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REVIEW

The links between the gut microbiome and non-alcoholic fatty liver disease (NAFLD)

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Abstract

NAFLD is currently the main cause of chronic liver disease in developed countries, and the number of NAFLD patients is growing worldwide. NAFLD often has similar symptoms to other metabolic disorders, including type 2 diabetes and obesity. Recently, the role of the gut microbiota in the pathophysiology of many diseases has been revealed. Regarding NAFLD, experiments using gut microbiota transplants to germ-free animal models showed that fatty liver disease development is determined by gut bacteria. Moreover, the perturbation of the composition of the gut microbiota has been observed in patients suffering from NAFLD. Numerous mechanisms relating the gut microbiome to NAFLD have been proposed, including the dysbiosis-induced dysregulation of gut endothelial barrier function that allows for the translocation of bacterial components and leads to hepatic inflammation. In addition, the various metabolites produced by the gut microbiota may impact the liver and thus modulate NAFLD susceptibility. Therefore, the manipulation of the gut microbiome by probiotics, prebiotics or synbiotics was shown to improve liver phenotype in NAFLD patients as well as in rodent models. Hence, further knowledge about the interactions among dysbiosis, environmental factors, and diet and their impacts on the gut–liver axis can improve the treatment of this life-threatening liver disease and its related disorders.

Keywords Gut microbiota · Non-alcoholic fatty liver disease · Germ-free animals · Dysbiosis · Metabolic syndrome · Bile acids · Intestinal permeability · Antibiotics · Probiotics · Prebiotics

Abbreviations

AMPK	AMP-activated protein kinase
ANGPTL4	Angiopoietin-like 4
CV	Conventional
FMT	Faecal microbiota transplantation
FOS	Fructooligosaccharides
FXR	Farnesoid X receptor
GF	Germ free
GI	Gastrointestinal
GLP	Glucagon-like peptide
HBV	Hepatitis B virus
HFD	High-fat diet
LPS	Lipopolysaccharides
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
PAMPs	Pathogen-associated molecular patterns
PEMT	Phosphatidylethanolamine methyltransferase
SCFA	Short-chain fatty acid
TJ	Tight junction
TMA	Trimethylamine

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The gut microbiome

Trillions of the microbes that colonize the human body, including bacteria, archaea, viruses, and eukaryotic microbes, are spread along the length of the gastrointestinal (GI) tract. At different sites of the GI tract, there are varied compositions and amounts of bacteria per gram content, including the stomach and duodenum (10–10^3), the small intestine (10^4–10^7) and the large intestine (10^{11} and 10^{12}), where the highest levels are found [1]. The dominant phyla in the large intestine are Firmicutes and Bacteroidetes. The Firmicutes:Bacteroidetes ratio was found to be correlated with individual susceptibility to disease states, including obesity [2]. However, the relevance of this ratio is disputable

as the composition of gut microbiome is not homogeneous and displays a considerable heterogeneity between individuals [3]. In addition, the human colon is home to important pathogens such as *Escherichia coli* (*E. coli*), *Campylobacter jejuni*, *Salmonella enterica, Vibrio cholerae*, and *Bacteroides fragilis*, normally at very low levels (< 0.1% gut microbiome) [3, 4]. The combination of a low abundance of pathogens and a high abundance of key genera including *Bacteroides, Prevotella* and *Ruminococcus* represents a healthy state for gut microbiota [5]. There are also axial differences in the gut microbiome composition from the lumen to the mucosal surface of the intestine. The frequent luminal microbial genera include *Bacteroides, Streptococcus, Bifidobacterium, Enterobacteriaceae, Enterococcus, Clostridium, Ruminococcus* and *Lactobacillus*; however, *Clostridium, Lactobacillus, Enterococcus* and *Akkermansia* are more frequent in the mucus layer as well as epithelial crypts of the small intestine [6]. Numerous factors can affect the composition and function of the gut microbiome. These factors include genetics, diet, mode of delivery at birth, geographic location, and exposure to medical treatments [7, 8]. As a consequence, the gut microbiome composition is unique to each individual and together with these influencing factors changes with age over the course of a lifetime. Conversely, the gut microbiota influences the metabolic phenotype of the host, takes part in food and drug metabolism, and improves the immune system [9].

One of the early studies on the interaction between host genetics and the gut microbiome reported the composition of the gut microbiota in different mice strains during a course of antibiotics. They observed differences in the bacterial communities, suggesting that the establishment of the gut microbiome does not occur by chance but is driven by various host-derived factors [10]. Kovacs et al. [11] studied several specific inbred mouse strains to understand the role of the host genotype in the composition of the gut microbiota. They found that genetic background is a strong determinant in shaping the mouse intestinal microbiota. Additionally, remarkable correlations were revealed between eighteen host quantitative trait loci and the abundance of particular microbial taxa [12]. Moreover, several studies reported that changes such as mutations in single host genes, i.e. APOA1, NOD2, Mediterranean Fever, and FUT2, influence the gut microbiota either by changing its composition or decreasing bacterial diversity [13–17]. However, there are discrepancies among human studies, as a general approach (e.g. using twins) did not show significant genotype effects on microbiome diversity [18, 19]. Therefore, unbiased approaches are required to study the heritability of the human gut microbiome. In overall, the gut microbiota and host genetics profoundly interact with each other, and it is speculated that changes in the gut microbiota content could supplement the specific genetic makeup of an individual.

In healthy conditions, the host and gut microbiome benefit from each other in a state referred to as eubiosis. Conversely, a disturbance in the microbiome structure or function that results from an abnormal ratio of commensal and pathogenic bacterial species is referred to as dysbiosis.

