Minireview

Nonalcoholic fatty liver disease and the gut microbiome: Are bacteria responsible for fatty liver?

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Impact statement

This invited minireview for the upcoming thematic issue on the microbiome addresses the role of the microbiome in nonalcoholic fatty liver disease (NAFLD). The incidence of NAFLD has increased greatly in recent years in parallel with the rise in obesity and is now believed to have a population prevalence of 20–40%. It is anticipated to soon become the primary cause of liver-related morbidity and mortality, and unfortunately, there are few treatment options. Therefore, there is a critical need for improved understanding of NAFLD pathophysiology to provide new avenues for therapeutic intervention. In this paper, we have reviewed evidence from human and animal model studies that have associated microbiome composition and microbial metabolites with development and progression of NAFLD. We have also discussed proposed mechanisms by which the microbiome could contribute to NAFLD pathogenesis and addressed future directions for this field.

Abstract

Over the last several years, a growing body of literature has linked the gut microbiome to human health and diseases such as obesity, metabolic syndrome, and nonalcoholic fatty liver disease (NAFLD). This paper will review the current literature investigating the influence of diets associated with metabolic disorders on the microbiome and how those changes promote susceptibility to metabolic disorders. It will then focus in-depth on the role of the gut microbiome in NAFLD. The review will highlight associations of microbial composition and function with progression of NAFLD in patients and discuss potential mechanisms that link the gut microbiome to NAFLD. Finally, it will address limitations of existing studies along with future directions for microbiome research in NAFLD, including potential microbe-related treatments.

Keywords: Microbiota, fatty liver, fibrosis, obesity, nutrition

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Introduction

The human microbiome represents all microorganisms residing on or within the human body, including bacteria, archaea, fungi, protozoans, and viruses. The human microbiome has the same number of cells and about 100 times more genes than the human body.[1–3] These genes encode a wide array of pathways that produce bioactive molecules derived from dietary or metabolic precursors.[4] While the gut microbiome has many beneficial functions such as the extraction of energy from otherwise indigestible dietary fiber, there is increasing evidence connecting the microbiome and its metabolites to the development of certain diseases including obesity, metabolic syndrome, and nonalcoholic fatty liver disease (NAFLD).[5]

The incidence of NAFLD is rapidly growing in conjunction with the epidemic of obesity and metabolic disorders.[6] The risk factors associated with NAFLD include central obesity, insulin resistance, hyperlipidemia, and metabolic syndrome. Epidemiological studies have suggested that NAFLD is more prevalent in men as compared to women and more prevalent in those with Asian or Hispanic

heritage relative to other racial/ethnic groups.[7,8] NAFLD is now the one of the most common causes of chronic liver disease in the Western world and the top two reasons for cirrhosis and liver transplantation.[9,10] NAFLD is a term that encompasses two distinct diseases: nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH). While patients with NAFL have only bland steatosis on liver biopsy, patients with NASH will also have lobular inflammation and/or hepatocyte ballooning, a sign of hepatocyte damage. Patients with NAFL will often remain stable for many years and will rarely ever progress further.[11,12] Patients with NASH, however, are more likely to progress to fibrosis, cirrhosis, and hepatocellular carcinoma.[11,13] The progression from NAFL to NASH is associated with metabolic syndrome and insulin resistance.[14] Because of the interplay between the microbiome and energy metabolism, there have been many recent studies that have investigated the relationship between the human microbiome and NAFLD development and progression.

This review will explore how diets associated with obesity and NAFLD affect the microbiome and how the microbiome in turn can influence the pathogenesis of these diseases. We will also review potential mechanisms and pathways that link the microbiome to the development and progression of NAFLD. Finally, we will discuss limitations of current research and explore potential future directions including therapeutic applications.

Methods

A comprehensive literature review was performed of studies published from 1995 to the present using the following key terms in PubMed: nonalcoholic fatty liver disease, nonalcoholic steatohepatitis, cirrhosis, fibrosis, obesity, metabolic syndrome, diabetes, fat, adipose, bacteria, and microbiome. Particular emphasis was given to articles published within the last five years.

