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Microbiome-Liver Crosstalk: A multihit therapeutic target for liver disease

Microbiome-Liver crosstalk in liver disease

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Abstract

Liver disease has become a leading cause of death, particularly in the West, where it is attributed to more than two million deaths annually. The correlation between gut microbiota and liver disease is still not fully understood. However, it is well known that gut dysbiosis accompanied by a leaky gut causes an increase in lipopolysaccharides in circulation, which in turn evoke massive hepatic inflammation promoting liver cirrhosis. Microbial dysbiosis also leads to poor bile acid metabolism and low short-chain fatty acids, all of which exacerbate the inflammatory response of liver cells. Gut microbial homeostasis is maintained through intricate processes that ensure that commensal microbes adapt to the low oxygen potential of the gut and that they rapidly occupy all the intestinal niches, thus outcompeting any potential pathogens for available nutrients. The crosstalk between the gut microbiota and its metabolites also ensures an intact gut barrier. These processes that protect against destabilization of gut microbes by potential entry of pathogenic bacteria are collectively called colonization resistance and are equally essential for liver health. In this review, we shall investigate how the mechanisms of colonization resistance influence the liver in health and disease and the microbial-liver crosstalk potential therapeutic target areas.

Core Tip: The influence of the gut microbiome on various body systems has important implications for health and disease, such as liver disease. While the exact mechanisms of how the microbiome contributes to liver disease are unknown, there is strong evidence that the translocation of various metabolites across the mucosal barrier plays a strong role, which is precipitated by dysbiotic gut microbiota. Considering the

importance of the microbiome in liver disease, powerful therapeutic options that can manipulate the gut microbiome are being explored. These approaches could have the potential for effective treatments for various stages of liver disease. In this review, we shall investigate how the mechanisms of colonization resistance influence the liver in health and disease, and finally explore potential therapeutic targets in the gut-liver axis.

INTRODUCTION

Introduction

A healthy gut microbiota plays a significant role in maintaining a homeostatic gut environment. One such role is colonization resistance, which is defined as the microbial capacity to resist invasion of exogenous microorganisms (for example, pathogens) and/or prevent uncontrolled overgrowth of endogenous microbes (for example, pathobionts). For gut homeostasis to be achieved, microbial alpha diversity must remain high, gut mucosal integrity must be maintained, and tolerance to the billions of microbial immunogens present in the gut must be established. This is all achieved through intricate microbe-to-microbe and microbe-to-host interactions mediated by microbial metabolites, such as short-chain fatty acids, or microbial cell wall components, such as lipopolysaccharides, lipoteichoic acid, peptidoglycans and flagellin. Homeostasis is also achieved through the production of antimicrobial peptides, resource and oxygen competition, host immunomodulation, and conjugation of bile acids. The mechanisms by which these inter/intramicrobial interactions mediate colonization resistance or how their perturbation leads to disease have not yet been fully elucidated. However, it is known that an imbalance in microbial composition, otherwise known as dysbiosis, which may arise from dietary changes, ingestion of exogenous toxins such as antibiotics or xenobiotics, or through infections that suppress the immune system, has serious and sometimes long-term clinical implications. Diseases such as diabetes, obesity, atherosclerosis, and liver disease are associated with dysbiosis and the translocation of gut microbial products into circulation. As the liver is the first organ to be exposed to the gut bacterial products and digested food

delivered through the portal vein, any leakage of microbial products into circulation will lead to hepatocellular immune activation, thereby promoting systemic and hepatic inflammation, which may lead to liver disease[1]. An understanding of the mechanisms involved in colonization resistance and its influencing factors is therefore crucial to establish their link to the etiology of liver disease as well as to identify possible hit points along the gut-liver axis that can be utilized as therapeutic targets for liver disease[2]. This review explores some of the mechanisms of colonization resistance and their importance to the etiology of the different stages of nonalcoholic fatty liver disease (NAFLD) from simple steatosis to liver inflammation, as well as alcohol-associated liver disease (ALD), and highlights potential targets that may be used as therapeutic targets for liver disease. A summary of the interplay between the microbiome, liver, immune system, and metabolome is presented in **Figure 1**.

Figure 1: During development of nonalcoholic steatohepatitis (NASH), several immunological and metabolic pathways intersect, thus promoting progression of liver injury and NASH. In healthy conditions, gut-liver axis homeostasis is guaranteed by intact intestinal epithelium barriers and proper liver-host immune functions that limit the translocation of bacteria and their metabolites. In NASH, on the one hand, the intestinal barriers are disrupted (thin mucus layer, decreased expression of tight junction proteins, altered ratio of Firmicutes to Bacteroidetes, dysbiosis, decreased short-chain fatty acids (SCFAs) that result in increased leakage of bacteria and their metabolites (LPS, MDP, flagellin, bacterial DNA) into the portal vein and systemic circulation, consequently stimulating the production of inflammatory cytokines in the systemic circulation. On the other hand, liver function is compromised because of the accumulation of fat, altered lipid metabolism, and increased microbial burden, which in turn elicits hepatic inflammation, hepatic stellate cell activation (HSC) and collagen deposition, Kupffer cell (KC) activation, and triggering of the TLR4

signaling pathway, which altogether contribute to the development of NASH. Abbreviations: Tj; tight junction, SCFAs: short-chain fatty acids, KC: Kupffer cell, HSC; hepatic stellate cell.