Comparisons between the gut microbiota compositions of healthy subjects and of patients suffering from diverse pathologies showed a possible direct association between dysbiosis and inflammatory and metabolic disorders including cardiovascular disease [20], obesity [18, 21], diabetes [22, 23], metabolic syndrome [24, 25] and liver diseases such as NAFLD [26–28].

In this review, we will describe the contribution of the gut microbiota to NAFLD development that has been demonstrated using animal studies, the association between gut microbiota dysbiosis and liver diseases in humans, the mechanisms by which the gut microbiota influences NAFLD, and the therapeutic potential of targeting the gut microbiota.

NAFLD

NAFLD comprises a spectrum of liver diseases from steatosis to non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis and eventually hepatocellular carcinoma. NAFLD is becoming a major health problem. The prevalence of NAFLD worldwide is reported to be 24% [29]. NAFLD is highly prevalent in South America and the Middle East, followed by Asia, the USA and Europe (31%, 32%, 27%, 24% and 23%, respectively), while there are fewer NAFLD patients in Africa (14%) [29]. The lower prevalence of NAFLD in African-Americans compared to Hispanic-Americans is interesting because obesity and hypertension are more prevalent in African-American people [30, 31]. These variations can be explained by differences in lifestyle, prevalence of metabolic syndrome, altered microbiota and genetic background, such as changes within the patatin-like phospholipase domain-containing 3 (PNPLA3) gene, which was shown to predispose to fat accumulation in the liver, leading to NAFLD [32, 33]. Moreover, some of the histological characteristics of NAFLD and NASH are similar to those of alcoholic liver disease (ALD), including steatosis, inflammation, hepatocyte ballooning (a type of hepatocyte injury), Mallory–Denk bodies, and ultimately fibrosis within the lobules [34, 35]. A "two-hit" hypothesis has been proposed for NAFLD development, with the "first hit" involving lipid accumulation in the hepatocytes [36]. In fact, the molecular mechanisms leading to lipid accumulation could be increased lipid uptake, increased de novo lipogenesis, deregulated lipoprotein synthesis, or diminished fatty acid oxidation. After the "first hit", the susceptibility of the liver to many factors, including oxidative stress and

subsequent lipid peroxidation, pro-inflammatory cytokine and adipokine signalling, and mitochondrial dysfunction, increases, which constitutes the "second hit" that promotes hepatic cell damage, inflammation and fibrosis.

The interactions between the gut and liver, called the gut–liver axis, play an essential role in NAFLD development and evolution. Portal blood flow connects the intestine to the liver. A large portion of the liver blood that comes from the intestine exposes the liver to the metabolic products produced by the gut microbiome, including phenols, acetaldehyde and ammonia [37], as well as pro-inflammatory bacterial components such as peptidoglycan and lipopolysaccharides (LPS). The liver has a wide variety of immune cells (such as lymphocytes, macrophages, dendritic cells and natural killer cells) [38]. The innate immune system responds to the cell damage or pathogens via the pattern recognition receptors (PRRs) that are expressed intracellularly or on the surface of hepatocytes [39]. The damage-associated molecular patterns (DAMPs) released by damaged cells or pathogen-associated molecular patterns (PAMPs) produced by bacteria are recognized by the PRRs [40, 41]. The function of Toll-like receptors (TLRs) is to induce gene transcription that streamlines the responses of the innate immune system [41]. Thus, their activation is important in NAFLD development. TLRs are expressed in stellate cells, Kupffer cells, and hepatocytes and are able to identify a broad range of PAMPs, which can induce the pro-inflammatory response.

It is known that different strains of mice have different susceptibility to NAFLD, indicating a genetic predisposition to NAFLD. For example, mice lacking the PEMT (phosphatidylethanolamine methyltransferase) gene and fed a methionine–choline-deficient diet developed severe steatosis in their liver due to a lack of phosphatidylcholine biosynthesis [42]; however; choline supplementation promoted partial recovery [43]. There is evidence showing the presence of loss-of-function mutations in the PEMT gene in some NAFLD patients [44]. However, different susceptibility to NAFLD is also found within a single mouse strain, suggesting that other factors, including the gut microbiome, contribute to the propensity to developing NAFLD. The mechanism of how the crosstalk between the microbiome and host genetics determines NAFLD development is not yet understood. The link between the microbiome and NAFLD was suggested for the first time in the early 1980s in patients who underwent intestinal bypass surgery. Hepatic steatosis was observed to occur in parallel with bacterial overgrowth, while the use of an antibiotic (metronidazole) improved the disease status [45], implying the involvement of gut bacteria in NAFLD phenotype.

Here, we aimed to review the parameters that support the association between NAFLD and the gut microbiota.