Diet and the microbiome

Diet plays a critical role in the development of obesity, metabolic syndrome, and NAFLD. Epidemiological studies have consistently shown associations of diets high in fat and refined sugar with incidence of obesity and NAFLD.[15] In experimental models, such diets have been shown to increase adiposity, hepatic steatosis, and inflammation.[16] Here, we will discuss how the diets most commonly associated with NAFLD and obesity affect the gut microbiome.

Western diet

A Western diet is often defined as a diet that is high in sugar, fat, processed meats, and simple grains while being low in fiber.[17] It has been linked to many negative health outcomes including obesity, insulin resistance, metabolic syndrome, and NAFLD.[17,18] There is now growing evidence that the negative effects of a Western diet may be mediated by shifts in the microbiome.[17] Patients on this diet have been reported to have significantly lower microbial diversity and species richness than those on a more agrarian diet, features that are associated with gut dysbiosis.[19] The Western diet microbiome is often described as having a higher abundance of Firmicutes with a relatively lower abundance of Bacteroidetes.[20] The high Firmicutes to Bacteroides ratio decreases in subjects who lose weight on either a carbohydrate-restricted or fat-restricted diet.[21,22] At a genus level, a Western diet is associated with depletion of *Bidobacterium* and *Lactobacillus* and enrichment of *Enterobacter*.[23] These perturbations of the microbiome may cause metabolic changes in the host by altering short-chain fatty acid (SCFA) production, release of gut hormones such as peptide YY and glucagon-like peptide, and Toll-like receptor (TLR) signaling induced by lipopolysaccharides (LPSs) and other bacterial products.[22] The role of the microbiome in mediating the link between a Western diet and obesity has been examined extensively in several mouse models. Colonization of germ-free mice with the microbiota of obese mice (induced by leptin-deficiency or a Western diet) results in increased body fat accumulation compared to colonization with microbiota from lean controls.[24,25] Similarly, germ-free mice colonized with feces from obese humans had increased adiposity on a high-fat diet compared to germ-free mice colonized with feces from lean humans in weight discordant twin pairs.[26] Mice deficient in TLR 5 and inflammasome components develop susceptibility to Western diet-induced obesity that can be transmitted to other mice by fecal transplantation, demonstrating that genetic factors can modulate the diet-microbiome interaction.[26]

High saturated fat

Similar to the Western diet, diets high in saturated fats can also have deleterious effects on health that may be attributable to the microbiome. Epidemiological studies have shown that diets high in saturated and trans-fat are associated with obesity, cardiovascular disease, and NAFLD.[27,28] Mice that are fed a high saturated fat diet develop similar hepatic steatosis and inflammation as seen in patients with NAFLD.[28] However, not all fats have similar consequences. Diets high in polyunsaturated fats, such as those seen in a Mediterranean diet, have been associated with reduced cardiovascular events and a lower prevalence of obesity.[26–29,31] To examine the role of different fats on the microbiome, one study randomized subjects with risk factors for metabolic syndrome to receive either saturated or monosaturated fats.[32] The authors found that a diet high in saturated fats led to an overabundance of *Faecalibacterium prausnitzii*, which was not seen in patients on the diet high in monounsaturated fats.[32] However, this association with *F. prausnitzii* should not be construed as negative since the introduction of *F. prausnitzii* was protective against hepatic steatosis and adipose tissue inflammation in mice fed a high-fat diet.[33] In one of the largest studies comparing the effects of saturated and polyunsaturated fats on the microbiome, Menni *et al.* demonstrated in 876 women that higher polyunsaturated fat intake was associated with higher microbial diversity and expansion of members of the Lachnospiraceae family.[34] These findings suggest that the specific composition of fat may be more critical than

the total amount of fat. This idea is supported by a recent study demonstrating that mice fed on a lard fat diet had a greater abundance of *Bacteroides* and *Bilophila*, and a lower abundance of *Lactobacillus* and *Akkermansia*, than mice fed a diet with an equal amount of fat derived from fish oil.[35] Mice on a lard fat diet also had higher TLR signaling, white adipocyte inflammation, and insulin resistance as compared to mice on a fish oil diet.[35] Fecal transplantation of the lard fat-associated microbiome into germ-free animals induced the donor's metabolic phenotype in recipients, suggesting that these pathways are in part mediated by the microbiome.[35]

