HHS Public Access

Author manuscript

Nat Rev Microbiol. Author manuscript; available in PMC 2024 November 01.

Published in final edited form as:

Nat Rev Microbiol. 2023 November ; 21(11): 719–733. doi:10.1038/s41579-023-00904-3.

The gut–liver axis and gut microbiota in health and liver disease

Cynthia Hsu[1], **Bernd Schnabl**[1,2,†]

[1]Department of Medicine, University of California San Diego, La Jolla, CA, USA,

[2]Department of Medicine, VA San Diego Healthcare System, San Diego, CA, USA

Abstract

The trillions of microorganisms in the human intestine are important regulators of health, and disruptions in the gut microbial communities can cause disease. The gut, liver, and immune system have a symbiotic relationship with these microorganisms. Environmental factors, such as high-fat diets and alcohol consumption, can disrupt and alter microbial communities. This dysbiosis can lead to dysfunction of the intestinal barrier, translocation of microbial components to liver, and development or progression of liver disease. Changes in metabolites produced by gut microorganisms can also contribute to liver disease. In this Review, we discuss the importance of the gut microbiota in maintenance of health and the alterations in microbial mediators that contribute to liver disease. We present strategies for modulation of the intestinal microbiota and/or their metabolites as potential treatments for liver disease.

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In this Review, Hsu and Schnabl examine the role of the gut microbiota in liver and intestine function, how gut microorganisms contribute to liver diseases, and explore different microbiota-based strategies for the treatment of liver disease.

Introduction

The gut and liver interact via several pathways. One way is their anatomical association — venous blood from the small and large intestine drains into the portal vein, which provides 75% of the liver's blood supply. These organs also interact via the microbiota — the liver is the first organ to encounter enterally absorbed nutrients and microbial metabolites. The liver, in turn, regulates the gut by secreting bile containing bile acids, immunoglobulin A,

† beschnabl@ucsd.edu.

Author contributions

The authors contributed equally to all aspects of the article.

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Competing interests

B.S. has been consulting for Ambys Medicines, Ferring Research Institute, Gelesis, HOST Therabiomics, Intercept Pharmaceuticals, Mabwell Therapeutics, Patara Pharmaceuticals and Takeda. B.S. is founder of Nterica Bio. UC San Diego has filed several patents with B.S. as inventor. B.S.'s institution UC San Diego has received research support from Artizan Biosciences, Axial Biotherapeutics, BiomX, CymaBay Therapeutics, NGM Biopharmaceuticals, Prodigy Biotech and Synlogic Operating Company. C.H. declares no competing interests.

and antimicrobial molecules directly into the small intestine, which completes the cycle by modulating the microbiota (FIG. 1).

An intact intestinal barrier is important for absorption of nutrients, but also keeps trillions of microorganisms contained in the lumen, to prevent them from spreading systemically. Microbial products and metabolites help maintain the gut barrier and liver health[1]. The disruption of intestinal microbial communities (dysbiosis), due to dietary or environmental changes such as high caloric intake or alcohol consumption, is a risk factor for liver disease. Although mechanisms of pathogenesis differ among hepatic diseases, intestinal dysbiosis has been associated with metabolic liver diseases such as alcohol-associated liver disease and non-alcoholic fatty liver disease (NAFLD). Dysbiosis of the microbiota has been shown to modulate liver diseases — liver disease development can be transmitted with fecal microbiota in preclinical models[2,3], and modest improvement in metabolic and liver pathologies are seen after limited follow-up in patients following fecal microbiota transplantation from healthy donors[4]. These observations indicate the importance of the state of the intestinal microbiota in liver function. In this Review, we explore how the gut, liver, and intestinal microbiota interact under healthy conditions, how a dysbiotic microbiota affects functions of the intestine and the liver, and potential interventional approaches to restore the intestinal ecosystem.

The gut microbiota and the gut–liver axis in health

The human gastrointestinal tract harbors over 100 trillion microorganisms including bacteria, fungi, viruses, and archaea that make up the gut microbiota[5–7]. Though the gut microbiota is often referred to as a uniform entity, there is considerable compositional variation between the different sections of the gastrointestinal tract from the mouth to the colon due to environmental differences such as pH and oxygen availability. For example, the proportion of obligate anaerobes increases from the duodenum (proximal small intestine) to the ileum (distal small intestine) and colon due to decreased oxygen availability, and the microbial density is highest in the colon due to the longer transit time, which allows for more proliferation[8]. Most of our understanding of the human gut microbiome is derived from the fecal microbiome, which cannot be extrapolated to the entire gastrointestinal tract. The phyla Bacteroidetes and Firmicutes predominate in the gastrointestinal tract, representing 90% of the gut microbiota[9,10]. At a genus level, the most abundant genera are *Bacteroides*, *Alistipes*, *Parabacteroides*, and *Prevotella* within the Bacteroidetes phyla, and *Faecalibacterium*, *Lachnospiraceae*, and *Roseburia* within the Firmicutes phylum. The Actinobacteria phylum is proportionally less abundant and mainly represented by the *Bifidobacterium* genus.

The gut microbiome is highly dynamic, with significant interindividual as well as intraindividual variation across time due to influences not limited to diet, activity, medications, and circadian rhythm. In a deeply symbiotic relationship, the gut microbiota carries out critical functions for their host, such as assisting with efficient nutrient extraction, modulating hormonal pathways via microbial-derived metabolites, metabolizing drugs and xenobiotics, and priming host immunity[11,12]. For example, intestinal microorganisms degrade fibers into short-chain fatty acids (SCFA) such as acetate, butyrate, and propionate,

which provide an important energy source for enterocytes in the colon and stimulate the antimicrobial activity of macrophages[13] and regulatory T cells (T_{Reg} cells)[14]. Meanwhile, the gut maintains a nutrient-rich, anaerobic luminal environment by consuming oxygen in the mitochondria of colonocytes to maintain an oxygen partial pressure of less than 7.6 mmHg[15], and by producing inducible nitric oxide and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 1 in the ileum[16].

The gut is also responsible for preventing the translocation of microorganisms and microbial antigens to the extraintestinal space, and this is regulated by several important barrier systems (FIG. 2). The mucus layer separates luminal bacteria from the underlying intestinal epithelial cells (IEC). It consists of mucins rich in glycoproteins and its composition is dictated by the gut microbiota[17]. The mucus layer is thickest in the colon and is divided into an inner and outer mucus layer[18]. Whereas the outer mucus layer contains bacteria, the inner mucus layer is largely devoid of bacteria. This is facilitated by secretion of antimicrobial molecules by intestinal epithelial cells and Paneth cells[19]. Plasma cells in the lamina propria produce immunoglobulin A (IgA), which is secreted as secretory IgA into the intestinal lumen[20]. IgA is essential for protection of the mucosal surface by binding and neutralizing harmful pathobionts and pathogens[20]. Besides secreting important antimicrobial defense molecules into the intestinal lumen, epithelial cells are also an important physical barrier. Epithelial cells are tightly bound together by adherens and tight junctions, which restrict the passage of viable bacteria or fungi, but allow paracellular passage of small molecules, such as SCFAs[21].

The next barrier comprises the immunologic defense systems in the intraepithelial compartment, lamina propria, and mesenteric lymph nodes. Under healthy conditions, the epithelial barrier allows microbial antigens and stimuli to pass into the lamina propria, where many different immune cells can respond. These interactions are critical for maintaining gut homeostasis and immune responses. For example, lamina propria M-cells and dendritic cells sample luminal and lamina propria microbial antigens and present them to naïve T cells in mesenteric lymph nodes to trigger an adaptive immune response and induce $CD4^+$ T_{reg} cells, which are crucial for developing tolerance and preventing chronic inflammation. Primed T-helper 17 (Th17) cells control the mucosal interface and prevent expansion of luminal fungi and other microorganisms[22]. Different T cell subsets and innate lymphoid cells secrete cytokines rapidly upon a microbial trigger. Type 3 innate lymphoid cells (ILC3) are activated by microbial metabolites including indoles as microbial-derived tryptophan metabolites, to produce interleukin 22 (IL-22). IL-22 signals via its receptor on intestinal epithelial cells to induce expression of antimicrobial genes and increase cell proliferation. Finally, neutrophils and macrophages in the lamina propria phagocyte viable bacteria that breach the mucus and epithelial cell layer.