Gut Microbial Eubiosis

The gut microbiome starts taking shape at birth, where it is initially influenced by the mode of delivery. Vaginally born babies will have a gut microbial composition very close to the maternal vaginal microbiota, while the caesarian born will adopt mainly the skin microbiota[3]. Mammals have five phyla that predominate the gut: Firmicutes (e.g., *Lactobacillus, Clostridium, Ruminococcus, Eubacterium, Fecalibacterium and Roseburia*), Actinobacteria (with *Bifidobacterium* as one of its most important members), Bacteroidetes (e.g., *Bacteroides, Prevotella, and Xylanibacter*), Proteobacteria (e.g.,*Escherichia* and *Desulfovibrio*) and Verrucomicrobia (e.g., *Akkermansia*)[4]. The earliest colonizers are mainly facultative aerobes of the phyla Firmicutes and Actinobacteria, which play a significant role in lowering the gut's oxygen level to allow for the colonization of obligate anaerobes. These aerotolerant microbes reside in the upper gut, where they continue to reduce the amount of oxygen in the gut for life. *Escherichia coli* and *Enterococcus faecalis* are the most abundant in the oxygen-high neonatal gut, and they rapidly expand in the early phase, leading to a gradual depression of oxygen levelsand allowing growth of the facultative anaerobes *Bifidobacterium, Bacteroides* and *Clostridium,* which colonize most of the lower gut [4,5]. The neonatal microbiota is also influenced by the mode of feeding, where breast-fed babies show a more stable microbiota that has a higher copy number of *Bacteroides* and *Bifidobacterium* but a lower abundance of *Enterococcus* and *Streptococcus* species, while formula-fed babies have a higher abundance of *Clostridium, Streptococcus* and *Enterococcus*[6]. The early life microbiota only begins to take a semblance of adult microbiota when solid food is introduced and will remain relatively unstable until 3-5 years after birth[7]. The rapid expansion of early life

microbiota and the adaptation to oxygen levels signify the earliest mechanisms for initiating gut microbial homeostasis[8].

The colon has the highest density of microbes in the gastrointestinal tract, harboring approximately 70% of all gut microbes, which are mostly members of the Firmicutes and Bacteroidetes phyla[9]. The Firmicutes to Bacteroidetes axis is important in maintaining gut homeostasis, as members of each phylum have specialized metabolic roles (i.e., metabolism of sugar vs. indigestible fibers) that impact the microbiome and the host. It is believed that the role in homeostasis is optimized when the relative abundance is 15% Firmicutes and 80% Bacteroidetes [8,10 11]. However, the significance of this value and the actual impact it has on the host have been questioned by some researchers,[12] emphasizing the importance of more research on the role of Firmicutes and Bacteroidetes in gut microbial homeostasis, health and disease. Nutrients, metabolic byproducts and the competition between exogenous microbes and commensals help prevent colonization of pathogens and maintain homeostasis. Different animal studies have shown that nutrient competition occurs between metabolically related microbiota members. For example, germ-free mice colonized with three human commensal strains of *Escherichia coli* (*E. coli* HS, *E. coli* Nissle 1917, *E. coli* MG1655) successfully prevented colonization of the cecum by the pathogen enterohaemorrhagic *Escherichia coli* (EHEC) EDL933, an *E. coli* 0157:H7 biotype, due to the three precolonized commensal biotypes outcompeting *E. coli* EDL933 for nutrients[13]. This colonization resistance was further shown to occur by the use of multiple sugars as metabolic substrates for probiotic *E.coli* Nissle 1917 and commensal subtype *E. coli* HS, whose rapid growth effectively limited the colonization of EHEC *E.coli* EDL933 in a mouse model[14]. Competition for a shared nutritional niche of proline was similarly demonstrated in a gnotobiotic mouse model colonized with early life microbiota where early-life *E. coli* 1 was shown to outcompete *E. coli* 0157:H7[15]. This colonization resistance was also thought to be attributed to the production of lactate and acetate by bifidobacteria and enterococci, which can suppress the motility of *E. coli* 0157:H7 under

cecal anaerobic conditions[15]. Colonization resistance is also aided by the production of toxic antimicrobial peptides by commensals. For example, many members of the phylum Bacteroidetes produce toxic antimicrobial peptides through their type 6 secretion systems (T6SS)[16], *E. coli* produces narrow-spectrum antibiotics called microcins that effectively kill competitors within their niche[17,18], and the probiotic *Bifidobacterium* secretes broad-spectrum bacteriocins[19].

Overall, any extrinsic or intrinsic factors that upset the stable microbial communities will in essence destabilize the colonization resistance mechanisms and lead to disease by allowing colonization of pathogenic microbes and/or leakage of microbes and microbial toxins into circulation.

Colonization resistance through microbial enhancement of gut barrier function
The gut is lined with a thick mucus layer made of a highly glycosylated mucin 2 protein (MUC2), which is densely packed and insoluble in the layer closest to the epithelium but loosely packed and soluble on the outer layer[20,21]. This mucus layer prevents direct contact of bacteria with the gut epithelium, thereby reducing the potential for pathogen colonization[20,21]. The development of the mucus layer is enhanced by the gut microbiota and depends on the intestinal microbial composition. It has been shown that germ-free rodents have a much thinner mucus layer than their conventionally colonized counterparts[22]. Petersson and colleagues have shown that a thin colon mucosal layer in a colitis germ-free mouse model can be restored by administering lipopolysaccharides or peptidoglycans to germ-free mice[22]. Bacteria enhance the mucus layer in numerous ways, such as through the production of secondary metabolites. SCFAs, such as acetate produced by *Bifidobacterium* or butyrate produced by gram-positive Firmicutes such as *Faecalibacterium prausnitzii, Roseburia sp, and Butyricicoccus pullicaecorum*[23,24], are known to strengthen gut barrier function, normalize permeability, improve intestinal epithelium defense, protect against pathogenic infections, and reduce inflammation [25–28].

