized in **Figure 8**). hBD-2, administered orally on each of the last 7 days of the chronic EtOH feeding, attenuated liver injury as evidenced by a significant reduction in ALT levels. Our observation that hBD-2 is protective in ALD is in line with previous studies demonstrating that other AMPs such as CRAMP and alpha defensins ameliorate alcohol-associated liver injury, steatosis, and inflammation (Li et al., 2020; Zhong et al., 2020). Importantly, our results showed that the beneficial effects of hBD-2 on EtOH-induced liver injury were independent of baseline host gut microbiota. We observed that hBD-2 had a unified

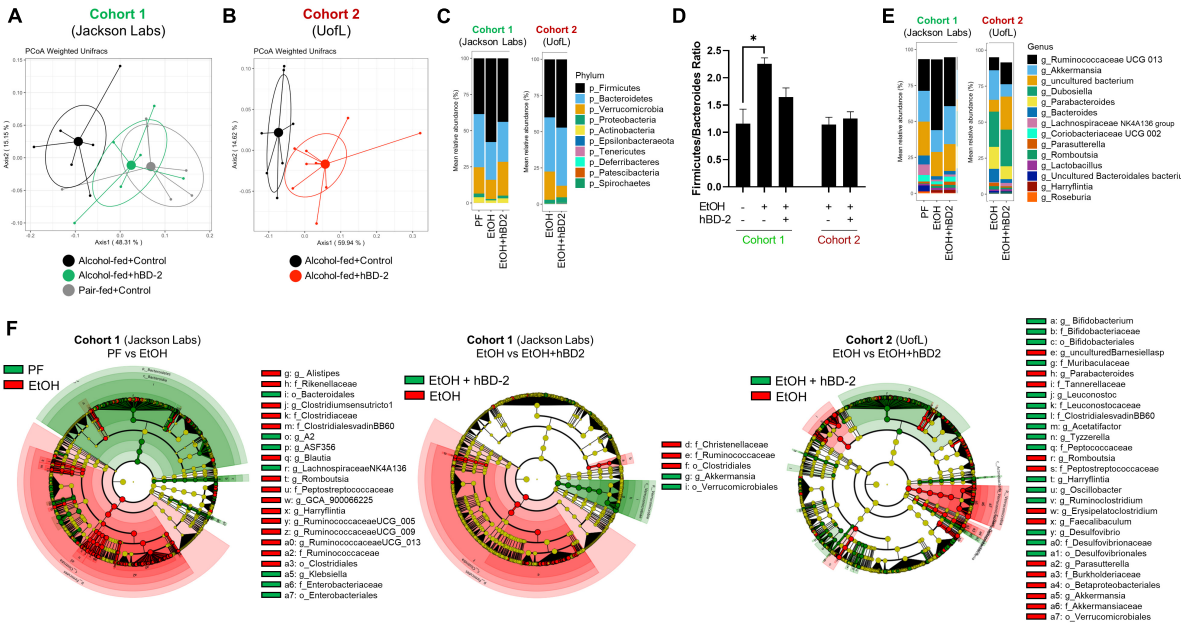

FIGURE 6 | Human beta defensin 2 alters the composition of the gut microbiota. **(A,B)** Principal coordinate analysis using weighted UniFrac distance at the conclusion of the study for Cohort 1 and 2, respectively. Individual data points are shown as points with indicated group mean and 50% confidence interval. PERMANOVA between EtOH and EtOH + hBD-2 $p = 0.037$ and $p = 0.002$ in panels **(A,B)**, respectively. **(C)** Phylum-level summary of mean relative bacterial abundance. **(D)** *Firmicutes/Bacteroides* ratio. Data are reported as mean ± standard error of the mean. $^*p < 0.05$. **(E)** Genus-level summary of mean relative bacterial abundance. **(F)** LEfSE cladograms showing taxonomical groups of bacteria associated PF or EtOH conditions in Cohort 1 (green and red, respectively; left), EtOH or EtOH + hBD-2 conditions in Cohort 1 (green and red, respectively; middle), and EtOH or EtOH + hBD-2 conditions in Cohort 2 (green and red, respectively; right).

protection against liver injury in two independent cohorts of mice (Cohort 1, vendor purchased, and Cohort 2, bred in-house) which had significant differences in baseline gut microbiota. These differences did, however, lead to distinct mechanisms governing the protective effects of hBD-2 against liver injury.

