



## Modulating the Intestinal Microbiota: Therapeutic Opportunities in Liver Disease

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### Abstract

Gut microbiota has been demonstrated to have a significant impact on the initiation, progression and development of complications associated with multiple liver diseases. Notably, nonalcoholic fatty liver diseases, including nonalcoholic steatohepatitis and cirrhosis, severe alcoholic hepatitis, primary sclerosing cholangitis and hepatic encephalopathy, have strong links to dysbiosis – or a pathobiological change in the microbiota. In this review, we provide clear and concise discussions on the human gut microbiota, methods of identifying gut microbiota and its functionality, liver diseases that are affected by the gut microbiota, including novel associations under research, and provide current evidence on the modulation of gut microbiota and its effects on specific liver disease conditions.

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### Identification and study of the gut microbiota (GM)

The microbiota is defined as all of the microbes associated with complex organisms such as humans, whereas the microbiome is the complete representation of these microbes and their genes. Initial methods to identify and characterize microbiota were predominantly based on culture techniques. High-throughput culturing, which combines controlled and automated cell-culturing systems to grow dozens of cultures at once over long periods, has essentially improved characterization of novel microbes and strains (also known as

culturomics). Apart from high-throughput culturing, the ‘continuous culturing’ utilizes an open system which is constantly supplied at one end with fresh growth medium and overflow allowed to drain from the vessel at the other end, diluting out toxic metabolites and dead cells and leading to a ‘steady-state’ of microbial activity that can be further studied.

These techniques have been overwhelmed with the advent of sequence-based approaches, in which, without the need for growing microbes in the lab, complete detail of the species present in the sample can be attained within a short time. Marker gene survey is the most common sequence-based approach used for microbial characterization. This method utilizes identification and comparison of the microbiome in the sample with universal marker genes, thereby identifying all known species within the sample based on unique conserved DNA. The universal marker gene that is most widely employed is the small subunit ribosomal RNA gene (16s rRNA gene for bacteria and archaea, 18s rRNA gene for eukaryotes).

Using the standard PCR technique, en masse sequencing of the extracted DNA is performed, and resulting data is clustered by comparative sequence similarity into Operational Taxonomic Units that are mapped against a comprehensive reference database to assign taxonomic classifications – thereby, representing an approximation of species within the sample. Whole-genome sequencing employs sequencing of multiple microbial genomes in a single run, by multiplexing samples through the addition of unique sequence tags. Shotgun-sequencing – in which extracted DNA is randomly fragmented before sequencing and then the resulting overlapping sequence data combined bioinformatically by mapping onto an existing reference genome into continuous stretches – is the standard method of whole-genome sequencing. Whole-genome-sequencing typically requires that the organism be grown in culture first before DNA extraction and sequencing.

With metagenomics, direct sequencing is performed on DNA extracted from a sample, followed by bioinformatical piecing together of the sequenced data into continuous data, allowing for study of the qualitative as well as functional aspect of the microbiota without culturing. Single-cell genomics utilizes isolation of individual microbes from the sample, after which whole genome amplification is performed. This powerful technique allows for recovery of genomic data of rare species and helps in the understanding of organisms that are capable of carrying out a particular metabolic function, even if such genomic and functional information is missing from comparative databases.

Metatranscriptomics, or RNA sequencing, in contrast to metagenomics, provides detailed information on the functional

**Keywords:** Microbiome; Metagenomics; NGS; Cirrhosis; Illumina.

**Abbreviations:** ACLF, acute-on-chronic liver failure; AH, alcoholic hepatitis; AIH, autoimmune hepatitis; ALD, alcoholic liver disease; APAP, acetaminophen; FMT, fecal microbiota transplantation; FXR, farnesoid-X receptor; GM, gut microbiota; HCV, hepatitis C virus; HE, hepatic encephalopathy; IL, interleukin; LPS, lipopolysaccharide; MELD, model for end-stage liver disease; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PSC, primary sclerosing cholangitis; TGR5, G protein-coupled bile acid receptor 1 and membrane-type bile acid receptor; TNF, tumor necrosis factor.

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activity of the microbiota at a given time and under prevailing environmental conditions, and not only the functional potential as seen with the latter. In metaproteomics, whole characterization of the entire microbiota protein complements at a given point in time is analyzed, which provides information that can be linked to species-based information. This enables the direct study of translated genes, revealing important metabolic information of the microbial community. Metabolomics provides metabolic profiling with regards to functional pathways of a given organism or groups in a given sample (Supplementary Fig. 1). A simplified summary of various mechanisms for identifying and studying GM is shown in Fig. 1.<sup>1-4</sup>

### Introduction to GM

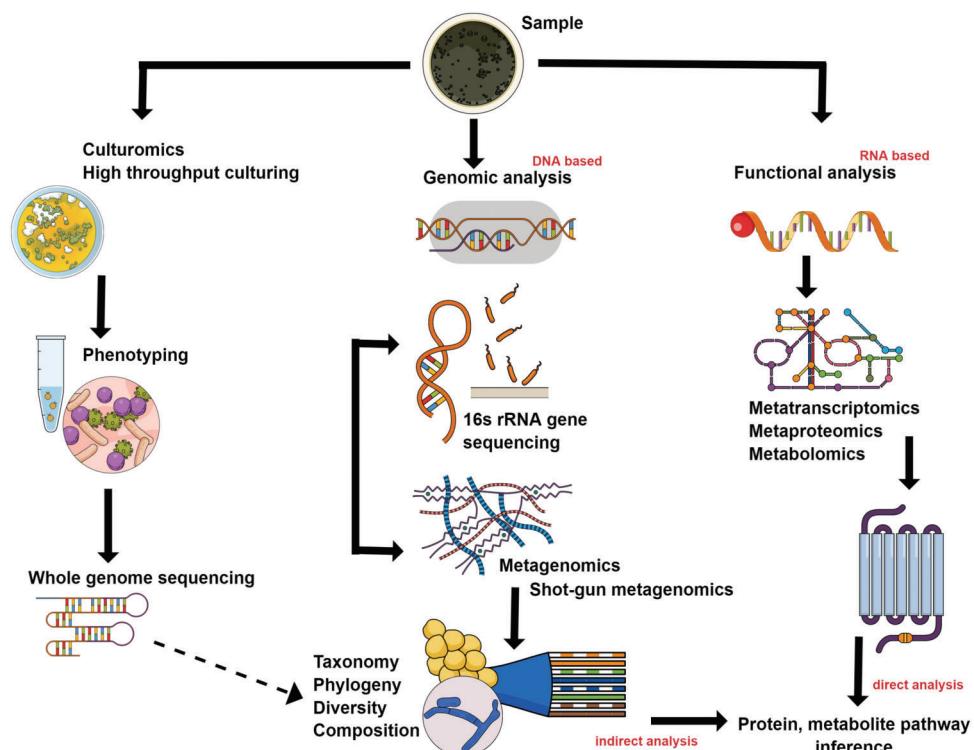
The Metagenomics of the Human Intestinal Tract (referred to as MetaHIT) and the Human Microbiome Project provided full detail of the human-associated microbial repository. The data classified bacterial communities into 12 different phyla, of which 93.5% belonged to Proteobacteria, Firmicutes (gram-positives), Actinobacteria and Bacteroidetes (gram-negative anaerobes). In the early stages of human development, the microbiota has low diversity, dominated by Actinobacteria and Proteobacteria. The main genera among Bacteroidetes in the gut include *Bacteroides* and *Prevotella*, while the major Firmicutes genera are *Clostridium*, *Blautia*, *Enterococcus*, *Faecalibacterium*, *Eubacterium*, *Roseburium*, *Ruminococcus*, *Streptococcus* and *Lactobacillus*. Actinobacteria mainly consist of *Bifidobacteria*, *Atopobium* and *Collinsella*, while Proteobacteria consist of Enterobacteriaceae like the *Escherichia* and *Klebsiella*. Verrucomicrobia is represented by only

one species in humans, *Akkermansia muciniphila*. Apart from these, the kingdom Archaea, predominated by *Methanobrevibacter* species and Eukarya, such as *Candida*, are also present. Lower representations by parasites, viruses and bacteriophages are also notable (Supplementary Fig. 2).

