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Review article: emerging role of the gut microbiome in the progression of nonalcoholic fatty liver disease and potential therapeutic implications

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Summary

Background: Nonalcoholic fatty liver disease (NAFLD) is a prevalent disorder associated with obesity and diabetes. Few treatment options are effective for patients with NAFLD, but connections between the gut microbiome and NAFLD and NAFLD-associated conditions suggest that modulation of the gut microbiota could be a novel therapeutic option.

Aim: To examine the effect of the gut microbiota on pathophysiologic causes of NAFLD and assess the potential of microbiota-targeting therapies for NAFLD.

Methods: A PubMed search of the literature was performed; relevant articles were included.

Results: The composition of bacteria in the gastrointestinal tract can enhance fat deposition, modulate energy metabolism and alter inflammatory processes. Emerging evidence suggests a role for the gut microbiome in obesity and metabolic syndrome. NAFLD is often considered the hepatic manifestation of metabolic syndrome, and there has been tremendous progress in understanding the association of gut microbiome composition with NAFLD disease severity. We discuss the role of the gut microbiome in NAFLD pathophysiology and whether the microbiome composition can differentiate the two categories of NAFLD: nonalcoholic fatty liver (NAFL, the non-progressive form) vs nonalcoholic steatohepatitis (NASH, the progressive form). The association between gut microbiome and fibrosis progression in NAFLD is also discussed. Finally, we review whether modulation of the gut microbiome plays a role in improving treatment outcomes for patients with NAFLD.

Conclusions: Multiple pathophysiologic pathways connect the gut microbiome with the pathophysiology of NAFLD. Therefore, therapeutics that effectively target the gut microbiome may be beneficial for the treatment of patients with NAFLD.

The Handling Editor for this article was Professor Stephen Harrison, and this uncommissioned review was accepted for publication after full peer-review.

1 | INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is characterised by fat accumulation (or steatosis) in >5% of hepatocytes in individuals who either consume little alcohol or have no other secondary causes of steatosis such as viral hepatitis, lipodystrophy or medications associated with the development of steatosis.[1,2] The increasing prevalence of NAFLD parallels rises in the incidence of obesity and insulin resistance.[3,4] NAFLD is among the most common causes of liver disease and liver transplantation in the Western hemisphere.[4,5] NAFLD can be sub-classified into two categories: the non-progressive form, nonalcoholic fatty liver (NAFL) and the progressive form, nonalcoholic steatohepatitis (NASH).[1,6] NASH is a clinic-pathologic entity that is typically characterised by the presence of zone 3 steatosis, ballooning and lobular inflammation; perisinusoidal fibrosis may or may not be also present.[1,7] Fibrosis progression rate is estimated to be higher in NASH than in NAFL and progression to cirrhosis may take up to 30 years; however, rapid progression to cirrhosis may occur in a small subset of patients.[8] In addition, NASH is associated with increased risk of hepatocellular carcinoma (HCC) and all-cause (cardiovascular and liver-related) mortality.[9-11]

NAFLD pathogenesis is related to multiple insults that occur simultaneously and may act synergistically,[12,13] including accumulation of triglycerides (TGs),[12] mitochondrial dysfunction and increased oxidative stress,[12-14] altered mechanisms of apoptosis and autophagy,[15,16] increased levels of toxic lipid-related factors (eg, free fatty acids)[12] and liver inflammation.[12] Genetics (eg, mutations in the patatin-like phospholipase domain-containing 3 gene)[17] and adverse consequences of dietary habits and sedentary lifestyle (eg, insulin resistance, central obesity, dyslipidaemia and hypertriglyceridemia) likely contribute to overall NAFLD pathophysiology and augment the underlying mechanisms of liver insult.[12]

Accumulating evidence also implicates the gut microbiota in the development and progression of NAFLD[18,19] and suggests that therapeutic agents that target the gut microbiota may be beneficial. This review examines the pathophysiologic implications of altered gut microbiota in NAFLD and highlights recent progress in the development of microbiota-targeting therapies for patients with liver disease.

2 | MATERIALS AND METHODS

A PubMed search was performed for English language articles published between January 1, 2002 and January 31, 2019. Search terms included “cirrhosis” or “insulin resistance” or “liver disease” or “metabolic syndrome” or “NAFLD” or “obesity” or “steatohepatitis” and “microbiome.” The search was initially limited to primary publications and those of human subjects; articles reporting animal studies were later retrieved to allow more thorough discussion of pathophysiology. An additional nonsystematic search was performed to gather data on the use of prebiotics, probiotics, symbiotics, synbiotics and antibiotics for NAFLD. A total of 230 articles were retrieved; of these, 60 articles were reviewed. In addition, the bibliographies of these 60 articles were reviewed for additional relevant articles to consider for inclusion.

3 | MICROBIOTA, MICROBIOME AND THE GUT

The term “microbiota” refers to a community of microbes in an organism, whereas “microbiome” is used to refer to both the microbiota and the collective genomes and gene products of the microbiota living in or on an organism. The gastrointestinal (GI) tract of humans contains 10 trillion to 100 trillion bacteria, with approximately 15 000-36 000 species.[20,21] Composition of the gut microbiota varies among individuals as the result of multiple intrinsic (eg, age[22,23]) and extrinsic (eg, method of feeding after birth[24,25] and geographic region inhabited[26]) aspects.

4 | ROLE OF GUT MICROBIOME IN MAINTAINING HOMEOSTASIS

Progressing along the human GI tract from the jejunum to the colon, the number and the diversity of bacteria increases,[27] and the predominant bacterial species change. In the upper GI tract (oesophagus and proximal small bowel), *Streptococcus* species predominate,[28,29] whereas in the colon, Firmicutes and Bacteroidetes are most prevalent.[20,21,30-32] These locational alterations likely reflect the overall functionality of the dominant species (eg, Firmicutes and Bacteroidetes convert dietary complex carbohydrates and insoluble oligosaccharides to short-chain fatty acids [SCFAs], which can be absorbed by the host within the intestines).[33]