Intestinal epithelial cells are held together by a set of tight junction proteins that are molecules situated at the tight junctions of epithelial cells. The integrity of these tight junctions can be influenced by commensal bacteria and their effects on tight junction proteins. For example, *Lactobacillus rhamnosus* (*L. rhamnosus*) GG induces claudin-3 expression, *L. acidophilus* and *L. plantarum* stimulate the expression of occludin, and *Bifidobacterium infantis* preservesclaudin-4 and occludin deposition at tight junctions[29,30]. In a mouse necrotizing enterocolitis model, *Bifidobacterium* was found to preserve claudin 4 and occludin localization in tight junctions, thereby preventing gut permeability[31]. In mouse models, probiotics have been shown to improve the integrity of the intestinal barrier, which has also been observed in Crohn's and colitis patients[28]. *In vitro* treatment of Caco-2 cells with the probiotic *E. coli* Nissle 1917 increases the expression and peripheral migration of ZO-2[32]. Treatment of Caco2 cells with the probiotic *Lactobacillus plantarum*MB452 increased occludin and cingulin gene expression[33]. These results indicate that *in vitro*, certain probiotics can improve gut barrier function. Maintaining the integrity of the intestinal barrier is essential due to the high levels of microbial LPS present within the lumen of the gut, as LPS is a potent immunological signal that can induce an inflammatory cascade if detected systemically, which a healthy intestinal barrier effectively prevents[34]. Leakage of LPS and other microbial polypeptides into circulation due to dysbiosis can lead to inflammation in the liver (among other organs), which can lead to the development of liver disease[34].

Colonization resistance and bile acid metabolism

Pericentral hepatocytes primarily produce bile acids from cholesterol[35]. In humans, these acids are then transported to the gut, where they are dehydroxylated, epimerized, or dehydrogenated into different secondary bile acids, such as deoxycholic acid (DCA), ursodeoxycholic acid (UDCA), ursocholic acid (UCA), or lithocholic acid (LCA)[35]. In mice,murideoxycholic acid (MDCA) and hyodeoxycholic acid (HDCA) are also produced[35]. Secondary bile acids are known to bind to the intestinal farnesoid X

receptor (FXR) and G-protein coupled receptor 5 (TGR5)[36]. Some bile acid metabolites have also been shown to have a contradictory effect on gut barrier tight junctions[36]. UDCA and LCA, for example, have opposing effects on the barrier of human colonic T84 cells[36]. Treatment of these cells with primary bile acid-chenodeoxycholic acid (CDCA) combined with lithocholic acid (LCA) leads to an increase in barrier permeability and the inflammatory cytokine IL-8[37]. Using a Caco-2 cell model, it was demonstrated that deoxycholic acid (DCA) led to an increase in the phosphorylation of epithelial growth factor receptor, which induced barrier dysfunction[38]. Prematurely weaned piglets treated with CDCA showed an improvement in the gut barrier with higher ZO-1 expression and increased expression of the proinflammatory cytokines TNF-α and interleukin (IL)-6 and the anti-inflammatory cytokine IL-10[39]. The authors speculated that the anti-inflammatory effects of both IL-10 and ZO-1 counteracted the inflammatory effects of IL-6 and TNF-a, thus precipitating a net improvement in the intestinal barrier[39]. These examples demonstrate that bile acid metabolism is a significant key player in gut health, and it can be utilized as a therapeutic target for liver disease and other metabolic disorders, as will be discussed later.

Microbial association with liver disease

Liver disease has been shown through preclinical and clinical trials to be accompanied by gut dysbiosis[40–44]. It has been shown that liver cirrhosis is also correlated with bacteremia, increased gut permeability and increased circulatory LPS[43]. Dysbiosis has been noted in many mouse models of liver disease, such as secondary biliary fibrosis (common) induced by bile duct ligation, alcoholic liver disease induced by alcohol uptake in drinking water and hepatotoxicity-induced liver cirrhosis using carbon tetrachloride (CCL_4) treatment[42,43]. In humans, several gram-positive bacteria, including members of the genera *Clostridium* XI, *Anaerobacter*, *Streptococcus*, and *Lactobacillus*, were found to be more abundant in the gut in NAFLD patient biopsies than in healthy volunteers[45]. In contrast, *Oscillibacter* and *Flavonifractor* of the family *Ruminococcaceae* were abundant in healthy volunteers relative to NAFLD

patients[45]. In severe fibrosis forms of NAFLD, the bacteria *Bacteroidetes vulgatus* and *Escherichia coli* were identified as the most abundant[46]. Although there has not yet been a general consensus on what microbial ratios of different strains exist in NAFLD patients, many research findings indicate that a lower Firmicutes to Bacteroidetes ratio is associated with liver disease[47,11]. Dysbiosis may be caused by a reduction in bile acids (which are bacteriostatic) of a cirrhotic liver, which precipitates inflammation and immunosuppression, factors that can positively feedback on cirrhosis[42]. Dysbiosis may also arise from increased saprophytic fungal growth in the alimentary canal. Cirrhotic liver patients who routinely receive antimicrobial treatment have an overgrowth of fungi, especially *Candida,* leading to fungal-bacterial balance in the gut and worseningdysbiosis[42]. Although cirrhosis is a systemic disease, it is believed to be worsened by dysbiosis both in the gut liver axis and outside this axis, such as in saliva and serum[42,48].

