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REVIEW

Gut Microbiota of Nonalcoholic Fatty Liver Disease

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Abstract The prevalence of nonalcoholic fatty liver disease has been rapidly increasing worldwide. It has become a leading cause of liver transplantation. Accumulating evidence suggests a significant role for gut microbiota in its development and progression. Here we review the effect of gut microbiota on developing hepatic fatty infiltration and its progression. Current literature supports a possible role for gut microbiota in the development of liver steatosis, inflammation and fibrosis. We also review the literature on possible interventions for NAFLD that target the gut microbiota.

Keywords NAFLD · NASH · Steatosis · Inflammation · Fibrosis · Microbiota and intestinal permeability

Introduction

Nonalcoholic fatty liver disease (NAFLD) ranges from simple steatosis (SS) to nonalcoholic steatohepatitis (NASH). Twenty to 30 percent of adults with NAFLD develop NASH. A subgroup of NASH patients develop severe morbidities such as cirrhosis, hepatocellular carcinoma and liver failure [1–3]. Prevalence of suspected NAFLD has rapidly increased over the past 20 years and currently affects about 11 % of adolescents [4] and 20 % of all ages [2]. This rapid increase among obese subjects, independent of body mass index (BMI), suggests that potentially modifiable risk factors other than obesity may have a role [4].

The number of bacterial cells present in the mammalian gut is a continuum that ranges from 10^1 to 10^3 bacteria per gram of contents in the stomach and duodenum, to 10^4–10^7 in the jejunum and ileum, culminating in 10^{11}–10^{12} in the colon [5]. The human intestinal microbiome (IM) has 150-fold more genes compared to the host [6]. Therefore, the IM has been referred to as the missing organ. The IM is essential for several physiological functions including but not limited to vitamin biosynthesis, bile acid degradation and complex carbohydrate digestion. It is also important for intestinal mucosal barrier (IB) integrity.

The liver receives 70 % of its blood supply from the portal vein, the direct venous outflow of the intestine. This anatomical connection makes the liver the first line of defense against gut-derived products such as antigens and toxins [7]. This strong connection between the intestine and liver is termed the gut-liver axis and has been linked to liver pathogenesis since first described in 1998 [8].

Gut Microbiota and Obesity

Obesity is a critical risk factor for NAFLD. Here we review the interaction of the gut microbiota and obesity.

Recent animal studies suggest a role for microbiota in the pathogenesis of obesity. Bäckhed et al. showed that

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germ-free (GF) mice were leaner than conventionally raised mice despite consuming more energy. GF mice were resistant to developing obesity when fed a high-fat and a high-sugar diet. Colonizing GF mice with cecal microbiota obtained from conventionally raised mice led to a 60 % increase in body fat content and increased insulin resistance (IR) within 14 days despite reduced food intake [9]. Also, microbiota from ob/ob mice had an increased capacity to harvest energy from the diet [10]. Interestingly, this property was transferable; GF lean mice gavaged with an obese-mouse microbiota developed increased total body fat compared to mice gavaged with lean-mouse microbiota [10]. Moreover, GF mice colonized with microbiota from a lean human twin developed lower body mass and adiposity compared with those colonized from the obese co-twin, despite comparable energy intake [11]. Although these studies show a role for fecal microbiota in obesity, the transplant includes other substances such as short chain fatty acids (SCFAs), bile acids, and possibly undigested or partially digested food that in the short term could affect body mass and adiposity.

In humans, the IM differs between obese and lean subjects. Firmicutes and Bacteroidetes are the most abundant distal gut phyla, constituting over 90 % of known phylogenetic categories [12, 13]. The Bacteroidetes/Firmicutes ratio is disturbed in obesity and NASH. When subjects were controlled for intake of medications such gastric acid suppressors, antibiotics, probiotics or prebiotics, increased Bacteroidetes were found in obese adults, children and pregnant women [14]. Le Chatelier and Cotillard et al. also showed that obese and overweight adults are more likely to have a low microbial gene count (LGC) than non-obese adults. LGC subjects were found to have increased Bacteroides, gain more weight over time and experience increased IR, dyslipidemia and elevated inflammatory markers. Although the authors in these two studies accounted for antibiotics, diet and other food supplements were not considered [15, 16]. When obese women were subjected to Roux-en-Y gastric bypass (RYGB) and achieved weight loss, their IM changed. This change included an increase in IM diversity with 58 new genera detected after surgery in all patients. There was an increase in Bacteroides and Alistipes (phylum: Bacteroidetes), decrease in Lactobacillus, Dorea and Blautia (phylum: Firmicutes), and increase in Escherichia (phylum: Proteobacteria) [17]. The results of this study need to be interpreted carefully since another factor that can affect IM such as antibiotics, probiotics and medical diseases was not considered.