The interconnection between the gut microbiome and the host is complex. The host provides both a suitable environment and nutrients for bacterial growth, and the host's diet, disease states and medications affect gut bacteria.[33] The gut microbiota can, in turn, affect host nutrient and drug metabolism, contribute to maintaining the mucosal barrier of the GI tract, affect mucosal immunity and contribute to disease states.[34] Bacteria in the GI tract synthesise host nutrients, such as vitamins and amino acids and conjugate primary bile acids (BAs) to form secondary BAs, such as deoxycholic acid and lithocholic acids.[35,36] The gut microbiota themselves derive sustenance mainly through the fermentation of dietary complex carbohydrates and indigestible oligosaccharides ingested by the host. Bacterial metabolism of these complex carbohydrates produces SCFAs (eg, butyrate, propionate and acetate), which the host can subsequently use as an energy source.[34,37,38] A study of 15 healthy women given diets with varying levels of choline for 2 months found that the composition of the gut microbiome assessed by pyrosequencing of 16S ribosomal RNA bacterial genes in stool samples was altered from baseline with varying levels of dietary choline.[39] Choline depletion was associated both with variations in the levels of Gammaproteobacteria and Erysipelotrichia

and variations in amount of liver fat.[39] The investigators hypothesised that the tendency to develop hepatic steatosis with a choline-deficient diet could be predicted by a model based on bacterial levels, presence of a single nucleotide polymorphism affecting choline metabolism and change in hepatic steatosis.[39]

Alterations in either the number or function of the tight junctions found on the GI epithelium can result in increased intestinal permeability, allowing for passage of antigens and microbes into systemic circulation. A growing body of research indicates that BA metabolism via epidermal growth factor signalling may affect these tight junctions.[40,41] Deoxycholic acid and chenodeoxycholic acid have been shown to interact with and phosphorylate the endothelial growth factor receptor, ultimately resulting in tight junction rearrangement (through alterations in occludin, a structural protein found in tight junctions) and increased paracellular permeability.[41] Interestingly, lithocholic acid has been shown to increase the integrity of tight junctions and to attenuate the production of reactive oxygen species, tumour necrosis factor alpha (TNF-α), interleukin-1β and interferon-γ.[42]

Gut microbiota play a key immunomodulatory role, interacting closely with macrophages, dendritic cells, gut-associated lymphoid tissues, B cells and T cells.[43] For example, a healthy gut microbiome is integral to the proper development and function of T regulatory cells through a variety of cellular signalling mechanisms,[44-47] such as *Clostridium butyricum* inducing transforming growth factor-β1 expression via toll-like receptor (TLR)-2 activation.[48] Bacteria in the GI tract also participate in maintaining intestinal villous function[34] and preventing intestinal epithelial cell apoptosis.[49] In addition, alteration of the gut microbiota appears to have a role in intestinal disease (eg, inflammatory bowel disease [IBD][50,51]) and extraintestinal disease (eg, obesity,[52,53] diabetes[43,52,53] and chronic liver disease[54,55]). Furthermore, accumulating evidence supporting the role of the gut microbiota in drug metabolism (and corresponding effects on efficacy or adverse events) suggests that assessing microbiome activity could impact pharmaceutical drug development.[56,57]

5 | GUT MICROBIOME AND METABOLIC SYNDROME

Metabolic syndrome is defined as the presence of any three of the following conditions: central obesity, hypertension, impaired glucose tolerance (or overt diabetes mellitus), hypertriglyceridemia and low levels of high-density lipoproteinemia (HDL).[58] Given the close association between NAFLD and obesity, insulin resistance and TG levels,[59] NAFLD is often considered the hepatic manifestation of metabolic syndrome.[60] Because gut microbiota play a key role in metabolism and energy production from dietary intake,[61] it stands to reason that they are closely related to components of metabolic syndrome (eg, obesity and diabetes).

One of the main factors in the development of obesity, diabetes and NAFLD is diet. Diet affects the composition of GI bacteria, which then influences host metabolism and inflammation.[62] Turnbaugh and colleagues demonstrated the effect of diet on the gut microbiota in a mouse model.[63] They humanised the gut microbiota of these mice by transplanting them with human faeces, and then they fed the mice either a high-fat, high-sugar diet ("Western diet") or a plant-based, low-fat diet.[63] The group that was fed a high-fat diet had a lower percentage of Bacteroidetes spp in its gut microbiota and a higher percentage of Firmicutes compared with mice fed a plant-based diet. Studies in humans and mice have shown an increased Firmicutes/Bacteroidetes ratio in individuals who are overweight or obese,[64,65] and a reduction in the Firmicutes/Bacteroidetes ratio with weight loss.[32]

In addition, higher colonic levels of SCFAs have been observed in obese individuals compared with their nonobese counterparts.[64,66,67] The higher production of SCFAs may result in energy accumulation and subsequent weight gain.[68] In a comparison between individuals who were morbidly obese and individuals of normal weight, increased numbers of both the H_2-producing bacteria Prevotellaceae and H_2-oxidising Archaea microorganisms were observed in the morbidly obese participants.[69] The authors hypothesised that interspecies H_2 transfer accelerated carbohydrate fermentation and the production of acetate, with an ensuing increased energy uptake by the host. An increase in SCFA levels may also alter other metabolic pathways (eg, lipid and glucose metabolism via activation of peroxisome proliferator-activated receptor gamma[70] and glucagon-like peptide-1 [GLP-1]).[71]

The gut microbiota can also directly affect factors that control adiposity. For example, alterations in the gut microbiota affect the epithelial cell product, fasting-induced adipocyte factor (FIAF, also known as angiopoietin-like protein 4), which inhibits lipoprotein lipase. Preclinical models have demonstrated that microbial suppression of the FIAF gene increases lipogenesis,[72] whereas administration of the bacterial strain *Lactobacillus paracasei* ssp *paracasei* F19 both induced FIAF gene expression and reduced body fat.[73] The gut microbiota may also inhibit activity of the enzyme, adenosine monophosphate-activated protein kinase, in muscle and liver, resulting in reduction of fatty acid oxidation and increased fat storage.[74,75]

The gut microbiome also has a role in type 2 diabetes mellitus (T2DM). For example, a correlation between the Firmicutes/Bacteroidetes ratio and plasma glucose concentrations was reported in patients with T2DM.[76] In addition, as stated above, the gut microbiota affect the production and release of GLP-1, which affects pancreatic β-cell function. SCFAs also appear to be closely interrelated with insulin resistance. Compared with patients without insulin resistance, patients with T2DM have a decrease in butyrate-producing bacteria.[77] SCFAs bind to G-protein (GPR)-coupled receptors (eg, GPR41 and GPR43),[78] which may lead to the secretion of factors such as protein YY that affect satiety, gastric motility and pancreatic function.[79-81]

