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The gut-liver axis and the intersection with the microbiome

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Abstract

In the past decade, an exciting realization has been that diverse liver diseases, ranging from non-alcoholic steatohepatitis, alcoholic steatohepatitis, and cirrhosis, to hepatocellular carcinoma, are not unrelated but fall along a spectrum. Recent work on the biology of the gut-liver communication axis has assisted in understanding the basic biology of both alcoholic and nonalcoholic fatty liver disease. Of immense importance is the massive advancement in understanding of the role of the microbiome, driven by high-throughput DNA sequencing and improved computational techniques that allow the complexity of the microbiome to be interrogated, together with improved experimental designs. Here, we review the gut-liver communications of these various forms of liver disease, explore the molecular, genetic and microbiome relationships, discuss prospects for exploiting the microbiome to determine the stage of liver disease, and to predict the effects of pharmaceutical, dietary, and other interventions at a population and individual level. We conclude that although much remains to be done in understanding the relationship between the microbiome and liver disease, rapid progress towards clinical applications is being made, especially in study designs that complement human intervention studies with mechanistic work in mice that have been humanized in multiple respects, including the genetic, immunological and microbiome characteristics of individual patients. These “avatar mice” may be especially useful for guiding new microbiome-based or microbiome-informed therapies.

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Competing interests

The authors declare no competing interests.

Introduction

The crosstalk between the gut and liver is increasingly recognized, strengthened by the parallel rise in liver diseases and gastrointestinal (GI) and immune disorders.[1,2] The most common type of liver disease, nonalcoholic fatty liver disease (NAFLD), alone affects more than 65 million Americans with a cost burden of $103 billion annually within the US itself.[3] To manage the socio-economic burden of GI-associated liver diseases by developing new therapeutic modalities, we must elucidate specific molecular events that facilitate interaction between the gut and the liver. As we begin to appreciate these links, animal models[4–6] as well as well-designed, clinical studies[7–9] are already revealing key components of these interactions.

The present understanding of the etiology of the spectrum of liver diseases (Figure 1) is underpinned by proinflammatory changes in the host. Intestinal dysbiosis and increased intestinal permeability leads to translocation of microbes and microbial products including cell-wall components (endotoxins from gram-negative bacteria, β-glucan from fungi) and DNA, together referred to as microbial- (or pathogen-) associated molecular patterns (MAMPs/PAMPs). These patterns are recognized by immune receptors on liver cells such as Kupffer cells and hepatic stellate cells and lamina propria (an immune cell-rich tissue beneath the intestinal epithelium) which initiate and maintain inflammatory cascades that ultimately lead to liver damage in the form of fibrosis.[10–13] This damage can progress from cirrhosis (severe fibrosis) to hepatocellular carcinoma (HCC), the most predominant form (more than 80%) of primary liver cancers.[14] Previously demonstrated associations between intestinal health and several different types of neoplasia suggest a potential role of the microbiome in HCC.[15,16] Additionally, the liver and microbiome engage in co-metabolism of xenobiotics including carcinogens, which can independently predispose the host to HCC.[17,18]

The missing links in the complex interaction network between host and microbes are being discovered piece-by-piece using various experimental designs (detailed later). These findings encourage microbiome-oriented therapeutic modalities to treat liver-associated as well as other metabolic diseases. Here, we review the current understanding of the etiology of liver diseases and discuss the open research questions (Box 3) to motivate focused research in this area with special attention to the role of the microbiome.

How do the liver and gut communicate?

The gut and liver communicate via tight bidirectional links through the biliary tract, portal vein and systemic circulation (Figure 2). The liver communicates with the intestine by releasing bile acids and many bioactive mediators into the biliary tract and the systemic circulation. In the intestine, host and microbes metabolize endogenous (bile acids, amino acids) as well as exogenous substrates (from diet and environmental exposure), the products of which translocate to the liver through the portal vein and influence liver functions.[19] Some crucial links between the gut and liver are discussed below.

Enterohepatic circulation of bile acids

Bile acids (BAs) are amphipathic molecules synthesized from cholesterol in the pericentral hepatocytes. These are conjugated to glycine or taurine and released in the biliary tract. On reaching the small intestine through the duodenum, BAs, together with other biliary components, facilitate emulsification and absorption of dietary fats, cholesterol, and fat-soluble vitamins. About 95% of the BAs are actively reabsorbed in the terminal ileum and transported back to the liver [20,21]. The remaining five percent are deconjugated, dehydrogenated and dehydroxylated by the intestinal microbiota to form secondary bile acids, which reach the liver via passive absorption into the portal circulation. The liver recycles BAs and secretes them back to the biliary tract completing the "enterohepatic circulation" i.e. a system of exchange between the gut and the liver.

A carrier-mediated process transports hydrophilic primary bile acids across cell membranes for uptake into intestinal epithelial cells. Regulatory effects of BAs have been best studied with respect to farnesoid X receptor (FXR) and Takeda G-protein-coupled receptor 5 (TGR5). BAs bind to FXR in the enterocytes and induce transcription of an enterokine, fibroblast growth factor 19 or FGF19 (FGF15 in mouse). FGF-19 reaches the liver through the portal vein and down-regulates *de novo* bile acid synthesis by inhibiting CYP7A1 in hepatocytes, forming a feedback system for modulating BA production.[22] FXR activation is known to affect glucose and lipid metabolism.[23,24] Additionally, BAs bind to TGR5 on the plasma membrane and act on tissues beyond enterohepatic circulation. This binding mediates host energy expenditure,[25,26] glucose homeostasis[27] and anti-inflammatory immune responses.[28,29]

BAs and the gut microbiota closely interact and modulate each other. BAs exert direct control on the intestinal microbiota. By binding to FXR, they induce production of antimicrobial peptides such as angogenin1 and RNAse family member 4, which are directly involved in inhibiting gut microbial overgrowth and, subsequently, gut barrier dysfunction. [30,31] Intestinal dysbiosis shifts the balance between primary and secondary bile acids and their subsequent enterohepatic cycling, the metabolic effects of which are not comprehensively understood. However, because of differences in the affinity of these two classes of BAs for the FXR, these shifts have been associated with increased hepatic bile acid synthesis and metabolic stress.[32–35] An imbalance in BAs and gut bacteria elicits a cascade of host immune responses relevant to the progression of liver diseases.