The link between the gut microbiome and NAFLD

Germ-free (GF) animal models have been used for decades to define the consequences of the absence of gut microbiota and, therefore, to establish the host physiological functions that are influenced by these bacteria. Among these functions, it has been shown using these animal models that the gut microbiota has a role in obesity and related metabolic diseases [46]. Indeed, it was found that GF animals are resistant to the obesity caused by different diets, including high-fat, Western-style, or even sugar-rich diets, and this effect is correlated with increased ANGPTL4 gene expression as well as the activity of AMP-activated protein kinase (AMPK) and its downstream target proteins, such as acetyl-CoA carboxylase (ACC) [47]. AMPK is an energy sensor that plays a substantial role in the switch between the metabolism of glucose and lipids in various organs. The study of Rabot et al. also confirmed the high-fat obesity-resistant phenotype in GF mice and showed decreased calorie usage and increased lipid excretion in these animals [48]. However, the obesity resistance phenotype in GF mice was later found to be strongly reliant on the sugar composition of the diet [47]. Fleissner et al. studied the effect of three different diets, namely low-fat, high-fat and Western (WD), on GF and conventional (CV) mice and disagreed about the role of Angptl4 in the protection of GF mice against obesity. More importantly, the GF and CV mice showed no difference in body weight gain on the low-fat diet. Strikingly, GF mice gained more body weight and body fat than CV mice with lower energy expenditure on the HFD. Finally, GF mice fed the WD showed significantly less body fat than GF mice on the HFD, suggesting that the absence of the gut microbiota does not generally protect the mice against diet-induced obesity [49]. Samuel et al. indicated that the short-chain fatty acid (SCFA)-binding G protein-coupled receptor Gpr41 may modulate the effects of the gut microbiota on host adiposity by comparing Gpr41-deficient and GF wild-type mice with and without a model fermentative microbial community [50]. The causative relationship between gut bacteria and obesity was further studied using microbiota transfer. For the first time, Bäckhed et al. demonstrated that transferring the normal caecum microbiota of conventional mice to GF C57BL/6 mice led to more fat deposition and insulin resistance in the body despite reduced food intake [51]. Turnbaugh et al. in line with previous findings indicated that the obese microbiome helps to harvest more energy from the diet and that this trait is transferable by faecal microbiota transplantation (FMT) [18].

Regarding liver diseases, the comparison of GF and CV mice has also been used to assess the role played by

the microbiota. The altered expression of several important hepatic genes including CAR (constitutive androstane receptor) has been found between GF and CV mice. Additionally, the absence of gut microbiota in GF mice results in elevated amounts and the accumulation of CAR ligands, including bilirubin, bile acids and steroid hormones, leading to altered liver xenobiotic metabolism, which could favour NAFLD development [52]. Comparisons of GF and CV mice also revealed that the intestinal microbiota protects against fibrosis upon chronic liver injury in mice [53] and could determine the predisposition to the liver injury [54].

In a nutshell, the absence or presence of bacteria under different treatment conditions results in variable phenotypes related to metabolic features and obesity as well as NAFLD. Altered hepatic gene expression, reduced cytokine production, dyslipidaemia, decreased calorie usage, increased lipid excretion, decreased insulin resistance and altered susceptibility to induced liver injuries have been observed in GF mice. Taken together, these studies showed that GF conditions impact the severity and/or incidence of disease, which highlights the role of the microbiome in liver disease development.

Gut microbiota transplantation in GF mice has also been used to assess the causality between microbiota composition and susceptibility to NAFLD. Le Roy et al. were the first to show that the gut microbiota composition determines NAFLD development in C57BL/6 strain mice. Indeed, by transplanting gut microbiota from mice with or without NAFLD to GF mice, we showed that the propensity to develop NAFLD features, including hyperglycaemia and steatosis, is transmissible by the gut microbiota. We further found that the gut microbiota evidently affects the lipid metabolism in the liver, independent of obesity [28]. Henao-Mejia et al. studied inflammasome-deficient mouse models (lacking pro-inflammatory multiprotein complexes) to investigate the possible role of the microbiome in NAFLD [27]. They reported that the gut microbiota changes due to NLRP6 and NLRP3 inflammasome deficiency were associated with aggravated hepatic steatosis and elevated TNF-α expression. In addition, this phenotype was transferable by co-housing the wild-type mice with the inflammasome-deficient mice, suggesting that inflammasome-mediated dysbiosis is involved in NASH progression [27]. This evidence provides insight and increased understanding of the role of the gut microbiome in the development and progression of NAFLD as well as the mechanisms involved. These results also bring into question whether the gut microbiota plays a part in NAFLD in humans and which bacteria are involved.

Dysbiosis

The healthy intestine is normally colonized by a broad array of bacteria, including over 1000 species. These bacteria are in a homeostatic balance with their host and contribute to the maintenance of a healthy state. Dysbiosis occurs if the intestinal bacterial homeostasis is disturbed. Any imbalances or changes in bacterial content or their metabolic functions, or any alterations in bacterial distribution within the gut, that are associated with a disease state is described as dysbiosis. There are growing numbers of studies revealing the association of gut microbiota dysbiosis with both intestinal (irritable bowel syndrome, inflammatory bowel disease, etc.) and non-intestinal disorders (metabolic syndrome, cancers, brain diseases, etc.). In several human and animal studies, dysbiosis was shown to be associated with NAFLD [55–57] and its severity [58–62]. Spencer et al. revealed that, during choline depletion, different levels of Erysipelotrichia and Gammaproteobacteria in different individuals were correlated with changes in the liver fat accumulation in each subject. They demonstrated that augmented numbers of Erysipelotrichia at baseline were correlated with a higher risk of NAFLD development; whereas higher levels of Gammaproteobacteria at baseline were correlated with a lower risk of developing fatty liver [63]. In a study comparing the gut microbiome in NAFLD patients and lean subjects, Gram-negative bacteria were observed to be higher in NAFLD patients with up to 20% elevated Bacteroidetes and 24% reduced Firmicutes in patients relative to healthy non-obese adult individual levels. This diminution of Firmicutes included bacteria such as the SCFA-producing *Lachnospiraceae*, *Lactobacillaceae,* and 7α-dehydroxylating *Ruminococcaceae,* and the rise in opportunistic pathogenic bacteria that produce LPS was also observed in patients with NAFLD [64]. Increases in Gram-negative bacteria were also found to be associated with NAFLD in children. Indeed, Michail et al. [65], using 16S rRNA gene analysis, identified microbial changes in obese NAFLD children compared to obese children without NAFLD and lean healthy children. Higher levels of Epsilonproteobacteria and Gammaproteobacteria were, therefore, observed in children with NAFLD compared to in healthy lean and obese children. Moreover, children with NAFLD also showed higher levels of *Prevotella* than did healthy controls. However, the results can be discordant, as Raman et al. found increased bacteria belonging to the phylum Firmicutes (such as *Dorea*, *Lactobacillus*, *Roseburia* and *Robinsoniella*) in NAFLD subjects compared with controls [66]. They also observed a non-significant underrepresentation of *Ruminococcus* in NAFLD cases compared to healthy controls, which was in line with the observation of Wang et al. [64]. In contrast, Jiang et al. and Del Chierco et al. [67, 68] observed increased levels of *Dorea* and *Ruminococcu*s in patients suffering from NAFLD.