IM affects energy harvest. The human genome lacks a variety of enzymes including glycoside hydrolases and polysaccharide lyases required to digest complex carbohydrates. Fascinatingly, IM compensates for this lack by encoding for these very enzymes [18, 19]. Obese subjects have an increased Bacteroidetes/Firmicutes ratio [20–24]. Bacteroidetes encode for higher levels of complex carbohydrate-digesting enzymes compared to Firmicutes [18]. This suggests that the microbiome of obese individuals might have the capacity to harvest more energy.

SCFAs (mainly acetate, butyrate and propionate) are the end products of polysaccharide digestion. Although complex carbohydrates are the main source for SCFAs, they are also produced from amino acids by reductive deamination [25]. Animal studies showed that SCFA supplementation prevents total parenteral nutrition-associated mucosal atrophy and increases the intestinal absorption of glucose [26, 27]. The SCFA concentration is 20 % higher in obese and overweight adults than in lean adults on a western diet [22]. For people living in developed countries, SCFAs produced in the colon contribute approximately 5–10 % of the energy requirements. This estimate was based on a typical British diet, where 50–60 g of carbohydrates (15 g fiber and 35–50 g sugar and starch) are fermented per day [28], and could be even higher in areas of the world where more dietary fiber is consumed. Dietary fiber intake in Africa is estimated to be seven times higher than in the UK [29]. SCFAs serve as a fuel source for colonocytes, with butyrate being preferred [30, 31]. For example, in vitro, about 70 % of oxygen consumed by human colonocytes was the result of butyrate oxidation [32].

The production of SCFAs by colonic microbiota is relevant to obesity as they lead to increased calorie extraction and glucose absorption from the diet. On the other hand, SCFAs increase anorexigenic peptides. For example, in mice, SCFA supplementation was shown to protect against high-fat-diet (HFD)-induced obesity and IR through induction of gut hormones and subsequent reduction of food intake [33]. SCFAs have a similar effect on gut hormones in humans, leading to attenuation of appetite. Obese adults were blindly randomized to receive either inulin-propionate ester in sachets or inulin without propionate sachets (controls) at a dose of 10 g/day for 24 weeks. The content of the sachets was bound to a carrier molecule to deliver it specifically to the colon. The inulin-propionate group showed a significant reduction in weight gain, liver fat content, improved dyslipidemia and liver function tests (LFTs) [34]. In the same report, the investigators showed that inulin propionate significantly increased the release of postprandial plasma peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) from colonocytes in vitro [34]. PYY and GLP-1 regulate appetite [35, 36].

In summary, there appears to be a link between gut microbiota and obesity, and this raises the possibility that alterations in the IM might have an effect on the development and sustainability of obesity.

Table 1 Intestinal microbiota in NAFLD and healthy controls

Reference	Participants	Diagnosis Location	Confounders before stool collection	Method to detect IM	Bacteroidetes/ Firmicutes ratio	Genus in NAFLD to HC	Comments
Zhu et al. [23]	Children 25 obese 22 NASH 16 HC	Biopsy USA	No antibiotics, probiotics, PPI, prebiotics, H2 antagonists for 3 months	16S rRNA pyrosequencing	Increased in NASH and obese to HC	↑*Escherichia* ↑*Prevotella* ↑*Peptoniphilus* ↓*Coprococcus* ↓*Eubacterium* ↓*Roseburia* ↓*Oscillospira* ↓*Alistipes* ↓*Bifidobacterium*	*Escherichia* is significantly higher in NASH compared to obese children
Michail et al. [69]	Children 11 Obese 13 NAFLD 26 HC	US USA	No antibiotics for 6 months	16S rRNA microarray	No difference in NAFLD or obese to HC	↑ *Prevotella*	*Prevotella* is not higher in obese to HC No histological diagnosis for NAFLD
Wong et al. [24]	Adults 16 NASH 22 HC	Biopsy China	No information about antibiotics or probiotics	16S rRNA pyrosequencing	Increased in NASH to HC	↑*Parabacteroides* ↑*Allisonella* ↓*Faecalibacterium* ↓*Anaerosporobacter*	NASH patients treated with probiotics or placebo with no significant difference in IM after 6 mon
Mouzaki et al. [120]	Adults 22 NASH 11 SS 17 HC	Biopsy Canada	No antibiotics, probiotics, or prebiotics for 6 months	Real-time qPCR	No difference in the 3 groups	No differences in *Escherichia coli* or Bifidobacteria in the 3 groups	NASH had a lower percentage of Bacteroidetes to SS and HC
Raman et al. [121]	Adults 30 NAFLD 30 HC	US USA	No antibiotics or probiotics for 3 months	Multitag pyrosequencing	No difference in NAFLD to HC	↑*Lactobacillus* ↑*Dorea* ↑*Robinsoniella* ↑*Roseburia* ↓*Oscillibacter*	No histological diagnosis for NAFLD