At around 3 years of age, the composition, diversity and functionality of a child's microbiota resemble that of an adult. Above 65 years of age, higher Bacteroidetes phyla and Clostridium cluster IV occur. It is important to note that the GM of an adult is in a state of constant remodeling based on sex, genetic make-up (the gut microbiome is heritable), environmental, dietary, habitual, objective and subjective interactions, and acute and chronic disease conditions as well as spatial differences within the subject. For example, the intestinal luminal and mucosal microbiota compositions are significantly different in the same person, while the intestinal luminal microbiota differs from person to person, from region to region, and between sexes. Normally, the abundance of Bacteroidetes is higher in luminal (fecal) samples than in the intestinal mucosa, in which the Firmicutes, specifically Clostridium cluster XIVa, is higher. The theory of 'core microbiota' – that is a fixed set of groups of organisms present in all individuals – was proposed, but recent observations have shown that the commonness lies at the level of the microbiome (and not microbiota), suggestive of a 'functional core microbiota', which remains to be wholly defined in healthy persons.<sup>5-11</sup>

### The functional microbiota

The gut metabolome is mostly derived from carbohydrate, protein, and lipid metabolism. The major metabolites



**Fig. 1. Various methods for identifying and studying the gut microbiota and microbiome.** Culturomics help in phenotyping the microbial communities, and further whole genome sequencing improves identification of microbial diversity up to the species and functional levels. DNA-based genomic analysis through 16s rRNA sequencing curtails time to identification of microbial species, while RNA-based analysis helps in studying microbial function at the transcriptome, proteome and metabolite levels.

produced in the gut include short-chain fatty acids, branched-chain fatty acids, branched-chain amino acids, biogenic amines, organic gases, and other secondary plant-derived compounds, such as choline, bacterial cell wall components, polyamines, and volatile organic compounds. The plethora of metabolites thus formed are further absorbed, distributed or excreted by multiple, highly dynamic processes and pathways that significantly affect human health by regulating pro- and anti-inflammatory processes, immunological landscape (innate and adaptive immunity), and detoxification. For maintaining homeostasis, the intestinal barrier limits exposure of the host immune system to the microbiota through multifactorial and dynamic processes that operate at the luminal and mucosal level.<sup>12,13</sup>

The GM caters to and provides multiple benefits to the host, such as nutrient metabolism and assimilation, protection and control over pathogenic species, and maintenance of immune functions. For example, the colonic bacteria express carbohydrate-active enzymes, which empower them to ferment complex carbohydrates, thereby generating metabolites which regulate cellular processes such as gene expression, chemotaxis, differentiation, proliferation, and apoptosis. On the other hand, specific anaerobes produce acetate while propionate and butyrate are produced by different subsets of gut bacteria through distinct molecular pathways. In the human intestine, propionate is mainly produced by Bacteroidetes, while the production of butyrate is mainly by Firmicutes. Starch fermentation by *Eubacterium rectale* or *Eubacterium hallii* that belong to Firmicutes, significantly contributes to butyrate production in the colon and *Akkermansia muciniphila* has been found to majorly contribute to propionate production through mucin degradation, the latter which is primarily absorbed by the liver. Propionate has been shown to reduce cancer cell proliferation and through its action on beta-cell function, ameliorates reward-based eating behavior though striatal pathways. In addition, butyrate is known for its anti-inflammatory activities in the liver microenvironment, acting by attenuating bacterial translocation and enhancing gut barrier strength by improving tight-junction function. Similarly, the short-chain fatty acids produced by the colonic GM regulate the immune system and inflammatory processes by influencing the production of interleukin (IL)-18, which is involved in maintenance and repair of mucosal epithelial integrity as well as in modulation of appetite regulation and energy utilization in the host, which are associated with metabolic syndrome and obesity.

Apart from carbohydrate metabolism, pertinent lipid metabolism in the host is also driven by the GM. For example, the facultative and anaerobic bacteria of the colon produce secondary bile acids which enter the systemic circulation to modulate hepatic and systemic lipid metabolism through nuclear or G protein-coupled receptors. *Akkermansia*, *Christensenellaceae*, *Tenericutes*, *Eggerthella*, *Pasteurellaceae*, and *Butyrimonas* are associated with body mass index in patients with metabolic syndrome as well as levels of triglycerides and high-density lipoproteins.<sup>14-18</sup> With regards to protein metabolism, the microbiota-derived metabolites produced from aromatic amino acids (tyrosine, tryptophan, and phenylalanine) affect host signaling pathways interacting with host immunity. *Bacteroides thetaiotaomicron*, *Proteus vulgaris*, and *Escherichia coli* act through tryptophanase activity, producing indole which is sulfated in the liver and resulting in the production of 3-indoxyl sulfate

and related compounds which promote systemic inflammation through transcription of IL-6.

Indole-3-propionate acts at the pregnane X receptor (referred to as PXR) and down-regulates tumor necrosis factor (TNF)-alpha production in enterocytes by limiting bacterial translocation and lipopolysaccharide (LPS) infiltration into the circulation, thereby reducing metabolic endotoxemia and host inflammation.<sup>19-22</sup> Various microbes or groups of microbes are associated with carrying out specific regulatory processes in the human gut, which is directly or indirectly associated with liver health (Supplementary Fig. 3).

### **Microbiota and the gut-liver-axis**

Since the liver is an organ that has privilege in placement with regards to maximal exposure to gut microbes and its metabolites, studies on 'healthy state' and diseases associated with the hepatobiliary system have been on the forefront in the current bench-to-bedside research. Changes associated with the GM are implicated in the pathogenesis of many liver diseases. This alteration in general is termed dysbiosis, in which there is an imbalance between the symbionts and pathobionts in the gut. The intestine and liver have a bidirectional communication mediated through the biliary tract, portal vein, and systemic circulation. The liver communicates with the gut through bile acids and other metabolic mediators. In the gut, the microbes metabolize endogenous and exogenous compounds, end-products of which translocate to the liver through the portal vein, influencing the liver microenvironment and functions. The liver receives and filters large amounts of nutrients, bacterial products, toxins and metabolites through the portal vein, with an efferent circulation via the biliary system. This 'metabolic endotoxemia', as described by Cani et al.<sup>23</sup> in patients with diabetes and metabolic syndrome promotes a steady-state of low-grade inflammation within the liver microenvironment, driven by unhealthy changes in the GM.