Indeed, while both cohorts were protected from liver injury, mechanistically, Cohort 2 mice had more profound changes in immunomodulatory cytokine levels. Specifically, hBD-2 administration in these mice led to significantly elevated levels of liver and intestinal IL-17A and IL-22. Interestingly, whereas IL-17A is typically considered a pro-inflammatory cytokine within the context of ALD (Lemmers et al., 2009), its expression is linked to increased anti-microbial peptide production, including defensins (Iwakura et al., 2008). IL-17 upregulates hBD-2 in the airway epithelium in a JAK/NFκB-mediated mechanism (Kao et al., 2004), suggesting a potential positive-feedback loop. hBD-2 also increased small intestine and liver IL-22 levels in our Cohort 2 mice. IL-22 is recognized as a beneficial cytokine in ALD (Ki et al., 2010; Sabat et al., 2014; Wang et al., 2014; Gulhane et al., 2016; Arab et al., 2020; Hwang et al., 2020; Xiang et al., 2020). Numerous reports demonstrate IL-22-mediated protection against alcohol-associated liver injury *via* upregulation of antioxidant and anti-apoptotic genes, downregulated lipogenesis, and increased liver regeneration and hepatocyte proliferation following injury (Ki et al., 2010; Hendrikx et al., 2019; Liu et al., 2020; Xiang et al., 2020). IL-22 also plays a defensive role in the gut mucosa against gram-negative microbes (Aujla and Kolls, 2009). hBD-2 may induce the expression of IL-17A and IL-22 through ROR-γt, a transcription factor which classically promotes Th17 differentiation (Ivanov et al., 2006) but has also been recently linked to Th22 differentiation (Plank et al., 2017; Sekimata et al., 2019), leading to downstream production of IL-17A and IL-22 by these T helper cell subtypes, respectively.

Whereas gut and liver IL-17A and IL-22 were unaffected by hBD-2 in Cohort 1, we did observe increased Treg abundance in these mice. Tregs are an immune-resolving T cell subset that plays a key role in a number of liver diseases (Van Herck et al., 2019), and is reduced in the liver in non-alcoholic fatty liver disease (Ma et al., 2007) and alcohol-associated hepatitis (Almeida et al., 2013). While we observed no difference in liver Tregs, we did notice a significant increase in mesenteric lymph node Tregs and a non-significant increase in large intestine Tregs. Gut Tregs may indirectly contribute to the attenuation of liver injury by maintaining gut homeostasis, preventing excess immune cell activation, and importantly, promoting gut barrier integrity *via* production of IL-10 (Corthay, 2009), although this mechanism needs to be further investigated. Further, while the exact mechanism by which hBD-2 increased gut Treg abundance is unclear, gut microbiota may have contributed to this increase, as some bacteria such as *Bacteroides fragilis* and *Clostridium rhamnosus* can induce the differentiation of some Treg phenotypes (Pandiyan et al., 2019). Apart from gut microbiota imprinting, a recent report elegantly demonstrated