NAFLD: nonalcoholic fatty liver disease; NASH: nonalcoholic steatohepatitis; SS: simple steatosis; IM: intestinal microbiota, HC: healthy controls; Diagnosis: method used to diagnose NAFLD; Location: geographic area where study subjects reside; USA: United States of America; US: ultrasonography; mon: months; PPI: proton pump inhibitors; H2: histamine receptor; ↑: increase; ↓: decrease

Gut Microbiota and Steatosis

Hepatic steatosis is likely the result of many factors, including increased energy intake, de-novo lipogenesis and influx of free fatty acids, IR and dysbiosis. Dysbiosis is a deviation of the enteric microbiome from that found in healthy individuals, which has been shown in multiple studies (Table 1). However, there is no consistency or specificity to this difference. This is likely due to multiple factors that could influence the IM. Age, location where the study subjects reside as well as the method used to identify stool microbiota and diagnose NAFLD, biopsy or ultrasonography (US), could be partially responsible for this inconsistency. Also, not all the studies accounted for other confounders that affect the IM before stool collection such as antibiotics, acid-suppressing agents, probiotics, prebiotics, and whether there were attempts to control weight with specific dietary habits.

Factors affecting the IM composition such as HFD, choline-deficient diet (CDD), high-fructose diet, changes in bile acids and altered intestinal epithelium integrity have been linked to hepatic steatosis.

Mice fed with a saturated HFD based on palm oil exhibit increased hepatic steatosis, weight gain and reduced microbial diversity compared to mice fed an unsaturated fat-based diet of olive and safflower oil [37]. This observation suggests that the microbial changes associated with HFD are due to saturated fat. Yet, in mice fed HFD, the gut microbiota composition alters the lipid metabolism in the liver so steatosis can develop independently from obesity [38].

There is a relationship among choline, IM and NAFLD. Choline is derived from food such as red meat and eggs, but also can be biosynthesized [39]. Colonic bacteria can hydrolyze choline to form dimethylamine and trimethylamine, which are precursors of dimethylnitrosamine (DMN) [40–42]. DMN is a potent hepatotoxin, and carcinogen [43]. Choline is important in very low-density lipoprotein (VLDL) secretion by the liver, thus hepatic lipid homeostasis [39]. Both deficiency and over nutrition could have a negative impact on the liver. CDD is used to create a mouse model of NASH. Interestingly, an HFD reduces the bioavailability of choline, mimicking the effect of CDD when given to mice susceptible to impaired glucose homeostasis and NAFLD [44]. Therefore, alteration of the choline metabolism linked to steatosis could be due to HFD depleting choline. In humans, steatosis associated with parenteral nutrition is thought to be partly due to choline deficiency since supplementation with choline reverses the steatosis [45].

Phosphatidylethanolamine N-methyltransferase (PEMT) is important in endogenous phosphatidylcholine synthesis. PEMT polymorphism in adults and children influences susceptibility to choline deficiency-induced fatty liver [46]. Spencer et al. studied the effect of a choline-manipulated diet in 15 healthy females in relation to liver fat. They suggested that Gammaproteobacteria and Erysipelotrichi abundance, particularly when combined with the PEMT genotype, could predict choline deficiency-induced fatty liver [47].

High fructose consumption has been linked to NAFLD and its progression. In animal models, a high-fructose diet promotes NAFLD, alters microbiota composition, and increases endoplasmic reticulum stress and apoptotic activity [48, 49]. In humans, there are no such studies showing a link between high fructose consumption, microbiota and NAFLD. Two recent large clinical studies linked high fructose consumption with NAFLD histopathology, but the mechanism was not examined. The first study included 427 adults with NAFLD. Less steatosis and increased fibrosis were observed in those who had increased fructose intake [50]. Since fructose consumption was self-reported, serum uric acid, which is known to be a biologic marker for fructose [51, 52], was measured and found to be higher in subjects who reported increased fructose intake [50]. The second study included 149 children with NAFLD. Although histopathology did not correlate with self-reported fructose consumption, uric acid was significantly higher in NASH children compared to those with SS [53]. Thus, high fructose consumption might be associated with more pronounced NASH. However, more studies are needed to validate this association and examine whether there is any role for IM as shown in animal studies.