While there is a knowledge gap on the use of microbial interventions for NAFLD therapy, there are data showing that nonalcoholic steatohepatitis (NASH) patients improve following treatment with the antibiotic rifaximin, which is used for the treatment of traveler's diarrhea caused by *Escherichia coli*[49]. In a study examining the gut microbiota of stage 4 hepatitis C virus (HCV) patients, *Prevotella* and *Faecalibacterium* were found to be more abundant in HCV patients than in healthy controls, while *Rhuminococcus* and some *Clostridium* species were more abundant in healthy controls than inHCV patients. *Bifidobacterium* was found only in healthy individuals[50]. Germ-free mice were shown to develop NAFLD following fecal microbial transplantation from donor hyperglycemic mice with systemic inflammation when fed a high-fat diet[51]. On the other hand, germ-free recipients that received fecal transplantation from normal donors (i.e., normoglycemic with negligible systemic inflammation) did not develop NAFLD and were normoglycemic when fed a high-fat diet[51]. Rabot *et al*. also showed that germ-free mice fed a high-fat diet were more resistant to hepatic steatosis than colonized controls[52]. In an experimental mouse model of cholestasis-induced liver fibrosis induced either through bile duct ligation or

by CCl[4] treatments, colonization with complex microbiota (specific pathogen-free mice) was protective against severe fibrosis when compared to limited colonization (Altered Schaedler Flora)[53]. How the gut microbiota induces a leaky gut, bacteriaemia and an inflammatory flare leading to liver disease has been the subject of intense research. Brown and colleagues fed mice a high carbohydrate diet to induce a leaky gut[54]. This high carbohydrate diet caused a sloughing of the intestinal villi and reduced tight junction integrity, which allowed bacteria to translocate into the circulatory system[54]. In cirrhotic patients, it has been shown that microbial components leaking through the intestinal barrier, such as LPS, lipoteichoic acid, lipopolypeptides, and peptidoglycans, activate Toll-like receptors (TLRs) in hepatic stellate cells, Kupffer cells, and hepatocytes (all of which are differentially populated with TLRs 1-9), inducing severe inflammatory responses and fibrosis in the liver[43,55] Microbial activation of TLR2 in monocytes has especially been identified as significant in liver fibrosis through the production of tumor necrotic factor (TNF) alpha, which initiates a cascade of reactions leading to increased gut permeability[43].

Microbial metabolites in liver disease

Gut microbiota-host crosstalk in liver disease remains widely unclear. However, in recent years, many studies have established a correlation between different microbial metabolites and liver disease[56]. LPS are gut microbiota-derived endotoxins that form the major component of the gram-negative bacterial outer cell wall. High plasma levels of LPS have been identified in NAFLD patients and are associated with gram-negative intestinal bacterial overgrowth and compromised gut lining epithelial tight junctions[57,58]. LPS induces an inflammatory response by activating hepatic Kupffer cells through TLR4. Apart from inducing proinflammatory cytokines and chemokines from hepatic Kupffer cells, LPS also activates hepatic stellate cells (HSCs) to differentiate into myofibroblast-like cells by producing extracellular matrix proteins, thus promoting liver fibrosis[59-61,49]. Other important metabolites are SCFAs from the fermentation of indigestible dietary fiber, which are mostly found in the colon, where most of them are produced and absorbed[62]. The major microbial fermentation products following

microbial degradation of fiber are the SCFAs butyrate, propionate, and acetate. The body utilizes approximately 10% of the energy supply from microbially derived SCFAs, meaning that 90% is stored in white adipose tissue[63]. Several studies have revealed that gut microbial dysbiosis is associated with chronic liver diseases such as NAFLD or ALD[64, 45]. In a metabolomic study in children with NASH, serum levels of 2-butanone and 4-methyl-2-pentanone were found to be elevated compared to those in healthy individuals[65]. Adults with NAFLD were found to have higher levels of fecal propionate and isobutyric acid, which are part of the fecal SCFA family[66]. Obese patients with NAFLD were also found to have high levels of propanoic acid and butanoic acid[67]. SCFAs such as acetate and butyrate modulate the host immune response by dampening the LPS-induced hepatocellular inflammatory response and restoring mucosal and systemic immunologic homeostasis, thus minimizing liver injury[68,69]. SCFAs can act as hormonal molecules by binding to G-protein-coupled receptors (GPCRs), which leads to activation of the GPCR pathway, slowing gut motility and increasing energy harvest[70-72]. Upon activation, glucagon-like peptide-1 (GLP-1) is secreted from epithelial L-cells, enters circulation and induces insulin release from the pancreas[70]. GPCR pathway activation also limits insulin-mediated hepatic and muscular fat accumulation and stimulates energy expenditure[71]. In adipocytes, SCFAs activate GPR41 and GPR43 to inhibit lipolysis and activate adipocyte differentiation[70]. SCFAs also regulate immune cell functions through GPR43, which is widely expressed in most immune cells[73-75]. SCFAs have also been shown to inhibit histone deacetylases (HDACs), which downregulate gene expression and reduce the production of inflammatory cytokines, particularly in macrophages and blood mononuclear cells during acute inflammatory hepatitis[69]. Therefore, it can be argued that dysbiosis that reduces microbial SCFA generation will result in a dysregulated inflammatory response and thus contribute to the progression of liver disease."