Most venous blood from the small and large intestine drains into the portal vein. The gut vascular barrier, comprised of tight and adherens junction proteins on endothelial cells, prevents the translocation of viable bacteria from the lamina propria into the portal circulation. Portal vein blood contains enterally absorbed nutrients such as carbohydrates, lipids, and amino acids, which are taken up and metabolized or stored in hepatocytes, as well as microbial-derived metabolites. The liver is the first major organ to face these

microbial-derived metabolites, which are important for the immune and metabolic functions of the liver. For example, commensal-derived D-lactic acid promotes killing of circulating pathogens by Kupffer cells, the resident macrophages in the liver[23]. Bacterial sphingolipids from the gut symbiont *Bacteroides thetaiotaomicron* transfer to the liver and reduce lipid accumulation by increasing beta-oxidation in mice[24]. Lipopolysaccharides (LPS) and other microbial products are important to support immune tolerance and xenobiotic metabolism locally. Complete absence of microbiota in germ-free mice renders the liver more susceptible to toxins[1]. Kupffer cells are an important protective barrier, as they phagocytose translocated microbial products and viable bacteria. Sinusoidal endothelial cells sense microorganisms and signal for Kupffer cells and natural killer T (NKT) cells to localize to the periportal area, as the entry point of invading microorganisms[25]. This immune zonation in the liver protects against translocating gut bacteria[25]. IgA-producing plasma cells are primed in the intestine, migrate to the liver, and secrete IgA[26].

Gut–liver interactions are often considered a one-way path from the gut to the liver, but the liver also directly communicates with the gut via the biliary system. Hepatocytes synthesize and secrete bile, a solution rich in IgA, bicarbonate, antimicrobial molecules, and bile acids. Bile acids have multiple functions, such as facilitating the digestion and absorption of lipids and lipid-soluble vitamins and shape the gut microbial composition. Bile acids exert bacteriostatic effects both directly via their detergent properties and indirectly via activation of the bile acid receptor farnesoid X receptor (FXR) to stimulate the production of antimicrobial molecules in intestinal epithelial cells[27] and maintain the gut vascular barrier[28]. In rats, interruption of bile flow to the gut by bile duct ligation or choledochovesical fistula (diversion of bile flow from the bile duct directly into the urinary bladder) resulted in an increase in the cecal Gram-negative aerobic population and increased bacterial translocation[29]. In healthy human volunteers, suppression of endogenous bile acid synthesis by obeticholic acid, a bile acid analog and FXR agonist, led to proliferation of small-intestinal Gram-positive bacteria[30]. Conversely, gut microorganisms can also shape bile acid supply and composition by deconjugating and dehydroxylating liver-derived primary bile acids into secondary bile acids, which are not as efficiently reabsorbed in the ileum[31]. In the ileum, bile acid activation of the FXR target gene fibroblast growth factor (FGF) 15 in mice and FGF19 in humans not only suppresses hepatic synthesis of bile acids in a self-regulating feedback loop[32], but also mediates metabolic effects such as regulation of insulin sensitivity and stimulation of hepatic protein and glycogen synthesis[33].

A third route of communication between the gut and the liver is the systemic circulation, through which metabolites, products, or inflammatory mediators derived from the liver reach the gut via the arterial blood supply. Taken together, the gut and liver have a multidirectional interaction that is vital for both organs.

The gut microbiota and the gut–liver axis in liver disease

Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) encompasses a spectrum of liver pathologies, including fatty liver, which progresses in a minority of patients to nonalcoholic steatohepatitis (NASH) with fibrosis[34] (BOX 1). Patients with NAFLD present with

changes in the intestinal microbiota composition (FIG. 3), though taxonomic differences specific to NAFLD are occasionally conflicting and difficult to parse from those caused by or resulting from comorbid conditions of obesity, diet, and metabolic syndrome. For example, bacterial dysbiosis is different in patients with NAFLD who are lean versus obese. A study of patients with NAFLD and without obesity in Asia found decreased bacterial diversity, reduced proportions of Ruminococcaceae and increased proportions of Veillonellaceae, and these changes were associated with fibrosis severity[35]. Similarly, patients with NASH or liver fibrosis in stages F2–F4 and without obesity showed a distinct fecal fungal microbiome (mycobiome) signature[36]. The degree of liver fibrosis also affects the microbiome composition, as fecal proportions of *Ruminococcus obeum* CAG:39 and *E. rectale* were significantly lower in patients with advanced fibrosis than mild or moderate NAFLD[37]. Patients with NASH with advanced fibrosis also had a decrease in intestinal viral diversity and a reduction in the proportion of bacteriophages (phages) compared with other intestinal viruses[38]. Specific viral taxa, such as several *Lactococcus* phages, were less frequently present in patients with more severe disease, though whether this is caused by gut bacterial dysbiosis or a result of dysbiosis is unknown[38].

Gut dysbiosis induces intestinal inflammation, which contributes to intestinal barrier dysfunction and translocation of microbe-associated molecular patterns (MAMPs), such as LPS, to the liver and systemic circulation[39]. This pathophysiology was seen in a high fat diet mouse model of NASH which demonstrated dysbiosis and increased epithelial and gut vascular permeability, suggesting a possible mechanism of NASH pathogenesis[28], and mouse models of diet-induced steatohepatitis demonstrate positive correlation between levels of serum LPS and liver injury[40]. However, only about one half of patients with NASH have increased intestinal permeability[41].

Dysbiosis is also associated with functional metabolic consequences. Anaerobic bacteria ferment carbohydrates to produce ethanol, which is increased in patients with NASH under conditions that are incompletely understood. Individuals with NAFLD and NASH exhibited higher ethanol concentrations in their portal vein blood compared with individuals without hepatic steatosis[42], and similar results were also seen in the peripheral blood of pediatric NAFLD patients[43]. In the most extreme case, the microbiota of patients with auto-brewery syndrome produce enough ethanol to make them drunk and incapacitated[44]. Hepatocytes metabolize ethanol into acetate and eventually triglycerides, resulting in steatosis. This is accompanied by oxidative stress resulting in death of hepatocytes and progression of liver disease[44]. The prevalence of patients who develop liver disease via this mechanism is not known, but it is likely that ethanol-producing bacteria contribute to liver disease in only a subgroup of NAFLD patients.

Other microbial metabolites such as phenylactetate and N,N,N-trimethyl-5-aminovaleric acid (TMAVA) have also been implicated in NAFLD pathogenesis. Serum phenylacetate was significantly associated with steatosis in women with obesity, and mice fed phenylacetate demonstrated increased hepatic triglyceride accumulation[45]. Plasma levels of TMAVA, metabolized from trimethyllysin by intestinal *Enterococcus faecalis* and *Pseudomonas aeruginosa*, were increased in patients with hepatic steatosis, and mice given TMAVA on a high fat diet demonstrated reduced carnitine synthesis and fatty acid oxidation and

increased hepatic steatosis[46]. Conversely, gut microorganism-derived tryptophan metabolites such as indole-3-acetate and indole-3-propionate have been shown to suppress inflammation and attenuate hepatic steatosis in NAFLD or NASH[47,48]. Tryptophan metabolites and their effects on the immune system in NAFLD have been thoroughly reviewed[49].

Patients with NAFLD or NASH also have higher serum levels of total bile acids compared with healthy controls, but a relative increase of secondary bile acids compared to primary bile acids along with a higher abundance of taurine and glycine-metabolizing gut bacteria[50,51]. These changes in the composition of the bile acid profile results in reduced FXR activity in enterocytes and a dysfunctional negative-feedback loop, via FGF15 or FGF19. Despite increased systemic levels of bile acid, de novo hepatic synthesis of bile acids is also increased in patients with NASH[52]. Interestingly, the role of FXR signaling in NAFLD pathogenesis is controversial. While one study demonstrated that high-fat diet-induced hepatic steatosis was reduced with intestinal FXR stimulation in mice[53], another showed that it was reduced in intestine-specific *Fxr*-knockout mice[54]. Because of the bidirectional relationship of bile acids in the gut–liver axis, it remains to be shown what is a cause and what is a consequence. Nevertheless, bile acids control immune system functions through their interaction between host and bacteria. Several secondary bile acids induce T_{reg} cells, either directly or by stimulating dendritic cells through FXR[55–57]. It is not known whether this mechanism is involved in modulating liver disease. In addition, bile acids are amphipathic detergents that can directly cause hepatocyte damage — especially in patients with increased bile acid levels due to cholestatic conditions (reduction in bile secretion and flow)[58]. Finally, while much of our understanding of bile acids in health and disease stems from rodent studies, it is important to consider some significant differences in bile acid composition between humans and mice[59]. For example, mice have more abundant hydrophilic bile acid species and 6-hydroxylated bile acids, which are quite rare in humans.