Intestinal permeability

The central components of the intestinal barrier are enterocytes that are tightly bound to adjacent cells by apical junctional proteins that include claudins, occludins, junctional adhesion molecules (JAMs) and E-cadherins.[36] This barrier restricts movement of microbes and molecules from the gut lumen, while allowing permselective, active transport of nutrients across the tight junctions. The intestinal barrier is further strengthened by several additional lines of defense:

(1) Mucins (heavily glycosylated protein aggregates) form a physical barrier between luminal bacteria and the underlying epithelial layer[36]

(2) Antibacterial lectins, such as regenerating islet-derived protein III-gamma (REG3G), which are produced by intestinal paneth cells to target bacteria associated with mucosal lining[37,38]

(3) Immunoglobulins, specifically sIgA, produced by plasma cells and transported into the lumen through the intestinal epithelial cells that neutralize microbial pathogens by blockading epithelial receptors[39]

(4) Commensal bacteria are closely associated with the gut mucosa, and reinforce barrier integrity by stimulating cell-mediated immunity via toll-like receptor mediated signaling[37,40] or by producing metabolites that directly strengthen tight junctions (short chain fatty acids)[41–43] and inhibit other microbes[44–46]

Breakdown of one or more of these barrier components compromises gut-barrier integrity. The major drivers of increased permeability include gut inflammation and dysbiosis[47,48] which have been linked to consumption of high-fat, Western diet,[49–51] chronic alcohol consumption,[52–54] prolonged antibiotic usage,[55] and immune-mediated inflammatory diseases such as IBD.[56] An important association between the gut microbiota, inflammation and gut-barrier integrity is provided by *Akkermansia muciniphila*, a gram-negative anaerobe that colonizes the intestinal mucus layer. Reduced abundance of *A. muciniphila* has been associated with thinning of mucus layer and increased inflammation promoting both, alcoholic and nonalcoholic liver damage.[57,58] When the gut barrier is compromised, microbes and microbe-derived molecules can translocate to the liver through the portal system, causing inflammation and hepatic injury. Some translocated intestinal products may also directly interact with host factors and contribute to exacerbation of liver disease.[59–64]

Systemic circulation

Bacteria and MAMPs—Intestinal permeability is characterized by compromised tight junctions between enterocytes, and is consistently seen across the spectrum of liver diseases.[65,66] Liver damage is associated with small intestinal bacterial overgrowth (SIBO) and microbial dysbiosis of the lower gastrointestinal tract.[67] Together, these lead to increased translocation of MAMPs into the portal circulation. On reaching the liver, MAMPs induce localized inflammation through pattern recognition receptors (PRRs) on Kupffer cells[68] and hepatic stellate cells.[69,70] Endotoxin-mediated activation of Toll-like Receptor-4 (TLR4)[69,68] along with TLR9 (activated by methylated DNA[70] and TLR2 (activated by gram-positive bacteria)[71] are the primary drivers of immune response in liver disease. TLR signaling in Kupffer cells activates downstream proinflammatory cascade, leading to MyD88 mediated activation of NF-kB.[13] Additionally, TLR4 signaling also promotes fibrosis by down regulating Bambi, a decoy receptor for TGF-β.[13] These lead to expression of inflammatory cytokines, oxidative and endoplasmic reticulum (ER) stress, and subsequent liver damage.[72]

Choline metabolites—Choline is a macronutrient that is important for liver function, brain development, nerve function, muscle movement, and maintaining a healthy metabolism.[73] (Rodents fed a choline-deficient diet have been used to model human nonalcoholic steatohepatitis.[74–76]) Choline is processed into phosphatidylcholine (lecithin) by the host, which assists in excretion of very-low density lipoproteins (VLDL) particles

from the liver. This prevents hepatic accumulation of triglycerides (liver steatosis). Additionally, choline can also be converted to trimethylamine (TMA) by intestinal bacteria. TMA can translocate to the liver through the portal circulation where it is converted to trimethylamine N-oxide (TMAO).[77]

The significance of methylamines is increasingly being recognized with respect to liver, cardiometabolic and more recently, mental disorders[77,78]. Increased systemic circulation of TMAO is concomitant with reduced levels of host-produced phosphatidylcholine, an imbalance characteristic of intestinal dysbiosis. This has been linked with liver damage due to increased triglyceride accumulation (hepatic steatosis)[9,77,79–81] and consequently, non-alcoholic fatty liver disease[9] and liver tumorigenesis.[82].

Free fatty acids—Free fatty acids include short-chain fatty acids (SCFA) and saturated long-chain fatty acids (LCFA). Butyrate and propionate (products of bacterial fermentation) are the dominant short chain fatty acids in the large intestine. Butyrate is an energy source for the enterocytes and facilitates maintenance of intestinal barrier.[41–43] Alcohol-induced liver injury is suggestively marked by reduced butyrate and propionate[83,84] and increased acetate (possibly produced by ethanol metabolism in the lumen, but predominantly derived from ethanol metabolism in the liver). Increased acetaldehyde can weaken gut barrier[59,85] and induce hepatic stress [86,87] on translocation of intestinal antigens to the liver. Butyrate supplementation in the form of a glycerol ester, tributyrin, reduced ethanol-induced intestinal permeability and subsequent liver injury in mice on a short-term alcohol diet. However, how tributyrin mechanistically protects intestinal barrier remains to be established.

Luminal species of LCFAs include pentadecanoic acid (C15:0), palmitic acid (C16:0), heptadecanoic acid (C17:0), and stearic acid (C18:0). In mice fed alcohol chronically, C15:0 and C17:0, which are only produced by bacterial fermentation,[88] are significantly reduced when compared to control on isocaloric diet.[83,89]. There is also an overall reduction in total saturated LCFAs highlighting the importance of bacterial contribution in LCFA homeostasis. Of the remnant LCFA species, C16:0 and C18:0 were in highest concentration which suggests lower microbiome involvement (which is disrupted by alcohol consumption) in their production.[83] *Lactobacillus spp.* are known metabolizers of saturated LCFAs and a reduction in their concentration is concomitant with decreased luminal *Lactobacilli*.[83] To our knowledge, restoring *Lactobacillus spp.* by LCFA supplementation has not been experimentally demonstrated. However, dietary supplementation of *Lactobacillus rhamnosus* has been shown to increase luminal LCFAs,[89] suggesting that *Lactobacillus*-induced increase in intestinal FFAs contribute to its probiotic effects.[90–96]

Ethanol and acetaldehyde—The mucosa of the GI tract absorbs ethanol by simple diffusion. Within the GI tract, the majority of ethanol from food and beverages is absorbed by the stomach (~ 20%) and small intestine (~ 70%).[97,98] Although, microbial fermentation contributes to luminal ethanol concentration, the biggest share of alcohol in the large intestine comes from the systemic circulation.[59]

Gut microbiota and enterocytes express alcohol-metabolizing enzymes such as alcohol dehydrogenase, aldehyde dehydrogenase co-metabolizing ethanol into acetaldehyde and, to

a lesser-studied extent, acetate[59,85,86]The liver also responds to circulating levels of ethanol by upregulating its ethanol metabolism pathway.[86,87] The importance of microbes for xenobiotics metabolism was underscored by a study that demonstrated an increase in hepatic expression of ethanol metabolizing genes in germ-free mice, and subsequent liver damage. [86,87]

Non-alcoholic and alcoholic liver diseases (Figure 3; Table 1) are characterized by increased luminal and circulating levels of ethanol and its metabolites, acetaldehyde and acetate.[64,99] These metabolites have independently been associated to liver damage.[61–63] Acetaldehyde has been implicated in weakening the intestinal tight junctions compromising the gut barrier and allowing translocation of microbial products.[100–105] It has also been associated with downregulating the expression of antimicrobial peptides (AMPs) in the intestine,[106,107] and eliciting inflammatory and adaptive host immune responses.[108–110] Additionally, ALD is marked by reduced intestinal butyrate[83,111,112] (an energy source for enterocytes) which is linked to weakening of intestinal tight junctions and hence, permeability.[84,113–115]