Indole and its derivatives are microbial metabolites of tryptophan breakdown. Indole upregulates tight junction proteins in the gut and downregulates colonic epithelium inflammatory genes through the aryl hydrocarbon receptor[76]. Indole-3-propionate

activates pregnane X receptor (PXR) to downregulate proinflammatory cytokine production and has been associated with protection against injury through oxidative stress signaling[76,77]. Indole-3-acetate has been shown to modulate hepatocyte lipogenesis, thus playing a protective role against NAFLD[78]. Microbial metabolism of dietary choline and L-carnitine produces trimethylamine (TMA), which is oxidized to trimethylamine N-oxide (TMAO) during hepatic detoxification of the blood through catalysis of the liver enzyme hepatic flavin monooxygenases[79]. TMAO is excreted in urine, and recent findings in animal NAFLD models fed a high-fat diet have shown increased urine levels of TMAO[80]. In a Chinese cohort study, the severity of NAFLD was closely associated with circulatory TMAO[81]. Bacteria are essential for the conversion of dietary choline to trimethylamine (TMA), which is oxidized in the liver through the catalysis of hepatic flavin monooxygenase to generate trimethylamine-N-oxide, whose accumulation has been associated with both cardiac and renal disease[82,83]. Phosphatidylcholine is also metabolized by gut microbes to generate TMA, whose oxidation in the liver yields TMAO and, as previously described, may lead to kidney and cardiac disease[84,85]. It is now thought that accumulation of TMAO in the liver causes NASH through the inhibition of farnesoid X receptor (FXR) and alteration of bile acid homeostasis[86]. SCFAs are significant microbial metabolites in the etiology of liver disease. More studies are required to target SCFAs as diagnostic or therapeutic tools for predicting or treating liver disease.

Diet and xenobiotics in liver disease

Liver disease is highly influenced by exposure to different environmental factors, which has recently been referred to as the exposome. It is now known that liver disease is impacted by an interaction between the genetic makeup of the host, exposome, and gut microbiome[87,88]. Certain types of gut microbiota have been associated with endogenous alcohol generation, which may in turn be hepatotoxic, leading to NASH[89]. The gut microbiota is important for the metabolism of bile acids, and in the absence or deficiency of bacteria that can convert primary bile acids to secondary bile acids, there is an accumulation of circulatory bile acids, which in turn activate Takeda-G-protein-

coupled receptor 5 (TGR5), leading to monocyte dysfunction, which may exacerbate the hepatic inflammatory response and lead to liver disease[90]. High circulatory bile acids reflect a dysfunctional FXR, the nuclear receptor responsible for bile acid homeostasis, whose function is to facilitate enterohepatic bile acid circulation[91]. Dysbiosis affecting 7α-dehydroxylation-rich Firmicutes, which convert primary bile acids to FXR-low-binding secondary bile acids, will inevitably affect the function of FXR, leading to liver disease[92]. The liver is a crucial filter for toxins that find their way into the body either accidentally or deliberately. Alcohol is by far the most significant xenobiotic causing liver disease in humans, and it has been identified as the cause of alcoholic fatty liver disease (ALD)[93]. It can be argued that alcohol consumption causes both destruction of microbial communities and rupture of the barrier wall integrity in the gut and leads to induction of inflammation during detoxification in the liver. A compromised gut barrier leads to leakage of LPS and other microbial ligands into circulation, triggering inflammation of liver cells.

A high-fat diet and environmental pollutants are further risk factors for liver disease, and their effects are exacerbated by microbial metabolites[94,95]. It is likely that most xenobiotics, in addition to being directly toxic to hepatic cells, will cause dysbiosis that favors changes in microbial composition that generate toxic liver disease-causing metabolites. The changes in these microbial metabolites may therefore be used as noninvasive diagnostic biomarkers for liver disease[96] but may also become significant therapeutic targets for the treatment of this disease[96,97,98]. A high carbohydrate diet has been demonstrated in environmental enteropathy animal models to lead to intestinal wall epithelial brush-border shortening and loosening of tight junctions[54]. Furthermore, small intestinal gram-negative bacterial overgrowth and high plasma LPS levels can lead to liver disease[56]. In the absence of dietary fibers, the gut microbiota cannot produce sufficient SCFAs, which may lead to a dysregulated inflammatory response and liver disease[99,100]. Most liver metabolism occurs through the catalysis of cytochrome P-450 (CYP-450), and it is known that many dietary biproducts

can influence the activity of CYP-450[101]. Dietary retinoids, for example, are metabolized by hepatic cells, including hepatic stellate cells. An alteration in the uptake and metabolism of retinoids may influence retinoic acid signaling, which may activate hepatic stellate cells, resulting in loss of retinoid stores, aberrant extracellular matrix generation and the onset of fibrosis, which inevitably precipitate liver disease[102]. Additionally, alcohol consumption affects hepatic retinoid metabolism through inhibition of retinoid oxidation, induction of CYP2E1 enzymes to increase retinoic acid metabolism, or increased peripheral tissue damping of retinoic acid, all of which lead to activation of HSCs and development of liver disease [103]. Retinoic acid is a gut microbial metabolite of vitamin A whose intestinal concentration is modulated by suppression of retinol dehydrogenase 7 expression by commensal *Clostridia* microbes[104]. Retinoic acid not only regulates bile acid homeostasis but also shares with it the receptors retinoid X receptor (RXR) and farnesoid X receptor (FXR) and therefore shares the functions of lipid metabolism and insulin sensitivity[105]. In a rat model, a high-fat diet in combination with high glucocorticoid treatment resulted in a fourfold hepatic lipid deposition and an almost threefold increase in circulatory alanine aminotransferase indicative of liver injury[106]. A high-fat diet also caused severe liver damage with high levels of circulatory alanine transaminases (ALT) and aspartate aminotransferases (AST) in a mouse model[107]. Mice fed a high-fat diet developed high intestinal gram-negative microbial growth and an increase in ethanol-producing bacteria when compared to mice fed normal chow[107]. This result is consistent with findings from clinical studies where it has been documented that microbial diversity rapidly changes with a change in diet[108]. Therefore, it can be concluded that diet, food additives, and xenobiotics affect liver disease by influencing gut microbial composition, gut permeability, and microbial metabolites. The liver plays a major role in metabolism and blood detoxification and is thus prone to damage from microbial endotoxins, environmental toxins, and microbial dietary metabolites, all of which work together in cascaded inflammatory responses to cause liver injury. Understanding the individualized microbial signatures and their

influence on gut permeability, immunologic inflammatory responses, and the hepatic response to insult will expose multientry avenues to precision liver disease therapy.