Studies of fecal microbiota transfer in preclinical models indicate that progression, and in some cases the onset, of hepatic steatosis and steatohepatitis is affected by the gut microbiota. Antibiotics and antifungals reduce diet-induced steatohepatitis in mice[36,39]. Furthermore, early-life exposure to microbiota from wild mice confers resistance to high-fat diet-induced obesity and hepatic steatosis[60]. It is not clear how much of the clinical NAFLD phenotype in patients is driven by dysbiosis and its functional consequences. Gut dysbiosis can be considered a risk factor in a subset of patients and might be an additional hit that causes progression of disease in patients who otherwise would not progress to more advanced disease. It will be important to better phenotype patients with NAFLD and identify which patients might benefit from a microbiome-centered therapy. Such a personalized medicine approach is likely to achieve better treatment outcomes than the current one-size-fits-all clinical trials.

Alcohol-associated liver disease

Hepatic steatosis will develop in most patients with alcohol use disorder, but less than 50% will progress to more advanced disease with fibrosis, cirrhosis, or alcohol-associated hepatitis (BOX 1). Patients with alcohol-associated liver disease have a dysbiotic gut microbiota that is generally characterized by decreased bacterial diversity, increased

proportions of pathobionts such as enterococci[61], and reductions in beneficial bacteria such as *Akkermansia muciniphila*[62] (FIG. 3). Stool samples from patients with alcohol-associated liver disease have decreased fungal diversity and increased *Candida albicans*[63,64]. One study of stool samples from patients with alcohol use disorder found higher abundance of *Enterobacteria* and *Lactococcus* phages in samples from patients with more progressive liver disease[65], and a higher abundance of *Lactococcus* phages was also seen in NAFLD patients who consumed low levels of alcohol[66]. Another study found that *Escherichia*, *Enterobacteria*, and *Enterococcus* phages were overrepresented in stool samples from patients with alcohol-associated hepatitis, whereas *Parabacteroides* phages were underrepresented[67]. Viral diversity and the proportion of mammalian viruses are increased in fecal samples from patients with alcohol-associated hepatitis.

Similar to NAFLD, an altered gut microbiota is considered a risk factor for progression of liver disease, but it is not clear if it is a causative factor. Increased intestinal permeability is present in less than half of patients with alcohol use disorder and early liver disease[68]. MAMPs such as LPS translocate to the liver and cause progression of liver disease via activation of pathogen recognition receptors such as toll like receptor 4 (TLR4) in the liver[69]. This more traditional view of pathogenesis has dominated the field for many years. However, we now know that increased permeability is only one piece of the puzzle. MAMP signaling via their receptors alone is necessary, but not sufficient to cause alcohol-associated liver disease[70].

Several dysregulated microbial metabolites might also contribute to gut barrier dysfunction. SCFAs are decreased in the stool of patients with alcohol-associated liver disease[71], which is associated with gut barrier dysfunction[72], though a limitation to the measurement of fecal SCFAs is their rapid metabolism by gut microbiota or absorption by colonocytes limiting the remaining measurable SCFAs pool in feces[73]. Microbial metabolites of tryptophan metabolism such as indoles are decreased in fecal samples of patients with alcohol-associated hepatitis[74]. Indoles are aryl hydrocarbon receptor ligands and stimulate IL-22 in ILC3 cells, which induces antimicrobial proteins including regenerating islet derived factor 3 (REG3)[74]. These molecules maintain an inner mucus layer devoid of bacteria to reduce bacterial translocation. Once this protective barrier is disrupted during chronic ethanol administration, translocation of viable bacteria occurs even during early liver disease in mice[70].

Bile acid metabolism is also dysregulated in patients with alcohol-associated liver disease. This is characterized by increased total systemic bile acids and suppressed hepatic synthesis of bile acids[58]. Intestinal inflammation can be reduced, gut barrier function restored, and ethanol-induced liver disease decreased following intestine-restricted FXR stimulation in mice[75].

Plasma levels of trimethylamine (TMA), a microbial product of choline metabolism, were elevated in patients with alcohol-associated hepatitis, whereas TMA n-oxide (TMAO), a co-metabolite generated in hepatocytes from TMA and linked to cardiovascular disease, was decreased in patients with alcohol-associated hepatitis. Small molecule inhibition of gut microbial CutC and CutD activity, which is involved in TMA synthesis, reduced ethanol-

induced liver disease in mice[76]. Further studies are required to test whether TMA affects the gut barrier or exacerbates disease at the level of the liver.

Exotoxins are actively secreted microbial toxins that target other bacteria or host cells. Pore-forming toxins often consist of multiple subunits that oligomerize and form pores in cell membranes. Cytolysin is a two-subunit exotoxin secreted by *E. faecalis*[77]. Approximately one third of patients with a severe form of alcohol-associated liver disease, alcohol-associated hepatitis, are colonized by cytolysin-positive *E. faecalis*, which is associated with high mortality[61]. Cytolysin appears to be specific for alcohol-associated liver disease, as the prevalence for cytolysin positivity was low in patients with NAFLD and there was no correlation with disease severity[78]. Cytolysin promotes ethanol-induced liver disease in preclinical models and causes direct hepatocyte death, presumably via direct pore formation in the cell membrane[61]. It is feasible that within-host evolution of cytolysin-positive *E. faecalis* allows colonization of the mucosal niche and facilitate bacterial translocation, as was shown for the pathobiont *Enterococcus gallinarium*[79].

Candidalysin is another pore-forming, cytolytic peptide secreted by *C. albicans*, and it is encoded by hyphae-associated gene *ECE1*[80]. Patients with alcohol-associated hepatitis and colonized with *ECE1*-positive *C. albicans* had increased mortality[63]. Candidalysin exacerbates ethanol-induced liver disease and damages hepatocytes in vitro[63]. Candidalysin-induced Th17 response[81] and intracellular activation of the Nlrp3 inflammasome in macrophages and dendritic cells[82] might further enhance tissue damage. Cytolysin and candidalysin are not associated with increased epithelial cell damage or gut barrier dysfunction in liver disease models. It is unknown how the peptide toxins get to the liver—whether by direct translocation of the encoding viable microorganisms to the liver or in synergy with other microbial virulence factors produced and released in the intestinal lumen.

Taken together, several microbiota-related pathways contribute to alcohol-associated liver disease, including gut barrier dysfunction.

Cirrhosis

Cirrhosis, characterized by a decrease in functional hepatocytes and an increase in fibrotic tissue, is the end stage of chronic liver disease and can have multiple etiologies (BOX 1). A common consequence of cirrhosis is decreased bile flow (cholestasis) due to remodeling and scarring of liver tissue. These changes, along with decreased gut motility[83], lead to the small intestinal bacterial overgrowth[84] and intestinal dysbiosis seen commonly in cirrhosis. In addition to decreased bacterial diversity, proportional increases in *Escherichia coli*, *Acidaminococcus sp. D21*, and *Klebsiella pneumoniae*; and decreases in *Eubacterium eligens*, *Eubacterium rectale, Dorea longicatena*, and *Faecalibacterium prausnitzii* were found in patients with cirrhosis[56] (FIG. 3). Patients with cirrhosis also present differences in the fecal virome. One study showed that *Bullavirinae*, *Felixounavirus*, *Streptococcus*, *Escherichia*, and *Pseudomonas* phages were positively linked with model for end-stage liver disease (MELD) score, whereas *Faecalibacterium* phages were negatively linked with MELD score[85]. Differences in intestinal fungal populations were found to be predictive of hospitalization in patients with cirrhosis; a lower ratio of the bacterial phylum Bacteroidetes

to the fungal phylum Ascomycota was associated with lower likelihood of 90-day hospitalization[86].

Most patients with cirrhosis have increased intestinal permeability, which allows MAMPs to translocate to the circulation and contribute to an increase in systemic inflammation[87]. Once disease progresses, patients with decompensated cirrhosis have translocation of viable bacteria, causing infections such as bacterial peritonitis. Cirrhosis-associated immune dysfunction, with a decrease in phagocytic activity of bacteria by neutrophils, monocytes, and macrophages, contributes to increased infections in patients with decompensated cirrhosis[88]. A vicious cycle that includes worsening gut dysbiosis, increased gut permeability, systemic inflammation, and deteriorating liver disease with less bile flow, exacerbates progression of disease.