Recently, gut microbiome compositions were characterized via whole-genome shotgun sequencing of DNA extracted from stool samples to differentiate between mild or moderate NAFLD and aggravated fibrosis [69]. Firmicutes and Proteobacteria were observed to have different frequencies in the mentioned groups. Firmicutes was more prevalent in mild or moderate NAFLD, whereas Proteobacteria was more highly represented in fibrosis. At the species level, *Bacteroides vulgatus* was highly represented in mild or moderate NAFLD as well as in advanced fibrosis. *Eubacterium rectale* was frequently observed in mild or moderate NAFLD, while *E. coli* was more abundant in advanced fibrosis. *Ruminococcus obeum* and *E. rectale* were significantly less abundant in advanced fibrosis than in mild/moderate NAFLD. Finally, these authors established a random forest classifier model based on microbiome analysis that had a strong diagnostic precision (AUC 0.936) for identifying advanced fibrosis [69]. Similarly, Boursier et al. [70] showed the association of gut microbiota to the level of disease aggravation from NAFLD to NASH. They found correlations between increased levels of *Bacteroides* and NASH and between increased *Ruminococcus* abundance and fibrosis development [70].

Zhu et al. reported a link between the amount of the endogenous ethanol produced in the gut and the pathogenesis of NASH in obese paediatric patients [57]. Accordingly, bacterial ethanol producers belonging to *Proteobacteria/Enterobacteriaceae/Escherichia* did not show a difference between healthy and obese microbiomes but were remarkably higher in the gut microbiome of NASH patients. This higher abundance of alcohol-producing bacteria in the microbiome of NASH patients was associated with elevated ethanol concentration in the blood. Based on the existing knowledge about the role of alcohol metabolism in oxidative stress and thus in hepatic inflammation, alcohol-producing microbiota may be involved in NASH development [57]. Differences among other members of the gut microbiota have been found in patients suffering from liver diseases and healthy controls. As an example, NASH patients harboured reduced amounts of *Anaerosporobacter* and *Faecalibacterium* but higher amounts of *Allisonella* and *Parabacteroides* [71]. Likewise, Mouzaki et al. has reported lower levels of Bacteroidetes in obese individuals with NASH versus healthy controls, but they did not observe any differences between simple steatosis versus healthy control microbiome [55].

Altogether, these human studies reveal measurable differences in the microbiome between healthy individuals and NAFLD or NASH patients. However, owing to factors such as the variability of study design, methods, and clinical endpoints, the interpretation of these differences in association with the liver diseases is challenging and requires further studies to define the liver disease-associated dysbiosis.

Table 1 summarizes the human studies that demonstrate a link between dysbiosis and NAFLD and provides details regarding the specific bacterial groups identified.

Mechanisms

Microbiota can improve or aggravate NAFLD through several mechanisms, including changing the permeability of the intestine, changing the amount of energy absorbed from diet, altering the expression of genes in the de novo lipogenesis and choline and bile acid metabolic signalling pathways, producing ethanol in the intestine and interacting with the innate immunity (Fig. 1). However, the associations between these factors and NAFLD development or progression are still controversial. These parameters are briefly described here.

Increased permeability

One of the main factors in the development and progression of NAFLD is gut permeability, which may be mediated by the microbiome (Fig. 2). Several factors, including the mucus layer, antimicrobial peptides and the network of tight junction (TJ) proteins, work together to maintain the function of gut barrier. Intestinal permeability has been associated with NAFLD severity; as Giorgio et al. reported, there is higher intestinal permeability in children diagnosed with steatohepatitis than in those with steatosis [78]. Approximately 39.1% of the patients recruited in a meta-analysis with 128 NAFLD patients exhibited enhanced intestinal permeability based on the urinary excretion of a measured compound, compared with only 6.8% of healthy controls. Almost 49.2% of NASH patients were found to have increased intestinal permeability [79]. This increased gut permeability could be due to a weaker TJ protein network, as decreased expression of one of major TJ proteins, ZO-1 (zona occludens), has been found in the intestinal mucosa of NAFLD patients [80]. The altered function of the gut barrier could lead to the passage of pro-inflammatory molecules, and several human studies revealed that the later stages of NAFLD (with or without fibrosis initiation) are often associated with high bacterial endotoxin levels in the blood [81–83]. Verdam et al. [84] found higher levels of plasma antibodies against LPS in NASH patients than in healthy controls, and this effect was enhanced with increasing severity of liver disease.