Bile acids might have a signaling function outside of the enterohepatic circulation, possibly involving glucose, lipid and energy homeostasis through activation of the farnesoid X receptor (FXR) and G-protein coupled receptor (TGR5). Animal studies showed that FXR is strongly expressed in the liver and intestine [54, 55]. FXR is known to have an important role in controlling hepatic de-novo lipogenesis, VLDL export and plasma triglyceride (TG) turnover [56]. TGR5 binds secondary bile acids and stimulates GLP-1 secretion, which plays a key role in glucose homeostasis [57]. Swann et al. [58] showed that the IM in rats affects the composition of the bile acid pool as well as the expression of genes controlled by FXR. They also observed that antibiotics could change the pool composition and lead to partial microbiota reduction [58]. HFD also affects bile acids. Mice fed with HFD have an altered bile acid composition that influences the gut microbial environment [59]. Human studies are lacking. However, FXR and TGR5 are activated by bile acids in humans [60–62], and they might serve similar functions. Collectively, these data show that bile acids can alter gut microbiota and vice versa, which could have a role in NAFLD.

IM homeostasis, including the abundance and diversity of phyla, families and genera, promotes a healthy IB, which is essential for liver health. Gut dysbiosis influences the integrity of the IB and exposes the liver to microbial products. For example, adults with biopsy-proven NAFLD ($n = 35$) compared to healthy controls (HC; $n = 24$) have small intestinal bacteria overgrowth (SIBO) and increased intestinal permeability with disturbed integrity of duodenal tight junctions. SIBO and intestinal permeability correlated with steatosis severity but not with NASH ($n = 17$) [63]. IB disruption in the pathogenesis of NAFLD is further supported by a recent study showing irregularly arranged duodenal microvilli and widened tight junctions in NAFLD adults compared to HC [64]. Similarly, intestinal permeability (measured by the lactulose/mannitol ratio) is increased in children with NAFLD compared to controls (children with asthma, upper respiratory tract infection and headache) [65]. Unlike the findings in adults, the only study performed on children showed that intestinal permeability was higher in children with NASH than in those exhibiting only steatosis. This association between increased permeability and NAFLD suggests a role for bacterial translocation exposing the liver to more microbial products and influencing the development and/or progression of NAFLD. The controversy with regards to gut permeability and its association with steatosis or NASH needs further study.

Thus, the microbiome is affected by many factors including diet and bile acids. It is possible that changes to the microbiota alter the intestinal barrier, allow intestinal contents access to the liver, and drive the development of steatosis and its progression to NASH.

Gut Microbiota and Inflammation

Inflammation in NASH is likely the result of many factors including, but not limited to, endogenous alcohol production, endotoxemia and inflammatory mediators. SCFAs might have antiinflammatory effects by helping to maintain a healthy IB.

Alcohol consumption is a known factor in alcoholic fatty liver disease and continued imbibing is associated with disease progression. Endogenously produced alcohol by IM may play a similar role in NASH. The enterobacteriaceae family including Escherichia exhibits mixed-acid fermentation, a major product of which is ethanol [66–68]. We reported that the abundance of Escherichia is significantly higher in adolescent NASH ($n = 22$) compared to obese subjects ($n = 25$) and HC ($n = 16$). Adolescents with NASH also had higher peripheral serum alcohol concentrations [23]. Higher ethanol metabolites are also observed in the stool of children with NAFLD diagnosed

by US compared to obese with no ultrasound evidence of NAFLD or healthy lean children [69]. We showed that alcohol dehydrogenase (ADH) and aldehyde dehydrogenase are among the most highly upregulated genes in livers of adolescents with NASH [70]. A recent study reported that blood ethanol levels positively associate with IR in children with NAFLD [71]. In the same report hepatic ADH activity was shown to be significantly lower in ob/ob mice compared to controls. Based on the results in mice, the authors suggested that increased ethanol in NAFLD may result from insulin-dependent impairments of hepatic ADH activity [71]. This hypothesis goes against the observations that ADH activity is highly elevated in livers of adolescents with NASH [70], and the microbiome intervention reduces alcohol levels in ob/ob mice [72].

Similarly, a higher serum alcohol level is observed in adults with histology-proven NAFLD compared to HC [73]. One could expect an even higher alcohol level in the portal blood. Ethanol is shown to increase hepatocyte TG accumulation [74] and is a well-known source for generating reactive oxygen species (ROS), which can lead to steatohepatitis [75] and affect the IB integrity. Intestinal permeability measured by urinary excretion of the lactulose/mannitol ratio is higher in alcoholic steatohepatitis compared to subjects with no evidence of liver disease or HC [76]. The alcohol metabolite acetaldehyde disrupts tight junctions and adherens junctions in human colonic mucosa [77]. Collectively, these data suggest that dysbiosis in NASH results in increased endogenous alcohol production, increased permeability of the IB and exposure of the liver to high concentrations of potentially harmful intestinal contents, similar to that seen in alcoholic steatohepatitis.