Dysbiosis changes the metabolism of intestinal bile acids, with less conversion of primary to secondary bile acids. Patients with advanced cirrhosis had the lowest amount of total and secondary bile acids in stool, compared with controls or patients with early cirrhosis[89]. Patients with cirrhosis had increased levels, in particular, of conjugated serum bile acids[89]. Total and conjugated serum bile acids correlated with liver disease severity in patients with alcohol-associated hepatitis, most of which had underlying cirrhosis[58]. Changes in luminal bile acids and in FXR activity are connected to gut barrier dysfunction and bacterial translocation. Oral treatment with FXR agonists reduced translocation of viable bacteria from the intestine to the liver in mice with cirrhosis by increasing expression of tight-junction proteins in epithelial cells and restoring the gut vascular barrier[90].

Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is a feared complication of advanced liver disease that develops in about one third of patients with compensated cirrhosis (BOX 1). By contrast, few patients develop HCC in the absence of advanced fibrosis or cirrhosis. The main risk factors for HCC are chronic hepatitis B or C virus infection, heavy alcohol use, diabetes, and more recently described, NAFLD[91].

A few studies have compared the gut microbiomes of patients with cirrhosis, with and without HCC. Fecal microbial diversity is decreased in patients with cirrhosis compared with healthy controls, but then diversity increases with progression of cirrhosis to HCC[92]. Other microbiota changes include decreased *Bifidobacterium* and *Akkermansia* species and increased *Enterococcus* species[92–94] in fecal samples from patients with HCC compared to patients with cirrhosis without HCC (FIG. 3). Further, butyrate-producing bacteria such as the genera *Ruminococcus, Oscillibacter, Faecalibacterium, Clostridium IV* and *Coprococcus* were decreased in samples from patients with early-stage HCC, whereas LPS-producers such as *Klebsiella* and *Haemophilus* were increased compared with controls[92].

Gut microbiota are believed to contribute to the pathogenesis of HCC. MYC-transgenic mice, which are predisposed to development of spontaneous HCC, were found to have fewer and smaller HCCs when they were given antibiotics[95]. This study further demonstrated that primary bile acids increased accumulation of hepatic NKT cells, whereas secondary bile acids reversed this; therefore, depletion of secondary bile acids by antibiotics led to

NKT cell accumulation and antitumor activity in the liver. One specific secondary bile acid, deoxycholic acid, was found to be more abundant in individuals with obesity; blocking production of deoxycholic acid with antibiotics prevented HCC development in obese mice[96].

In addition to modulating immune reactions in the liver, secondary bile acids may also affect gut permeability. One proposed mechanism is via the proteolytic enzyme gelatinase E (GelE), secreted by *E. faecalis*. GelE induces loss of barrier function in mice with colitis[97]. Patients with chronic liver disease have increased colonization by GelE-positive *E. faecalis*, through altered deoxycholic acid production. GelE-positive *E. faecalis* increase gut permeability, and their translocation to the liver promotes liver carcinogenesis[98]. GelE-positive *E. faecalis* therefore have synergistic detrimental effects in intestine and liver.

Previous studies have linked gut permeability-induced activation of inflammatory signaling via toll-like receptors (TLRs), gut translocation of MAMPs, and development of liver disease and HCC. Rats with diethylnitrosamine-induced HCC have increased serum levels of LPS, and administration of antibiotics decreased tumor size and number[99]. The same study showed that TLR4-knockout mice given injections of diethylnitrosamine have significantly decreased tumor burdens compared with wild-type mice. Gut sterilization significantly reduced tumor burden in mice given diethylnitrosamine plus carbon tetrachloride to induce HCC. However prolonged administration of low-dose LPS after gut sterilization significantly increased HCC development[100]. In humans, higher circulating levels of serum anti-LPS and anti-flagellin immunoglobulins, consistent with chronic exposure to bacterial products LPS and flagellin, was significantly associated with increased risk of HCC[101].

Translating microbiota research into novel therapies for liver disease

Lifestyle modifications such as alcohol cessation and weight loss remain the cornerstones of treatment for alcohol-associated and non-alcoholic liver disease, respectively, and gut microbiota play a key role. The gut microbiota is critical for nutrient absorption – germ-free mice have lower body fat than conventionally raised mice despite consuming more calories[102], and obesity is transmissible via colonization of germ-free mice with gut microbiota from obese mice[103]. Similarly, alcohol use behavior may be modulated by gut microbiota. Several bacteria are capable of producing neurotransmitters such as γ-aminobutyric acid, serotonin, and dopamine[104,105] and gut microbiota influence blood-brain barrier permeability[105]. Given the scope of these topics, this section will not cover the exciting preclinical work focused on diet and lifestyle-based therapies for liver disease.

Here, we will review emerging therapies that manipulate the gut–liver interaction for treatment of liver disease. Small clinical trials of untargeted therapies such as fecal microbiota transplantation (FMT), probiotics, and antibiotics (Table 1) have had encouraging outcomes. Preclinical studies of engineered bacteria or phages have revealed novel therapeutic approaches. We discuss the results from studies of untargeted therapies and provide a detailed summary of recent randomized clinical trials (Table 1) and a comprehensive overview of targeted therapies (FIG. 4).

Fecal microbiota transplantation

Fecal microbiota transplantation, which introduces stool from a healthy donor into a patient's gastrointestinal tract, is a Food and Drug Administration-approved therapy for recurrent *Clostridioides difficile* infection and has had promising results in clinical trials for NAFLD, alcohol-associated liver disease, alcohol-associated hepatitis, and hepatic encephalopathy (Table 1). Although the exact mechanisms of FMT are unclear, it has been shown to restore gut barrier function and increases synthesis of SCFA[106,107]. However, there are still several outstanding issues surrounding broader implementation of FMT in the treatment of chronic liver diseases. For example, the route and frequency of fecal microbiota administration varies among clinical trials, and includes via oral capsules, nasogastric or nasojejunal tubes, upper endoscopy, colonoscopy, or rectal enemas, performed once in some studies, or through several daily or monthly instillations in others (Table 1)[108]. While there are no head-to-head clinical trials comparing efficacy and engraftment achieved by the different routes and frequencies for FMT, one FMT trial in patients with type 2 diabetes mellitus and obesity showed improved microbial engraftment with repeated FMTs and association with improved liver stiffness[109]. A recent study performed metagenomic analysis of the stool microbiomes of 226 triads of donors, pre-FMT recipients, and post-FMT recipients to compare methods of FMT in a systematic meta-analysis of 24 studies that used FMT in different clinical settings[110]. This study found that strain engraftment rate positively correlated with clinical response to FMT, and antibiotic treatment prior to FMT greatly improved strain engraftment. Additionally, the route of FMT delivery was most significantly associated with engraftment rate, with mixed route of FMT administration (combining upper and lower gastrointestinal tract administration) yielding the highest engraftment. Bacteroidetes and Actinobacteria species demonstrated higher average strain engraftment rates than Firmicutes and Proteobacteria species, and Gram-positive bacteria were less likely to have high rates of engraftment compared with more resistant Gram-negative species. These findings emphasize the importance of characterizing and possibly even standardizing FMT donor stool. Some trials attempt to provide more targeted FMT therapy by selecting a donor sample enriched in *Lachnospiraceae* and *Ruminococcaceae*[106,111], but concern regarding transfer of multidrug-resistant bacteria after FMT still exists[112]. SER-109, an oral therapeutic composed of live purified Firmicutes bacterial spores, circumvents this concern and effectively reduced risk of *C. difficile* infection recurrence in patients with history of recurrent *C. difficile* infection[113]. Similar purified bacterial formulations may be developed in the future for the treatment of liver diseases.

Phages

Phages are the natural predators of bacteria and make up the majority of the intestinal virome[114]. Recent metagenomic studies demonstrated changes in intestinal phage composition in patients with liver disease[65,67]. There have been preclinical studies of the ability of phages to selectively target pathogenic bacterial species and their effects in models of liver disease. Colonization of gnotobiotic mice with fecal microbiota from patients with alcohol-associated hepatitis containing cytolysin-positive *E. faecalis* resulted in exacerbation of liver disease; administration of phages that target cytolytic *E. faecalis* reversed liver disease progression[61]. Steatohepatitis develops in healthy mice transplanted with fecal microbiota from gnotobiotic mice that had received fecal microbiota from a patient with

NASH; but pre-treatment with phages that selectively target an ethanol-producing strain of *Klebsiella pneumoniae* prevented the development of diet-induced steatohepatitis[44]. Although the target selectivity of phages can be a therapeutic advantage, it also hinders widespread clinical use, because only patients with specific bacterial strains in the intestinal microbiota are likely to respond. The development of phage cocktails or genetic engineering of a phage species to target multiple receptors might extend its host range for broader therapeutic applications.