SCFAs also regulate various aspects of GI inflammation, such as neutrophil migration, T-cell differentiation and macrophage expression of proinflammatory cytokines.[82] Insulin resistance is often accompanied by low-grade inflammation.[83,84] The movement of the GI bacterial product lipopolysaccharides [LPS] into intestinal capillaries may contribute to inflammation and insulin resistance.[84] TLRs are a

type of innate immune receptor that are thought to recognise LPS and other products of invading pathogenic bacteria.[83] TLR knockout studies in mice are helping to elucidate the role of the gut microbiota in metabolic syndrome and insulin resistance. For example, mice deficient in TLR-5 developed features of metabolic syndrome, including insulin resistance.[85] The authors of this report suggested that changes in the composition of gut microbiota, resulting from loss of TLR-5, induced the low-grade inflammation that contributed to the symptoms of metabolic syndrome. Another report described mice with elimination of TLR-4 specifically in hepatocytes.[86] When these mice received a high-fat diet, they became obese, but compared with control mice, these mice displayed enhanced insulin sensitivity and reduced hepatic steatosis. Thus, hepatocytes are centrally involved in the effects of inflammation on metabolic control, with the gut microbiota making up a significant part of that inflammatory signal.[86]

6 | GUT MICROBIOME AND NAFLD

The major NAFLD risk factors (ie, diet, obesity and insulin resistance) are closely connected with the gut microbiome (Figure 1).[14,62,87-96] It is reasonable to speculate that the gut microbiota and the pathophysiology of NASH are closely intertwined. One study found that the gut microbiota play a large role in the development of NAFLD, by transplanting the gut microbiota from mice with diet-induced NAFLD into germ-free mice; NAFLD developed in the initially germ-free mice.[18] In humans, characterisation of the faecal microbiomes of 86 patients with biopsy-proven NAFLD (n = 72, stages 0-2 fibrosis; n = 14, stages 3-4 [advanced] fibrosis) revealed that patients with NAFLD and advanced fibrosis had increased levels of Proteobacteria, whereas patients with mild fibrosis had increased levels of Firmicutes.[19] In addition, 37 of 40 features that were predictors of advanced fibrosis in patients with NAFLD were related to the gut microbiota.

Patients with NAFLD have increased intestinal permeability related to disrupted tight junctions.[97] As mentioned previously, increased GI "leakiness" may allow bacterial translocation and entry of bacteria-derived products into the portal circulation.[98,99] Once in the liver, these factors may initiate proinflammatory cascades (eg, production of interleukin-6 [IL-6] and TNF-α) via interaction with the TLR present on a variety of cell types (ie, Kupffer cells, stellate cells and hepatocytes).[100] In fact, more than half of patients with NAFLD may have small-intestinal bacterial overgrowth,[97] and the presence of this comorbid condition parallels cirrhosis severity (ie, Child-Turcotte-Pugh class).[101,102]

Inflammation is a key factor in the development of NASH, and LPS produced by GI bacteria trigger proinflammatory cytokine cascades that involve TLR-4 and nuclear factor kappa B (NFκB).[92] LPS are also, as mentioned earlier, a key factor in the activation of Kupffer cells. Kupffer cells may release inflammatory cytokines in response to leptin, a hormone associated with adipocytes, thereby indirectly activating hepatic stellate cells and potentially perpetuating