Links between the microbiome and specific liver diseases

Nonalcoholic fatty liver disease (NAFLD)

NAFLD refers to a spectrum of liver disease that can be broadly classified into two categories: nonalcoholic fatty liver (NAFL), the non-progressive form of NAFLD, and nonalcoholic steatohepatitis (NASH), the progressive form of NAFLD.[116] NASH is generally linked to type 2 diabetes, cardiovascular risk factors and obesity[117, 118], although incidences have also been reported in lean individuals, emphasizing that genetic and environmental factors also contribute to disease development. [119,120,121,122]

Several studies have stressed on the role of the gut microbiota in NAFLD, but, causality is yet to be established[123]. Patients with NAFLD have a higher prevalence of small intestinal bacterial overgrowth (SIBO)[65,124] and microbial dysbiosis. Using 16S amplicon sequencing, Boursier et al.[125] found that the bacterial genera, *Bacteroides* and *Ruminococcus* were significantly increased, and *Prevotella* was reduced in NASH patients with stage 2 fibrosis or higher. Loomba et al. (2017)[7] utilized whole genome metagenomics to characterize the gut microbiota in NAFLD patients with and without advanced fibrosis (stages 3 and 4) and showed an increased abundance of *Escherichia coli*, and *Bacteroides vulgatus* in advanced fibrosis patients. An enrichment of *Escherichia* (genera) was also seen in pediatric NASH patients compared to obese controls.[64] Consistent with preclinical studies, these studies indicate an association between gram-negative bacteria and progression of liver fibrosis.[126]

Genetically modified mouse models have been used to study NAFLD-associated gut dysbiosis and permeability for mechanistic insights in liver disease progression. Rahman et al. (2016)[127] used JAM-A (junctional adhesion molecule-A protein) knockout mice to demonstrate that deficiency in this tight junction protein is linked to increased intestinal permeability and liver inflammation. This inflammation could be alleviated by administering antibiotics, underscoring the importance of microbial translocation in promoting immune response in the liver. Another group used muc-2 knockout mice and found that there was a

compensatory increase in intestinal levels of Reg3b and Reg3g genes leading to an overall protective response against NAFLD.[107]

The contribution of liver-damaging inflammation in response to translocation of microbes and MAMPs was elucidated by Henao-Mejia and colleagues (2012)[48]. Using NLRP3- and NLRP6- (inflammasome-) deficient mice models, they demonstrated an increase in influx of TLR4 and TLR9 in portal circulation, which enhanced the expression of hepatic tumor-necrosis factor (TNF)-α driving NASH progression. Furthermore, cohousing inflammasome-deficient mice with wild-type controls exacerbated hepatic steatosis and obesity in healthy cage mates, suggesting transferability of disease via the microbiome.

Increasing links between NAFLD and the gut microbiome at both the observational and mechanistic levels have gut microbiota an attractive source of biomarkers for early diagnosis of NAFLD. In a comparison between obese children with and without NASH, Zhu and colleagues[64] observed significantly elevated gut microbial production of ethanol in NASH patients. NAFLD patients also show increased systemic TMAO[9] and hepatic bile-acid synthesis[35], and decreased production of phosphatidylcholine.[128] Recently, Loomba et al. further observed differences in carbon and amino acid metabolism in gut microbiome of patients with NAFLD-associated advanced fibrosis. This proof of concept study provides preliminary evidence to support the utility of a microbiome-derived metagenomics signature to detect advanced fibrosis and as well as candidacy for anti-fibrotic treatment trials in NAFLD.

Alcoholic liver disease (ALD)

The manifestation of ALD in chronic alcohol abuse patients is a consequence of multifactorial interactions involving genetics, immune system, gut microbiome and environmental factors.[100,129,130,131] Like NAFLD, non-progressive form of ALD is characterized by accumulation of fat inside liver (fatty liver or steatosis), while it's progressive form is marked by inflammation and liver injury (alcoholic steatohepatitis or ASH).

Our understanding of the compositional and mechanistic contributions of the gut microbiota in ALD is improving with the increasing number of studies investigating this link. As in NAFLD, SIBO has been demonstrated as an important hallmark of alcohol-associated liver disease in humans[35] and mice.[106,131] Intestinal dysbiosis in alcohol-abuse patients is characterized by significant enrichment of *Enterobactericaea* (family) and reduction in *Bacteroidetes* and *Lactobacillus* (genera). [132,106,133,134] It has also been demonstrated that alcohol-induced dysbiosis is only partially reversible by alcohol-withdrawal or probiotic treatment.[94,113] Interestingly, alcohol-dependent patients also displayed reduced fungal diversity and *Candida* overgrowth, presenting the first evidence of the role of gut mycobiome in pathogenesis of liver diseases.[8]

Genetically-modified murine models have advanced our mechanistic understanding of the contribution of various components of the gut-barrier in the etiology and progression of ALD. Using Reg3b(−/−) or Reg3g(−/−) mice, it was found that REG3 lectins protected against alcoholic steatohepatitis by reducing mucosa-associated microbiota, thereby

preventing translocation of viable bacteria.[135] Muc-2 deficient mice were protected against alcohol-induced inflammation (similar to HFD-induced inflammation in NAFLD model) due to a compensatory increase in Reg3g and Reg3b lectins.[107] Furthermore, IgA knockout in mice led to increased levels of IgM and a net protective effect against ASH progression.[136]

In response to ethanol-induced gut-barrier dysfunction and translocation, TLRs and other pathogen recognition receptors activate hepatic Kupffer cells and macrophages, as was demonstrated in male Wistar rats.[137] This initiates inflammatory cascades releasing TNF-alpha, IL-1, IL-10, IL-12, and TGF-beta. [138,139,140] Using TLR-4 chimeric mice, it was shown that endotoxin-induced release of TGF-beta is mediated by MyD88-NF-kappaB-dependent pathway providing explanatory mechanism of inflammation-induced liver damage.[68] Furthermore, increased translocation of fungal β-glucan also induced liver inflammation via CLEC7A receptor on hepatic Kupffer cells such that treatment of mice with antifungal agents reduced intestinal fungal overgrowth, decreased β-glucan translocation, and ameliorated ethanol-induced liver disease.[8]

Concomitant with immunological responses to barrier dysfunction, ALD is also marked by system-wide changes in many bioactive compounds. Alcohol consumption leads to an increase in hepatic bile acid synthesis humans and mice. [141,142] This could be explained by dysbiosis-associated disruption in FXR activation in the enterocytes as FXR deficient mice were more likely to develop ethanol-induced steatohepatitis[143], and treatment with an FXR agonist (WAY-362450) had protective effects against liver damage.[144] Alcohol-associated dysbiosis in mice was further linked to reduced LCFA biosynthesis such that LCFA supplementation restored eubiosis. In fact, a significant correlation between *Lactobacillus spp.* and bacterial LCFA (C15:0 and C17:0) was found in ALD patients but not in healthy controls.[83] Butyrate (SCFA) production was also negatively altered following ethanol exposure and administration of butyrate in the form of tributyrin mitigated alcohol-induced liver injury in mice.[84]

With increasing evidence of mechanistic links between the gut microbiota and liver disease progression, fecal microbiota transplant (FMT) is being explored as a therapeutic option for ALD. However, larger, carefully designed trials across multiple ethnic groups are needed before FMT can be considered safe in routine clinical practice for managing ALD.