Engineered bacteria

Probiotics (live bacteria thought to promote health) have been known to have beneficial effects for decades. Some clinical trials of probiotics, mostly from the *Lactobacillus* and *Bifidobacterium* genera, in patients with liver diseases such as NAFLD, alcohol-associated liver disease, or hepatic encephalopathy, have had moderate success while others have had equivocal results (Table 1). Due to the wide variability of probiotic formulations and their untargeted nature, results from clinical trials have been difficult to replicate.

The idea of using genetically engineered bacteria as targeted therapies has gained traction. For example, expression of IL-22, a cytokine that regulates the production of REG3G lectins to mediate an intestinal immune response against pathogens, is lower in ethanol-induced liver disease. One study showed that mice fed *Lactobacillus reuteri* engineered to produce IL-22 along with an ethanol diet developed less severe liver damage, inflammation, and bacterial translocation to the liver as compared with mice fed a wild-type strain of *L. reuteri*[74]. In another study, oral administration of a genetically engineered *E. coli* strain (SYNB1020) that metabolized ammonia and converted it to L-arginine in the gut to mice with thioacetamide-induced liver injury lowered the systemic levels of ammonia[115]. Unfortunately, a phase Ib/IIa randomized controlled trial in patients with cirrhosis and elevated blood ammonia levels did not show a change in systemic ammonia or other endpoints, compared with placebo (ClinicalTrials.gov Identifier: NCT03447730)[116]. Nevertheless, the concept of using genetically engineered bacteria to treat liver diseases remains attractive and may be facilitated by the development of methods for genetically engineering native host bacteria as a chassis for transgene delivery[117]. In this study, authors engineered the native host *E. coli* to express bile salt hydrolase and demonstrated stable engraftment and improved insulin sensitivity and glucose tolerance in treated mice.

Prebiotics and postbiotics

An alternative to viable microorganisms is the use of prebiotics, non-digestible dietary substances, or postbiotics, inactivated microbial cells or cell components[118] that confer health benefits. For example, the prebiotic lactulose, a non-absorbable disaccharide, is a clinical staple for the treatment of hepatic encephalopathy (BOX1). Preclinical trials suggest that supplementation with prebiotics such as inulin, fructooligosaccharides, and pectin may attenuate hepatic steatosis and liver injury in diet- and ethanol-induced liver injury models, respectively[119–121]. In addition to modifying the composition and function of gut microbiota, prebiotics are fermented by the gut microorganisms into SCFA[122]. In a mouse model of ethanol-induced liver injury, supplementation of the SCFA butyrate in the form of tributyrin protected mice from disrupted intestinal permeability and liver injury[117].

However, the benefits of SCFA supplementation for liver disease are controversial. One study demonstrated that increased SCFA production in response to supplementation of the soluble fiber inulin in dysbiotic TLR5-deficient mice that were fed a high-fat diet led to development of HCC[123]. There are no clinical trials to date testing the therapeutic efficacy of SCFA in patients with liver disease.

Postbiotics previously encompassed a broader definition[118], but some preclinical and clinical trials of postbiotics under the new stricter definition do show promise. A small trial of 32 insulin-resistant volunteers with obesity or who are overweight showed that oral supplementation of pasteurized *Akkermansia muciniphila* improved metabolic parameters compared with control[124]. Preclinical trials using exopolysaccharide derived from lactic acid bacteria or postbiotics prepared from *Lactobacillus paracasei* demonstrated benefits in insulin sensitivity and NAFLD[125,126]. Further research is needed to understand what specific postbiotic components are most likely to benefit patients with liver disease and in what context.

Implications and future directions

The composition of the intestinal microbiota affects all aspects of gut–liver interactions, from the intestinal epithelium, to the gut vascular barrier, to the liver sinusoids, and back to the gut via the biliary tree and the systemic circulation. In a healthy state, the gut microbiota is contained within the gut lumen, but its metabolites act locally and systemically to maintain homeostasis of the gut–liver axis. Disruption of the homeostasis at any of these interfaces can contribute to liver disease. Hence, therapeutic strategies for liver diseases that modulate the gut microbiota, such as fine-tuning of fecal microbiota transplantation, or tailored approaches that specifically target the mechanisms by which dysbiosis leads to liver injury, are being developed.

An important aspect to the success of therapeutics that aim to target the gut–liver axis is the ability to personalize the intervention to specific patients within a disease population. One relatively unexplored avenue of research into more individualized microbiome risk factors for liver disease is to study the interplay between individuals' genetics and their gut microbiome. For example, variants in the patatin-like phospholipase 3 (PNPLA3), transmembrane 6 superfamily member 2 (TM6SF2), and membrane bound O-acyltransferase domain-containing 7 (MBOAT7) genes are some of the strongest genetic contributors to NAFLD and alcohol-associated liver disease[127] and their effects on the microbiome needs further study.

Another avenue that will open new opportunities for both patient research and diagnostic testing is the development of better technologies to measure microbial communities and metabolites in vivo. Currently, our standard methods for investigating patients' gut microbial communities or gut microbial metabolite concentrations are from fecal samples, which limits investigation of the whole gastrointestinal tract, or from endoscopic evaluation, which requires an invasive procedure. Development of non-invasive methods to detect concentrations of microbial metabolites in vivo will allow researchers to understand where

in the gastrointestinal tract these metabolites are being produced and allow for increased ease of diagnostic testing in patients.

Finally, development of more precise therapeutic options will improve both the safety and efficacy of gut microbiota-modulating treatments. Preclinical studies have evaluated new precision approaches, such as the use of bacteriophages or engineered bacteria, but these results have yet to be replicated in humans. If they are found to be safe and effective in clinical trials, these therapies have the potential of transforming the way liver disease is managed in clinical practice, including more precise interventions with fewer side effects. Safety remains a concern for translating these therapies into humans. The durability of treatment has been another concern, though more widespread use of genetic engineering of native (host) bacteria may facilitate stable engraftment and durable results. Although preclinical studies have provided valuable proof of concept, translating their findings into humans is critical, as rodent microbiomes, even those of gnotobiotic mice implanted with human microbiota, cannot fully recapitulate the intricacies of the human gut–liver axis. Nevertheless, our growing understanding of the gut–liver axis confirms that the intestinal microbiota is a compelling target for novel liver disease therapies.

Acknowledgments

C.L.H. is supported by a Pilot and Feasibility Award from the Southern California Research Center for ALPD and Cirrhosis P50 AA011999 and by T32 DK007202. The review was supported by services provided by NIH center P30 DK120515.

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BOX 1.

Liver disease overview

Liver diseases have different etiologies, including viral (hepatitis A, B, or C viruses), autoimmune (acute autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis), metabolic (nonalcoholic fatty liver disease), and toxic causes (alcohol, acetaminophen). Over time, all forms of chronic liver injury progress from fibrosis (hepatocyte death and scarring of the liver) to cirrhosis (advanced fibrosis and liver failure). Liver fibrosis and cirrhosis are clinically diagnosed by liver stiffness measurement, using elastography (transient elastography or magnetic resonance elastography) or characteristic imaging and serum laboratory analyses. Patients with cirrhosis are susceptible to decompensation events secondary to increased portal pressures such as the development of ascites, esophageal variceal bleeding, hepatic encephalopathy, or hepatocellular carcinoma, which are associated with large decreases in patients' prognoses and quality of life.

Clinically, the gut microbiota is intimately linked with the pathogenesis of increased portal pressures and every aspect of cirrhotic decompensation. For example, one serious complication of ascites associated with significant morbidity and mortality is spontaneous bacterial peritonitis, caused by translocation of gut bacteria into the sterile peritoneal space. For patients with cirrhosis who develop variceal bleeding, prophylactic antibiotics are standard of care, and 30% to 48% of patients who did not receive antibiotics developed bacterial infections[149,150]. Patients with cirrhosis may also accumulate nitrogenous waste products, such as ammonia, in the systemic circulation, leading to hepatic encephalopathy. The mainstays of hepatic encephalopathy therapy are lactulose, a disaccharide that acts as a laxative and has been shown to enrich for beneficial gut microorganisms that reduce ammonia production and acidify the colonic contents, allowing for excretion of ammonia. Hepatic encephalopathy is also treated with rifaximin, a non-absorbable broad-spectrum antibiotic[151].

BOX 2.

Microbial metabolites and molecules involved in bidirectional communication between gut and liver

Ethanol.

Ethanol can be produced by the gut microbiota via saccharolytic fermentation and has been implicated in liver disease progression in a subset of patients with nonalcoholic steatohepatitis who were found to have increased abundance of ethanol-producing bacteria in their stool and increased concentrations of ethanol in their systemic circulation[42–44]. Ethanol and its metabolite acetaldehyde cause hepatic damage, directly and indirectly, via disruptions in gut barrier function.