Role of the microbiome in obesity and insulin resistance

One of the early pivotal studies that linked the microbiome to the development of obesity came from Turnbaugh *et al.* in 2006.[24] They used 16s rRNA sequencing to demonstrate an increased ratio of Firmicutes to Bacteroidetes in obese humans and experimental mice on a high-fat diet and found that colonization of germ-free mice with this obesity-associated microbial profile could induce an obese phenotype in the recipients. Since then, several other studies have shown that the microbiome can influence weight gain by affecting host gene expression, metabolism, and ingestive behavior.[36–39] Pathways implicated in these metabolic changes included short-SCFA signaling and LPS activation of TLRs, which can induce altered gene expression, hormone secretion, and energy consumption in adipocytes.[40] Additionally, several papers have shown that the efficacy of surgical weight loss interventions may be in part mediated by shifts in the gut microbiome.[41] For example, a study in mice found that gastric bypass led to a persistent increase in *Escherichia* and *Akkermansia*, and that microbial transplantation from these mice into nonoperated germ-free mice successfully transferred the donor phenotype.[42]

Insulin resistance is another risk factor for NAFLD that is associated with obesity and may also be affected by the microbiome. In support of this concept, two small randomized controlled trials of probiotics for NAFLD—alone or in combination with metformin, an insulin sensitizer—have reported improvement in insulin resistance, hepatic inflammation, and hepatic steatosis.[43,44] This association between the microbiome and insulin resistance led Wu *et al.* to examine the effects of metformin on the microbiome of diabetic patients.[45] They found that metformin greatly alters the microbiome and that some of its metabolic effects on the host could be recapitulated by transferring this altered microbiome into germ-free animals. Wu's and prior studies support the concept that the microbiome influences insulin sensitivity and demonstrates how the microbiome can potentially alter the course of NAFLD through insulin-related pathways.

Microbiome associations across the spectrum of NALFD

The growing evidence linking the microbiome to obesity spurred interest in the potential role of the microbiome in other metabolic diseases including NAFLD. Here, we will review evidence from human studies for microbiome associations with NAFL-, NASH-, and NAFLD-related advanced fibrosis.

NAFL

Studies that have examined the microbiome profile of patients with NAFL as compared to either healthy controls or weight-matched controls have yielded variable results. Pediatric NAFL patients have been reported to have more *Prevotella* and less *Oscillospira* than matched controls.[46–48] In studies of adult NAFL patients, *Lactobacillus* and *Escherichia* have been enriched while *Coprococcus* and *Prevotella* have been depleted (Table 1).[49–52] These studies utilized 16s rRNA sequencing, which can only provide insight into composition (what bacteria are there) but not function (what products are made by bacteria that may affect disease). Three studies took a multi'omics approach combining microbiome sequencing with metabolomics analysis to evaluate potential microbial metabolic pathways promoting the development of NAFL.[51–53] Raman *et al.* found 18 differentially abundant stool metabolites associated with NAFL in adults, including elevated levels of derivatives of butanoic, propanoic, and acetic acid.[53] Similarly, Da Silva *et al.* found enrichment of propionate and isobutyric acid in the feces of NAFL patients.[51] These differences were associated with an increase in serum 2-hydroxybutyrate and L-lactic acid. The most convincing data to date that links the microbiome to the development of NAFL comes from Hoyles *et al*.[52] This study assessed the hepatic transcriptome, gut metagenome, and serum/urine metabolome of a cohort of nondiabetic obese women. NAFL was associated with increased serum levels of several branched-chain and aromatic compounds. Administration of one of these, phenylacetic acid, to mice colonized with human fecal microbiota triggered hepatic steatosis.