In animal studies, treatment with both a HFD and high-sucrose diet in rats resulted in higher levels of LPS; reduced expression of occludin, which is an important intestinal TJ protein; and increased deposition of hepatic fat [85]. LPS, which is produced by Gram-negative bacteria, are known to be involved in the development of metabolic features and insulin resistance through TLR4-dependent activation of the NF-ĸB pathway. LPS can cross the gastrointestinal

Table 1 Comparison of microbiota in healthy subjects vs patients suffering from different liver diseases using ● qPCR or ◊16S rRNA sequencing

	Disease	Phylum	Family	Genus	Population/technique
1	HBV cirrhotic patients vs. healthy subjects		*Enterobacteriaceae* ↗ *Firmicutes*↘	*Bacteroides–Prevotella*↘ *Enterococcus faecalis* ↘ *Faecalibacterium prausnitzii* ↘ *Clostridium clusters XI*↘ *clusters XIV*↘ *Lactic acid bacteria*↘ *(including Lactobacillus, Pediococcus, Leuconostoc, and Weissella)* *Bifidobacterium* ↘	Healthy ($n=32$), HBV cirrhosis ($n=31$) [72] ●
2	Cirrhotic patients vs. healthy subjects	*Bacteroidetes* ↘ *Proteobacteria*↗ *Fusobacteria*↗	*Bacteroidaceae* ↘ *Streptococcaceae*↗ *Lachnospiraceae* ↘ *Veillonellaceae* ↗ *Enterobacteriaceae*↗ *Pasteurellaceae*↗	*Enterococcus faecalis* ↗ *Clostridium clusters XI*↗ *Fusobacteriaceae*↗	Healthy ($n=24$), HBV cirrhosis ($n=24$), alcoholic cirrhosis ($n=12$) [73] ◊
	Alcoholic cirrhotic patients vs. healthy subjects		*Prevotellaceae*↗		
	Alcoholic cirrhotic patients vs. HBV cirrhosis patients		*Prevotellaceae*↗		
3	HBV cirrhotic patients vs. healthy subjects			*Bifidobacterium catenulatum group* ↘	Healthy ($n=15$), HBV cirrhosis ($n=16$) [74] ●
4	HBV cirrhotic patients vs. healthy subjects			*Lactobacillus acidophilus*↘ *Lactobacillus rhamnosus*↘ *Lactobacillus reuteri*↘ *Lactobacillus gasseri*↗	Healthy ($n=38$), HBV cirrhosis ($n=61$) [75] ●
5	Cirrhotic patients vs. healthy subjects		*Leuconostocaceae* ↗ *Lactobacillaceae* ↗ *Enterobacteriaceae*↗ *Alcaligenaceae*↗ *Fusobacteriaceae*↗ *Lachnospiraceae* ↘ *Ruminococcaceae* ↘ *Clostridium-Incertae sedis-XIV* ↘		Healthy ($n=10$), cirrhosis ($n=25$) [76] ◊●
6	Mucosal samples—cirrhotic patients vs. healthy subjects			*Clostridium*↗ *Dorea*↘ *Subdoligranumum*↘ *Acidaminococcus*↗ *Enterococcus*↗ *Burkholderia*↗ *Ralstonia* ↗ *Proteus*↗	Healthy ($n=17$), cirrhosis ($n=36$) [77] ◊
7	NASH patients vs. healthy subjects			*Faecalibacterium*↘ *Anaerosporobacter* ↘ *Parabacteroides*↗ *Allisonella*↗	NASH ($n=16$), controls ($n=22$) [71] ◊
	NASH/obese vs. healthy children	*Actinobacteria*↘ *Bacteroidetes*↗ *Firmicutes*↘ *Proteobacteria*↗	*Bifidobacteriaceae*↘ *Prevotellaceae*↗ *Rikenellaceae*↘ *Lachnospiraceae*↘ *Ruminococcaceae*↘	*Bifidobacterium*↘ *Prevotella*↗ *Alistipes* ↘ *Blautia* ↘ *Escherichia coli*↗	Healthy ($n=16$), obese ($n=25$), NASH ($n=22$) [57] ◊
8	NAFLD patients vs. healthy subjects		*Lactobacillaceae*↗ *Lachnospiraceae*↗ *Ruminococcaceae* ↘	*Lactobacillus*↗ *Robinsoniella*↗ *Roseburia* ↗ *Dorea* ↗ *Oscillibacter*↘	Healthy ($n=30$), NAFLD ($n=30$) [66] ◊

Table 1 (continued)

	Disease	Phylum	Family	Genus	Population/technique
9	NAFLD patients vs. healthy controls			*Alistipes*↘ *Prevotella*↘ *Escherichia coli*↗ *Odoribacter*↘ *Lactobacillus*↗ *Oscillibacter*↘ *Anaerobacter*↗ *Clostridium XI*↗ *Streptococcus*↗ *Flavonifractor*↘	Healthy ($n=32$), NAFLD ($n=53$) [68] ◊
10	NAFLD children vs. healthy/Obese children with no NAFLD		*Gammaproteobacteria (class)*↗	*Prevotella*↗	Healthy ($n=26$), NAFLD ($n=13$), Obese ($n=11$) [65] ◊
11	Significant fibrosis vs. mild fibrosis		*Bacteroidaceae*↗ *Prevotellaceae*↘	*Ruminococcus*↗ *Bacteroides*↗ *Prevotella*↘	NASH ($n=35$), No NASH ($n=22$) [70] ◊
	NASH vs. no NASH (NAFLD)		*Bacteroidaceae*↗ *Prevotellaceae*↘	*Bacteroides*↗ *Prevotella*↘	
12	Paediatric NAFLD, NASH, or obesity vs. healthy	*Actinobacteria*↗ *Bacteroidetes*↘	*Rikenellaceae*↘	*Ruminococcus*↗ *Blautia*↗ *Dorea*↗ *Bradyrhizobium*↗ *Anaerococcus*↗ *Peptoniphilus*↗ *Propionibacterium acnes*↗ *Oscillospira*↘	Paediatric NAFLD, NASH, or obese ($n=61$); healthy ($n=54$) [67] ◊