Microbiome-host interaction in liver disease

The intestine is heavily colonized with microbiota, yet the surrounding tissues remain sterile. This barrier is maintained by intricate crosstalk between gut microbes, the gut wall epithelium and the innate immune system[109,110]. The expression of intercellular tight junction proteins between the intestinal epithelium is regulated by cytokines such as interferon gamma (IFNγ) and tumor necrosis factor (TNF) and other regulatory cytokines that interact with immunoglobulin A (IgA)-coated gut microbiota to maintain gut and immune homeostasis[111]. A change in diet or intake of xenobiotics such as alcohol, prescription/over-the-counter drugs, or other environmental chemicals may lead to destabilization of the intestinal homeostatic environment either through selective overgrowth or reduction of specific microbial strains or injury to the mucosal lining. A destabilization of the homeostatic environment will give way to a shift in the immunological signaling molecules protective to the gut tight junctions and a sloughing of intestinal villi. This breach in the barrier allows leakage of microbial endotoxins into circulation and microbial translocation into the liver, thus triggering an immunological inflammatory response once the microbial products are detected by the liver's pathogen recognition receptors, mainly the TLR and nucleotide oligomerization domain (NOD)-like receptors (NLR)[109,112]. HSCs are endowed with TLR 2, 4, and 9, which are associated with promoting TLR4 fibrosis[60, 43]. Kupffer cells are lined with TLR 2, 3, 4, and 9 and are hepatic macrophages that form the main targets of microbial ligands within the liver[43]. Furthermore, hepatocytes express TLR 1-9 and are the most abundant cells in the liver, playing a critical role in the acute phase of the immunologic response through cytokine-like interleukin 6 (IL-6)[113]. The inflammatory response of the liver to leaked gut-microbial endotoxins is not yet fully understood. However, it is known that upon activation, Kupffer cells release proinflammatory and profibrogenic cytokines, such as TNFα, TGFβ and IL-1β, and a few more members of the inflammasome whose effect is

to induce inflammation and accumulation of lipids in the liver, and if this is not resolved, it leads to fibrosis (NAFLD)[114]. Therapeutic target efforts are geared toward minimizing the hepatic inflammation seen after proinflammatory cytokine release. Chemokine receptor antagonists such as CCR2 and CCR5 (Cenicrinivoc CVC) have been used with some success to decrease leukocyte infiltration, and when used in a diet-induced NASH mouse model and a thioacetamide-induced fibrosis rat model, liver fibrosis was effectively reduced[115,116]. This outcome has since been replicated in phase 2 clinical trials with a remarkable reduction in fibrosis[117]. Several other proinflammatory cytokines, including IL-17, IL-11, and IL-1, are still under investigation. A clinical trial therapy utilizing an IL-1 pathway anti-inflammatory drug, diacerelin, achieved a remarkable reduction in fibrosis in NAFLD patients with diabetes[118].

The gut microbiome as a diagnostic biomarker for liver disease

The dynamics of the gut microbiome could be used as a noninvasive diagnostic tool for liver cirrhosis and hepatocellular carcinoma (HCC)[119]. In a cross-regional prospective validation study in China, human fecal samples analyzed for microbial diversity revealed a significant rise in diversity as the liver condition advanced from cirrhosis to HCC with cirrhosis[119]. There was also a high level of butyrate-producing bacteria in healthy controls relative to early cirrhosis patients and a notable rise in LPS-producing bacteria in HCC patients[119]. In a different experiment, gut microbiota known to originate from the oral cavity were found to be enriched in liver cirrhosis patients relative to healthy volunteers[120]. In an Asian NAFLD cohort, *Ruminococcaceae* and *Veillonellaceae* species were found to be more predominant in NAFLD patients relative to healthy individuals[121]. These microbiome changes could not be associated with genetic predispositions known to influence NAFLD and were thought to be environmentally driven[121]. *Bacteroides* and *Escherichia* spp. have, on the other hand, been associated with liver fibrosis in NAFLD patients[122]. Overall, these multiregional studies indicate that there is great potential for the gut microbiota as a noninvasive diagnostic biomarker for liver disease with distinct indications of the

staging of fibrosis and inflammation[121,123]. There is also great potential for the gut microbiota and associated metabolites to be utilized as therapeutic biomarkers[119-121]. It must, however, be appreciated that as of yet, a single microbial signature indicative of liver disease does not exist mainly because disease outcome is influenced by multiple factors such as diet, genetic background, age, and lifestyle (such as alcohol consumption), all of which must be considered while interpreting data on the predictive value of fecal microbiota on liver disease[124].