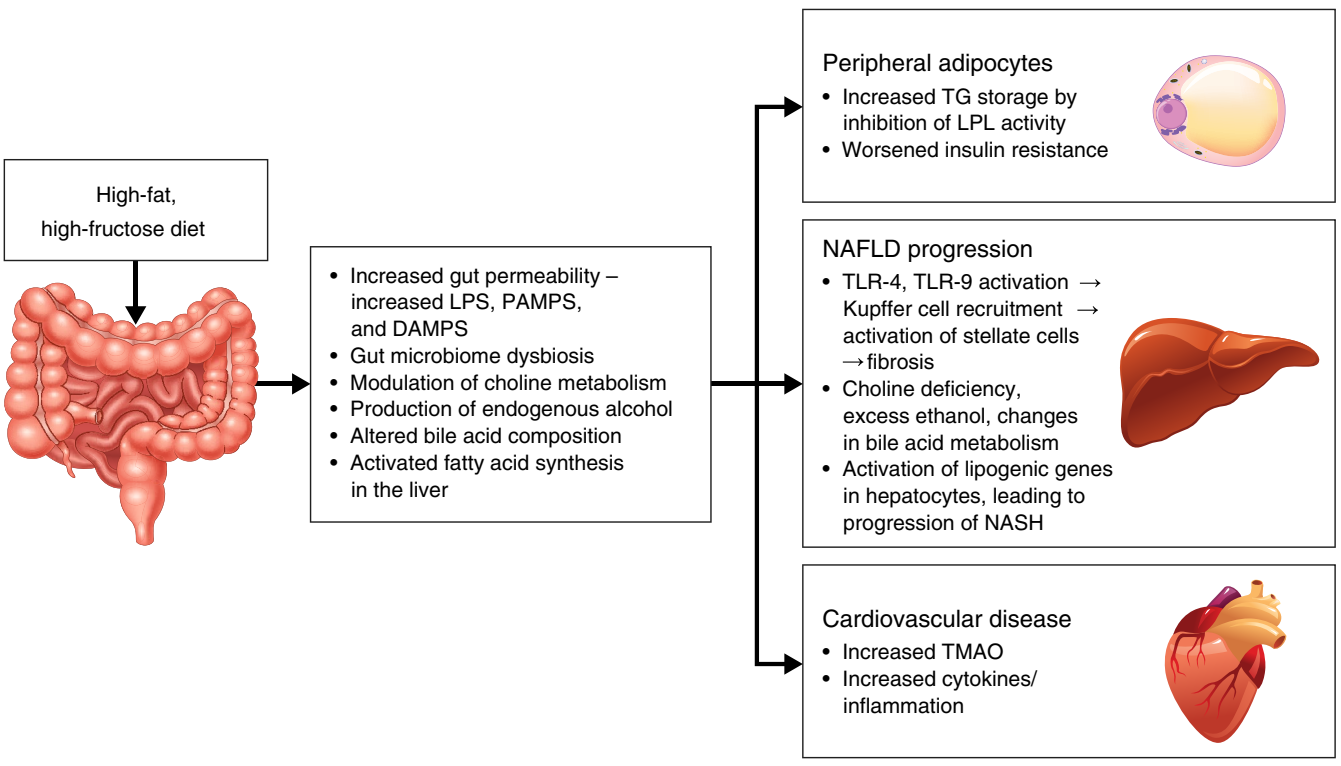

FIGURE 1 Role of the gut microbiome in NAFLD progression.[14,62,87-96] DAMPS, damage-associated molecular patterns; LPL, lipoprotein lipase; LPS, lipopolysaccharides; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PAMPs, pathogen-associated molecular patterns; TG, triglyceride; TLR, toll-like receptors; TMAO, trimethylamine-N-oxide

liver fibrosis.[103] In a mouse model, obesity-induced leptin increased liver responsiveness to LPS and enhanced progression of NASH.[104] Results from a meta-analysis of patients with NAFLD or NASH showed that circulating levels of leptin were higher in patients with NAFLD compared with healthy controls (standardised mean difference 0.64; 95% CI 0.42-0.86) and in patients with NASH compared with patients with simple steatosis (standardised mean difference 0.21; 95% CI, 0.02-0.40).[105]

Non-inflammatory bacterial products also have been implicated in the development of hepatic steatosis. Monosaccharides produced by microbial fermentation of carbohydrates in the GI tract may activate carbohydrate-responsive element-binding protein (ChREBP) and sterol-response element-binding protein 1 (SREBP1) pathways, which regulate lipid accumulation. In an obese mouse model, deficiency of ChREBP reduced hepatic fat levels, suggesting that inhibiting ChREBP could be beneficial in patients with hepatic steatosis.[106] Using stool samples and 16S ribosomal RNA gene pyrosequencing, Zhu et al[88] examined the gut bacteria of three groups of paediatric patients—healthy, obese without NASH and those with biopsy-proven NASH. The study found an association between health status and gut microbiome composition (at the phylum, family and genus levels). Both the obese and NASH groups demonstrated increased abundance of Bacteroidetes (specifically species of the genus *Prevotella*) and decreased abundance of Firmicutes compared with the healthy group. In addition, levels of species in the Proteobacteria phylum increased with progression from the healthy to obese to NASH groups, while the abundance of species in the Actinobacteria phylum (specifically those of the genus *Bifidobacterium*) decreased with worsening health status. The gut microbiome composition of obese patients and patients with NASH was similar, except for increased levels of Proteobacteria (specifically those of the Enterobacteriaceae family and the genus *Escherichia*; $P < 0.05$ for all three levels of classification) in the NASH group compared with the obese group. Increased abundance of ethanol-producing bacteria in the NASH microbiome prompted the investigators to measure serum alcohol levels in the three groups. While little to no difference was noted in ethanol levels between the healthy and obese groups, ethanol levels were significantly increased in the NASH vs the obese groups ($P < 0.01$). Combined with the demonstration of increased abundance of *Escherichia* in patients with NASH, the authors theorised a pathophysiologic mechanism linking the altered microbiome in NASH and the development of hepatic inflammation.

Although other studies have demonstrated increased blood ethanol levels associated with NASH and obesity,[94,107] the study conducted by Zhu et al was the first to demonstrate that patients with NASH have higher blood ethanol levels than obese patients, and related this finding to alcohol-producing bacteria in the gut microbiome of NASH patients.[88] The results from this study suggest that patients with NASH may be differentiated from healthy and obese patients by assessing the gut microbiome using stool samples. The authors postulated that the constant presence of bacteria-derived ethanol in the patients with NASH supplied a source of reactive oxygen species that could, in turn, increase inflammation and fibrosis.[88] Figure 2 summarises the effects of various factors involved in the development of fibrosis and cirrhosis in patients with NAFL.[19,40,41,88,108,109]

7 | ROLE OF THE GUT MICROBIOME IN DIFFERENTIATING NAFL FROM NASH AND ADVANCED FIBROSIS

Although animal studies have linked gut dysbiosis to the severity of hepatic inflammation and/or fibrosis, the findings of preclinical studies do not necessarily translate to humans; however, few human studies have examined this connection in the NAFLD setting.[110,111] In a study comparing the gut microbiomes of 50 patients (healthy controls, patients with NAFL/simple steatosis and patients with NASH), patients with NASH had lower levels of Bacteroidetes and higher levels of *Clostridium coccoides* compared with both healthy controls and patients with NAFL ($P < 0.05$).[110] In another study, examining the taxonomic composition of gut microbiota using 16S ribosomal RNA gene sequencing with stool samples from 57 patients with biopsy-proven NAFLD, 30 patients had F0/1 fibrosis stage disease and 27 patients had advanced fibrosis (defined as ≥ F2 fibrosis stage).[111] Ten patients with F0/1 stage and 25 patients with ≥ F2 stage had NASH. Patients with NASH had higher levels of *Bacteroides* and a lower abundance of *Prevotella* compared with patients without

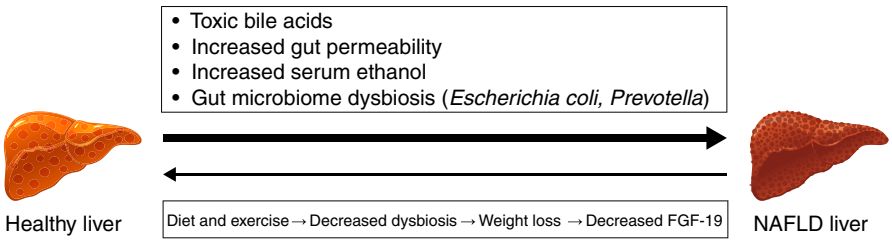

FIGURE 2 Association between gut microbiome and NAFLD. Patients with NAFL can progress to fibrosis and cirrhosis through different mechanisms, including toxic bile acids,[40,41,124] increased gut permeability,[41] increased endogenous ethanol[88] and gut microbiome dysbiosis[19,88] (with higher levels of *Escherichia coli* and *Prevotella*). However, patients can have an improvement in hepatic inflammation and fibrosis with lifestyle modifications that include exercise and diet (which improves gut microbiome dysbiosis) and weight loss, as both of these conditions decrease FGF-19.[108,109] FGF-19, fibroblast growth factor-19; NAFL, nonalcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease

NASH. In comparison to patients with F0/1 fibrosis stage, patients with advanced fibrosis (≥F2 fibrosis stage) had increased levels of *Bacteroides* and *Ruminococcus* and lower levels of *Prevotella*. A multivariate analysis revealed that NASH was associated with increased *Bacteroides*, whereas findings of increased *Ruminococcus* were associated with advanced fibrosis.