epithelium through leaky TJ or infiltrating chylomicrons [86]. Infusion of low doses of LPS was observed to result in steatohepatitis development in genetically obese mice [87] by enhancing the production of pro-inflammatory cytokines. LPS injections in mice also mimic HFD effects such as weight gain, IR, and NAFLD development. Moreover, mice lacking TLR-4 are not only resistant to LPS-induced obesity and NAFLD but are also resistant to HFD-induced obesity and NAFLD [88], as well as NAFLD and NASH in various rodent models [89–91], demonstrating the essential role of the TLR4–NF-ĸB pathway in NAFLD pathophysiology. Similarly, inflammasome-deficient mice display aggravated steatosis and inflammation in the liver due to TLR-4 and TLR-9 activation via their altered gut microbiota [27]. The activation of the TLR-9 signalling pathway induces the production of IL-1β by Kupffer cells, resulting in hepatic steatosis, inflammation, and fibrosis [92].

Increased dietary energy harvest

NAFLD is one of the well-known comorbidities of obesity, and the gut microbiota has been proposed to be involved in its development. Indeed, the gut microbiota is a crucial regulator of energy harvest from dietary food and can result in increased fat deposition via different mechanisms, such as developing the gut epithelium [93, 94] by enhancing the density of small intestinal villi and impacting gut physiology and motility via producing SCFAs that interact with G protein-coupled receptors (GPCRs) [95]. Bacterial enzymes extract calories from otherwise indigestible polysaccharides in the diet [94]. Finally, it has been shown that enteric bacteria decrease the synthesis and secretion of small intestinal angiopoietin-like 4 protein, leading to enhanced activity of lipoprotein lipase and augmented liver fat storage [47, 51].

Regulation of choline metabolism

Dietary choline is essential for VLDL production and hepatic lipid transfer. Therefore, diets lacking choline are commonly used to induce NAFLD in animal models. These diets lead to lowered VLDL levels and beta oxidation, causing deposition of fatty acids and cholesterol, oxidative stress and alterations in cytokines and adipokines, as well as slight inflammation and fibrosis in the liver [67, 96, 97]. The gut microbiota is involved in the conversion of choline to dimethylamine (DMA) and trimethylamine (TMA) [98], which can lead to choline deficiency with consequences for liver physiology. Indeed, Dumas and co-workers analysed urinary metabolites in different mice strains fed high-fat diets. They found that, in strain 129S6, the conversion of choline into methylamines by the gut