Similarly, a 'tip of the balance' towards a more pathogenic profile of microbiota leads to increased exposure of the liver to pathogen and microbe-associated molecular patterns through an increase in bacterial translocation and leakiness of the gut. This exposure results in proinflammatory cellular signaling within the hepatic environment driven by major cytokines, such as IL-1, IL-6 and TNF-alpha. Continuous proinflammatory state, in the presence of persistence of factors that promote it (such as alcohol, drugs, obesity, diabetes) leads to production of reactive oxygen species which promote liver injury and fibrosis.

Liver, a highly active site of metabolism and immune homeostasis, handles and secretes multiple immunogenic molecules and metabolites into the gut, which affects the microbiota and vice-versa. Secretory immunoglobulin A, bile acids and fatty acids processed by the liver activate various nuclear receptors, such as the G-protein coupled receptor and farnesoid-X receptor (FXR), that regulate glucose and lipid metabolism and homeostasis as well as conjugation and detoxification of exogenous and endogenous toxins. Gut-derived hormones (for example, fibroblast growth factor, glucagon-like-peptide-1 and serotonin) also play an important role in maintaining homeostasis and energy assimilation and balance by affecting the steady metabolic state, via their action on appetite and food intake. Dysbiosis has been shown to initiate, promote or cause progression of liver diseases, such as alcoholic liver disease (ALD), alcoholic hepatitis (AH),

nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH), drug-induced toxic liver injury, liver fibrosis, cirrhosis and its complications, hepatocellular carcinoma, and chronic cholestatic and autoimmune hepatobiliary disease (Fig. 2).<sup>24–27</sup>

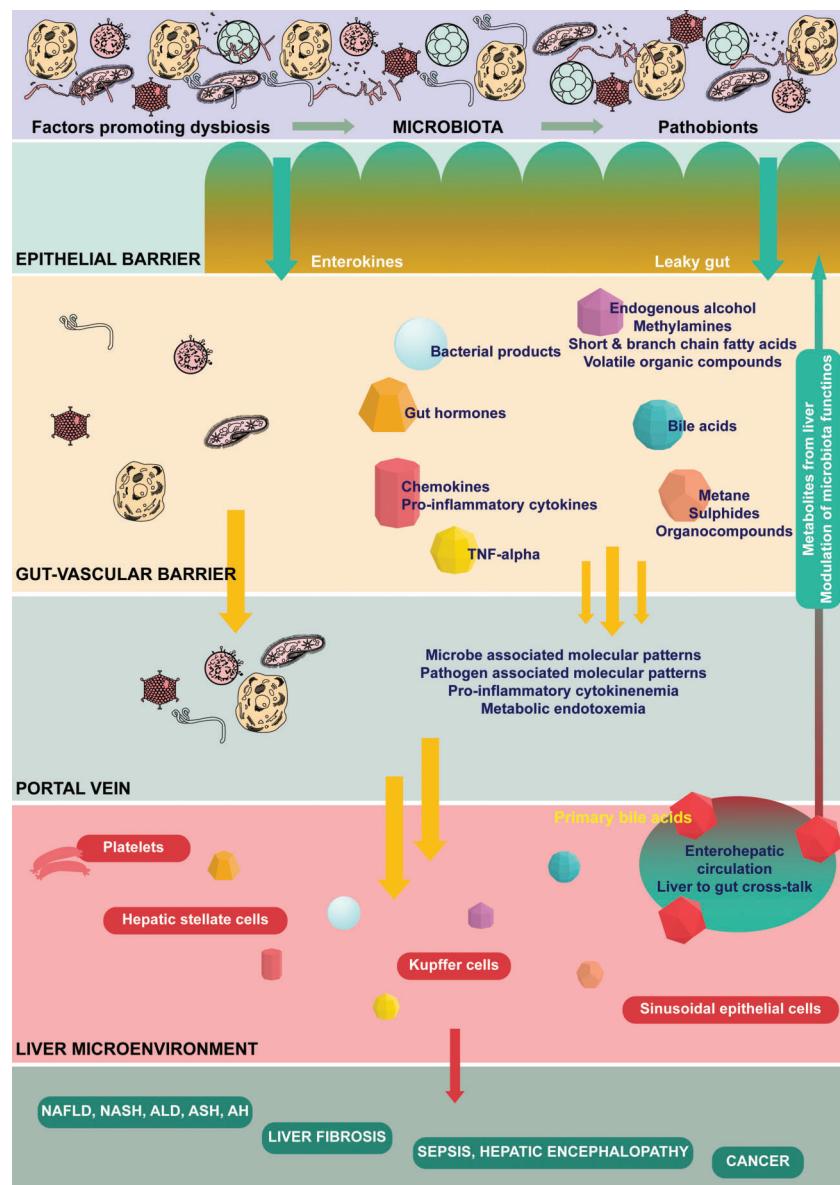
## GM and diseases of the liver

### NAFLD and NASH

NAFLD encompasses steatosis, steatohepatitis, advanced fibrosis, cirrhosis and related hepatocellular carcinoma. High-quality studies in humans with and without NAFLD/NASH have shown a strong correlation of GM in the initiation

and progression of NAFLD-associated conditions.<sup>28,29</sup> Lower levels of *Bacteroides* are associated with obese patients with NASH, while the lower abundance of *Firmicutes* was demonstrated in non-obese NASH patients. Reduction in *Lachnospira*, *Ruminococcus* and *Lactobacillus* was notable in lean patients with NASH. In adolescents, the abundance of *Bacteroides* followed a 'U' pattern, based on the dietary pattern of fat intake. In those with high fat intake, low and high abundances were noted, while in those with low fat intake, a moderate level of abundance was found.

*Bilophila*, *Paraprevotella* and *Suteraella* are associated with higher hepatic fat content, in contrast to *Oscillospira* and *Varibaculum* for which a negative association with steatosis has been demonstrated. Higher levels of *Bilophila wadsworthia*



**Fig. 2. The gut-liver axis and microbiota related cross-talk.** In the presence of factors that disrupt microbial diversity and function (alcohol, metabolic diseases, drugs, environmental toxins, diet), pathobionts that promote disease causation or progression evolve in the dysbiotic milieu. This leads to gut barrier disruption, enhancement of local proinflammatory cascade, endotoxemia and ultimately systemic inflammation, leading to end organ adverse events.

were found to be associated with T helper-1-mediated gut inflammation. Lower levels of *Oscillospira* were associated with an increase in the metabolite 2-butanone, which was related to the onset of NAFLD. Increased levels of *Ruminococcus*, *Dorea*, *Robinsoniella* and *Roseburia* were found to be associated with progression of inflammation and fibrosis in NASH. Patients with NASH fibrosis  $\geq 2$  were found to have higher abundance of *Bacteroides* and *Ruminococcus*, and lower levels of *Prevotella*; while, in those with advanced fibrosis and cirrhosis, *Proteobacteria* were relatively higher. At the species level, it was shown that *Eubacterium rectale* and *Bacteroides vulgaris* were relatively abundant in patients with mild to moderate NASH, while *Bacteroides vulgatus* and *E. coli* were predominant among patients with NASH-related advanced fibrosis and cirrhosis.<sup>30–32</sup>

Changes in GM in NAFLD patients have been shown to have positive correlation with small-intestinal bacterial overgrowth, defined as total bacteria growth of more than  $10^3$  colony-forming-units of coliform bacteria per milliliter of proximal jejunal fluid, which is directly related to endotoxemia, circulating bacterial DNA and leakiness of the gut, and associated with worsening of steatosis and inflammation. Intestinal dysbiosis results in lower levels of colonic junctional adhesion molecule A expression, increase in intestinal permeability, leading to liver exposure to higher levels of bacterial LPS, and endogenous ethanol, acetone and butanoic acid, that leads to hepatic inflammation. The GM also modulates choline metabolism, in which dietary choline is converted to dimethylamine and trimethylamine — increased levels of which result in hepatic inflammation. Lower levels of dietary choline lead to changes in GM and its functionality, which promotes fatty liver disease which, however, can reverse with high choline diet in small animal models. *Escherichia*, which increases production of endogenous ethanol utilizing the 'mixed-acid-fermentation' pathway was shown to be significantly associated with NASH and higher grades of fibrosis. The end metabolites that form out of ethanol degradation include acetate, which takes part in fatty acid synthesis, and acetaldehyde, which promotes cytotoxic effects within the liver microenvironment. Gut dysbiosis and small-intestinal bacterial overgrowth in NASH patients have been shown to be associated with higher levels of circulating serum TNF-alpha and IL-8, through signaling mediated by toll-like receptors -9 and -4.