Therapeutic approaches

As we have discussed above, dysbiosis and a dysfunctional gut barrier promote the leakage of microbial endotoxins and components, as well as bile acid metabolites, into circulation, which can eventually lead to liver injury. Various therapeutic approaches (which are at various stages of testing) could be used to address these different factors for the treatment or prevention of liver disease, which will be highlighted below. Although SCFA supplements could be an attractive therapeutic approach in liver disease, their taste is normally not well tolerated. However, methods such asmicroencapsulation[125], either as soft gels or liquid capsules, are available that mask the taste of bitter medications and could be used for oral delivery of SCFA, which has the added benefit of being slow release and helps prevent evaporation of some volatile SCFAs, such as butyrate. Butyrate enemas have been used in a rat model, with the treatment group showing improved mucosal repair and reduced colonic damage compared to the untreated control groups[126]. However,butyrate enemas did not show any improvement in clinical studies with ulcerative colitis patients [127]. There is potential for the use of SCFA as a therapeutic approach, but more research is required to develop an optimal approach. Prebiotics such as inulin represent a substitute approach for the supply of SCFAs[98]. Multiple agonists of FXR are under investigation, including GS-9674 and LJN452, in phase 2 trials for NASH[98]. Some fibroblast growth factors (FGFs), such as FGF19 and FGF21, have shown encouraging results for NAFLD therapy[128,129]

Probiotic interventions

Treating dysbiosis and restoring homeostasis is complicated due to the wide range of associated factors that lead to a loss of important microbial populations or diversity in the first place. In most cases, treating dysbiosis with a single approach usually gives discouraging outcomes. However, studies involving probiotics have shown encouraging results in terms of safety, tolerance and efficacy[130]. In a Phase 1 clinical trial, *Lactobacillus rhamnosus* GG administered to cirrhotic patients resulted in reduced *Enterobacteraceae* and increased relative abundance of *Clostridiales incertae* Sedis XIV and*Lachnospiriceae* with reduced endotoxemia and decreased pathogenic bacterial growth indicative of improved health[131]. In another study using multiple probiotic strains, a reduction in inflammatory cytokine flares in cirrhotic patients was observed[132]. In obese, sonographically identified NAFLD children, treatment with a probiotic combination of *Bifidobacteria* (*B. bifidum and B. lactis)* and two *Lactobacilli* (*L. rhamnosus* DSMZ 21690 and *L. acidophi*lus) strains significantly lowered intrahepatic fat content and alanine aminotransferase (ALT) levels as well as aspartate amino transferase (AST) relative to the placebo treatment[133]. This reduction in hepatic steatosis was replicated in NAFLD patients treated with a multistrain probiotic[134]. In another study, a twelve-week treatment of 30 NAFLD volunteers with six strains of bacteria containing *Bifidobacterium breve* and *B. lactis, Lactobacilli rhamnosus, L. acidophilus* and *L. pacasei* and *Pediococcus pentosaceus* in a randomized, double-blind, placebo-controlled study led to an improvement in proinflammatory cytokines, a reduction in cholesterol and a decrease in body weight[135]. When probiotics are mixed with compatible prebiotics, better outcomes have been achieved in clinical trials, but more studies are needed to determine the most effective combinations[136,137]. Hepatic steatosis has, for example, been reported to decrease in patients with NASHfollowing symbiotic and prebiotic treatment. Serum alkaline phosphatase (ALP) was decreased following treatment with probiotics, prebiotics and synbiotics[136] However, it is noteworthy that the outcomes are dependent on the composition of probiotics, the exposure time and the dosage[136]. Studies in animal models have shown similar

outcomes as in human studies. In rats fed a high-fat diet, treatment with *Bifidobacteria longum* or *Lactobacillus acidophilus* significantly reduced hepatic fat accumulation[138]. There was also a strong negative correlation between fat liver content and probiotic concentration in the stool [138]. In addition, hepatic steatosis was markedly reduced after 12 wk of treatment with *B. longum*, but this was not the case with *L. acidophilus* treatment[138]. In a diabetic rat model, treatment with *Akkermansia muciniphila* led to a decreased inflammatory response and improved liver function[139]. In hepatic encephalopathy, a mixture of *Lactobacillus plantarum*, *L. casei*, *L. delbrueckii* subsp. Bulgaricus, *Bifidobacterium infantis*, *B. longum*, *B, breve and Streptococcus salivarius* subsp. Thermophilius has been associated with both primary and secondary prophylaxis[140,141]. Yogurts containing *L. bulgaricus*, *S. thermophilus*, *L. acidopilus* La5 and *B. lactis* Bb12 as well as a prebiotic mixture of fruco-oligosaccharides and *L. casei*, *L. rhamnosus*, *S. thermophilus*, *B. breve*, *L. acidophilus*, *B.longum*, and *L. bulgaricus* have been shown to improve aminotransferase in NAFLD patients[142-144]. In NASH patients, probiotics containing *L. bulgaricus* and *S. thermophilus* have also shown improvement in aminotransferase[145]. A combination of *B. longum* W11 and fructooligosaccharides, on the other hand, has shown improvement in aminotransferase and the histological score activity of NASH patients[146]

Fecal microbiota transplantation

Fecal microbiota transplantation (FMT) is the administration of a solution containing fecal material from a "healthy" donor into the intestinal tract of a recipient to modify that recipient's gut microbial composition for targeted health benefits[147]. To date, FMT has been successfully used in the treatment of recurrent *Clostridium difficile* infection, and there is growing evidence that FMT can be used to treat noninfectious diseases such as inflammatory bowel disease, obesity, and other metabolic disorders [147]. FMT has also been tried as a therapeutic option for liver disease. In a diet-induced steatohepatitis mouse model, FMT-treated mice showed increased SCFAs, improved expression of tight junction proteins, reduced proinflammatory cytokines and less intrahepatic lipid

deposition compared to controls (i.e., no FMT)[148]. There have also been several human clinical trials but with mixed outcomes, with some achieving a significant reduction in proinflammatory cytokines and improved gut barrier function and others not responding to therapy[149,150]. Future experiments should address the question of who qualifies as a healthy donor, how should we deal with the variation in gut microbial diversity among the recipients, and how best to package the product for better acceptability.