NASH

Studies characterizing the microbiome profile of NASH compared to NAFL or obese controls have found more consistent differences than has been seen for NAFL.[54] In children, patients with NASH generally had more *Ruminococcus*, *Dorea*, *Streptococcus*, and *Escherichia* as compared to their obese counterparts.[46–48,55] In adults, patients with NASH had lower levels of *Faecalibacterium*, *Ruminococcus*, and *Bidobacterium*[51,56] and a higher level of *Lactobacillus*.[57] Few studies have examined fecal or serum metabolites distinguishing NASH from simple NAFL, most likely due to the fact that a diagnosis of NASH often requires a liver biopsy in order to distinguish it from NAFL. Del Chierico *et al.* showed higher levels of 4-methyl-2-pentanone and 2-butanone in the serum of children with NASH.[47] Higher levels of 2-butanone were seen in the serum of adults with NAFL,[52] but the functional significance of this metabolite is still unknown. In a cohort of 16 adults with biopsy-proven NASH, patients with NASH had an increased ratio of primary to secondary bile acids, which the author correlated to an increased risk of hepatic injury.[58]

Table 1. Bacteria genera and fecal/serum metabolites associated with different stages of nonalcoholic fatty liver disease in human studies.

NAFLD subtypes	Community composition (genera)	Fecal metabolites	Serum metabolites
NAFL	↑↓*Bifidobacterium*[41,47,a] ↑↓*Lactobacillus*[41,45,46,48,a] ↑↓*Oscillobacter*[42,45,47,48,a] ↑↓*Prevotella*[43,44,a] ↑*Roseburia*[48] ↑↓*Ruminococcus*[42,44,46,a] ↑*Blautia*[42,44] ↑*Clostridium*[45] ↑*Dorea*[42,48,a] ↑*Escherichia*[44,47] ↑*Streptococcus*[45] ↓*Alistipes*[45] ↓*Coprococcus*[46,47] ↓*Faecalibacterium*[46] ↓*Odoribacter*[45] ↓*Oscillospira*[42,a]	↑Acetic acid[48] ↑Butanoic acid[48] ↑Cholic acid[53] ↑Ethanol[43,a] ↑Isobutyric acid[46] ↑Propanoic acid[48] ↑Propionate[46] ↓2-butanone[48]	↑2-butanone[42,a] ↑2-hydroxy-butyrate[46] ↑Isoleucine[47] ↑Leucine[47] ↑L-lactic acid[46] ↑Phenylacetic acid[47] ↑Valine[47]
NASH	↑↓*Ruminococcus*[41,46,52,a] ↑*Allisonella*[51] ↑*Blautia*[42,44] ↑*Clostridium*[53] ↑*Dorea*[41] ↑*Escherichia*[43] ↑*Lactobacillus*[41,52] ↑*Parabacteroides*[51] ↓*Bifidobacterium*[41,52] ↓*Coprococcus*[46] ↓*Faecalibacterium*[46,51,52] ↓*Oscillospira*[41]	↑Chenodeoxycholic acid[53] ↑Cholic acid[53] ↑Lithocholic acid[53]	↑2-butanone[42,a] ↑4-methyl-2-pentanone[42,a] ↑Ethanol[43]
NAFLD-related advanced fibrosis	↑↓*Prevotella*[55,56] ↑↓*Ruminococcus*[55–57] ↑*Bacteroides*[55–58] ↑*Blautia*[56] ↑*Enterococcus*[56] ↑*Escherichia*[57,58] ↑*Klebsiella*[55] ↑*Lactobacillus*[56] ↑*Parabacteroides*[56] ↑*Roseburia*[56] ↑*Streptococcus*[56] ↓*Akkermansia*[56]		↑3-(4-hydroxyphenyl)lactate[58] ↑3-phenylpropanoate[57]

[a]Denotes an association that has been reported only in pediatric cases of NAFLD.
NAFLD: nonalcoholic fatty liver disease; NAFL: nonalcoholic fatty liver; NASH: nonalcoholic steatohepatitis.