Loomba et al[19] characterised the gut microbiome of patients with biopsy-proven NAFLD using whole-genome shotgun sequencing for stool samples. Of 86 patients in the study, 72 had early fibrosis (F0/2) and the remaining 14 patients had F3/4 fibrosis. The median abundance for species in patients with early fibrosis was 2.5% *Eubacterium rectale* and 1.7% *Bacteroides vulgatus*, while *E coli* (1.0%) and *B vulgatus* (2.2% were predominant in patients with advanced fibrosis. Interestingly, a statistically significant increase in Proteobacteria levels was apparent as patients progressed to advanced fibrosis. Given that the increase in *E coli* levels preceded any clinical measures of fibrosis, they postulated that dysbiosis precedes the development of portal hypertension. However, larger studies are needed to determine if this finding is simply a correlation, or if a causal association is possible.

Exposure of liver Kupffer cells to bacterial LPS results in a release of proinflammatory cytokines through activating pathways that involve TLRs, myeloid differentiation factor 88 and NFκB,[100,112,113] which may activate stellate cells and fibrogenesis. These factors are integral in the promotion of inflammation and the progression of fibrosis to cirrhosis in many diseases, such as viral hepatitis, biliary liver disease and NAFLD.[112,114] In addition, a case-control/cross-sectional study has shown that trimethylamine-N-oxide, the liver product of a bacterial metabolite of choline, is associated with hepatic steatosis and inflammation.[87]

Neither of these studies had any subgroup analysis of patients with and without insulin resistance or other confounding variables that may also affect gut microbiota.[19,110,111] However, there may be a future role for the gut microbiome to be used as a non-invasive marker to determine the presence of NASH and advanced fibrosis. However, further studies, with larger cohorts, are needed before this marker can be recommended for diagnosis and prognosis of NASH. Thus, while additional studies are warranted to validate the promise of gut microbial profiling, the gut microbiome in these patient populations may one day serve as an emerging tool for non-invasive diagnosis of disease severity and monitoring progression.

8 | GUT MICROBIOTA AND BILE ACIDS

BA composition is influenced by gut microbiota, and BAs are also thought to play a role in the development of NAFLD.[115] Compared with healthy controls, patients with NASH have been shown to have higher concentrations of total faecal BA, cholic acid, chenodeoxycholic acid and BA synthesis, and a higher ratio of primary BA to secondary BA.[95] BAs such as chenodeoxycholic acid bind to the farnesoid X receptor (FXR) in the intestines.[116,117] FXR is a member of the nuclear receptor superfamily and plays a key role in the absorption and transport of BA into the liver, as well as de novo hepatic lipogenesis, very low-density lipoprotein transport and TG metabolism.[96,115,118] Mice deficient in FXR demonstrated increased hepatic TG and cholesterol content,[119] whereas FXR stimulation has been seen to suppress NFκB signalling, leading to decreased hepatic inflammation.[120] Of note, findings using the high-fat diet murine model of NAFLD demonstrated that intestinal antagonism of FXR through the manipulation of gut microbiota resulted in decreased hepatic lipogenesis.[115] Stimulation of FXR has also been shown to alter carbohydrate metabolism, phosphoenolpyruvate carboxykinase gene expression and gluconeogenesis regulation.[121]

Glucose homeostasis is also governed by GLP-1, which is stimulated by G-protein coupled receptor 5 (TGR5). Because the ligands for TGR5 are gut bacteria-derived secondary BAs, the gut microbiome may play a large part in both lipid metabolism (through FXR) and glucose homeostasis (through TGR5).[96,122] Animal models of NAFLD have shown reductions in hepatic steatosis after exposure to BA derivatives that are FXR agonists[123]; in the FXR Ligand Obeticholic Acid in NASH Treatment trial, improvement of hepatic steatosis and inflammation was observed in patients with NAFLD who received obeticholic acid.[124]

9 | TREATMENTS FOR NAFLD THAT TARGET GUT MICROBIOTA

Historically, the mainstay of treatment for patients with NAFLD has been lifestyle modification (eg, diet, exercise and weight loss) and the correction of underlying risk factors (eg, tight control of T2DM).[108] Most pharmacologic treatments for NAFLD are designed to improve insulin sensitivity (eg, metformin, thiazolidinediones liraglutide and sitagliptin), reduce oxidative stress (vitamin E, ursodeoxycholic acid and pentoxifylline) or downregulate fibrosis mechanisms (angiotensin receptor blockers).[125] Unfortunately, these medications have not demonstrated consistent improvement in liver fibrosis.[125-127] Alternatively, data are accumulating on the potential role of therapies that alter gut microbiota in the treatment of patients with NAFLD and NASH.

9.1 | Prebiotics

Prebiotics are indigestible foods that promote the growth of beneficial GI bacteria through the fermentation of the prebiotic into SCFA.[128] Preclinical studies of prebiotics have shown improvement in biochemical and histologic markers of NAFLD.[129] One randomised trial (placebo crossover design) has been published to date.[130] In patients with biopsy-proven NASH (n = 7), prebiotic administration (ie, oligofructose 16 g/d) significantly reduced hepatic levels of aspartate aminotransferase (AST; $P < 0.05$ vs placebo) and nonsignificantly decreased TG concentrations compared with placebo after 8 weeks of treatment. However, a systematic review that included four clinical studies of patients with obesity-related

NAFLD did not support the use of prebiotics, due to a lack of study quality.[131]