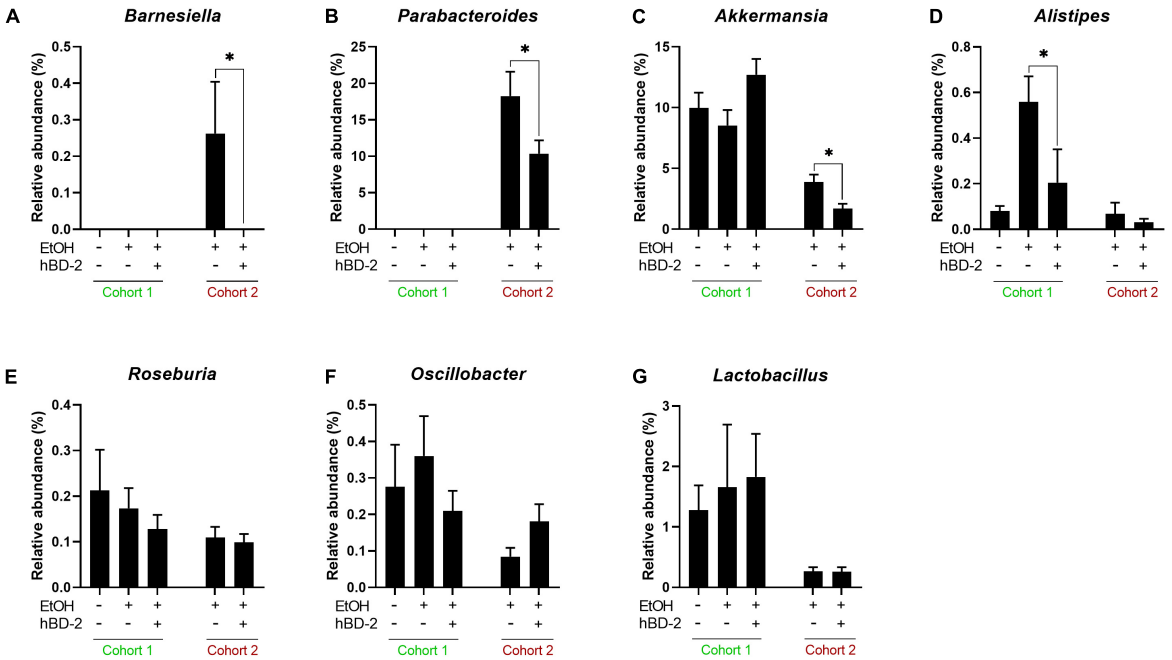

FIGURE 7 | Genus-level effects of hBD-2. Relative abundance (%) is shown for seven genera of interest in EtOH-associated liver disease, specifically, *Barnesiella*, *Parabacteroides*, *Akkermansia*, *Alistipes*, *Roseburia*, *Oscillobacter*, and *Lactobacillus* (**A–G**, respectively). Data are reported as mean ± standard error of the mean. $^{*}p < 0.05$.

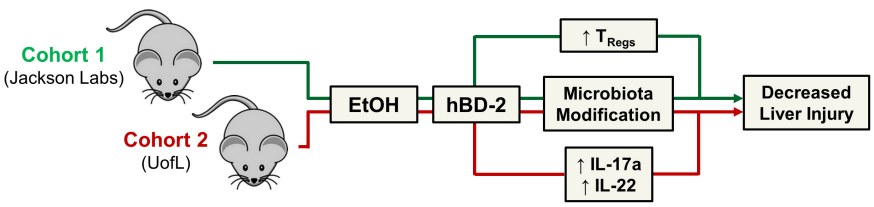

FIGURE 8 | Summary figure. Schematic representation of study findings. hBD-2 ameliorated experimental alcohol-associated liver disease in two independent cohorts of mice with distinct gut microbiota.

how CCR2 agonism enhances CD25 expression by FoxP3^{+} Tregs, thereby boosting their abundance (Zhan et al., 2020). Because hBD-2 curbs LPS-induced inflammation by CCR2 engagement (Koeninger et al., 2020), it seems possible that hBD-2 may potentially interact directly with gut Tregs *via* Treg-expressed-CCR2 to boost their abundance in the draining mesenteric lymph nodes. These results collectively suggest hBD-2 may function to modulate host immunity by upregulating beneficial cytokines and modulating Treg abundance to shift the liver and gut toward a less pro-inflammatory environment. This is in line with the growing concept that AMPs can act as signaling molecules with beneficial immunomodulatory functions, a role which beta defensins are thought to have acquired over evolutionary time (Meade and O'Farrelly, 2018). It is also possible that hBD-2 may function through other beneficial immunomodulatory effects. For example, hBD-2 was shown to enhance recruitment of circulating immature dendritic cells and memory T cells to sites of inflammation, improve antigen presentation *via* formation of defensin-antigen complexes to deliver antigens to immune cells (Yang et al., 2002; Auvynet and Rosenstein, 2009), and prevent toll like receptor-dependent pro-inflammatory pathway activation in dendritic cells (Koeninger et al., 2020).