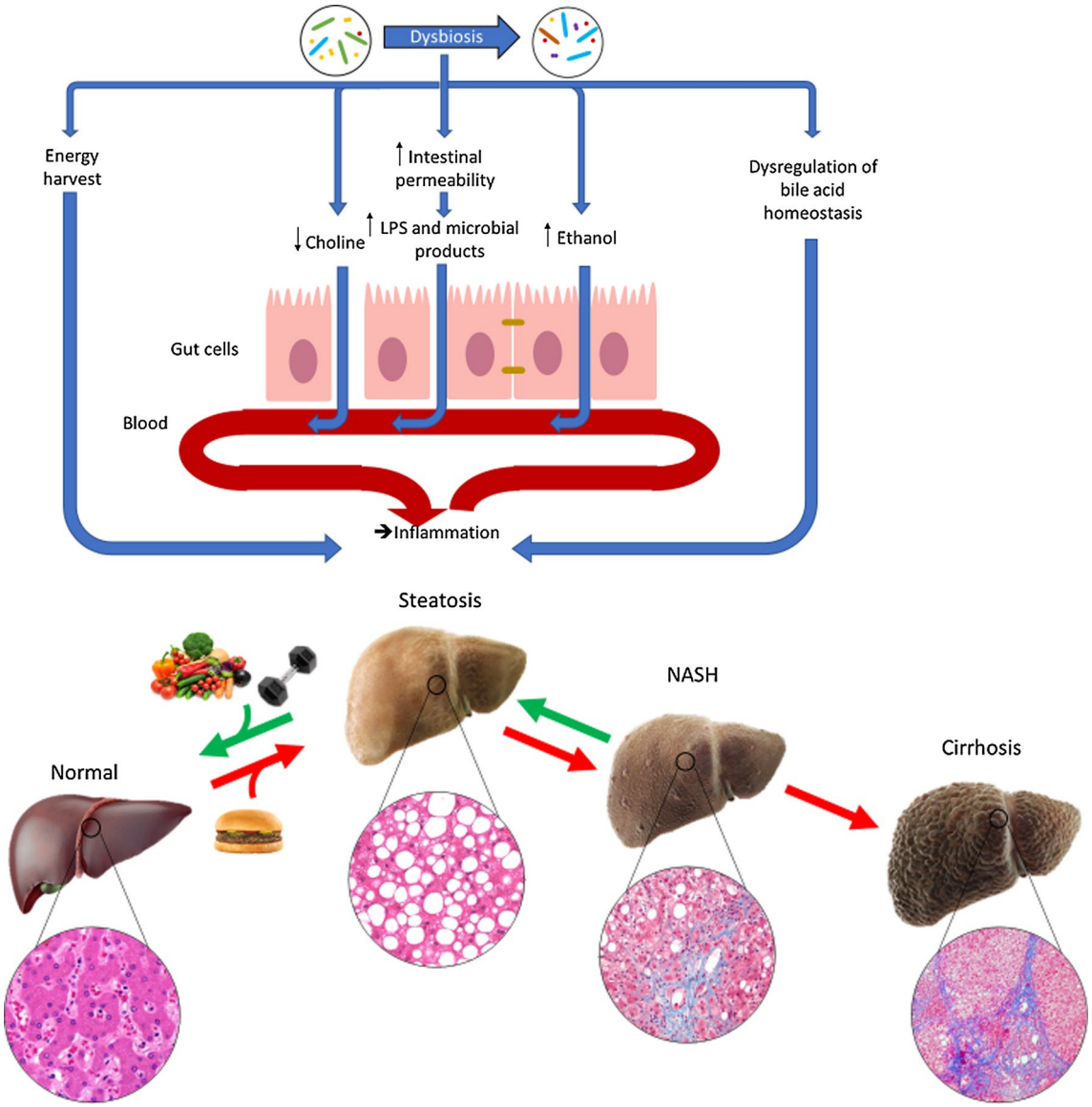

Fig. 1 The mechanisms linking the microbiome to NAFLD development. Perturbation in the intestinal microbiota composition or function can result in increased gut permeability and facilitation of the passage of LPS and other inflammatory factors to the blood, decreased choline availability, changes in bile acid composition and increased ethanol production in the intestine. These factors and metabolites together with dietary lipids can result in liver steatosis, inflammation and, eventually, NASH development

microbiota decreases the bioavailability of choline and simulates the effect of choline-deficient diets, resulting in NAFLD and insulin resistance [99].

In humans, Spencer et al. [63] explored the effect of a choline-deficient diet on the composition of the gut microbiome and the consequences for NAFLD development. Patients received 10 days of a normal diet (baseline) and then 42 days of a choline-depleted diet, which led to changes in Gammaproteobacteria and Erysipelotrichia abundances. Interestingly, the baseline levels of these taxa combined with a polymorphism in *N*-methyltransferase (PEMT), a vital enzyme in the metabolism of choline, could determine the susceptibility of individuals to fatty liver disease induced by a choline-deficient diet. [63].

Bile acids

Bile acids are saturated, hydroxylated C24 cyclopentanephenanthrene sterols that streamline the absorption of lipids in the gastrointestinal tract. Primary bile acids (cholic and chenodeoxycholic acids, in humans) are made from cholesterol in the liver. They are conjugated to either taurine or glycine via an amide bond at the C24 carboxyl [100, 101]. Later, the primary bile acids are converted

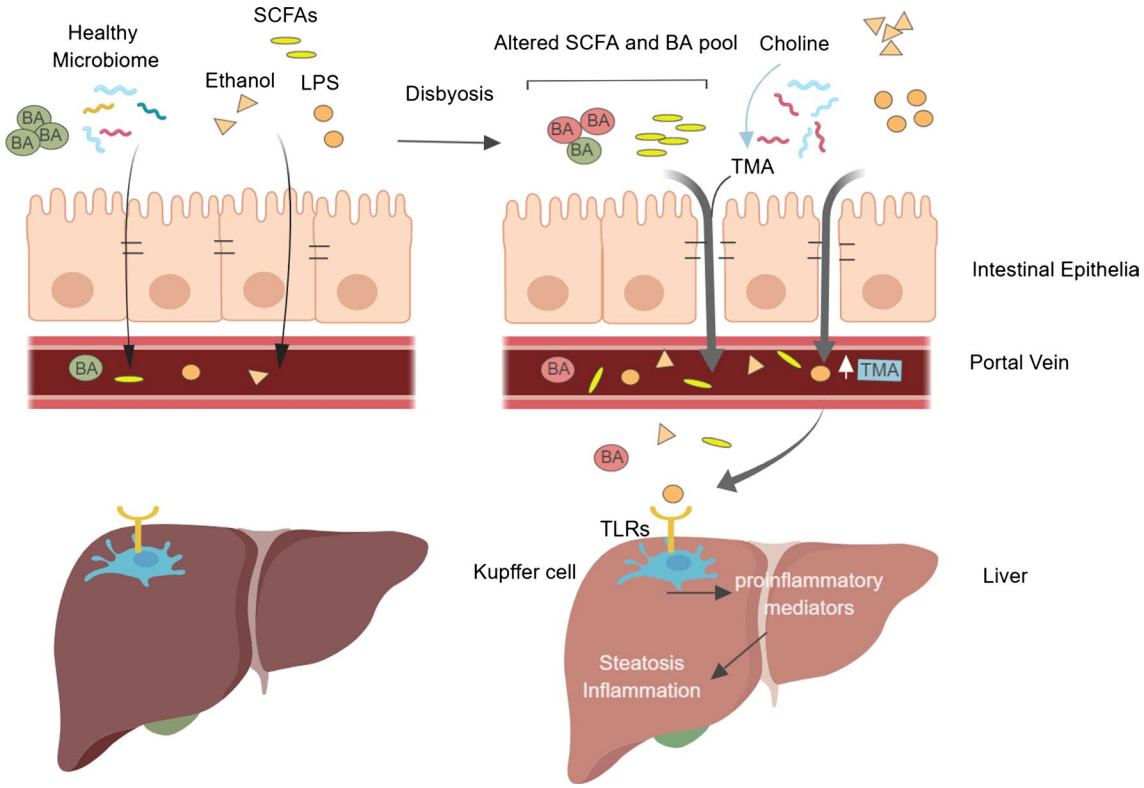

Fig. 2 Schematic view of the role of the microbiome in gut permeability and NAFLD development. On the left side, the gut–liver axis components are operating normally; on the right side, NAFLD status is shown. The dysbiotic microbiome, together with the changed intestinal barrier due to the malfunction of the tight junctions, facilitates the translocation of some bacterial products into the portal vein. These bacterial products interact with Toll-like receptors (TLRs) on the surface of the hepatic cells, which leads to inflammation and NAFLD development