High energy intake is associated with increased levels of circulating LPS in humans and animals [78]. HFD might facilitate LPS uptake through elevated chylomicron production in intestinal epithelial cells [79]. Enhanced responsiveness to low-dose LPS, leading to liver injury and severe fibrosis, was observed in mice on an HFD compared to controls [80]. Taken together, these observations suggest a potential effect of HFD on altering the gut microbiota in favor of LPS-producing bacteria and enhancing the LPS impact on liver injury.

Animal studies suggest that endotoxemia alone might induce steatohepatitis [81–83]. In adults, some investigators have suggested a link between endotoxemia and NAFLD. There was a higher endotoxin level in all subjects with NAFLD compared to controls, and it was higher in NASH compared to subjects with steatosis [84–86]. However, one adult study showed that 17 % of NAFLD patients were free from endotoxemia [87]. We observed that endotoxemia is not necessarily found in NASH [88]. In our study of adolescents, none of the control subjects had a high endotoxin level. Only 28 % of obese individual and

42 % of NASH patients had high endotoxin levels; 71 % of obese and 58 % of NASH subjects had low endotoxin levels similar to HC [88]. This observation was supported by an earlier study showing that 22.5 % children with NAFLD had low levels of endotoxin [89]. Table 2 shows the human studies that looked at endotoxemia in NAFLD in further detail. To properly interpret these studies, it is necessary to consider the method used to measure LPS, how the patients were grouped, and whether there were any associated medical diseases that could affect the results. It is also important to consider that other confounders such as antibiotics and energy intake might have biased the results.

LPS and other microbial products are recognized by the Toll-like receptors (TLRs), which are expressed by liver cells, such as Kupffer cells (KCs), hepatic stellate cells (HSCs) and hepatocytes [90]. TLR-4 and TLR-9 receptors are the most studied and known receptors associated with liver injury [91, 92]. KCs produce inflammatory cytokines and enhance the activity of HSCs, which promote liver injury [91, 93, 94]. Cytokines, tumor necrosis factor α (TNF-α) and tumor growth factor-$β$1 (TGF-$β$1) have been strongly linked to more advanced NAFLD. TNF-α liver expression was shown to be increased in adults with NASH compared to obese adults with normal livers or only steatosis [95]. This observation was further supported by a larger scale study showing that hepatic TNF-α expression in adults with NAFLD ($n = 92$) is higher than in controls ($n = 25$) and even higher in NASH ($n = 57$) compared to subjects with only steatosis ($n = 35$) [80]. In children ($n = 72$), serum TNF-α correlated with the NAFLD activity score (NAS) [96]. TGF-$β$1 enhances HSC activation and promotes fibrogenesis [93, 94] by activating TLR-4, which is highly expressed by HSCs [91].

SCFAs might serve a protective role in inflammation. Butyrate was shown to have antiinflammatory effects on human monocytes by enhancing interleukin-10 (IL-10) secretion, inhibiting IL-12 and interferon-α (IFN-α) release [97]. Decreased fecal concentrations of butyrate and propionate have been reported in patients with ulcerative colitis (UC) [98]. UC is a chronic inflammatory bowel disease that affects the mucosal surface of the large intestine, mainly the sigmoid colon and rectum. SCFA administration in UC has beneficial clinical and laboratory outcomes [99–102]. These data suggest a role for SCFAs in maintaining IB integrity and a protective role against NAFLD by an antiinflammatory effect.

Gut Microbiota and Fibrosis

HSCs are the major producers of the fibrotic matrix [103–105]. These cells could be influenced by the IM through expression of TLR9 [92], which is the only known receptor for bacterial DNA [106, 107]. Hepatic fibrosis was shown to be significantly lower in TLR9−/− compared to TLR9+/+ in the bile duct ligation (BDL) mouse model [92, 108].