Bile acids.

Primary bile acids are produced from cholesterol in the liver, conjugated with glycine or taurine, and released into the intestine, where they are deconjugated and dehydroxylated by gut microbiota to secondary bile acids. Primary and secondary bile acids are reabsorbed and returned to the liver via the portal vein. In addition to aiding nutrient absorption, bile acids also serve as signaling molecules, by binding to cellular receptors such as FXR to regulate bile acid synthesis, lipid metabolism, and hepatic gluconeogenesis via fibroblast growth factor 15 (FGF15) and FGF19[33].

Short chain fatty acids (SCFA).

SCFA such as acetate, propionate, and butyrate are synthesized via anaerobic fermentation of non-digestible proteins and fibers by the gut microbiota and delivered to the liver via the portal circulation. In addition to serving as an energy source, SCFA also act on signaling pathways to affect hepatic metabolism. Known prolific butyrate producers belong to the *Lachnospiriaceae* and *Ruminococcacecae* families and include *Eubacterium rectale*, *Faecalibacterium prausnitzii*, and several *Roseburia* and *Anaerostipes*. species[152].

Indoles and tryptophan metabolites.

Indole is produced from L-tryptophan by gut microbial enzymes and decreases gut inflammation, prevents gut barrier dysfunction, and reduces lipopolysaccharide-induced hepatic inflammation.

Trimethylamine (TMA) and trimethylamine N-oxide (TMAO).

Choline is converted to TMA by gut microbiota and can then be oxidized to TMAO by hepatic monooxygenases[153]. Serum levels of TMAO have been positively correlated with severity of hepatic steatosis. TMAO is thought to increase the risk for cardiovascular disease by promoting endothelial dysfunction and thrombus formation, reducing glucose tolerance, and increased foam cell production[154,155].

Microbiota-derived hepatotoxins.

Bacterial lipopolysaccharides, found in the outer membrane of Gram-negative bacteria, activate microbe-associated molecular pattern receptors such as toll-like receptors.

Microorganism-derived exotoxins such as cytolysin from *E. faecalis* and candidalysin from *C. albicans* have hepatoxic effects, forming pores in cell membranes.

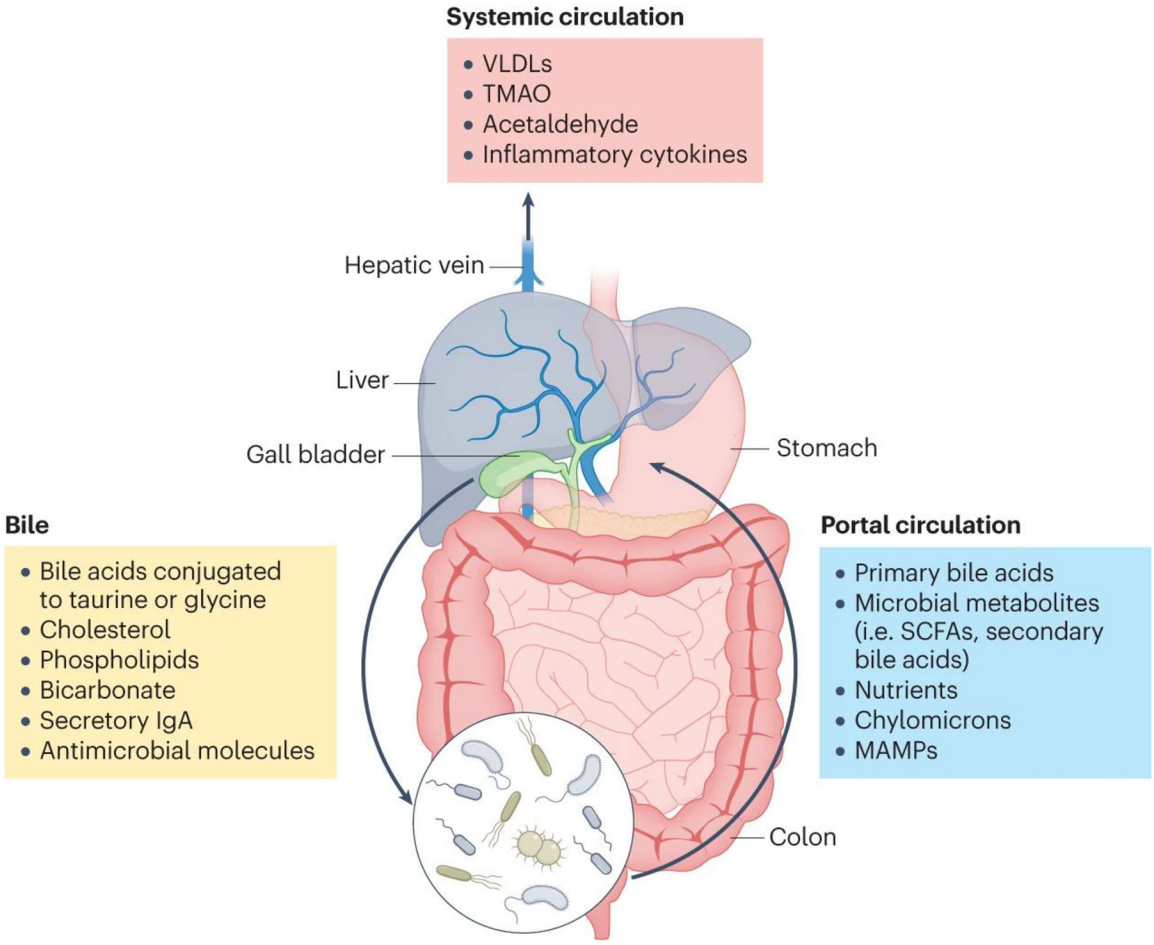

Fig. 1. The gut–liver axis.

The liver produces bile, which is composed of cholesterol, phospholipids, proteins, bicarbonate, and bile acids that have been conjugated by the liver to taurine or glycine (yellow box). Bile is directed into the proximal small intestine via the bile ducts, where it aids in lipid digestion and absorption. In the colon, gut bacteria convert primary bile acids into secondary bile acids via dihydroxylation, and eventually both primary and secondary bile acids are reabsorbed back into the liver via the portal circulation (blue box). The portal circulation also carries absorbed nutrients, lipids, microbial products such as lipopolisaccharide, and microbial metabolites such as short-chain fatty acids (SCFAs) back to the liver. Toxic metabolites such as acetaldehyde and inflammatory cytokines produced by the liver then continue into the systemic circulation (red box). IgA, immunoglobulin A; SCFAs, small-chain fatty acids; MAMPs, microbe-associated molecular patterns; VLDLs, very low-density lipoproteins; TMAO, trimethylamine N-oxide.

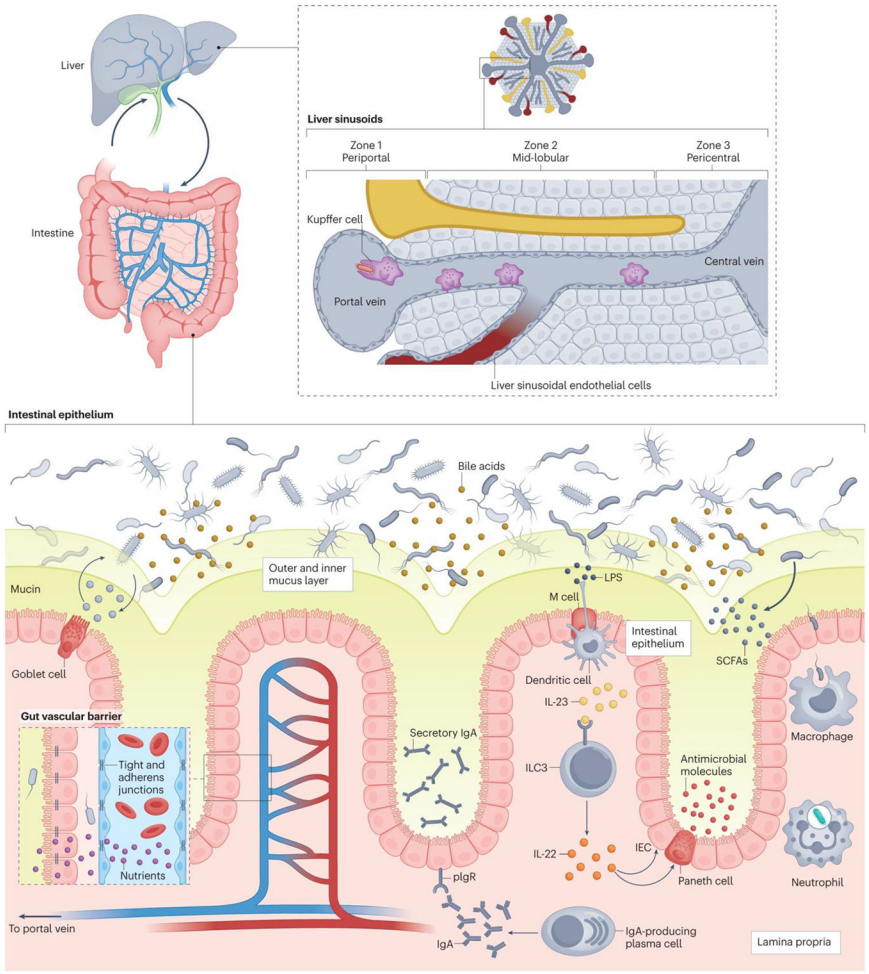

Fig. 2. Barrier systems against translocation of microorganisms.