Bile acids have significant effect on GM and have great impact on the metabolism of bile acids. Taurine- and glycine-conjugated primary and secondary bile acids were found to be higher in patients with NASH compared to healthy controls. Bile acids, through regulatory effects on FXR and G protein-coupled bile acid receptor 1 and membrane-type bile acid receptor (referred to as TGR5), affecting the natural history of NAFLD. Agonists of FXR and TGR5 have been shown to reduce liver fat, improve NASH histology, and promote weight loss in a small animal model of NAFLD.<sup>33–35</sup>

Intestinal bacteria have been shown to reduce expression of fasting-induced adipocyte factor on the enterocytes, leading to induction of lipoprotein lipase activity and accumulation of hepatic triglycerides. Gut bacteria also promote increased production of short-chain fatty acids, acetate, propionate and butyrate, contributing to obesity, metabolic disease and increase in liver steatosis. LPS production by specific microbiota groups has been shown to promote liver carcinogenesis in mouse models, while in the germ-free mouse model, hepatocarcinogenesis was reduced. The occurrence of

liver cancer in obese mouse models has been linked to the persistence of low-grade systemic inflammation that is initiated from dysbiotic microbiota. Thus, there is robust evidence that GM plays a central role in steatosis, inflammation and progression of fibrosis in NAFLD patients.

GM targeted therapies are upcoming strategies for treatment of NAFLD, NASH and NASH-related HCC. Currently, the focus of such treatments has been solely on probiotics and prebiotic supplementation — most commonly utilizing *Lactobacillus* and *Bifidobacterium*. Administration of probiotics has been shown to reduce liver enzymes, total cholesterol, TNF-alpha, serum endotoxin levels, liver fat and NASH activity index in small-animal as well as human models. Probiotics increase PPAR-alpha activity and reduce metalloproteinases 2 and 9 and cyclooxygenase expression. *Lactobacillus casei* strain Shirota reduced the development of NASH in methionine- and choline-deficient diet mouse models by lowering serum LPS concentration; whereas *Bifidobacterium* and *Lactobacillus rhamnosus* GG ameliorated liver steatosis by acting on the sirtuin-1-mediated signaling pathway and reducing nuclear factor- $\kappa$ B inhibitor protein expression.<sup>36–38</sup> Meta-analysis of randomized controlled trials on probiotics and prebiotics have shown that supplementation led to reduction in aminotransferase level, reduced body fat, and improved glucose metabolism.<sup>34,39–41</sup>

Fecal microbiota transplantation (FMT), generally called 'stool transplant', utilized the transfer/transplantation of fecal microbiota from a healthy individual into a patient with dysbiosis, aiming to restore intestinal microbial diversity. There have been strong notions challenging the current terminology of FMT. The term 'fecal/stool' as a treatment modality has negative implications within the scientific community, pharmaceutical industry and funding sources as well as among patients and their families with regards to acceptance. Khoruts et al.<sup>42</sup> proposed the term 'intestinal microbiota transplantation', while Bajaj et al.<sup>43</sup> proposed that FMT be renamed 'microbiome restoration therapy'. However, both these terms feature inadequacies. Even though considered by some as 'an organ', the fecal microbiota is not an organ and is a highly different, unexplored entity, which differs in accordance with sex and region, and even between individuals. Hence, the term transplantation is inaccurate. We are yet to define a 'matching/healthy' microbiota donor. Microbiome, as discussed, is the genetic totality associated with the microbiota, which remains unique to the person. Studies have mostly looked at bacterial communities, even though eukaryotes, protozoa, viruses and phages are also part of the restoration, and which remain undefined. Hence, the term microbiome becomes vaguely general and does not hold well as a replacement to current terminology of FMT.

The term Microbiota Restoration Therapy™ has been patented by Rebiotix Inc (Ferring, Saint-Prix, Switzerland; [www.rebiotix.com](http://www.rebiotix.com)) to define their microbiota-based therapies. In this regard, an ideal, general, novel terminology for FMT, for utilization in discussions and in trials, in the light of current studies, would be 'intestinal microbiota reinstitution therapy', which can be modified accordingly, to further characterize different sites and specific components of the microbiota in future studies. We propose 'reinstitution' and not 'restoration' because the latter means 'to bring something back to the original form/former position or condition', when in reality, we are unaware of 'original' condition of microbiota within the recipient prior to the disease state; moreover, FMT actually modifies the microbiota more towards the donor

profile and is not technically a 'restoration'. 'Reinstitution' means the act of establishing something again – and with FMT, we aim to establish a healthy microbiota. Studies on FMT in animal models have demonstrated amelioration of steatohepatitis in high-fat diet mice.<sup>44</sup> Utilization of FMT for metabolic syndrome in humans was first performed by Vrieze *et al.*<sup>45</sup> They found that FMT from lean donors temporarily increased peripheral insulin sensitivity and reduced hepatic steatosis without statistical significance. Xue *et al.*<sup>46</sup> presented preliminary data on the effects of FMT (200 mL/day for 3 days) in human NAFLD. They showed that change in a fat-attenuated parameter, as measured by FibroTouch™, was significantly lower after treatment with FMT compared with the control group. Clinical trials assessing the efficacy and safety of FMT for NASH (NCT02469272) and NASH-related cirrhosis (NCT02721264) are ongoing.

## ALD

Continuous or binge alcohol use over long periods results in ALD, which comprises liver steatosis, AH, alcoholic cirrhosis and acute-on-chronic liver failure (ACLF). Characteristic changes in intestinal microbiota have been shown to predispose to severe forms of ALD. Dysbiosis is associated with AH in animal models, which were reversed with healthy donor FMT. Similarly, progressive worsening of dysbiosis is associated with the progression of alcoholic cirrhosis and its complications. Severe AH was associated with the higher fecal abundance of *Bifidobacteria*, *Streptococci* and *Enterobacteria*. Germ-free mice (C57BL/6) demonstrated higher susceptibility to alcohol-induced liver injury than conventional mice.<sup>47</sup> This impresses the fact that complete absence of intestinal microbiota as well as an imbalance in microbiota both predispose to alcohol-related liver injury; hence, a 'eubiosis state' properly defines protection against alcohol-induced liver injury.