9.2 | Probiotics

Probiotics are living microorganisms that are ingested and improve the mucosal integrity of the GI tract through alteration of the gut microbiota (via competitive colonisation and by acidification of the GI lumen).[128] To date, six double-blind, randomised controlled trials,[132-137] one open-label, randomised controlled trial[138] and one open-label, single-treatment trial[139] have examined the effect of probiotics in patients with NAFLD (Table 1). The studies have reported improvement in several biochemical markers (eg, alanine aminotransferase [ALT], AST and TNF-α). A systematic review that included three clinical studies examining the efficacy of probiotics in patients with NAFLD did not support their use in this patient population, due to a lack of high-quality studies.[131]

Unfortunately, few studies have examined the effect of probiotics on histologic markers of NAFLD and NASH. In a 2013 meta-analysis of randomised controlled trials, only four studies were available when patient inclusion was limited to those with histologically or radiologically diagnosed NAFLD.[140] However, Alisi et al[133] reported that obese children with histologically diagnosed NAFLD who received sachets of eight probiotic strains daily for 4 months (n = 22) had a significantly lower risk of "more severe" steatosis (vs "less severe" steatosis) compared with children who received placebo (n = 22).

9.3 | Synbiotics

Synbiotics (or symbiotics) are a combination of both a prebiotic and a probiotic, and represent an emerging area of therapeutic research in NAFLD. Malaguarnera et al[141] evaluated 66 patients with histologically diagnosed NASH who were randomly assigned to receive 24 weeks of a synbiotic (*Bifidobacterium longum* plus a prebiotic [fructooligosaccharides]) or placebo. Both groups underwent lifestyle modification and a B vitamin regimen. Compared with the placebo arm, the active treatment arm had significantly lower TNF-α and C-reactive protein (CRP) levels, as well as histologic improvement (decreased hepatocellular injury, inflammation and steatosis) after treatment ($P < 0.05$).

In the largest double-blind, placebo-controlled trial to date, 80 patients with ultrasound-diagnosed NAFLD were randomly assigned to receive either a synbiotic (probiotics [*Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus bulgaricus*, *Bifidobacterium breve*, *B longum* and *Streptococcus thermophilus*] and fructooligosaccharides) or placebo for 8 weeks. At the end of the intervention period, patients who received synbiotics had significantly reduced steatosis (as measured by ultrasound) vs baseline, whereas no significant improvement was observed in patients who received placebo.[142] No significant differences in CRP, ALT or AST levels were observed between groups (adjusted for energy intake). In contrast, a study of 50 lean patients (ie, low or normal body mass index [BMI]) with NAFLD (patients had steatosis and elevated ALT) demonstrated significant reductions in fibrosis and hepatic steatosis, fasting blood sugar, TG levels and markers of inflammation after 28 weeks of synbiotic supplementation compared with placebo ($P < 0.05$).[143]

9.4 | Antibiotics

Several small trials, mostly in animal models, have analysed the effect of antibiotics on NAFLD. The mechanism of action for antibiotics is multifactorial and may include alterations in the gut microbiota composition, bacterial virulence and/or bacterial metabolic function,[128] although the specific pathway differs with each antibiotic. In a murine model, improvement in NAFLD was observed after the administration of an antibiotic cocktail (bacitracin, neomycin and streptomycin).[115] This improvement was hypothesised to be the result of alterations in gut microbiota and BA metabolism and reductions in intestinal FXR signalling, serum ceramides and fatty acid synthesis. Reduced levels of bile salt hydrolase, a bacterial enzyme that metabolises the BA, result in the retention of tauro-beta-muricholic acid in the ileum, which then inhibits FXR signalling within the intestinal wall, leading to decreased ceramide production.[115] Decreased ceramide production causes hepatic SREBP1C inhibition and decreased hepatic fatty acid accumulation. These findings were replicated in a rat study that showed alterations in the tissue BA profile, steroid biosynthesis and FXR signalling pathways after streptomycin and penicillin administration.[144] As in the previous study, elevated levels of tauro-beta-muricholic acid were observed, although this time in the liver.

Several human studies of antibiotics in NAFLD have assessed the effect of rifaximin, a nonsystemic antibiotic (Table 2).[145-147] Rifaximin is currently indicated in the United States to prevent overt hepatic encephalopathy recurrence and to treat travellers' diarrhoea and irritable bowel syndrome (IBS) with diarrhoea.[148,149] Although one may hypothesise that rifaximin exerts its effect by altering the composition of the gut microbiota, data have shown only modest changes in the components of the gut microbiome in patients with cirrhosis and hepatic encephalopathy after rifaximin treatment.[150] Additionally, preclinical studies have indicated that the efficacy of rifaximin may be attributable to its beneficial effects on host cell physiology and bacterial gene expression.[151-153]

9.5 | Faecal transplantation

Faecal transplantation has been used successfully in the treatment of patients with refractory and recurrent *Clostridium difficile*.[154,155] Although no human studies have examined the role of faecal transplantation for NAFLD, this strategy may be a potential avenue for exploration. In mouse models of NAFLD, animals that underwent a faecal transplantation from wild-type mice donors showed decreased hepatic gluconeogenesis[156] and reduced intestinal permeability[157] (Table 3). However, faecal transplantation is not without