NAFLD-related advanced fibrosis

In contrast to NAFL and NASH, which have data from both children and adults, NAFLD-related fibrosis has only been studied in adults due to the slow progression of fibrosis. Advanced fibrosis, defined as a fibrosis stage > 2, is associated with a higher incidence of mortality and liver cancer.[59] Microbiome association studies of NAFLD-related advanced fibrosis have generally reported a decrease in microbial diversity, often due to expansion of Gram-negative bacteria.[60–62] Multiple studies have found an association between advanced fibrosis and an overabundance of *Bacteroides* and *Escherichia*,[60–63] while associations with other genera such as *Prevotella* have been less consistent.[61,64] Utilizing metagenomic sequencing, which allows for species level resolution, Loomba *et al.* showed that *Escherichia coli* and *Bacteroides vulgatus* were higher in patients with NAFLD-related advanced fibrosis.[62] They also examined serum metabolites and showed that 3-phenylproanoate was the metabolite with the highest fold increase in advanced fibrosis, though it did not reach significance. Recently, Caussy *et al.* found an association between 3–(4-hydroxyphenyl)lactate, a microbial metabolite involved in amino acid metabolism, and NAFLD-related advanced fibrosis.[63] This metabolite was also strongly correlated with several bacterial species that were associated with hepatic fibrosis, including *Escherichia coli*, *Bacteroides caccae*, and *Clostridium sp.*[62]

Potential mechanisms that link the microbiome to fatty liver disease

While recent human studies have provided meaningful insights into the composition and possible function of the microbiome in each stage during the development and progression of NAFLD, the findings are largely correlative and do not provide conclusive evidence of whether the microbiome is a critical driver of NALFD or simply responds to

the altered diet and host environment associated with NAFLD. Mechanistic investigation supporting a causative role for the microbiome in NAFLD pathogenesis has largely depended upon animal models. The results of studies evaluating microbial composition and metabolites in animal models of NAFLD are summarized in Table 2.[24,65–72] Overall, inflammatory pathways such as TLR signaling, choline deficiency, and bile acid metabolism have been linked to NASH, while SCFAs and amino acid metabolism have been linked more to NAFL. Here we will review the potential mechanisms by which the microbiome influences NAFLD development.

Epithelial barrier function, TLR signaling, and endotoxemia

Adult patients with NAFLD as well as healthy patients on a Western diet have both been shown to have a "leaky gut" characterized by higher intestinal permeability and altered tight junctions.[73,74] This disruption in the epithelial gut barrier leads to an increased translocation of bacterial products such as LPS into the portal circulation, potentially inducing hepatic inflammation. One of the first studies to causally link the microbiome to NAFLD demonstrated that mice lacking inflammasome components—which are important to intestinal barrier defense—developed dysbiosis and NASH. Transfer of this dysbiosis to wild-type recipients could induce NASH via an influx of TLR agonist, specifically TLR4 and TLR9, into the portal circulation.[68] Rahman *et al.* showed that fibrotic steatohepatitis induced by a high-fat, high-cholesterol, and high-fructose diet was exacerbated in mice lacking a gene involved in junctional adhesion molecules, an important component of the intestinal barrier. Administration of antibiotics improved liver histology in these knockout mice, suggesting that products of microbial metabolism crossing an impaired intestinal barrier mediated the phenotype.[75,76] There is also a significant role of the host immune system in modulating gut permeability. Beta7 integrin-deficient mice, which are deficient in intestinal immune populations requiring this integrin for chemotaxis, show decreased insulin resistance on a high-fat diet.[77] Treatment of wild-type mice on a high-fat diet with a local gut anti-inflammatory medication, 5-aminosalicyclic acid, reversed diet-induced bowel inflammation and improved metabolic parameters.[77] The downstream effects of LPS translocation are mediated through induction of TLR signaling in the liver. In several studies, LPS has been shown to induce TLR4, leading to increased NF-κB activation and cytokine production important to the progression from NAFL to NASH.[78,79] Unfortunately, a recent phase 2 trial did not show any significant benefit of TLR4 antagonism in NASH patients. Therefore, the clinical relevance of this pathway remains unclear.[80]

Table 2. Bacterial genera and fecal/serum metabolites associated with NAFL and NASH development in animal models.