Defining the microbial communities that promote this eubiosis is still a matter of research. Change in GM has also been implicated in alcohol-induced damage to the liver through modulation of immune responses, expression of alcohol metabolism, oxidative stress, fat metabolism and endotoxemia. Alcohol use was shown to be associated with decreased levels of butyrate-producing Clostridiales and increased levels of pro-inflammatory Enterobacteriaceae and Proteobacteria. Lower abundance of *Ruminococcus* was associated with increased intestinal permeability and dysbiosis, which was reversed with abstinence. In AH, reduction in the relative abundance of *Clostridium leptum* and *Fecalibacterium prausnitzii* has been demonstrated.<sup>46</sup> Philips *et al.*<sup>48</sup> demonstrated that patients with severe AH had a higher relative abundance of *Enterobacter*, *Megasphaera*, *Dialister*, *Prevotella* and *Klebsiella*, while in healthy controls, *Akkermansia*, *Veillonella*, *Oscillopsira*, *Lachnospira*, *Bacteroides*, *Eggerthella*, *Coriobacterium* and *Bifidobacterium* were higher. At the functional level, LPS biosynthesis, glycosyl transferase and valine-leucine-isoleucine degradation pathways were affected in patients with AH, while in healthy controls, alanine-aspartate-glutamic acid metabolism and non-aromatic amino acid metabolism were significantly up-regulated. The gut microbiota composition in healthy and ALD and its effect on intestinal permeability in ALD pathogenesis point toward emerging evidence on GM modulation in ALD as a mode of treatment from preliminary clinical and non-clinical studies.<sup>49</sup>

Grander and colleagues<sup>50</sup> showed that *Akkermansia muciniphila* abundance reduced with increasing severity of ALD and was lowest in AH. Ciocan and colleagues<sup>51</sup> showed that in patients with cirrhosis and AH, total plasma bile acid levels were higher, while levels of total and secondary bile acids were lower compared to those without cirrhosis or AH. The relative abundance of Actinobacteria was higher and that of Bacteroidetes which was lower in alcoholic cirrhosis with AH. Puri *et al.*<sup>52</sup> found that in alcohol-consuming patients, there was an enrichment of bacteria with genes related to methanogenesis and denitrification. Both heavy drinking controls and patients with severe AH demonstrated activation of a type III secretion system associated with gram-negative bacterial virulence. In patients with AH compared to non-alcohol consuming controls, there was an increase in isoprenoid synthesis through upregulation of the mevalonate and anthranilate degradation, which are known modulators of gram-positive bacterial growth and biofilm production, respectively. Bluemel *et al.*<sup>53</sup> investigated the microbiota in the jejunum, ileum, cecum, feces and liver of mice subjected to chronic ethanol feeding; they found that chronic ethanol administration modified alpha diversity in the ileum and the liver, largely driven by an increase in gram-negative phyla, resulting in endotoxemia. Specifically, the gram-negative *Prevotella* increased in the mucus layer of the ileum and also in liver tissues, suggesting the central role of dysbiosis and bacterial translocation leading to liver injury with alcohol use.

In an open-label randomized controlled trial, probiotic-rich in *Bifidobacterium bifidum* and *Lactobacillus plantarum*, compared to placebo in patients with AH, led to a reduction in hepatic inflammation in the form of improvement in liver biochemistry, while the addition of *Lactobacillus casei* Shirota 3 strain thrice daily for 30 days improved neutrophilic phagocytic capacity in ALD patients, when compared with baseline.<sup>54</sup> A placebo-controlled trial showed that supplementation with 1.5 g of *Bacillus subtilis* and *Enterococcus faecium* daily for 7 days improved liver function and reduced systemic inflammation and endotoxemia in AH.<sup>55</sup> The first pilot study of FMT in steroid ineligible severe AH demonstrated an improvement in 1-year survival in FMT-treated patients compared to historical controls (87.5% vs. 33.3%). The relative abundance of Proteobacteria was high and that of Actinobacteria low in patients with severe AH at baseline. Post-FMT, this was significantly reversed, along with coexistence of protective symbiotic donor and recipient species at 12 months. Reduction in relative abundance of pathogenic species [*Klebsiella pneumonia* (10% to <1%)], and increase in non-pathogenic species [*Enterococcus villorum* (9% to 23%) and *Megasphaera elsdenii* (10% to 60%)] was demonstrated. After FMT, reduction in methane metabolism, bacterial invasion of the epithelial cells, inflammatory and cytotoxic pathways, and aromatic amino acid generation was noted.<sup>48</sup>

In a retrospective observational study comparing FMT to other conventional modalities of treatment for AH, the proportions of patients surviving at the end of 3 months in the steroids, nutrition, pentoxifylline, and FMT group were 38%, 29%, 30%, and 75% ( $p = 0.036$ ).<sup>56</sup> In patients with severe AH and non-responders to steroids with ACLF grades 0, 1 and 2 + 3, the 90-day survival rates were 68.1%, 45.8% and 36.7%. Philips and colleagues<sup>57</sup> showed that, at the end of 548 days follow up, the proportion of ACLF-AH patients surviving, after FMT, in the lower (ACLF 0 + 1) and higher grade (ACLF 2 + 3) groups were 72.7% and 58.3% respectively, which was higher than what is seen with current medical therapies and

comparable to liver transplantation. Future studies on FMT in AH could identify better methods for fecal transfer, refine targeted therapy, and utilize precision metabolomics to modulate the intestinal milieu to improve outcomes.

#### **Role of GM in cirrhosis and its complications**

In liver cirrhosis, the presence of portal hypertension results in structural changes to the intestinal mucosa and vasculature, leading to an increase in intestinal permeability that worsens with gut microbial changes and associated functional metabolism. Altered microbiota has been found in the intestinal mucosa, stool and saliva samples from patients with cirrhosis of variable etiologies. The dysbiotic microbiota in patients with cirrhosis reveal a reduction in *Bacteroides* and *Lachnospira* and increase in *Proteobacteria*, *Enterobacteria* and *Veillonella*. The progressive increase in Enterobacteria correlates with complications of cirrhosis, especially hepatic encephalopathy (HE). The severity of cirrhosis with regards to Child-Pugh class correlated positively with *Streptococcus* and negatively with *Lachnospiraceae* (*Coprococcus*, *Pseudobutyryrivibrio*, *Roseburia*). The decrease in autochthonous taxa such as *Lachnospiraceae*, *Ruminococcaceae* and *Clostridiales XIV*, and the relative increase in *Staphylococcaceae*, *Enterococcaceae* and *Enterobacteriaceae* were found to be associated with progressive liver failure and endotoxemia. The cirrhosis to dysbiosis ratio, between indigenous and non-indigenous taxa, negatively correlated with endotoxemia, was highest among healthy controls and lowest in patients with decompensated cirrhosis. A higher proportion of bacteria of buccal origin (*Streptococcus* and *Veillonella*) within the gut microbiome of patients with cirrhosis suggested that the oral microbiota invaded the gut, thereby contributing to the progression of the disease. Composition of the microbiota differed between patients with and without HE, only in mucosa but not in stool samples. *Veillonella*, *Megasphaera*, *Bifidobacterium* and *Enterococcus* were prevalent in HE, whereas *Roseburia* was more abundant in the non-HE groups. Minimal HE was associated with higher levels of *Streptococcus salivarius*, while in overt HE, fecal levels of *Alcaligenaceae* and *Porphyromonadaceae* were associated with poor cognition. Salivary dysbiosis was greater in patients with cirrhosis who developed 90-day hospitalizations. Stool *Bacteroidaceae* and *Clostridiales XIV* predicted 90-day hospitalizations independent of such clinical predictors as Child-Pugh class and model for end-stage liver disease (commonly known as MELD) score.<sup>58–60</sup>

Bajaj et al.<sup>61</sup> demonstrated distinct gut microbial profiles associated with ACLF in hospitalized patients. The cirrhosis-to-dysbiosis ratio was lower in those with ACLF and also those with renal failure. *Enterobacteriaceae*, *Campylobacteriaceae*, *Pasteurellaceae*, *Enterococcaceae* and *Streptococcaceae* were associated with the development of poor outcomes, while *Lachnospiraceae* and *Clostridiales* were associated with a reduction in poor outcomes. Changes in the microbiota and dysbiosis had an independent and significant association with extrahepatic organ failure, intensive unit admissions, ACLF, and death in cirrhosis patients in-hospital.