Bile acid metabolism

A recent study in mice indicated that during antibiotic-induced dysbiosis, the homeostasis of bile acids was equally destabilized[151,152]. Treatment of these mice with flavanones and total phenolic extracts of citrus aurantium L. (TPE-CA) restored bile acid homeostasis and gut barrier integrity[152]. TPE-CA also regulates the enterohepatic circulation entry of bile acids through the farnesoid X receptor-fibroblast-growth factor 15 pathway[152]. The effects of dysbiosis and increased intestinal unconjugated bile acid that are observed in ALD were reversed through improved farnesoid X receptor (FXR) activity and gut barrier function following treatment with fexaramine, which is an intestine restricted FXR agonist. These results indicate that modulation of cyp7a1 and lipid metabolism can be achieved in a mouse model and thereby minimize ethanol-derived liver damage by targeting the bile acid-FXR-fibroblast-growth factor 15 signaling pathway[153]. Future experiments to verify these findings in higher mammals and translate the results to therapeutic interventions for human liver disease are warranted.

Precision microbial engineering

The mechanisms by which the intestinal microbiota influences the development and/or progression of liver disease are only beginning to unfold, but to fully elucidate the microbiome role in liver disease, a more comprehensive picture of the dynamics of the gut ecosystem is needed. Unfortunately, most of our knowledge about the intestinal

microbiota arises from fecal or biopsy sample analysis, which is not representative of the entire gut microbiome. However, novel technologies are being developed to address this knowledge gap. One such innovation is a capsule sampler and drug delivery system that is swallowed and utilizes mechanical gut peristaltic movements to guide the capsule down the entire length of the gut as the capsule collects samples[154]. Recently, a capsule robot was designed from a shape memory alloy spring with a chamber of a storage capacity of 500 µL, which showed enhanced sample preservation[155]. Another approach consists of an inexpensive 3D-printed sampler containing a hydrogel whose swelling ability seals and protects the liquid gut samples[156]. Such strategies that analyze small samples from various sites will provide information on microbiota distribution and will make microbial engineering and microbial targeting more feasible.

One such microbial engineering approach being developed is the use of CRISPR Cas-based instructions to precisely cut off targeted genetic sequences of the microbial genome and thus change their function *in vivo*[157]. A conjugative plasmid, TP114, was recently used as a delivery vehicle for CRISPR-Cas9, targeted at drug-resistant *Escherichia coli* and *Citrobacter rodentium*, which led to full clearance of these organisms in a mouse model four days after administration[157]. More recent delivery systems for CRISPR-Cas9 have been designed to utilize probiotics as a genetically engineered conjugative vehicle that are more efficient and practical to use than bacteriophage-based systems[158,157]. The use of CRISPR-Cas9 as antimicrobial therapy is still in its early stages but has the potential to be an effective therapy for targeting specific, undesired microbes in the dysbiotic gut of liver disease. Other approaches to manipulate the gut microbiome are mucosal vaccines. IgA is the predominant antibody in the gut that binds to pathogens and commensals, preventing their translocation across the mucosal barrier. Using a probiotic-based mucosal vaccine with *Lactobacillus acidophilus*, Fox *et al* showed that a potent, diverse IgA response could be elicited[159,] which could help with colonization resistance. In another study, Slack and

colleagues designed an oral vaccine using genetically modified *Salmonella enterica* capable of setting evolutionary traps for prophylaxis treatment in a mouse model[160,161]. While this technology was advanced into a pig model and is currently being tested on human neonates to treat neonatal sepsis and necrotizing enterocolitis, it has hallmarks to be equally beneficial as therapeutic approaches for liver disease.

Diet and lifestyle changes as therapeutic targets

There are many therapeutic options for NAFLD that are being explored, some of which are in advanced levels of clinical trials; however, no treatment is yet available[124]. Diet and lifestyle changes remain the most effective methods of managing liver disease[162]. Low caloric diets, low carbohydrate intake and low protein diets have all been shown to be effective in the management of liver disease [163, 162]. It should, however, be noted that dietary changes alone cannot achieve the intended long-term weight loss goals to reduce liver inflammation. It is rather a combination of correct diet and exercise that is most effective against NAFLD[162]. The response to dietary changes and exercise on both gut microbiota that are negatively associated with liver disease and the amount of fat in the liver is different between individuals and between races [164] The amount of *Bacteroides,* for example, is lower in Chinese NAFLD individuals after diet and exercise compared to people from the West, and this is correlated with lower hepatic fat[164]. It has also been noted that Bacteroides increases in obese volunteers but decreases in lean volunteers following exercise and diet intervention[165].This is suggestive of personalized intervention approaches of diet and lifestyle changes[164].

CONCLUSION

Conclusion

The influence of the gut microbiome on various body systems has important implications for health and disease, such as liver disease. While the exact mechanisms by which the microbiome contributes to liver disease are unknown, there is strong evidence that translocation of various metabolites across the mucosal barrier

plays a strong role, which is precipitated by a dysbiotic gut microbiota. Considering the importance of the microbiome in liver disease, powerful therapeutic options that can manipulate the gut microbiome are being explored. These approaches could have the potential for effective treatments for various stages of liver disease. More research needs to be done to understand the crosstalk between the microbiome and host as it relates to liver disease so that more effective and targeted preventative and therapeutic options can be developed.

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