Dysbiosis can lead to the progression from normal liver to steatosis, inflammation and finally fibrosis. Fibrosis can occur independent of steatosis, and it is associated with dysbiosis itself. In mice, an HFD model subjected to BDL showed an increase in the percent of gram-negative bacteria, a reduced Bacteroidetes/Firmicutes ratio, complete disappearance of Bifidobacteriaceae (Phylum: Actinobacteria), a dramatic increase of gram-negative Proteobacteria, especially Enterobacteriaceae, and a reduction in microbial diversity in comparison to controls [109]. These microbiota changes were associated with decreased intrahepatic TG, increased hepatic mRNA expression of TLR4 and TLR9, increased HSCs activity and increased liver fibrosis. To assess the role of Proteobateria in fibrosis, De Minicus et al. transplanted selected gram-negative or -positive bacteria from the more fibrogenic HFD/BDL mouse model to the controls. The transplantation was done by oral gavage of donors' cecal content. They found that control mice receiving the selected gram-negative flora showed a higher increase in liver injury compared to controls receiving the gram-positive fraction [109].

In humans, Boursier et al. recently showed that the type and function of gut microbiota in French adults with NAFLD ($n = 57$) is different based on the stage of fibrosis. Patients with stage 2 or greater had higher abundance of Bacteroides and Ruminococcus and a lower abundance of Prevotella compared to patients with fibrosis stage 0–1 [110].

Based on animal and human studies, microbiota dysbiosis could lead to fibrosis and contributes to its severity.

In summary, NASH patients have a different IM compared to healthy individuals. This altered microbiota can promote liver inflammation and fibrosis through different mechanisms, including but not limited to generating more alcohol and endotoxins that have increased access to the liver through disrupted IB. Overwhelming the liver with gut microbial products can activate an inflammatory cascade leading to hepatic injury. Manipulating this dysbiosis could have a preventive and therapeutic effect on NASH.

Interventions for NAFLD-Targeting Gut Microbiota

Diet

The diet can contribute to the contents of the gut microbiota. As discussed above, an HFD, high-energy diet, high-fructose diet, and decreased choline and its bioavailability

Table 2 Endoxemia in NAFLD

Reference	Participants	Diagnosis Location	Method to evaluate endotoxemia	High endotoxin	Low endotoxin	Comments
Yuan et al. [88]	Children 19 NASH 14 obese 18 control	Biopsy USA	LAL assay to measure LPS	8 NASH 4 obese 0 control	11 NASH 10 obese 18 control	Subjects with steatosis were included in the obese group Controls had abdominal pain or dyspepsia with normal EGD No correlation between NASH severity and endotoxin level
Alisi et al. [89]	Children 40 NAFLD 9 HC	Biopsy Italy	LAL assay to measure LPS	31 NAFLD 0 control	9 NAFLD 9 control	24 subjects in the NAFLD group had NAS <5 Endotoxin level positively correlates with NAS
Ruiz et al. [84]	Adults 40 NAFLD 10 HC	Biopsy Spain	Immunometric assay with a chemiluminescent substrate to measure LBP	40 NAFLD 0 HC	0 NAFLD 10 HC	LBP is higher in NASH compared to steatosis LBPs correlate with liver TNF-α mRNA expression
Thuy et al. [87]	Adults 12 NAFLD 6 control	Biopsy Germany	LAL assay to measure LPS	10 NAFLD 1 control	2 NAFLD 5 control	Endotoxin correlates with liver TLR4 mRNA expression Controls had partial liver resection because of liver metastasis or other medical reasons
Harte et al. [85]	Adults 155 NAFLD 23 HC	Biopsy UK	LAL assay to measure LPS	155 NAFLD 0 HC	0 NAFLD 23 HC	NFLD included patients with or without DMT2 No significant difference between endotoxin levels in NAFLD and NASH Endotoxin correlates with IR
Verdam et al. [86]	Adults 21 NASH 9 obese	Biopsy The Netherlands	ELISA for human EndoCab to measure IgG levels against endotoxin	21 NASH 0 obese	0 NASH 9 obese (lower compared to NASH)	Both groups included patients with or without DMT2 Obese controls had liver biopsy with <5 % steatosis or a NAS score ≤2 IgG levels against endotoxin correlate with the inflammation grade

NAFLD: nonalcoholic fatty liver disease; NASH: nonalcoholic steatohepatitis; NAS: NAFLD activity score; IM: intestinal microbiota; HC: healthy controls; Diagnosis: method used to diagnose NAFLD; Location: geographic area where study subjects reside; USA: United States of America; UK: United Kingdom; EGD: esophagogastroduodenoscopy; LAL: limulus amebocyte lysate; LBP: lipopolysaccharide-binding protein; IgG: immunoglobulin G; DMT2: diabetes mellitus type 2; IR: insulin resistance; TLR4: *Toll-like receptor 4*; TNF-α: tumor necrosis factor α

lead to altered microbiota, which has been shown to be associated with NAFLD [37, 39, 48–50, 53, 78]. Moreover, an HFD and high-energy intake are linked to changes in the bile acid pool and increased LPS, which contribute to the development and promotion of NAFLD [59, 78]. Collectively, these data suggest that diet is the most potent tool to target NAFLD. Only 5 % loss of body weight can improve steatosis and 10 % can improve steatohepatitis [111]. Since it is not always possible to change dietary habits and maintain weight loss, other interventions that manipulate the IM might be beneficial.