In a randomized controlled trial, rectal enema-based FMT improved cognition among cirrhotic patients with recurrent HE, significantly higher than seen in the control group. The MELD score transiently worsened post-antibiotics but reverted to baseline after FMT. Antibiotic therapy in the control group reduced beneficial taxa and decreased microbial diversity concurrent with *Proteobacteria* expansion, which was again

reversed towards a beneficial pattern after FMT, leading to stable liver disease severity scores. The same group studied the utility of FMT capsules in patients with recurrent HE. In this phase 1 study, they found that oral capsule-based FMT treatment was safe and well-tolerated in patients with cirrhosis and recurrent HE and was associated with improved duodenal mucosal alpha diversity, reduced dysbiosis, antimicrobial peptide expression, reduced LPS binding protein level and improved EncephalApp performance.<sup>62,63</sup>

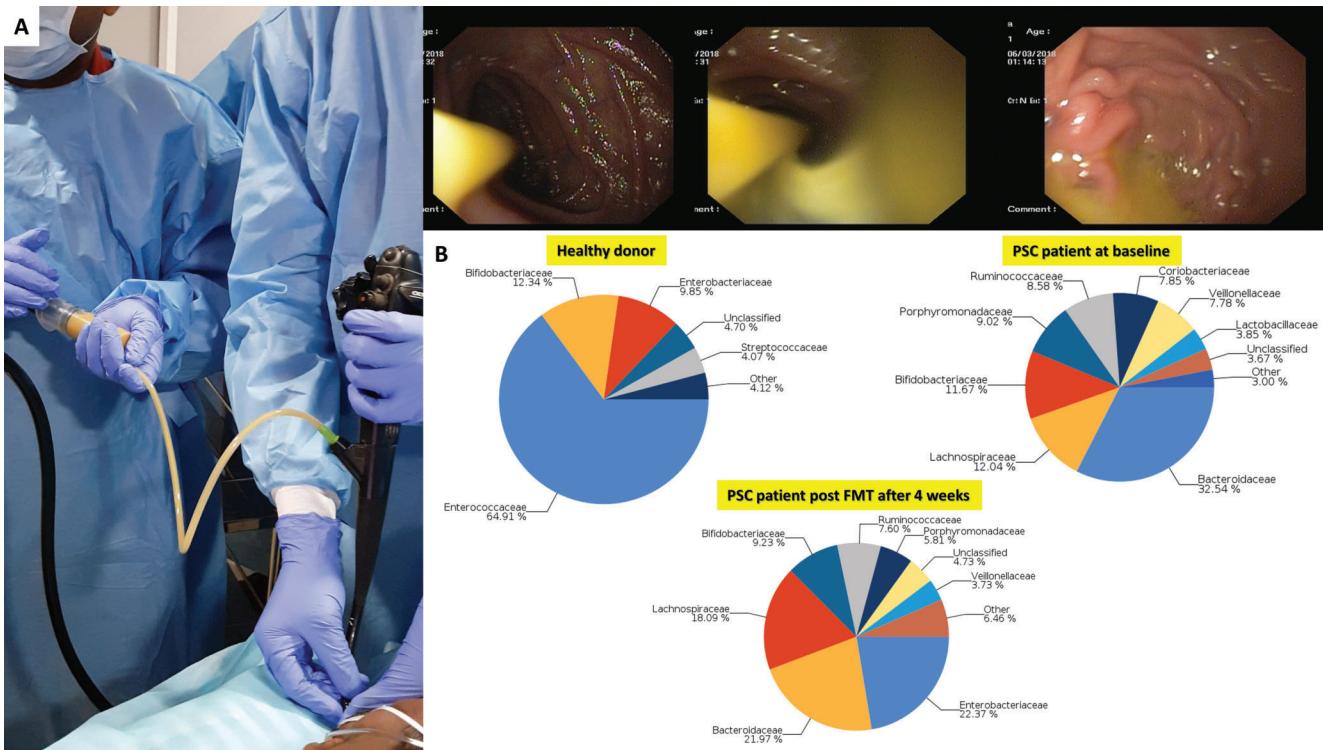
Even though circulating bacterial DNA, plasma endotoxin levels, and inflammatory and vasoactive markers in ascites and blood have been linked to infections in cirrhosis, especially spontaneous bacterial peritonitis, no direct linkage to dysbiosis or specific patterns of bacterial community changes have been studied.

#### **GM in liver cancer**

High-quality studies concerning experimental animal models supporting the role of GM changes and hepatocarcinogenesis are well known in the literature. In earlier studies, gut sterilization by antimicrobials in carcinoma animal models was shown to reduce tumor incidence and growth. *Helicobacter hepaticus* co-administration in the AFB-1 model of hepatocarcinogenesis revealed greater tumor number and size compared to AFB-1 alone. Chronic administration of diethylnitrosamine decreased the abundance of *Lactobacillus*, *Enterococcus* and *Bifidobacterium* species, leading to the promotion of tumor development and growth, which was then arrested by probiotic supplementation. LPS administration also resulted in increased number and size of HCC in animal models, which was attenuated via antibody and antimicrobial use. High-fat diet-related increase in gut dysbiosis and subsequent increase in deoxycholic acid resulted in hepatic tumor formation, which decreased with antibiotic treatment. Higher abundance of *Atopobium*, *Bacteroides vulgatus*, *Bacteroides acidifaciens*, *Bacteroides uniformis*, *Clostridium cocleatum*, *Clostridium xylanolyticum* and *Desulfovibrio spp* in a NASH mouse model was found to be associated with HCC development, which was again associated with a change in bile acid fractions in the liver tissue and plasma. The number and size of tumors was ameliorated using cholestyramine in the small animal model of NASH-related HCC. *Prevotella* and *Oscilibacter*, that are producers of anti-inflammatory metabolites, were found to be negatively associated with liver tumor formation. Studies linking dysbiosis and specific bacterial communities in human HCC is lacking, but targeting GM and its metabolites in patients with chronic liver disease is an exciting frontier for HCC management in the future.<sup>64,65</sup>

#### **Role of GM in other liver diseases – emerging indications**

Studies have shown that the intestinal microbiome could affect the development of autoimmune hepatitis (AIH) in predisposed individuals. A decrease in fecal *Bifidobacterium* and *Lactobacillus* abundance along with an increase in plasma LPS was notable in patients with higher severity of AIH.<sup>66</sup> In germ-free mice, protection against experimental AIH was notable, in the presence of lower levels of leukocyte infiltration and inflammatory cytokines and absence of hepatocyte apoptosis due to the deficiency in activation of natural killer T cells in the liver microenvironment, that predisposes to auto-antibody-mediated liver injury. Humans studies on the



**Fig. 3. Healthy donor microbiota restitution therapy through upper gastrointestinal endoscopy.** Given to a patient with primary sclerosing cholangitis (A). The bacterial communities at the family level in the donor, the patient and the patient after 4 weeks (B). The modification of gut bacterial communities is evident, associated with improved clinical outcomes.

microbiome in AIH are lacking. Wei *et al.*<sup>67</sup> showed that the gut microbiome of steroid treatment-naïve AIH had lower alpha-diversity with distinct overall microbial composition when compared with healthy controls. Reduction in obligate anaerobes and increase in pathobionts such as *Veillonella* was associated with AIH – of which, *Veillonella dispar* was the most significantly disease-associated taxa with positive correlation with the elevation of aspartate aminotransferase. Thus, microbiota-based biomarkers could help identify AIH disease severity as well as being potential therapeutic targets.