Cirrhosis

Cirrhosis (or end-stage liver disease) is an extreme manifestation of chronic liver injury characterized by loss of liver cells, thick fibrous scar, and regenerating nodules. This topic has been extensively reviewed recently so we only provide a brief discussion here.[145] NAFLD, ALD, primary biliary cholangitis (PBC; Box 1), primary sclerosing cholangitis (PSC; Box 2) or hepatitis can each progress to cirrhosis and constitute its subtypes. Currently, NASH is the second leading cause of adult cirrhosis in the USA.[146] Depending upon the etiology of cirrhosis, there is a variable risk of developing HCC.

Alterations in the gut microbiome including dysbiosis and SIBO have been associated with cirrhosis and its complications.[147–149] Treatment for portal systemic encephalopathy and

decompensated cirrhosis includes treatment with nonsystemic antibiotics to reduce intestinal microbiota overgrowth.[150–152] Gut microbiome alterations were observed in alcohol- and hepatitis-associated cirrhotic patients in a Chinese cohort,[153] which observed an invasion of the lower intestinal tract by oral bacteria. Concordant with these findings, Chen and colleagues (2016) also found an overrepresentation of genera including *Veillonella*, *Megasphaera*, *Dialister*, *Atopobium*, and *Prevotella* in the duodenum of cirrhosis patients. The genera, *Neisseria* and *Gemella* were discriminative between hepatitis-B-virus- and PBC-related cirrhosis.[154] Recently, Bajaj and colleagues observed significant fungal dysbiosis in cirrhosis patients and showed that *Bacteroidetes* to *Ascomycota* ratio could independently predict hospitalization in these patients.[155]

All experimental models of liver fibrosis result in gut microbial dysbiosis and increased intestinal permeability and treatment of GI tract with nonabsorbable antibiotics decreases liver fibrosis. Mice with genetic ablations of the receptors for bacterial product ligands, TLR2, TLR4, TLR9, and NLP3, are protected from experimental liver fibrosis.[156] The current treatment philosophy involves decreasing the bacterial product ligands or blocking their receptors, which results in decreased inflammatory and fibrogenic signaling in the liver, although no antifibrotic drug is currently available for routine clinical practice.

Hepatocellular carcinoma (HCC)

The etiology of non-viral HCC follows a “multiple-hit” pathway, whereby liver steatosis followed by oxidative stress, endoplasmic reticulum (ER) stress together with intestinal dysbiosis and inflammation contribute to the final manifestation of cancer.

The gut microbiota changes in composition dramatically in hosts suffering from HCC. *Clostridium* species have been found to be enriched in obesity-induced mouse models of HCC[157,158], but clinical studies with HCC patients detect an overgrowth of intestinal *Escherichia coli.*[159] Murine models as well as human studies have reported a migration of *Helicobacter* species to HCC tumor tissues.[160–163] Notably, members of this genus are known to promote tumor-development by activating NF-kB and WNT signaling and suppressing anti-tumor immunity, and might play a potential role in HCC development.[160,164]

To get insights into the molecular events explaining the progression of liver disease to HCC, various murine models (diet-based, toxin plus diet-based and genetic plus diet-based models) have been explored. However, most of these have proven suboptimal because they either do not develop all intermediate pathological & metabolic stages, or they manifest HCC incompletely (Febbraio and Karin, submitted). We have highlighted some frequently-used rodent models, their usage and caveats in Table 2 to aide future research.

Accumulating evidence suggests that HCC-associated dysbiosis is accompanied by gut-barrier dysfunction, bacterial translocation, systemic circulation of their tumor-promoting metabolites and activation of proinflammatory and oncogenic signaling pathways.[165] The intestinal poly-immunoglobulin receptor (PIgR) regulates the transport of IgA into the intestinal lumen and maintains microbial homeostasis.[166] A recent study showed that PIgR $^{-/-}$ mice modelling NASH-induced HCC had increased systemic and liver IgA, and a

concomitant increase in hepatic tumorigenesis due to localized inhibition of liver cytotoxic T cells that prevent HCC development. Further, the application of broad spectrum antibiotics has been shown to attenuate liver inflammation and HCC-development in mice[157,167] highlighting the role of the intestinal microbiome in liver tumorigenesis. In another mouse model where HCC was induced by diethylnitrosamine (a carcinogen), activation of TLR4 due to LPS translocation upregulated the hepatic mitogen EREG in HSCs and activated NF-kB, resulting in enhanced tumor cell proliferation.[167] Additionally, deoxycholic acid (DCA), a gut bacterial metabolite was shown to upregulate proinflammatory genes, such as IL6 and TNFa to provoke a senescence-associated secretory phenotype in HSCs.[157,168–170]

In addition to its role in HCC development, the gut microbiome also modulates pro-tumorigenic adaptive immune response via Th17 cells, which produce the proinflammatory cytokine IL-17A.[171–173] The therapeutic efficacy of the anticancer drug cyclophosphamide depended on the interplay between Th17 signaling and gut microbiome such that germ-free tumor bearing mice or mice given non-absorbable antibiotics had reduced Th17 response and a subsequent resistance to therapeutic effects of cyclophosphamide was seen.[174]

Increased understanding of the role of the gut microbiota has motivated successful microbiome-based therapeutic modalities for HCC, such as treating with synthetic bile acids to reduce HCC risk in NAFLD patients,[175] non-selective beta-blockers in the intestinal mucosa to prevent bacterial translocation and liver inflammation[176] and administering probiotics in rodents modeling HCC to slow tumor growth and reduce tumor size.

Experimental design of microbiome studies

Given the intense recent interest in links between the microbiome and liver disease, we provide a brief overview of experimental models useful for researchers entering this field.

Much of our knowledge of the human microbiome comes from association studies that use either a cross-sectional or case-control design. Well-designed case-control studies are critical to demonstrate there may be a relationship between microbes and a disease of interest. However, these studies cannot establish causality, and are often subject to confounding variables. Most studies are conducted at a single time point in a population with the disease, and no long term follow up is performed. Consequently, these studies can only identify microbes that differentiate individuals with the disease and the control population. While these may have been causative agents, it is nearly impossible to separate this from secondary effects associated with the condition. For example, medication plays a major role in shaping the microbiome; a study of Type II diabetics found that treatment with Metformin had a larger effect on the microbiome than the disease.[177] Similarly, we hypothesize the physiology of the disease may also contribute to changes in community structure.

Association studies are also often confounded by the selection of poor controls. The microbiome is dynamic,[178,179] and cumulative exposures over an individual's life, shaped by their diet,[180] lifestyle,[181] medical history[177,182,177,182], genetics,[183] and other factors[184] create a unique community. Therefore, if cases and controls are not correctly selected, association studies may detect differences due to confounding factors. Matching cases and

controls based on age and sex is often not sufficient. In cases where this is not possible, it is critically important to collect information about potential confounding factors.