NAFLD animal models	Community composition (genera)	Fecal metabolites	Serum metabolites
NAFL (high-fat diet or leptin-deficient mice)	↑*Bacteroides*[65] ↑*Barnesiella*[67] ↑*Bilophila*[64,66] ↑*Dorea*[66] ↑*Helicobacter*[65] ↑*Oscillospira*[65] ↑*Roseburia*[67] ↑*Sutterella*[66] ↓↑*Allobaculum*[67] ↓↑*Lactobacillus*[62,67] ↓*Akkermansia*[64–66] ↓*Bifidobacterium*[66] ↓*Flavobacterium*[65] ↓*Marinitoga*[65] ↓*Parabacteroides*[65,66] ↓*Ruminococcus*[66]	↑Butyrate[21] ↓Deoxycholic acid (relative abundance)[66] ↓Hyodeoxycholic acid (relative abundance)[66]	Taurine-conjugated bile acid[66]
NASH (NASH inducing diet, i.e., methionine-choline deficient diet)	↑(f) Bacteroidaceae[63] ↑(f) Erysipelotrichaceae[63] ↑(f) Porphyromonadaceae[63] ↑(f) *Clostridiaceae*[63] ↑*Alistipes*[60] ↑*Bacteroide*[60,61,63] ↑*Bilophila*[61] ↑*Blautia*[61] ↑*Parabacteroides*[63] ↑*Turicibacter*[63] ↓*Akkermansia*[61] ↓*Bifidobacteriu*[60,61] ↓*Desulfovibrio*[61] ↓*Enterorhabdus*[61] ↓*Lactobacillus*[63]	↑Hexadecane[60] ↑Tetracosane[60] ↓Arachidic acid[60] ↓Cholic acid[60] ↓Stearic acid[60]	

Wild-type mice on a control diet serve as the reference group. NAFLD: nonalcoholic fatty liver disease; NAFL: nonalcoholic fatty liver; NASH: nonalcoholic steatohepatitis

Choline deficiency

The relationship between choline deficiency and NAFLD development has been well established.[81] Deficiency in choline leads to abnormal phospholipid synthesis and alterations in very-low-density lipoprotein secretion, eventually leading to hepatic steatohepatitis.[81] Recently, dietary choline bioavailability was shown to be reduced by the gut microbiome through the production of metabolites such as trimethylamine (TMA).[30,82] Several gut microbes are high utilizers of choline and only low abundance of these microbes is required to greatly reduce host choline levels.[83] Mice fed a high-fat diet have been shown to have increased levels of gut microbes that metabolize choline and produce TMA.[84] The liver converts gut-derived TMA to trimethylamine-N-oxide (TMAO) via flavin containing monooxygenase 3.[85] Elevated levels of TMAO are associated with cardiovascular disease, which potentially links the extrahepatic manifestations of NAFLD to microbial-derived metabolites.[86] However, the role of circulating TMAO in NAFLD has not been well studied.

Short-chain fatty acids

One of the major functions of the human microbiome is the fermentation of indigestible carbohydrates (e.g., fiber) to produce SCFAs. These SCFAs include acetate, propionate, and butyrate, and they act as a major energy source for intestinal epithelial cells. SCFAs also facilitate a wide array of biological activities including hormone production and gene regulation.[87] Obese individuals as well as individuals with NAFL have higher total levels of gut SCFAs as compared to lean controls.[51,53,88] The administration of inulin-type fructan prebiotics was associated with a reduction in SCFAs in obese women along with a reduction of other metabolic markers.[89] Conversely, certain SCFAs may be beneficial against obesity and NAFLD. One mechanism by which SCFAs can affect the host is by binding to highly specific G-protein coupled receptors (GPR), which mediate distinct effects of each SCFA. For example, in a mouse model of diet-induced obesity, a mixture of SCFA predominantly made up of butyrate reduced hepatic expression of GPR41 and GPR43, two receptors that have been shown to promote hepatic lipid accumulation.[90,91] The positive effect of butyrate was further highlighted by Mattace Raso *et al.* when they demonstrated that butyrate supplementation was able to improve hepatic steatosis induced in mice by a high-fat diet.[92] Furthermore, fecal microbial transplantation from lean human donors to obese patients resulted in improved insulin sensitivity, which was associated with increased abundance of butyrate-producing bacteria.[93] The inconsistent findings on SCFAs are most likely due to the distinct biological effects of individual SCFAs on host metabolism.