GM-driven pathophysiological progression of primary sclerosing cholangitis (PSC) and primary biliary cholangitis are well documented in animal models. Bile acid metabolism is heavily handled by the GM and is central for the pathogenesis of PSC and primary biliary cholangitis. Functional gut microbial activities associated with bile acid metabolism, such as dehydrogenation, conjugation and deconjugation and degradation of primary and secondary bile acids, and subsequent metabolism and toxin generation play an important role in autoimmune-mediated cholestatic inflammatory disease. Germ-free mice were found to have severe PSC features, in comparison to conventional mice, as the complete lack of microbiota resulted in alteration of useful bile acid metabolism that is associated with worsening fibrosis and liver injury. Experimental animal studies have demonstrated that enteric but not colonic dysbiosis was associated with hepatobiliary inflammation, and small bowel bacterial overgrowth in rats resulted in hepatobiliary inflammation resembling histological and cholangiography features of PSC.

Human studies have revealed that increased abundance of *Escherichia* and *Megasphaera* and lower levels of *Prevotella*,

*Roseburia* and *Bacteroides* were associated with PSC and inflammatory bowel disease.<sup>68</sup> Even though a single-case study, done longitudinally over 12 months, Philips *et al.*<sup>69</sup> demonstrated that endoscopic FMT (250 mL, distal duodenum) repeated weekly for 4-weeks improved symptoms, liver biochemistry, bile acid fractions and survival in tandem with beneficial changes in bacterial communities and functional metabolites in a patient with advanced PSC and recurrent-cholangitis listed for liver transplantation (Fig. 3). Allegretti *et al.*<sup>70</sup> performed the first pilot study on FMT in 10 patients with PSC, of whom 9 had ulcerative colitis, and 1 had Crohn's colitis. The mean baseline alkaline phosphatase level was 489 U/L. Overall, 30% experienced a ≥50% decrease in alkaline phosphatase levels post-FMT. The bacterial diversity increased in all patients post-FMT, in the first week itself and abundance of engrafted microbial communities after FMT also correlated with the decrease in alkaline phosphatase levels.

Functional and compositional changes of GM have been demonstrated in patients with chronic hepatitis B virus infection-related cirrhosis, in the form of reduced abundance of *Bifidobacteria* and *Lactobacillus*, and high levels of *Enterococcus*. In another study, it was shown that, in hepatitis B virus-related cirrhosis reduction in *Bacteroidetes* and increased levels of *Proteobacteria* compared to the healthy group was notable. Healthy donor FMT, in addition to standard antivirals, was shown to be significantly more effective in clearance of the hepatitis B e antigen when compared to anti-viral therapy alone.<sup>71,72</sup>

In patients with hepatitis C virus (HCV)-related cirrhosis, the microbial diversity was found to be lower when compared to healthy controls. HCV can alter the GM through

**Table 1. Summary of association of gut microbiota in various liver diseases**

Disease	Pertinent associated microbiota and metabolites	Comments
NAFL	Increase <i>Blautia, Dorea, Streptococcus, Clostridium</i> Butanoic acid, Propanoic acid, Phenylacetic acid, Isobutyric acid, Unconjugated cholic acid, Ethanol	In animal models, reversing microbiota changes reversed hepatic steatosis in the absence of weight loss
	Decrease <i>Oscillospira, Coprococcus, Fecalibacterium</i> 2-butanone	
NASH	Increase <i>Escherichia, Blautia, Dorea, Lactobacillus, Clostridium, Allisonella, Bacteroides</i> Chenodeoxycholic acid, Unconjugated cholate, Lithocholic acid, Ethanol, 4-Methyl-2-pentanone	
	Decrease <i>Oscillospira, Coprococcus, Fecalibacterium</i>	
NASH-related advanced fibrosis	Increase <i>Blautia, Roseburia, Streptococcus, Lactobacillus, Enterococcus, Bacteroides, Escherichia, Klebsiella</i> 3-Phenylpropanoate, 3-4-Hydroxyphenyl-lactate	
	Decrease <i>Ruminococcus, Akkermansia</i>	
NAFLD-related HCC	Increase <i>Enterococcus, Oscillospira, Bacteroides</i>	
	Decrease <i>Blautia, Bifidobacterium</i>	
NASH in obese children	Increase <i>Prevotella, Escherichia coli</i>	
	Decrease <i>Bifidobacterium, Alistipes, Blautia</i>	
Alcoholic liver disease without cirrhosis	Increase Proteobacteria Threonine, Glutamine, Guanidino-succinate, Propionate, Isobutyrate, Dimethyl disulfide, Dimethyl trisulfide, Urinary 3-hydroxytetradecanedioic acid, and so-citric acid	
	Decrease Bacteroidetes, Ruminococcaceae Urinary sebacic acid	
Alcoholic cirrhosis with abstinence	Increase Enterobacteriaceae	
	Decrease Lachnospiraceae and Ruminococcaceae	
Alcoholic cirrhosis with active drinking	Increase Oral-origin microbiota and Lactobacillaceae	
	Decrease Citrate, Malate, Phosphate, Threonine, Ornithine, Serine, Ribosine, Orotic acid, Hexanoate	
Alcoholic hepatitis	Increase Enterobacteriaceae, Streptococcaceae, <i>Actinobacterium, Bifidobacterium, Fusobacteria</i> Eicosapentaenoate, Docosapentaenoate, Benzoic acid metabolites	Higher total serum bilirubin in patients with higher fecal abundance of Enterobacteria Lower total serum bilirubin in patients with higher fecal abundance of Clostridiales <i>Akkermansia muciniphila</i> abundance reduced with increasing severity of alcoholic liver disease; lowest in alcoholic hepatitis
	Decrease <i>Akkermansia muciniphila</i> Monoacylglycerols, Malate, Fumarate, Citrate, Glycodeoxycholate	
Cirrhosis (any etiology)	Increase Proteobacteria, Fusobacteria, Clostridium clusters XI Enterobacteriaceae, Streptococcaceae, Leuconostocaceae, Lactobacillaceae, Alcaligenaceae <i>Acidaminococcus, Enterococcus, Burkholderia, Ralstonia, Proteus</i>	Reduction in levels of <i>Lachnospira</i> and increase in level of <i>Streptococcus</i> associated with higher Child-Pugh scores Enterobacterium associated with spontaneous bacterial peritonitis Bacteroidaceae and Clostridiales XIV predictors of 90-day hospitalization and higher Child-Pugh and MELD scores
	Decrease Bacteroidetes Lachnospiraceae, Ruminococcaceae <i>Clostridium-Incertae sedis - XIV</i> <i>Dorea, Subdoligranumum</i>	