Comparisons across current cross sectional studies are also challenging due to large effects due to technical parameters, including sample collection, storage, primer selection, and analysis techniques.[184] Differences across studies increase the challenge of meta-analysis and increase the challenge of identifying causative clades.[185] Some of these problems can be ameliorated by using consistent methodology.[184,185]

Twin studies provide a potential antidote to some of the problems with association studies. Twin pairs are naturally controlled for age and some early life exposures.[186] Monozygotic twin pairs also share the same genetic background, further limiting potential confounders.[186] Twin studies can be leveraged in two ways. First, identifying differences between discordant and concordant twin pair represent more powerful association studies, due to the partial internal control. Although these are particularly useful in young children, the approach can also be used with adults.[187] Second, twin studies are critical to examine genetic control of the microbiome. A recent study of the UK Twins cohort suggested strong association of the microbiome and genes, including those associated with dietary preference and serum lipids.[188]

As the cost of microbiome analysis decreases, longitudinal studies are becoming more common. Understanding temporal fluctuation in the microbiome, and the role of microbes in contributing to disease etiology will rely on studies over time. Recent work suggests that community instability may, in and of itself, be a characteristic of an unhealthy ecosystem.[189,190] Prospective studies, such as a recent study to looking at death from hepatocellular carcinoma in individuals with nonalcoholic fatty liver disease help identify the role of exposures and etiological factors in contributing to disease outcomes.[191] Incorporating microbiome samples into these long term studies will help look at the role of microbial communities - either at a single time point or the community dynamics - as a contributing factor to complex conditions.[192]

Model animals also play an important role in shaping our understanding of the microbiome in disease (Table 2). Although rodent microbial communities are distinct from the human microbiome, there are some shared physiological and microbial-shared traits.[193] Both rodent and human communities are dominated by the same set of bacterial phyla, although a smaller percentage of genera are shared. As such, experimental findings implicating individual organisms or genera in rodents should be taken with caution until they are validated in humans. Instead, rodent models can show phenotypic consequences of microbiome manipulation. This makes mice a useful model system to investigate causality, explore interactions, and test early interventions.

Both antibiotics and probiotics have been used to study the effect of changing the conventional murine microbiome on a phenotypic outcome. Antibiotics decrease the total bacterial load, as well as causing major perturbations in the microbial communities.[194] In some cases, such as in liver disease models, this can demonstrate the role of bacterial products like lipopolysaccharide (LPS) in modulating inflammation.[127] In other cases, like a

recent addiction model, it can be used to demonstrate the importance of an intact microbiome in regulating behavior.[195] Probiotics can also be used to look at the effect of a specific bacteria or bacterial cocktail within a controlled environment. A study of alcoholic fatty liver disease demonstrated an attenuation of the microbiome-mediated inflammation when a probiotic was used.[106]

Gnotobiotic, or germ free mice, can be used in multiple contexts. Comparisons of specific pathogen free laboratory mice and germ free mice can be used to examine the role of the microbiome in modulating an expressed or induced phenotype.[196,197] More importantly, gnotobiotic mice can be humanized with a donor's stool. This creates a system in which an individual's microbiota can be tested, either for its ability to modulate a disease phenotype or as a target for intervention.[187,196] For instance, in a recent small study, mice received their microbiome from either a donor with severe alcoholic hepatitis or no liver disease. Following alcohol treatment, the mice with the microbiome from the patient with alcoholic hepatitis showed greater liver damage than mice that received stool from the healthy donor.[198]

Well-designed mouse models that combine our current understanding of liver disease with humanized microbiomes offer some of the greatest potential for preclinical interventions. Avatar, or sometime called Patient-derived Xerograph (PDX) mice, are widely used in the cancer community to test the efficacy of chemotherapeutics for individual tumors, including HCC.[199,200] This model better re-capitulates the complexity of a tumor than cell culture. Avatar mice can be further personalized by introducing an human immune system into an immuno-compromised mouse, along with the tumor.[199] Generating this model in germ-free mice with a humanized microbiome as well as immune system expands our capacity to understand the role of the microbiome in modulating cancer. For example, this model could be used to study whether the microbiome of a patient with alcoholic liver disease leads to more tumor growth than the microbiome from a healthy control.

The use of well-designed experiments in both mice and humans promise to expand our understanding of the role of the microbiome in the development and progression of liver disease.

Conclusions

An accumulating body of research suggests that the disparate observations in liver disease-related studies could be unified and explained by the microbiome. It is now widely accepted that liver damage is a result of an extensive interplay between gut microbiota via specialized molecules such as TMA, acetaldehyde, LPS and host-immune system via Kupffer cells-mediated liver inflammation. However, a comprehensive understanding of the exchange between the microbiome and the liver still evades us. Animal models, particularly rodents, have been instrumental in elucidating many important mechanistic pathways in disease etiology. The introduction of the microbiome into these models will provide a more complete view of the cancer ecosystem. Because microbiome research is sensitive to technical variability that often masks underlying biological signal, there is a need for consistency in technical platforms and standardized protocols, so that findings from different

laboratories (and model organisms) can be replicated and validated. Additionally, it is also critical to use an animal model that mimics human disease as closely as possible in all its physiological and metabolic manifestations.

We are slowly advancing from observation-based studies in human patients as recent research establishes grounds for microbiome-based therapeutic modalities such as fecal microbiota transplant (FMT) and probiotic interventions. However, effectively translating and applying findings accrued through animal models to humans requires well-designed, large-scale clinical trials spanning multiple disease etiologies and patient ethnicities. As the role of microbiota in liver disease development, prognosis and treatment is increasingly recognized, we emphasize on the need for focused, microbiome-aware efforts to efficiently tackle the socio-economic burden of this spectrum of liver diseases.

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Key points

- The liver and intestine communicate extensively through the biliary tract, portal vein and systemic mediators. Liver products primarily influence the gut-microbiome composition and gut barrier integrity, whereas intestinal factors regulate bile acid synthesis, glucose and lipid metabolism in the liver.
- Diverse liver diseases (ALD/ASH, NAFLD/NASH, PBC, PSC) are not unrelated, but converge along a common path of progression. Proinflammatory changes in the liver and intestine mediate development of fibrosis, cirrhosis and ultimately, hepatocellular carcinoma (HCC).
- Alcoholic and nonalcoholic liver diseases share key characteristics such as intestinal dysbiosis, gut permeability and shifts in levels of bile acids, ethanol and choline metabolites.
- Precise contributions of the microbiome to liver diseases may differ based on etiology. Improvements in experimental design and development of animal models is rapidly elucidating causal mechanisms.
- Recent advances in understanding the gut-liver axis encourage research into microbiome-based, diagnostic, prognostic, and therapeutic modalities to improve management of liver diseases.

Box 1: Primary Biliary Cholangitis (PBC)

Primary biliary cholangitis (PBC) is characterized by inflammation-mediated damage to the small bile ducts inside the liver gradually progressing to liver fibrosis and cirrhosis. Previously considered as a typical autoimmune disorder, the modified etiological understanding of PBC considers proinflammatory changes in the gut-microbiota, intestinal bile acid disruptions, and gut-barrier dysfunction.[201–204] Consequently, MAMPs ascend within the biliary duct, perpetuating infection. An immune attack against the biliary epithelial cells is mediated by antibodies that recognize E2 subunit of pyruvate dehydrogenase complex (PDC-E2) due to cross-reactivity with conserved proteins in *Escherichia coli*,[201] *Lactobacillus delbrueckii*,[205] and *Novosphingobium aromaticivorans*.[206] In fact, genetically susceptible mouse strains developed liver lesions mimicking PBC when infected with *Novosphingobium aromaticivoransm,* which further grounds the implications of microbiome associations in this disease.[207] Ursodeoxycholic acid, a tertiary bile acid produced by *Ruminococcus* has been approved for PBC-treatment.[208] Thus, microbiome-based treatment modalities hold promise for managing PBC and should be studied further.