TABLE 1 Human studies of probiotics for NAFLD

Publication	Study population	Study design/treatments	Primary outcomes
Miccheli et al 2015[132]	Obese children with elevated ALT and ultrasonographic and histologic evidence of NAFLD	DB, RCT; patients received placebo or the probiotic medical food VSL#3[a] qd (1 package for patients aged < 10 y and 2 packets for patients aged > 10 y) for 4 mo	• Among patients who completed the study (n = 22 for both groups), BMI, AST, total and active GLP-1 levels, and presence of fatty liver at 4 mo significantly improved in the probiotic group vs the placebo group ($P < 0.05$) • No significant differences in TGs, cholesterol, HDL, LDL or glycometabolism indices (glucose, insulin and HOMA-IR) were observed between groups at 4 mo • Key metabolites (valine, 3-aminoisobutyrate, alanine, tyrosine and pseudouridine) were significantly lower in the probiotic group vs placebo group at month 4 ($P < 0.05$), indicating an effect on BCAA and AAA metabolism, oxidative stress and microbiota metabolic pathways
Alisi et al 2014[133]	Obese children (median age, 10-11 y) with histologically diagnosed NAFLD	DB, RCT; patients received placebo or VSL#3[a] (1 sachet/d for patients aged < 10 y or 2 sachets/d for patients aged > 10 y) for 4 mo	• At 4 mo, risk of severe steatosis was significantly lower in VSL#3 groups (n = 22) vs placebo (n = 22) ($P < 0.001$) • BMI was significantly reduced with VSL#3 vs controls at 4 mo ($P < 0.001$) • At 4 mo, there was a trend towards a reduction in GLP-1 in the VSL#3 group • No significant changes were observed with VSL#3 vs placebo for TGs, HOMA-IR or ALT levels
Nabavi et al 2014[134]	Patients with NAFLD (unspecified diagnosis)	DB, RCT; patients received 300 g/d of conventional yogurt containing *L bulgaricus* and *S thermophilus*, or yogurt enriched with *B lactis* Bb12 and *L acidophilus* La5 for 8 wk	• Serum levels of ALT, AST, TC and LDL-C were reduced significantly with probiotic-enriched yogurt (n = 36) vs normal yogurt (n = 36) • Probiotic-enriched yogurt significantly reduced ALT, LDL-C, AST and TC serum levels vs baseline ($P < 0.05$ for all)
Shavakhi et al 2013[135]	Adults with histologically confirmed NASH, persistent elevation of ALT, and alcohol consumption < 20 mg in men or < 10 g in women	DB, RCT; patients received 2 tablets of metformin 500 mg and either probiotic supplement daily or placebo for 6 mo	• After 6 mo of treatment, levels of ALT and AST were significantly reduced with metformin and probiotic treatment (n = 31) compared with metformin and placebo (n = 32) ($P < 0.001$ for each) • At 6 mo, BMI, TG and TC levels were significantly reduced with metformin and probiotic treatment compared with metformin and placebo ($P \leq 0.02$ for all)
Wong et al 2013[138]	Adults with histology-proven NASH	OL, RCT; patients received "usual care" or 1 sachet of Lepicol®[b] b.i.d. for 6 mo	• At 6 mo, a significant between-group change in AST level was noted ($P = 0.02$) • Patients in the probiotic group (n = 10) tended to have greater reductions in IHTG at 6 mo than the usual care group (n = 10) • No significant alterations in TG, BMI, ALT, fasting glucose, TC, HDL-C, LDL-C, hepatic TG or liver stiffness were observed between groups at 6 mo
Aller et al 2011[136]	Patients with biopsy-proven NAFLD	DB, RCT; patients received placebo or 1 tablet containing *L bulgaricus* and *S thermophilus* qd for 3 mo	• After 3 mo, significant reductions from baseline in ALT, AST and GGT were observed in the probiotic group (n = 14) ($P < 0.05$) but not the placebo group (n = 14) • Neither treatment had any effects on glucose, TC, LDL-C, HDL-C, TG, insulin, HOMA-IR, IL-6 or TNF-α levels

(Continues)

TABLE 1 (Continued)

Publication	Study population	Study design/treatments	Primary outcomes
Vajro et al 2011[137]	Paediatric patients with a BMI > 95th percentile for their age and sex who had liver abnormalities (eg, increased ALT levels) associated with ultrasound evidence of fatty liver (n = 20)	DB, RCT; patients received either *Lactobacillus* GG 12 billion CFU/d (n = 10) or placebo (n = 10) for 8 wk	• No significant between-group differences in the number of patients who achieved ALT values < 40 U/L • ALT levels and concentration of peptidoglycan-polysaccharide were significantly higher with probiotics vs placebo (P = 0.03 for each) • No significant differences in BMI, visceral fat, TNF-α levels or hepatorenal ultrasonographic ratio
Loguercio et al 2005[139]	Patients with biopsy-proven NAFLD (n = 22) alcoholic cirrhosis (n = 20), HCV (n = 20) or HCV-related cirrhosis (n = 16)	OL; patients received VSL#3[a] for 3 mo	• After 3 mo, plasma levels of AST and ALT were significantly improved in all patients (P < 0.01) vs baseline • In patients with NAFLD or alcoholic cirrhosis, VSL#3 significantly reduced markers of oxidation (MDA and 4-HNE) from baseline at 3 mo (P < 0.01) • Levels of S-NO were significantly reduced in all groups (P < 0.05)

Abbreviations: 4-HNE, 4-hydroynonenal; AAA, aromatic amino acids; ALT, alanine aminotransferase; AST, aspartate aminotransferase; b.i.d., twice daily; BCAA, branched chain amino acids; BMI, body mass index; CFU, colony-forming unit; CRP, C-reactive protein; DB, double-blind; GGT, gamma-glutamyl transferase; GLP-1, glucagon-like peptide 1; HCV, hepatitis C virus; HDL, high-density lipoprotein; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance; IHTG, intrahepatic triglycerides; LDL, low-density lipoprotein; LDL-C, low-density lipoprotein cholesterol; MDA, malondialdehyde; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; OL, open label; qd, once daily; RCT, randomised controlled trial; S-NO, S-nitrosothiol; TC, total cholesterol; TG, triglyceride; TNF-α, tumour necrosis factor alpha.
[a]VSL#3 (Alfasigma USA, Inc; Covington, LA, USA) is a probiotic mixture containing *S thermophilus*, *B breve*, *B infantis*, *B longum*, *L acidophilus*, *L plantarum*, *L paracasei*, and *L delbrueckii* ssp *bulgaricus*.
[b]Lepicol® (Healthy Bowels Company Ltd; Birmingham, UK at the time of the study) contains *L plantarum*, *L delbrueckii ssp bulgaricus*, *L acidophilus*, *L rhamnosus*, *B bifidum*, and fructooligosaccharides.

risks. For example, a 2015 case report documented that a previously lean patient with recurrent *C difficile* infection developed obesity after a faecal transplant from an obese donor.[158]

Both murine knockout studies[85,86] and population-based studies[19,88] have examined alterations in gut microbiota in both NAFLD/NASH and in non-NAFLD subjects with risk factors for NAFLD. In the murine model, faecal transplantation from human obese adult twins into germ-free lean mice resulted in the development of obesity in these mice,[159] indicating that dysbiosis may lead to the development of obesity even in the absence of poor diet or genetic predisposition.