There are multiple layers of defense that prevent translocation of gut bacteria into the systemic circulation. The intestinal epithelium is the first interface, as it separates the bacteria in the gut lumen from the lamina propria below. A mucus layer (green), composed of mucins produced by goblet cells, separates the bacteria from the underlying intestinal epithelial cells. In the lamina propria (beige), whole bacteria that cross the intestinal epithelium are phagocytosed by macrophages and neutrophils. Plasma cells produce immunoglobulin A (IgA), which binds to pIgR, a receptor on enterocytes, for transport across the epithelium and secretion into the mucus layer, where this secretory IgA binds and neutralizes harmful pathobionts and pathogens. M cells in the intestinal epithelium relay microbial antigens to dendritic cells, which activate Type 3 innate lymphoid cells (ILC3) (via interleukin 23 (IL-23)) to produce interleukin 22 (IL-22). IL-22 ultimately signals Paneth cells and intestinal epithelial cells (IEC) to secrete antimicrobial molecules into the mucus layer. Short-chain fatty acids (SCFAs), produced by bacterial microbial degradation of fiber, are an energy source for enterocytes and enhance the antimicrobial activity of macrophages. Another physical barrier against bacterial translocation is the gut vascular barrier (inset box). Tight junctions and adherens junctions on endothelial cells regulate the permeability of the gut vascular barrier, allowing passage of nutrients and small molecules

into the enteral circulation but restricting the passage of viable bacteria and microbial antigens. Finally, gut microorganisms that do translocate into the enteral circulation will face the liver barrier via the portal vein. In the liver, blood from the portal vein flows into the central vein via sinusoids (grey), which are lined with liver sinusoidal endothelial cells that release chemokines in response to microorganisms and microbial products. This creates a chemokine gradient that recruits Kupffer cells to the periportal zone, where they can neutralize translocated microorganims. LPS, lipopolysaccharide.

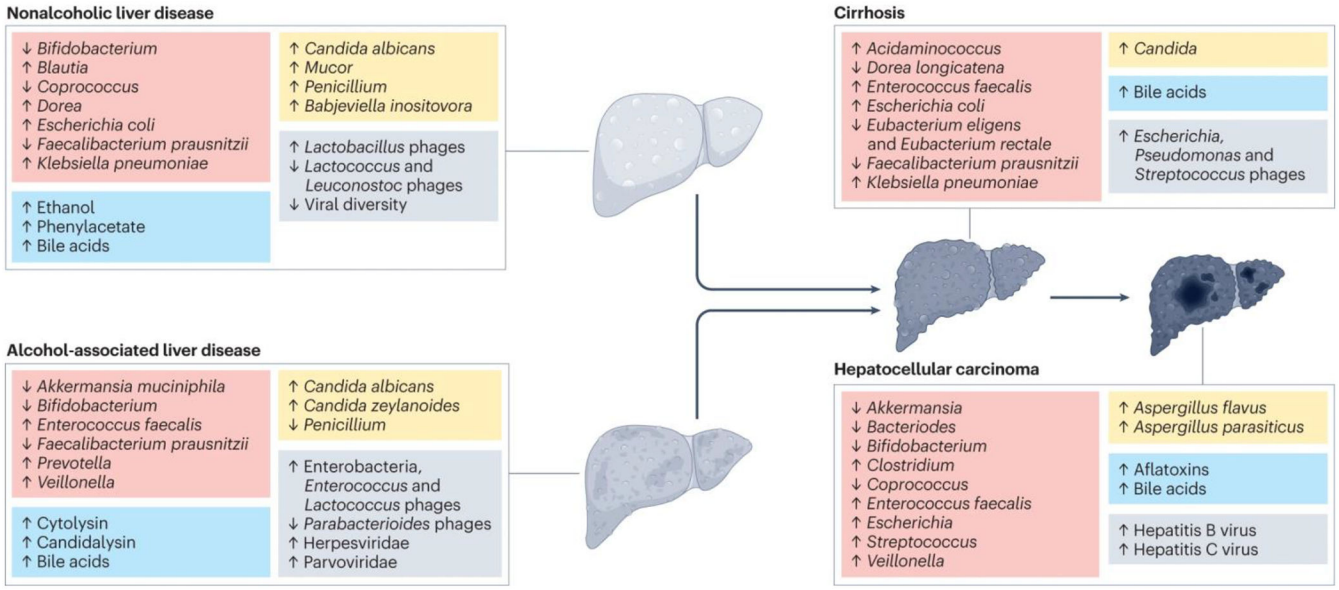

Fig. 3. Gut microbiomes differ with etiology of liver disease.

Non-alcoholic liver disease and alcohol-associated liver disease are associated with different changes in the composition of bacteria, fungi, viruses, and metabolites in the gut. Liver disease progression to cirrhosis and subsequent hepatocarcinogenesis (development of hepatocellular carcinoma) are associated with further changes in the gut microbiome and metabolites. The red boxes represent bacteria, blue boxes metabolites, yellow boxes fungi, and grey boxes viruses.

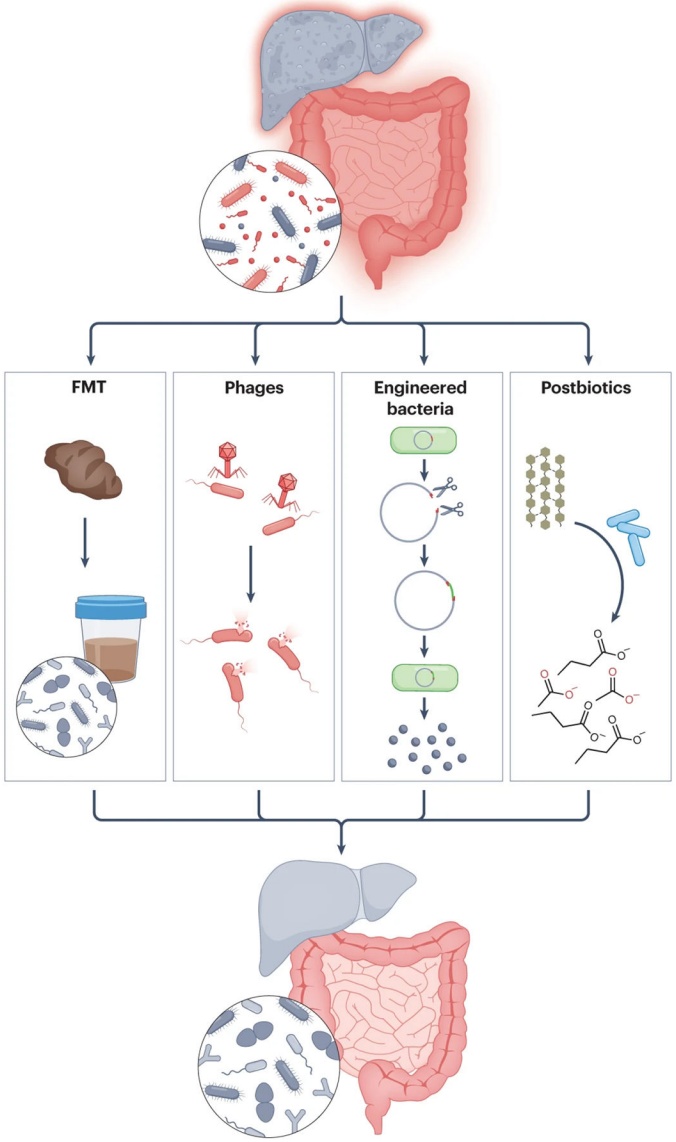

Fig. 4. Targeted approaches for treatment of liver disease.