Box 2: Primary Sclerosing Cholangitis (PSC)

Primary sclerosing cholangitis (PSC), is also an immune-mediated disease of the bile ducts. However, unlike PBC, PSC can affect bile ducts, both inside and outside of the liver. Gut dysbiosis-mediated bile dysregulation, intestinal permeability and translocation of proinflammatory molecules in the portal vein characterizes PSC.[201,209,210] The immune reaction in PSC is mediated by autoantibodies including p-ANCAs (perinuclear antineutrophil cytoplasmic antibody) that recognize the ubiquitously expressed bacterial antigen, FtsZ.[211] Furthermore, increase in microbe-associated TLR expression and T helper type 17 (Th17) cells has been reported in PSC which strongly suggests microbiome involvement in disease pathogenesis.[212,213] PSC is closely associated with inflammatory bowel disease (IBD), in particular ulcerative colitis and shares some of its characteristic features (such as increased Th17 cells). Thus, a common disease mechanism may be at play, and novel treatment avenues by targeting microbe-associated immune pathways can be explored.

Box 3: Open research questions

Mounting evidence implicates the gut microbiome in the development and progression of different forms of liver disease. However, several questions remain open and must be answered to advance the field.

- Is there a set of microbes (beneficial or harmful) that can read out the current extent, or predict the future extent, of disease progression in patients with ALD and NAFLD?
- Can microbiome research using a consistent set of methodologies, including multi-omics profiling, provide a consistent mechanistic picture that unifies our understanding of the relationships among forms of liver disease?
- Can fecal microbiota transplant, or collections of probiotic strains isolated from human feces, be expanded as a therapeutic modality for liver disease?
- Does introducing a humanized microbiome into an HCC avatar mouse improve its fidelity in terms of responding to therapeutic options like an individual patient?

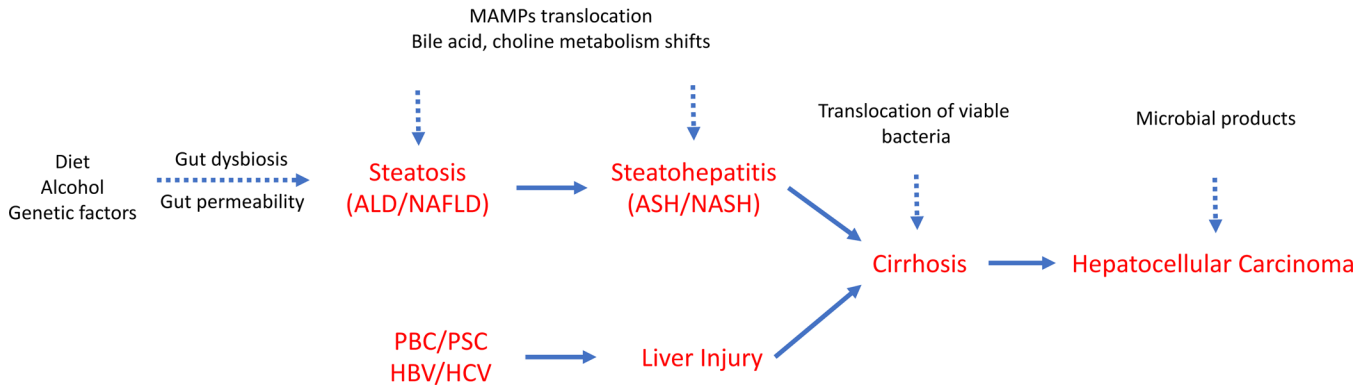

Figure 1: Physiological manifestations of liver injury along a spectrum of progression.

Risk factors such as alcohol abuse, unbalanced diet, infection (HBV/HCV) or immune dysfunction (PBC/PSC) can independently lead to liver injury. Alcohol-abuse patients and obese individuals often develop steatosis (fatty liver), which is characterized by increased intestinal permeability and dysbiosis. Subsequently, bile acid and choline homeostasis is disturbed along with increased translocation of MAMPs across the gut-barrier, leading to steatohepatitis, the progressive form of liver damage. Both, steatosis-dependent and steatosis-independent liver damage can progress to cirrhosis (end-stage liver damage), which is marked by translocation of viable bacteria to the liver and severe inflammation. As liver function is progressively compromised, tumor-promoting metabolites and xenobiotics accumulate. These could activate oncogenic pathways causing hepatocellular carcinoma, the most predominant form of primary liver cancers.

(MAMPs: Microbial-associated molecular patterns; ALD: Alcoholic liver disease; NAFLD: Nonalcoholic fatty liver disease; ASH: Alcoholic steatohepatitis; NASH: Nonalcoholic steatohepatitis; HBV: Hepatitis B virus; HCV: Hepatitis C virus; PSC: Primary sclerosing cholangitis; PBC: Primary biliary cholangitis)

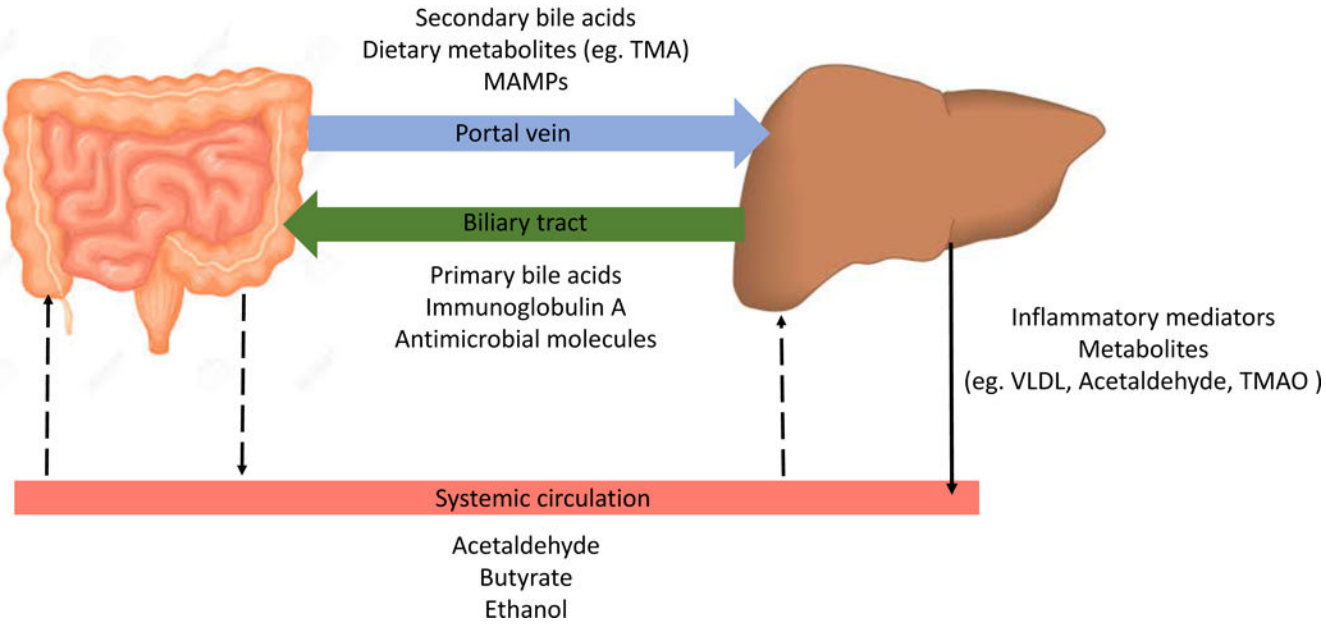

Figure 2: Bidirectional communication between gut and liver.