Probiotics and Symbiotics

Table 3 shows the effect of probiotics and symbiotics on NAFLD. At this time, the data on the treatment of NAFLD with probiotics or symbiotics are not compelling. There is no standardized dose or strain or a clear understanding of treatment duration. There was no follow-up on any of the intervention groups. The trials focused only on the commercially available probiotics, and none of the trials targeted the specific microbiota depletion described in NAFLD. Most important, however, the content and concentrations of those preparations cannot be assured because they are exempt from FDA oversight (https://ods.od.nih.gov/About/DSHEA_Wording.aspx and http://thomas.loc.gov/cgi-bin/query/z?c103:S.784: See section 4).

Antibiotics

Antibiotics alter the IM by decreasing the growth of some bacteria and promoting the growth of others. There have been attempts to treat human metabolic syndrome and NAFLD with antibiotics. For example, 20 Dutch adults with metabolic syndrome were blindly randomized to either vancomycin (group A) or amoxicillin (group B) at a dose of 1500 mg/day for 7 days. Group A showed improved insulin sensitivity compared to no change in B. Group A showed a decrease of gram-positive bacteria (mainly Firmicutes) and a compensatory increase in gram-negative bacteria (mainly Proteobacteria) and decreased microbial diversity [112]. Regretfully, there was no assessment of the microbiota after discontinuation of the antibiotics. Decreased microbial diversity might not favor human health [15, 16]. Proteobacteria has been shown to be higher in NASH compared to HC [23]. Also increased Proteobacteria and reduced microbial diversity have been associated with increased liver fibrosis in mice [109].

A recent study examined the effect of oral rifaximin at a dose of 1200 mg/day for 28 days on Turkish adults with steatosis ($n = 15$) and NASH ($n = 27$). The NASH group showed a significant reduction in the mean BMI, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), low-density lipoprotein (LDL), LPS and IL-10 compared to the baseline. However, the steatosis group showed a significant reduction only in ALT. In both groups, there was no significant change in the levels of blood glucose, insulin, cholesterol, TG, C-reactive protein (CRP), homeostasis model of insulin resistance (HOMA IR), TNF-α, IL-1, IL-6 and IL-12 levels [113]. Although there were some favorable laboratory results with treatment, it is hard to accept them as a result of rifaximin. This was an open-label, observational study with no placebo control. The results could be biased by other factors such as diet and exercise, which were not considered. More importantly, IM, which was proposed as the mechanism behind this effect, was not examined.

Thus, antibiotics do not appear to offer a promising role in targeting microbial alteration promoting NAFLD.

Bile Acids

In animal models, bile acids were shown to have antimicrobial activity and an effect on the gut microbiota composition [114]. They also preserve the integrity of the IB [115]. BDL leads to bacterial overgrowth, mucosal injury and bacterial translocation [116, 117]. These observations inspired some investigators to test the effect of bile acid derivatives as a potential adjuvant treatment for NASH. In adults, a multicenter, randomized, placebo-controlled trial tested the effect of obeticholic acid (OCA), an FXR agonist, on NASH histopathology for 72 weeks. Less than 50 % of the OCA group showed histological improvement that was evident in patients with diabetes. Weight, ALT, AST and GTT improved during OCA treatment but returned to baseline after it was discontinued [118]. Although the NAS improved, OCA does not seem to be a promising treatment since dyslipidemia and pruritus were major side effects. Dyslipidemia is not a newly described result of OCA as it was reported in an earlier study that examined the effect of only 6 weeks of OCA treatment of diabetic patients with presumed NAFLD [119].

Conclusion

Patients with NAFLD have specific enteric microbial alterations that could promote liver injury through multiple routes. Diet seems to be the driving force behind the development of such dysbiosis. There is an increase in the Enterobacteriaceae family (phylum: Proteobacteria), especially Escherichia, which produces alcohol and expresses endotoxins. Enterobacteriaceae is also shown to be the main bacteria associated with fibrosis in an HFD/BDL animal model. Proteobacteria is abundant in obese and