Studies in humans indicate that even in healthy individuals who donate stool for a faecal microbiota transplant (FMT), faeces from a select few patients (called "super donors") may yield more FMT success than stool obtained from other healthy donors.[160,161] FMTs have been studied in the setting of *C. difficile* infection[162] and in chronic illnesses, such as IBD,[160,161] IBS,[163] constipation[164] and neurologic conditions.[165] Two separate studies assessing the efficacy of FMT in the treatment of IBD found that patients who had received stool transplants from a particular donor had a higher success rate in inducing clinical and endoscopic remission compared with patients who did not receive stool from the "super donors."[160,161] FMTs using stool obtained from lean donors transplanted into patients with metabolic syndrome led to a greater degree of improvement in peripheral insulin sensitivity compared with autologous FMT.[166,167] Thus far, the only factor that seems to predict a successful FMT is the diversity of gut microbiota in the donor; conversely, recipients who are able to increase their faecal microbiome diversity to a higher degree in response to FMT were more likely to have successful outcomes in treatment of the underlying disease.[168,169]

10 | CONCLUSIONS

The GI tract and the liver develop from the same embryologic origins in the foregut, and this close interrelationship is maintained. Obesity, diet and insulin resistance are common risk factors for the development of NAFLD,[15,83,84] and these risk factors seem to have a strong connection with the gut microbiome.[12] While diet and obesity play a role in the modification of bacteria in the gut microbiota, the bacteria, in turn, affect the ability of host cells to produce and absorb nutrient-derived energy.[34] SCFAs and the ratios of the different fatty acids produced by bacteria are affected by the predominant type of bacteria in the GI lumen. Excess production of certain SCFAs can lead to the accumulation of excess energy in the form of adipose tissue, and obese patients are found to have altered ratios of SCFAs compared with their lean counterparts.[64,66-69] These GI bacteria also affect host lipid metabolism and insulin sensitivity.[77,80,170]

TABLE 2 Human studies of nonsystemic antibiotics for NAFLD

Publication	Study population	Study design/ treatments	Primary outcomes
Cobbold et al 2017[145]	Adults with histologically confirmed NAFLD	OL rifaximin 400 mg b.i.d. for 6 wk (n = 15)	• No significant alterations from baseline in ALT, hepatic lipid content, hepatic insulin sensitivity or serum cytokine (TNF-α and IL-1β) after 6 wk • ALT (P = 0.017), HDL (P = 0.004) and HOMA-IR (P = 0.05) levels significantly increased from baseline to week 12 (ie, during the 6-wk post-treatment period) • No consistent differences were observed in faecal microbiota composition at the phylum level • Significant reduction in urinary hippurate levels with rifaximin treatment (P = 0.048) was reported, indicating possible alteration in gut microbiota metabolism
Gangarapu et al 2015[146]	Adults with histologically confirmed NAFLD (steatosis, n = 15; NASH, n = 27)	OL rifaximin 1200 mg/d for 4 wk (n = 42)	• At 4 wk post-treatment, rifaximin significantly reduced levels of ALT (P = 0.01) and ferritin (P = 0.004) from baseline in patients with steatosis • In patients with NASH, rifaximin significantly reduced BMI, ALT, AST, GGT, LDL and ferritin levels, plasma endotoxin concentrations and serum IL-10 levels from baseline to 4 wk post-treatment ($P \leq 0.01$ for all) • No changes in serum TNF-α, IL-1, IL-6, IL-12 or TLR-4 levels were observed in either patient group
Kakiyama et al 2013[147]	Adult patients with "early" cirrhosis (Child-Pugh Class A without history of decompensation)	Longitudinal sub study; rifaximin 550 mg b.i.d. for 8 wk (n = 6)	• Reduction in ratio of secondary BAs to primary BAs after rifaximin treatment • No significant change in bacteria composition of the gut microbiota, except for reduction in Veillonellaceae

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; b.i.d., twice daily; BA, bile acid; BMI, body mass index; GGT, gamma-glutamyl transferase; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment-insulin resistance; IL, interleukin; LDL, low-density lipoprotein; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; OL, open label; TLR, toll-like receptor; TNF-α, tumour necrosis factor alpha.

TABLE 3 Animal studies of faecal microbiota transplantation for NAFLD

Publication	Study population	Study design/treatments	Primary outcomes
Nicolas et al 2017[156]	Donors: • WT mice fed a high-fat diet • WT mice fed a normal calorie diet • Genetically obese (ob/ob) mice Recipients: WT mice	Recipients were gavaged with gut microbiota obtained from cecum of (a) WT mice fed a normal diet; (b) WT mice fed a high-fat diet; (c) genetically obese mice	• Gut microbiota from both WT mice fed a high-fat diet and genetically obese mice reduced hepatic gluconeogenesis and adiposity elicited by a high-fat diet
Li et al 2015[157]	Donors: WT mice Recipients: WT mice that received ceftriaxone b.i.d. for 7 d to induce gut microbiota dysbiosis	Recipients gavaged for 3 d with faecal microbiota from WT mice or cultured bacteria initially isolated from donor mice faeces	• Gavage with faecal microbiota from WT mice or cultured bacteria improved inflammatory cell infiltration, tissue architecture distortion and vascular congestion elicited by ceftriaxone • Ceftriaxone-induced intestinal permeability was significantly improved with administration of faecal microbiota or cultured bacteria after 1 and 2 wk vs untreated animals ($P < 0.05$)

Abbreviations: b.i.d., twice daily; WT, wild type.

Obesity and insulin resistance are also associated with increased intestinal permeability[171-173] and, therefore, increased rates of bacterial translocation, which activates proinflammatory cascades.[84,170,174,175] Kupffer cell activation by bacterial products such as LPS results in oxidative stress and the development of hepatocyte inflammation and fibrosis. LPS also promote the development of hepatic TG accumulation and hepatic steatosis.[12,100,112,113] There is a strong association between bacterial translocation and the degree of hepatic decompensation in the setting of cirrhosis of any aetiology,[174] and among gut microbiota, BA metabolism, FXR and hepatic steatosis and inflammation.[115,120,121,124]

In addition, activation of TLRs by bacterial products results in increased systemic and hepatic inflammation, a major stimulus in the development of NASH and the progression to fibrosis.[100,112,113] Thus, treatment options aimed at targeting the gut microbiome, or the downstream cell-signalling effects of the microbiome, continue to be therapeutic targets for the treatment of patients with NAFLD.

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