The liver transports bile salts and antimicrobial molecules (IgA, angiogenin 1) to the intestinal lumen through the biliary tract. This maintains gut eubiosis by controlling unrestricted bacterial overgrowth. Bile salts also act as important signaling molecules via nuclear receptors (such as FXR, TGR5) to modulate hepatic bile acid synthesis, glucose metabolism, lipid metabolism and energy utilization from diet. On the other hand, gut-products such as host and/or microbial metabolites and MAMPs translocate to the liver via the portal vein and influence liver functions. Additionally, systemic circulation extends the gut-liver axis by transporting liver metabolites from dietary, endogenous or xenobiotic substances (eg. FFAs, choline metabolites, ethanol metabolites) to the intestine through the capillary system. Owing to this medium of transport and ease of diffusion of systemic mediators across blood capillaries, these could affect the intestinal barrier both, positively (eg. butyrate) or negatively (eg. acetaldehyde)

(TMA: Trimethylamine; TMAO: Trimethylamine N-oxide; MAMPs: Pathogen-associated molecular patterns; VLDL: Very low-density lipoprotein; FXR: Farnesoid X receptor; TGR5: Takeda G-protein coupled receptor 5; FFA: Free fatty acid)

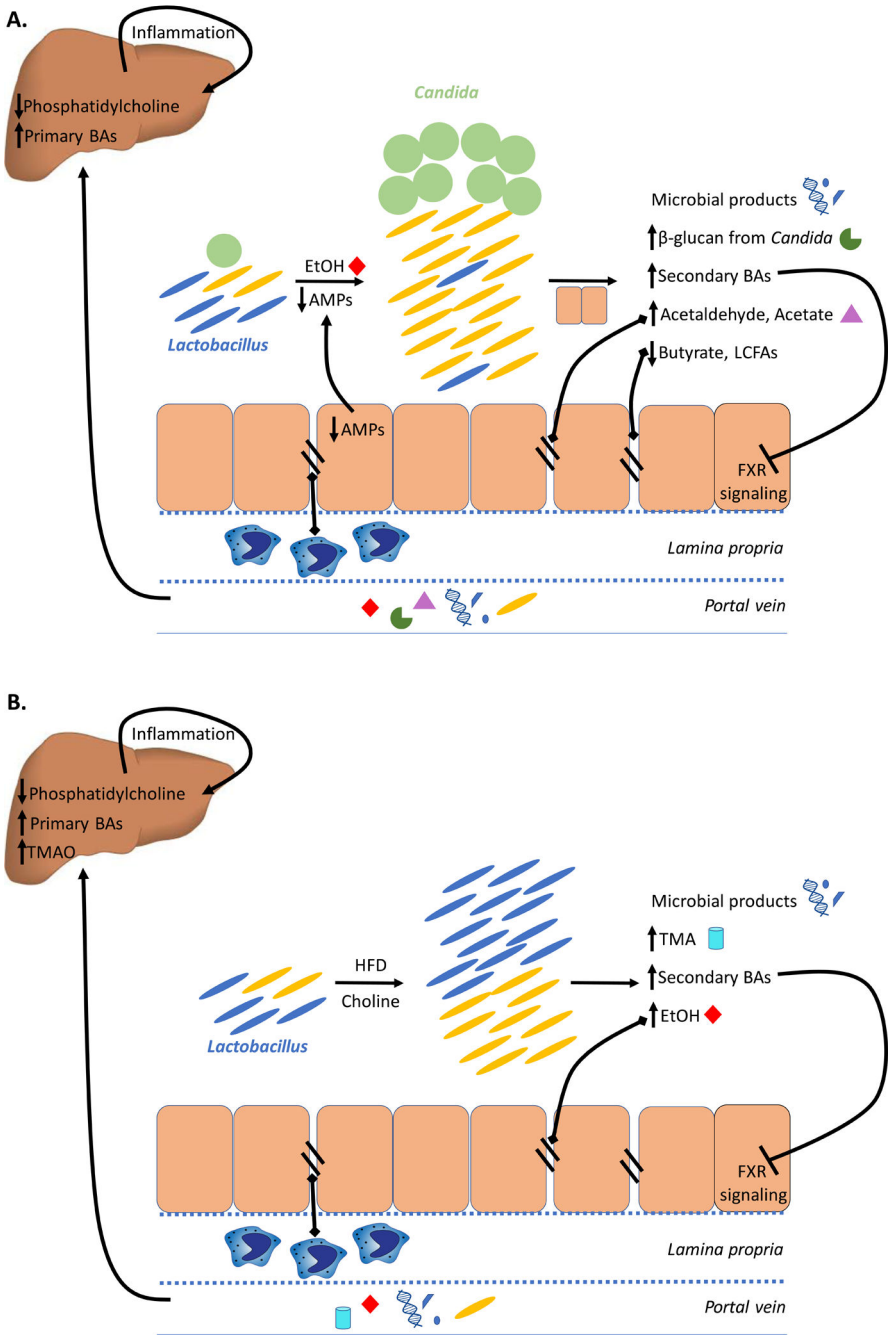

Figure 3. Interplay between the liver and gut microbiome in (A) Alcoholic liver disease (ALD) and (B) Nonalcoholic fatty liver disease (NAFLD).

Intestinal dysbiosis and bacterial overgrowth is observed in both, ALD and NAFLD. Bacterial overgrowth causes an increase in secondary BAs which disrupts FXR-mediated modulation of BA levels, leading to an overall increase in hepatic BA synthesis. A reduction in hepatic phosphatidylcholine is also seen in both ALD and NAFLD, which causes triglyceride accumulation in the liver (fatty liver). While ALD-associated dysbiosis is characterized by reduction in *Lactobacillus* and *Candida* overgrowth, NAFLD patients have higher abundance of *Lactobacillus* (effects on fungal population remain to be investigated).

Both, in ALD and NAFLD, increased ethanol and its metabolite acetaldehyde in the intestinal lumen mediates weakening of intestinal tight junctions. Consequently, increased translocation of MAMPs (seen in ALD and NAFLD) and gut metabolites such as acetaldehyde, acetate (seen in ALD) and TMA (seen in NAFLD) elicits intestinal and hepatic inflammatory responses, leading to progressive liver damage.
(AMP: Antimicrobial peptides; BA: Bile acids; EtOH: Ethanol; FXR: Farnesoid X receptor; HFD: High-fat diet; LCFA: Long-chain fatty acids; TMA: Trimethylamine; TMAO: Trimethylamine N-oxide)

Table1.