Fecal microbiota transplantation (FMT) involves transplantation of the entire gut microbial community. More targeted treatment approaches include use of bacteriophages (phages) to target specific bacterial strains that produce toxic metabolites, genetically engineered bacteria to produce beneficial metabolites, and supplementation of beneficial microbially produced metabolites or postbiotics.

Table 1.

Clinical trials of microbiome-based therapies for liver diseases

Reference	Intervention	Subjects	Results and conclusions
Fecal microbiota transplantation			
128	Oral probiotics (*Bifidobacterium* and *Lactobacillus acidophilus*) versus heterologous donor FMT via colonoscopy	75 NAFLD patients: 28 in probiotic arm, 47 in FMT arm. Of the 47 FMT patients, 15 were lean and 32 had obesity	FMT was associated with improvement in hepatic steatosis as measured by Fibroscan compared to oral probiotics and more improvement in lean NAFLD
129	Standard of care versus FMT via nasojejunal tube	33 patients with severe alcoholic hepatitis and acute-on-chronic liver failure; 20 received standard of care, 13 received FMT	Survival at 28 and 90 days; hepatic encephalopathy and ascites resolution was significantly better in the FMT group.
106	Placebo versus FMT enema from a donor enriched in Lachnospiraceae and Ruminococcaceae	20 men with AUD-related cirrhosis, randomized 1:1	FMT is safe and associated with short-term reductions in alcohol craving and consumption, with favorable microbial changes, as well as reduction in alcohol use disorder-related events over 6 months
130	Allogenic or autologous FMT delivered by upper endoscopy to distal duodenum	21 patients with NAFLD, randomized 3:1 to allogenic vs autologous FMT	No significant changes in insulin resistance or hepatic steatosis in patients who received the allogenic or autologous FMT. Patients with elevated small intestinal permeability at baseline who received allogenic FMT had a significant reduction after 6 weeks
131	Three FMT via colonoscopy, each 8 weeks apart, from autologous vs lean, vegan donors	21 overweight patients with metabolic syndrome and hepatic steatosis, randomized 1:1	Allogenic FMT from lean, vegan donors showed a trend towards decrease in necro-inflammation score compared with autologous FMT, though study was underpowered for significance
111	Placebo versus 15 FMT oral capsules from a single donor enriched in Lachnospiraceae and Ruminococcaceae	20 patients with cirrhosis and recurrent hepatic encephalopathy, randomized 1:1	Oral FMT capsules were safe and well-tolerated in patient with cirrhosis and recurrent hepatic encephalopathy, and associated with improved duodenal mucosal diversity, dysbiosis, and duodenal anti-microbial peptide expression
132	Standard of care (no treatment) versus 5 days of antibiotics followed by FMT enema from a single donor enriched in Lachnospiraceae and Ruminococcaceae	20 cirrhotic patients with recurrent hepatic encephalopathy	FMT treatment is safe and associated with reduction in hospitalizations due to liver-related and portal hypertensive complications and improvement in cognition
Probiotics			
133	Lactulose, rifaximin, or probiotic *Escherichia coli Nissle* (EcN) 1917 strain for 1 month	45 patients with cirrhosis and hepatic encephalopathy, randomized 1:1:1	Patients given rifaximin or the EcN probiotic had more significant reduction in serum ammonia and proinflammatory cytokines, normalization of fecal Bifidobacteria and Lactobacilli abundance, and improved cognition compared with lactulose treatment
134	Rosuvastatin only versus rosuvastatin plus *Clostridium butyricum* capsules for 6 months	96 patients with NAFLD, randomized 1:1	Probiotic treatment was associated with significantly lower serum total cholesterol, triglycerides, free fatty acids, total bilirubin, direct bilirubin, ALT, AST, and inflammatory markers compared with controls
135	Placebo versus probiotics sachet containing six *Lactobacillus* and *Bifidobacterium* species at a concentration of 30 billion CFU for 6 months	39 patients with NAFLD, randomized 1:1	No significant changes in hepatic steatosis, fibrosis, or liver-specific lab parameters between the two treatments.
136	Placebo versus VSL#3 probiotic supplementation for 10 weeks	35 patients with NAFLD, randomized 1:1	VSL#3 did not significantly improve markers of cardiovascular risk or liver injury in patients with NAFLD.

Reference	Intervention	Subjects	Results and conclusions
[137]	Probiotic capsule + placebo of prebiotic versus oligofructose + placebo of probiotic vs placebo of probiotic + placebo of prebiotic	111 patients with NAFLD, randomized 1:1:1	Probiotic supplementation was associated with decreases in triglyceride, ALT, AST, GGT, and alkaline phosphatase compared with placebo
[138]	Placebo versus symbiotic capsules (fructo-oligosaccharides plus *Bifidobacterium animalis lactis BB-12*) for 10–14 months	104 patients with NAFLD, randomized 1:1:1	1 year of administration of a synbiotic combination (probiotic and prebiotic) altered the fecal microbiome but did not reduce liver fat content or markers of liver fibrosis in patients with NAFLD
[139]	Placebo versus probiotic mixture (*Lactobacillus acidophilus, L. rhamnosus, L. paracasei, Pediococcus pentosaceus, Bifidobacterium lactis*, and *B. breve*) for 12 weeks	68 patients with NAFLD and obesity, randomized 1:1	12 weeks of probiotic treatment significantly reduced hepatic fat and BMI in NAFLD patients with obesity
[140]	Placebo versus probiotic mixture (14 probiotic bacteria genera *Bifidobacterium, Lactobacillus, Lactococcus, Propionibacterium*) for 8 weeks	58 patients with NAFLD and type 2 diabetes, randomized 1:1	Probiotic treatment was associated with reduction in fatty liver index, AST, and GGT, but not liver stiffness, compared with placebo
[141]	Placebo versus probiotic mixture (*Lactobacilli, Bifidobacteria*, and *Streptococcus thermophilus*) for 12 weeks	75 patients with NASH were randomized 1:1	Probiotic treatment was associated with significant reduction in serum ALT and cholesterol, liver stiffness, and BMI as compared with placebo
[142]	Placebo versus probiotic mixture (*Lactobacillus acidophilus, Bifidobacterium lactis, Bifidobacterium bifidum*, and *Lactobacillus rhamnosus*) for 12 weeks	64children with obesity and sonographic NAFLD, randomized 1:1	Probiotic treatment was associated with more normal liver sonography and significant decrease in serum ALT, AST, cholesterol, triglycerides, and LDL as compared with placebo
Bile acids and metabolites			
[143]	Placebo versus 50 mg or 80 mg of MET409 (a non-bile acid FXR agonist) for 12 weeks	58 patients with NASH, randomized 1:1:1	MET409 lowered liver fat content, but was associated with decrease in HDL and increase in LDL compared with placebo
[144]	Placebo versus aldafermin (an engineered analog of FGF19) for 24 weeks	78 patients with NASH, randomized 1:2	Aldafermin was associated with a significant reduction in absolute liver fat content and serum ALT and AST compared with placebo
[145]	Placebo versus 5, 10, or 20 mg volixibat (an inhibitor of the apical sodium-dependent bile acid transporter) for 48 weeks	197 patients with NASH without cirrhosis, randomized 1:1:1:1	Volixibat had no effect on steatosis, serum level of ALT, or liver histology in adults with NASH
[146]	Inulin control versus inulin-propionate ester for 42 days	18 patients with NAFLD, randomized 1:1	Intrahepatocellular lipid was significantly increased in the inulin-control group but not observed in the inulin-propionate ester group.
[147]	Placebo versus 500 mg or 1500 mg norursodeoxycholic acid capsules for 12 weeks	198 patients with NAFLD and increased serum ALT, randomized 1:1:1	1500 mg norursodeoxycholic acid resulted in a significant reduction of serum ALT within 12 weeks of treatment compared with placebo
[148]	Placebo versus 3 mg or 6 mg of subcutaneous NGM282 for 12 weeks	82 patients with biopsy-confirmed NASH, randomized 1:1:1	NGM282 was associated with significant reduction in liver fat content compared with placebo

FMT, fecal microbiota transplantation; NAFLD, nonalcoholic fatty liver disease; AUD, alcohol use disorder; EcN, Escherichia coli Nissle 1917; CFU, colony-forming unit; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyltransferase; BMI, body-mass index; NASH, nonalcoholic steatohepatitis; LDL, low-density lipoprotein; HDL, high-density lipoprotein; FXR, farnesoid X receptor.