Table 3 Effect of probiotics and symbiotics on NAFLD

Reference	Diagnosis Design	A/B Location	Intervention	Duration	Outcome in A compared to B	Comments
Malaguarnera et al. [122]	Biopsy-proven NASH Double blind RCT	34/32 adults Italy	B. longum + FOS versus placebo Lifestyle	24 weeks	Greater improvement in liver histology, US echotexture, AST, LDL, CRP, TNF-α, HOMA-IR and serum endotoxin No significant difference in BMI, ALT, glucose, insulin or TG	The only study to evaluate liver histology before and after the intervention No long-term follow-up No dose specification
Aller et al. [123]	Biopsy-proven NAFLD Double-blind RCT	14/14 adults Spain	L. bulgaricus and S. thermophiles versus placebo	3 months	Significant reduction in ALT, AST and GGT No significant difference in BMI, LDL, TG, glucose, insulin, HOMA-IR or TNF-α	No histology at the end of the trial No long-term follow-up Relatively small sample size
Wong et al. [124]	Biopsy-proven NASH Open label RCT	10/10 adults China	L. plantarum L. delbrueckii L. acidophilus L. rhamnosus and B. bifidum + FOS and cellulose versus usual care Lifestyle	6 months	Significant reduction in AST No significant difference in BMI, ALT, LDL, TG or glucose Group A showed significant reduction in IHTG measured by MRS	No histology at end of the trial Not blinded No long-term follow-up Small sample size
Eslamparast et al. [125]	ALT, US and FibroScan Double-blind RCT	26/26 adults Iran	L. casei L. rhamnosus L. acidophilus L. bulgaricus B. breve B. longum and S. thermophiles + FOS and cellulose versus placebo Lifestyle	28 weeks	Greater reduction in fibrosis (measure by FibroScan), AST, ALT, GGT, CRP, TNF-α, NF-κB, glucose and HOMA-IR No significant difference in BMI or waist-to-hip ratio	No histology No long-term follow-up
Vajro et al. [126]	ALT and US Double-blinded RCT	10/10 children Italy	L. rhamnosus versus placebo	8 weeks	Significant reduction in ALT No significant difference in US liver echogenicity, BMI or TNF-α	Lifestyle modification was not encouraged No histology Short intervention period No long-term follow-up Small sample size

Table 3 continued

Reference	Diagnosis Design	A/B Location	Intervention	Duration	Outcome in A compared to B	Comments
Alisi et al. [127]	Biopsy-proven NAFLD Double-blinded RCT	22/22 children Italy	L. acidophilus L. plantarum L. paracasei L. delbrueckii B. breve B. infantis B. longum and S. thermophilus versus placebo Lifestyle	4 months	Significant reduction in liver fat (assessed by US) and BMI No significant difference in ALT, TG or HOMA-IR	No histology at the end of the trial No long-term follow-up

NAFLD: nonalcoholic fatty liver disease; NASH: nonalcoholic steatohepatitis; IM: intestinal microbiota; Diagnosis: method used to diagnose NAFLD; Location: geographic area where study subjects reside; A/B: treatment group/controls; US: ultrasonography; RCT: randomized controlled trials; CFU: colony-forming unit; B: *Bifidobacterium*; L: *Lactobacillus*; S: *Streptococcus*; FOS: fructo-oligosaccharides (a non-digestible oligomer of beta-D-fructose); IHTG: intrahepatic triglyceride content; MRS: proton-magnetic resonance spectroscopy; BMI: body mass index; TG: triglycerides; LDL: low-density lipoprotein; HOMA-IR: homeostasis model assessment of insulin resistance; CRP: C-reactive protein; TNF-α: tumor necrosis factor α; NF-κB: nuclear factor kappa B; ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: gamma-glutamyltransferase

NASH adolescents compared to healthy controls. It is also observed to be the only abundant phylum exhibiting a significant difference between the obese and NASH groups. There is an increase in the Bacteroidetes/Firmicutes ratio. There is depletion of certain taxa such as Bifidobacteriaceae, Lachnospiraceae, Veillonellaceae and Ruminococcaceae [23]. Although probiotics appear to be promising aids in the management of NAFLD, more effort is needed to target the specific NAFLD microbiota alteration, standardize the dose and determine the length of the intervention. Diet appears to be the overwhelming factor influencing the microbiome, and dietary change to reduce fat is the safest and most effective treatment for NASH.

Key Messages

- Diet plays an essential role in NAFLD and contributes to its progression, possibly by its effect on the enteric microbial composition.
- Stool microbiota in obese and NAFLD patients is different from that in healthy lean individuals.
- Intestinal microbiota composition affects the intestinal barrier function, which is important for liver health.
- Diet is the strongest tool to overcome NAFLD.
- Targeting NAFLD-specific microbial alterations is a promising adjunct intervention.

Compliance with ethical standards

Conflict of interest None of the authors had any relevant personal or financial conflicts of interest.

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