Comparison of alcoholic and nonalcoholic liver disease

	Alcoholic Liver Disease (ALD)	**Nonalcoholic Liver Disease (NAFLD)**
SIBO	Observed[106,214,215]	Observed[65]
Gut microbiota	↑ Enterobactericeae (humans)[133, 132] ↓ *Lactobacillus*[215, 133] (humans and mice), Bacteroidetes (humans)[132,134], *Akkermansia muciniphila* (humans and mice)[57] Gut microbiota protects against alcohol -induced liver injury[86] Reduced fungal diversity; *Candida* overgrowth[8]	↑ Enterobactericeae (humans)[216,64], *Lactobacillus* (humans)[216, 217], *Bacteroides* (humans and mice)[158, 125], *Ruminococcus* (humans)[125] ↓ *Prevotella* (humans)[125, 216], *Akkermansia muciniphila* (mice)[58] Gut microbiota mediates HFD-induced liver steatosis[218, 219] (Fungal dysbiosis not demonstrated)
Reversibility of gut dysbiosis	Partial reversibility on abstinence[113, 94]	(Reversibility not demonstrated)
Inflammation	↑ Intestinal TNF-α (mice)[103] ↑ Systemic inflammatory markers (humans)[47, 220]	↑ Intestinal TNF-α, IFNγ, IL-6 (humans and mice)[221, 216] ↑ Systemic inflammatory markers (humans)[222]
Transferability via microbiome	FMT from alcoholic hepatitis patients caused severe liver inflammation and injury in mice[198] FMT from ALD resistant to ALD susceptible mice prevented liver injury in recipient[131]	Cohousing inflammasome deficient, NASH mice with WT mice exacerbated liver steatosis WT cage mates[48] FMT from NAFLD-susceptible mice promoted liver injury in recipient[223]
Translocation	↑ PAMPs translocation (endotoxins[224, 47, 225, 226], β-glucan[8], viral/bacterial DNA[227, 224] (humans and mice))	↑ PAMPs translocation(endotoxins[225, 228], viral/bacterial DNA[229] (humans and mice))
Bile acids	↑ Total plasma bile acids (humans)[230] ↑ Hepatic bile acid synthesis (humans and mice)[141, 142]	↑ Total serum bile acids (humans)[231] ↑ Hepatic bile acid synthesis (humans)[35] ↑ Total fecal bile acids, primary to secondary bile acid ratio (humans)[35]
Choline	↓ Phosphatidylcholine in plasma and liver (rats)[232, 233] (Changes in trimethylamine not demonstrated)	↓ Phosphatidylcholine in plasma (mice)[234] ↑ Intestinal trimethylamine (mice)[234]
Free-fatty acids	↓ Bacterial fatty-acid biosynthesis (mice)[83] LCFA and SCFA supplementation reduced ethanol-induced liver injury (mice)[83,115]	↑ Free-fatty acids in the liver[235]
Ethanol	↑ Blood ethanol, luminal acetaldehyde[130] ↑ Systemic acetate[234, 83]	↑ Blood ethanol[64, 236, 237]

Table 2.

Experimental Mouse models for liver disease

Model	Description	Liver pathology	Microbiome Features
Diet			
High Fat diet	Diet using higher saturated fat, or supplemented with cholesterol compared to chow	Induces fatty liver and hepatic steatosis. Associated with metabolic syndrome phenotype.[238]	Common model for inducing dysbiosis; associated with changes in the microbiome
Choline Deficient Diet	A high fat diet with choline and methionine omitted.	Induces fatty liver, steatosis and inflammation and fibrosis. The model does not contribute to metabolic syndrome.[5]	Small study suggests diet-induced changes[239]
Ethanol supplemented liquid diet	A model of chronic alcohol abuse administered as an isocaloric diet where ethanol or maltose and dextrose are supplemented. Diet can be administered orally (Lieber-DeCarli[240]) or intragastrically (Tsukamoto-French[241])	Oral supplementation leads to inflammation and fatty liver, representing a good model for early ALD Intragastric administration leads to severe steatosis and mild fibrosis[4]	Diet affects the abundance of several taxa and is associated with changes in the microbiome[57]
Genetic Manipulations			
Knock out model	A mouse line where both copies of a gene have been removed	This depends on the gene. For example, $FxR^{-/-}$ mice have more fatty liver accumulation on a high fat diet.[31] $Muc2^{-/-}$ are protected from diet-induced liver injury.[107] $GSTA4^{-/-}$, PPAR-$\alpha^{-/-}$ double knockout mice have increased inflammation and fibrosis compared to either single mutant or WT[242]	The microbiome of lineage-derived mice is distinct from wild type mice. This is likely to be an effect of microbiome drift within the colonies, rather than a direct effect of the genotype.[243]
Littermate controls	Mice from a heterozygous cross that lead to Wild Type and knockout littermates.		Much of the mouse microbiome is acquired through vertical transmission; littermates are better microbial controls.[244]
Cre-Lox localized mutation	A genetic cross that allows for tissue-specific knockout of a gene	This is gene dependent on the gene. A Cre/Lox model of liver specific E-cadherin knockout shows pathology like primary sclerosing cholangitis, and increases susceptibility to cancer.[245] The loss of TLR 5 in hepatocytes leads to increased inflammation and fibrosis in a high fat diet induced model of NASH[246]	Microbiome considerations depend on the how the controls are selected.
Avatar Mice	Mice transplanted with solid state tumors from cancer patients.	Human hepatocellular carcinoma can be transplanted into the mouse.[247]	There is no specific effect on the microbiome.
Microbiome			
Antibiotic treatment	Treatment with a broad-spectrum antibiotic	No direct effect on liver disease; Antibiotics can moderate the effect of other interventions.	Antibiotics can have off target effects and significantly alter the microbial community in addition to decreasing the bacterial load[194]
Probiotic manipulation	Microbial supplementation to modify the microbiome	No direct effect on liver disease; probiotics can modulate the effect of other treatments: Lactobacillus to ameliorate alcohol-induced liver	Can lead to the over-abundance of a specific organism or correct defects in the community. However, not all probiotics

		injury)[106]	colonize.
Germ-Free Mice	Raised without any bacterial community	Germ free mice have immune defects.[248] These mice are also more susceptible to alcohol-induced liver injury.[86]	Useful to demonstrate the importance of bacterial communities for a phenotype.
Monoculture gnotobiotic mice	Germ free mice that have been colonized with a single bacterium or defined bacterial community	No direct effect; depends on the community transplanted and challenge	Can test whether the defined community can modulate the phenotype
Mouse transplant	Bacterial communities from mice transplanted into germ free mice	No direct effect; depends on the community transplanted and challenge	Demonstrates whether mouse phenotype is transferable or can be modulated through the microbial community.
Humanized mice	Germ free mice which have been gavage with the microbiome from a human donor	No direct effect; depends on the community transplanted and challenge	Demonstrates whether human phenotype is transferable or can be through the microbial community.