(continued)

**Table 1.** (continued)

Disease		Pertinent associated microbiota and metabolites	Comments
Acute-on-chronic liver failure	Predictors of poor outcomes	Enterobacteriaceae, Campylobacteriaceae, Pasteurellaceae, Enterococcaceae, Streptococcaceae	
	Reduction in poor outcomes	Lachnospiraceae, Clostridiales	
Hepatic encephalopathy	Increase	Alcaligenaceae, Porphyromonadaceae, <i>Veillonella</i> , <i>Megasphaera</i> , <i>Bifidobacterium</i> , <i>Enterococcus</i> , <i>Streptococcus salivarius</i>	
	Decrease	<i>Roseburia</i>	
HCC	Increase	<i>Escherichia coli</i> , <i>Escherichia-Shigella</i> , <i>Enterococcus</i> , <i>Proteus</i> , <i>Veillonella</i> , <i>Actinobacterium</i> , <i>Gemmiger</i>	
	Decrease	<i>Fecalibacterium</i> , <i>Rumonococcus</i> , <i>Ruminoclostridium Pseudobutyryvibrio</i> , <i>Lachnospiraceae Phascolarctobacterium</i> , <i>Parabacteroides</i>	
Chronic hepatitis B virus-related cirrhosis	Increase	Proteobacteria, <i>Enterococcus</i>	
	Decrease	<i>Bifidobacterium</i> , <i>Lactobacillus</i>	
Chronic hepatitis C virus-related cirrhosis	Increase	<i>Prevotella</i> , <i>Fecalibacterium</i>	
	Decrease	<i>Acinetobacter</i> , <i>Veillonella</i> , <i>Phascolarctobacterium</i>	
Autoimmune hepatitis	Increase	<i>Veillonella dispar</i>	<i>V. dispar</i> associated with elevation of aspartate aminotransferase levels and severity of autoimmune hepatitis Additionally, increased proportion of fungi <i>Exophiala</i> and a decreased proportion of <i>Saccharomyces cerevisiae</i> notable in patients with primary sclerosing cholangitis and inflammatory bowel disease
	Decrease	<i>Bifidobacterium</i> , <i>Lactobacillus</i>	
Primary sclerosing cholangitis	Increase	<i>Barnesiellaceae</i> , Lachnospiraceae, <i>Blautia</i> , <i>Escherichia</i> , <i>Ruminococcus</i> , <i>Megasphaera</i>	
	Decrease	Uncultured Clostridiales II, <i>Prevotella</i> , <i>Roseburia</i> , <i>Bacteroides</i>	
Drug-induced liver injury		Increase in <i>Mucispirillum</i> , <i>Turicibacter</i> and <i>Ruminococcus</i> associated with higher risk of toxicity to acetaminophen Metabolite 1-phenyl-1,2-propanedione associated with diurnal variation of acetaminophen induced hepatotoxicity	
Post-liver transplantation		Higher fecal levels of <i>Klebsiella</i> , <i>Escherichia</i> , <i>Shigella</i> in post-transplantation period associated with infections	Fecal microbiome index consisting of <i>Staphylococcus</i> and <i>Prevotella</i> useful in identifying patients post-liver transplant who develop abnormal liver tests

\* Pertinent metabolites associated with prominent bacterial communities in the given liver disease condition

Abbreviations: HCC, hepatocellular carcinoma; NAFL, nonalcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.

immunoglobulin A produced by infected B-lymphocytes. In the GM of Egyptian patients with HCV, higher abundance of *Prevotella* and *Faecalibacterium* and lower levels of *Acinetobacter*, *Veillonella* and *Phascolarctobacterium* were notable. The role of *Prevotella* or *Faecalibacterium* to *Bifidobacterium* ratio has been demonstrated as a biomarker for fibrosis progression in HCV-infected patients. However, in patients with HCV-related cirrhosis, gut dysbiosis can persist, regardless of long-term sustained viral response.<sup>73</sup>

The intestinal microbiota influences drug and xenobiotic metabolisms, that can affect drug efficacy and toxicity. Microbiota-related drug metabolism is important for activation of some prodrugs.<sup>74</sup> The GM also takes part in additional metabolic reactions associated with some drugs, such as acetylation, decarboxylation, dihydroxylation and demethylation. Microbiota-derived metabolites can indirectly affect xenobiotic metabolism pathways. It was demonstrated that the intestinal microbiota modulated susceptibility to

acetaminophen (APAP)-induced acute liver injury. The relative abundance of *Mucispirillum*, *Turicibacter* and *Ruminococcus* before APAP dosing was found to be associated with increased hepatotoxicity.<sup>75</sup>

APAP-induced liver injury has diurnal variation. APAP causes more hepatotoxicity during consumption at night compared to morning. It was demonstrated that the gut microbial metabolite, 1-phenyl-1,2-propanedione was involved in the rhythmic hepatotoxicity induced by APAP, by depleting hepatic glutathione levels. The anti-inflammatory drug, salicylazosulfapyridine, underwent degradation in conventional rats and when cultured with human gut bacteria but not in germ-free rats, demonstrating the role of GM in drug transformations. Wang et al.<sup>76</sup> showed that healthy donor FMT prevented HE in rats with carbon tetrachloride-induced acute liver failure.

The intestinal microbiota has been shown to play a central role in sensitization of sepsis-induced liver injury, and microbiota-associated granisetron production resulted in amelioration of liver injury during sepsis development in a mouse model of cecal ligation puncture.<sup>77</sup>

A recent case series showcased beneficial outcomes with FMT-related reconstitution of the gut microbiome on immune checkpoint inhibitors in colitis associated with a relative increase in regulatory T-cells within the colonic mucosa.<sup>78</sup>

Lu et al.<sup>79</sup> showed that there was a higher relative abundance of *Klebsiella*, *Escherichia* and *Shigella* and reduced levels of beneficial butyrate-producing bacteria among liver transplant recipients. The authors established a fecal microbiome index (specific alterations in *Staphylococcus* and *Prevotella*) that could be used for assessing liver recipients at risk of liver dysfunction. A summary of pertinent GM association with various liver diseases is shown in Table 1.

## Conclusions

GM plays a central role in the initiation and progression of certain liver diseases and changes in GM drive the pathophysiology of select liver diseases. There are robust data on the role of GM in the development of NAFLD and NASH, severe AH, complications of cirrhosis and especially HE and PSC. High-quality studies have also shown specific roles played by the GM in the progression of diseases such as drug-induced liver injury, AIH and chronic viral hepatitis. The benefits of therapeutic modulation of GM in liver diseases such as AH, HE and PSC are well documented but high-quality randomized trials are lacking. The future of liver disease management could well include microbiota modulation based on the high-quality bench-to-bedside research in the coming years.

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## Author contributions

Designing the research study (CAP, PA, PKY, GNR), collecting the data (CAP, RA, SR, TG, SK), writing the manuscript (CAP,

PA, SR, RA), critically reviewing and revising the manuscript (CAP, PA, PKY, GNR, RA, SR, TG, SK).

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