Bile acid metabolism

The recent development and marketing of obeticholic acid, a farnesoid X receptor (FXR) agonist, underscores the importance of bile acids for host metabolism and health. Gut microbes play a critical role in the regulation of the bile acid pool through conversion of primary bile acids to secondary bile acids, which have distinct functional properties mediated by differential binding to bile acid receptors including FXR and G-protein coupled bile acid receptor 1 (GPBAR1).[94] In a murine model of NAFLD, animals with intestine-specific FXR disruption developed changes in their gut microbiome that were associated with reduced triglyceride accumulation in response to a high-fat diet as compared to controls.[95] In mice treated with antibiotics, there was an increase in conjugated bile acid metabolites that inhibited intestinal FXR signaling.[95] GPBAR1 signaling was also found to be necessary for sustained weight loss and improved fatty liver in mice undergoing sleeve gastrectomy.[96] In humans, a phase 2 clinical trial with obeticholic acid in patients with NASH showed improvement by histology after 72 weeks of treatment.[97] The administration of obeticholic acid also led to a reversible induction of Gram-positive bacteria in the human small intestine and increased proportion of Firmicutes in mice.[98] While initial results are promising, ongoing studies and phase 3 trials are underway in order to better understand the complex relationship between the gut microbiome, bile acid synthesis, and FXR signaling.

Amino acid metabolism

The gut microbiome can also affect the synthesis and metabolism of aromatic and branched-chain amino acids (BCAAs). In patients with insulin resistance, *Prevotella copri* and *Bacteroides vulgatus* were identified as the main species associated with increased BCAAs and insulin resistance.[99] The authors also showed that mice gavaged with *P. copri* developed increased insulin resistance when fed a high-fat diet as compared to controls.[99] In a recent study, Hoyles *et al.* demonstrated that phenylacetic acid, an aromatic amino acid derived from microbial metabolism, was strongly associated with hepatic steatosis in humans.[52] They also showed that the addition of phenylacetic acid in both primary human hepatocyte cultures and in mice models could trigger hepatic steatosis, implying a causal effect in NAFL.[52]

Therapeutic implications, limitations, and future directions

The growing evidence that links the human microbiome to NAFLD progression has motivated interest in the development of novel microbiome-related therapies for NAFLD. Microbiome-related interventions include gut-specific antibiotics, probiotics, prebiotics, and fecal microbial transplant (FMT).[54] However, large well-designed clinical studies examining microbiome-related interventions in NAFLD are lacking. Several randomized controlled trials involving probiotics in NAFLD have yielded conflicting results due to the lack of standardization across studies.[100] As of yet, no randomized controlled trial involving probiotics has shown any significant changes in body mass index (BMI).[100] Several small trials have shown a potential benefit of probiotics on important markers including insulin resistance, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and

Table 3. Summary of randomized control trials involving NAFLD and probiotics.

Study	Number of patients	Intervention	Major findings
Aller *et al.*[101]	30	*Lactobacillus delbrueckii subsp. bulgaricus* + *Streptococcus thermophilus* vs. placebo for 3 months	Decrease in ALT, AST, GGT
Alisi *et al.*[102]	44[a]	VSL#3 (*Bifidobacterium longum*, *Bifidobacterium infantis*, *Bifidobacterium breve*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus delbrueckii subsp. bulgaricus*, *Lactobacillus plantarum*, *Streptococcus thermophilus*) vs. placebo for 4 months	Ultrasound improvement in fatty liver
Eslamparast *et al.*[103]	19	Protexin (*Bifidobacterium breve*, *Bifidobacterium longum*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii subsp. bulgaricus*, *Streptococcus thermophilus*) vs. placebo for 28 weeks	Decrease in ALT, AST, GGT, CRP, TNFα, fibrosis score by transient elastography
Malaguarnera *et al.*[44]	66	*Bifidobacterium longum* + fructo-oligosaccharides + life-style modification vs. life-style modification alone	Decrease in AST, LDL, CRP, TNFα, HOMA-IR, steatosis, and NASH activity index
Shavakhi *et al.*[43]	64	Protexin + Metformin vs. Metformin alone	Decrease in ALT, AST, ultrasound grading of steatosis
Wong *et al.*[109]	20	Lepicol (*Bifidobacterium bifidum*, *Lactobacillus plantarum*, *Lactobacillus delbrueckii subsp. bulgaricus*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*) vs. nothing	Decrease in intrahepatic triglyceride content as measured by proton-magnetic resonance spectroscopy