While immunomodulation is increasingly recognized as a mechanism of action for AMPs, anti-microbial activity and gut microbiome modification is widely accepted as the primary mechanism for these molecules (Mahlapuu et al., 2016), as supported by several previous studies showing altered gut microbiota following AMP treatment (Li et al., 2020; Zhong et al., 2020). Similarly, in each of our cohorts, hBD-2 changed the composition of the luminal gut microbiota on multiple taxonomical levels, including the phylum, genus, and species levels. In Cohort 2, hBD-2 caused a decrease in abundance of bacteria within the genera *Parabacteroides* and *Barnesiella*, two gram-negative taxonomical groups within the phylum Bacteroides. While this phylum is generally considered beneficial in ALD (Ferrere et al., 2017), some evidence points to Bacteroides

as a contributor to colonic inflammation in ulcerative colitis (Shen et al., 2018). We also noted that hBD-2 decreased the EtOH-induced rise in the Firmicutes/Bacteroides ratio, an indicator of gut microbial dysbiosis. Within phylum Firmicutes, hBD-2 had a distinct modulating effect on numerous ASVs within the *Ruminococcaceae* family. Bacteria within this genus are generally thought to be beneficial in ALD, as both mice fed alcohol and patients with alcohol-associated cirrhosis have decreased *Ruminococcaceae* (Hartmann et al., 2015; Bajaj, 2019). Transplant of fecal material rich in *Ruminococcaceae* and *Lachnospiraceae* into patients with alcohol use disorder has been shown to decrease alcohol craving in a phase I clinical trial (Bajaj et al., 2020). *Ruminococcaceae* is also a major producer of the short-chain fatty acid butyrate (Vital et al., 2017), a beneficial molecule which attenuates liver injury and improves barrier function in experimental ALD (Cresci et al., 2017) and may potentially contribute to the beneficial effects of hBD-2 in our model.

Apart from these mechanistic effects of hBD-2, the question remains whether the beneficial effects of this peptide in the liver were mediated directly (i.e., host cell receptor-mediated *via* CCR2 or others) or indirectly (i.e., *via* the gut microbiota). Whether hBD-2 can cross the intestinal barrier to exert direct effects on the liver currently remains largely unknown. Potentially, in disease states with increased intestinal permeability, including ALD (Szabo, 2015), hBD-2 may be able to translocate to the systemic circulation. However, in general, peptides are considered to have low bioavailability after intestinal absorption due to rapid proteolytic cleavage (Diao and Meibohm, 2013). Despite this general assumption of diminished pharmacokinetics, it is worth noting that both intranasal and oral administration of hBD-2 alleviated experimental asthma (Borchers et al., 2021; Pinkerton et al., 2021), just as systemic administration of this peptide mitigated gut inflammation in three different colitis models (Koeninger et al., 2020), corroborating the pronounced extraintestinal efficacy of hBD-2. Alternatively, assessing the efficacy of hBD-2 in gut microbiota-depleted mice may also help to address this question. However, microbiota-deficient mice, e.g., germ free mice, have altered immunity and ethanol metabolism which confound the protective effects of hBD-2 (Chen et al., 2015). A previous report regarding the use of another AMP, fungal lysozyme, showed that in an experimental colitis model the presence of gut microbiota was required for the therapeutic effects of the treatment (Larsen et al., 2021), suggesting that the effects of hBD-2 may similarly be microbiota-dependent.

Overall, our data showed the beneficial effects of hBD-2 administration in experimental ALD *via* mechanisms associated with gut and liver immunomodulation and gut microbiota modification. Our data benefit from the use of two independent cohorts of mice with distinct gut microbiota which both showed protection against chronic alcohol-induced liver injury, albeit by different mechanisms. Future studies should, however, address the efficacy of hBD-2 in other experimental models of ALD, given that the chronic EtOH feeding model used here recapitulates mostly early onset features of human ALD (Lamas-Paz et al., 2018). The ability of hBD-2 to ameliorate more severe liver injury, inflammation, and fibrosis remains undetermined. Future work should also further examine the role of specific bacterial taxa such as *Ruminococcaceae* in hBD-2-mediated benefits in ALD.

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: ENA, PRJEB49248.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Louisville Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

All authors have reviewed and approved the manuscript. IK and JW: conceptualization. DW, JW, YS, and IL: methodology. JW and IL: software, writing—original draft preparation, and visualization. JW, IL, BJ, IK, and DW: formal analysis. IK, JW, IL, BJ, DW, and JH: investigation. IK, CM, and BJ: resources and funding acquisition. IL: data curation. JW, JH, DW, IL, BJ, CM, and IK: writing—review and editing. IK and BJ: supervision. IK: project administration.

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SUPPLEMENTARY MATERIAL

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