[a]Denotes a pediatric trial.
NASH: nonalcoholic steatohepatitis; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: Gamma-glutamyl transferase; CRP: C-reactive protein; TNF: Tumor necrosis factor; LDL: low-density lipoprotein; HOMA: Homeostatic model assessment.

histology grade (Table 3).[43,44,101–103] For example, a small randomized trial with 66 patients showed that supplementation with *Bidobacterium longum* and fructo-oligosaccharides improved insulin resistance, hepatic steatosis, and NASH activity index after 24 weeks of treatment.[44] These findings have not yet been reproduced in larger published studies. There is also no data available about the role of FMT in NAFLD, though there are now two actively recruiting clinical trials designed to address this question.[104,105] However, until there is a better understanding of the key mechanistic pathways by which the microbiome promotes NAFLD, the development of microbiome-related therapies will be limited. Nonetheless, a recent multi'omics study has provided initial support for the potential application of microbes and their metabolites as noninvasive biomarkers for diagnosis and prognostication of NAFLD.[62]

Despite major recent advances in microbiome research, the field is still in its infancy with many areas that can be improved upon. One of the main challenges to interpreting the existing literature on the microbiome and NAFLD is heterogeneity in study design. In particular, there has been wide variation in selection of healthy controls (including inconsistent BMI-matching), age range of study populations, incorporation of diet into the analyses, and sample collection/processing. In addition, early studies examining the gut microbiome and NAFLD have been predominantly association studies. These studies are unable to differentiate whether the microbial profile described was a potential cause of NAFLD or rather a byproduct of the environment. Moreover, relevant microbial metabolites that reach the liver may be produced primarily in the small intestine and/or proximal colon, which may not be well represented by the microbiome and metabolome of feces. At this time, studies are shifting away from these types of analysis and are moving towards studies focusing on mechanistic pathways.[106] This can be achieved in human studies by using a multi'omics approach combining microbiome analysis with other modalities including metabolomics, proteomics, and host transcriptomics to develop a systems level understanding of NAFLD development and progression. Such studies are complemented by experiments involving transplantation of human microbiota into germ-free or antibiotic-treated animals to establish causal relationships between dysbiosis observed in human cohorts and metabolic outcomes.[41,52]

Currently, 16s rRNA sequencing is the most common method for microbiome analysis.[106] It is effective for defining microbial composition and taxonomy to the genus and to some extent species level but does not provide functional data (i.e., presence of bacterial genes and their expression level). In order to achieve this level of specificity, shotgun metagenomic sequencing and/or metatranscriptomics are required.[107] Unfortunately, due to high cost, the sequencing of bacterial metagenomes and metatranscriptomes is still out of reach for many investigators. With ongoing advances in sequencing technology, it is likely that the price of these services will decrease sufficiently to allow for more widespread use in the future, similar to the widespread adoption of 16s rRNA sequencing after the dramatic decrease in sequencing costs early this decade.[108]

Conclusions

In summary, both animal models and human studies have supported the relationship between the gut microbiome and development and progression of NAFLD. By affecting gut barrier function, TLR signaling, choline metabolism, bile acid synthesis, SCFA, and amino acid production, the

gut microbiome appears to play a critical and multifactorial role in NAFLD development. But despite advances in technology and bioinformatics analysis, specific mechanistic pathways are not yet clearly defined. Future large, longitudinal, prospective studies incorporating multi-omics analysis and humanized animal models are needed to better define the multifactorial host-microbiome relationship involved in fatty liver pathogenesis.

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DECLARATION OF CONFLICTING INTERESTS

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