to more than 20 different secondary bile acids by the gut microbiota [100]. In addition to facilitating fat absorption, bile acids also function as signalling molecules in their own metabolism, as well as energy, glucose, and lipoprotein metabolism via Farnesoid X receptor (FXR) and G protein-coupled bile acid receptor 1 (TGR5) [68]. Hence, the involvement of the gut microbiota in bile acid metabolism can improve health or favour diseases depending on the form and amount of the secondary bile acids that are generated. Indeed, FXR and TGR5 have different affinities for individual bile acids: the stronger natural FXR agonists are CDCA > DCA > CA > LCA, while Tα, TβMCA and UDCA are antagonists. Similarly, bile acids activate TGR5 with different potencies (LCA > DCA > CDCA > CA). This implies that the alterations in the composition of bile acids due to gut microbiota dysbiosis may impact host metabolism by modifying these signals. In addition, it is well known that the secondary bile acids formed by the gut microbiota are usually found in peripheral tissues, including liver, heart and kidney, highlighting their possible impact on the homeostasis of mammalians [102]. The gut–liver axis has a crucial role in bile acid metabolism. The gut microbiota affects bile acid production, pool size and structure as well as the enterohepatic circulation of bile acids, while bile acids control the gut microbiota size and content. Altogether, these mutual relationships between bile acids and the gut microbiota strongly influence host metabolism as well as metabolic diseases [103–105].

Indeed, conventionally raised mice displayed a decline in tauro-conjugates (FXR antagonists) compared to GF mice, but the CV mice maintained levels of the more toxic cholic acid [106]. The activation of FXR via specific agonists prevents bile acid and fatty acid production and increases glucose and insulin sensitivity in obese and diabetic mice. Specific FXR activation was also shown to improve primary biliary cirrhosis and NASH through the reduction of the bile acid pool and the attenuation of fibrosis [107, 108]. This has been demonstrated using natural ligands (CA or CDCA), a semi-synthetic derivative of CDCA [obeticholic acid (OCA)], and synthetic non-steroidal molecules (GW4064 and WAY-362450). Specifically, OCA has demonstrated great potential in the treatment of a number of hepatic diseases [109] and has now entered into phase II and III clinical studies. However, OCA treatment was also found to induce

side effects including pruritus, ascites or jaundice, highlighting the complexity of the host response to FXR activation. The importance of bile acid–microbiota interactions in NAFLD was further highlighted by a study by Janssen et al. that modulated microbiota by guar gum. The addition of this fermentable dietary fibre to the mouse diet increased hepatic inflammation and fibrosis and markedly elevated plasma and hepatic bile acid levels, while it reduced adipose tissue mass and inflammation. Depletion of the gut bacteria using oral antibiotics diminished portal secondary bile acid levels and protected against NAFLD [110]. Therefore, the researchers proposed that the causal link between changes in the gut microbiota and hepatic inflammation and fibrosis is through alterations of bile acids.

Taken together, these studies show that the interactions between bile acids and intestinal microbes play indispensable roles in host metabolism [111] and metabolism-related diseases, including NAFLD.

Ethanol production

The fermentation of carbohydrates by intestinal bacteria leads to endogenous ethanol production that could promote NAFLD [57]. In a study performed on obese mice, ethanol was detected in exhaled breath, though the mice had not ingested any alcohol [112]. Children with NASH were shown to have increased blood ethanol concentrations compared to healthy individuals or children with NAFLD, suggesting that endogenous ethanol production may contribute to worsened liver damage by stimulating inflammatory signals [57].

Therapeutic potential of the gut microbiota

There are different ways to modulate the gut microbiota, including antibiotics, prebiotics, probiotics, or a combination of both prebiotics and probiotics (synbiotics). These modulators can influence the microbiome through the following different mechanisms, all of which potentially impact NAFLD susceptibility [113–115]: exerting anti-inflammatory effects by inhibition or elimination of invading bacteria or their products, reducing energy salvage, increasing Angplt4 production, improving the epithelial barrier function, decreasing ethanol production by the gut microbiota, and modulating bile acid and choline metabolic signalling.

Antibiotics

Antibiotics must be used cautiously because they may eliminate important species associated with healthy status and cause the appearance of antibiotic-resistant strains [116]. However, a few studies have evaluated the effect of antibiotic treatment of NAFLD in humans and in animal models. Six months of treatment with an alternating regimen of norfloxacin and neomycin was observed to decrease small intestinal bacterial overgrowth and to improve the liver function of patients with liver cirrhosis [117]. Additionally, chronic oral use of antibiotics was found to suppress the gut bacteria, decrease the amount of portal secondary bile acid, and attenuate inflammation in the liver as well as fibrosis [110] in a NAFLD mouse model. Furthermore, the combined administration of neomycin, bacitracin and streptomycin for 4 months was associated with reduced liver triglycerides, lipid accumulation and serum ceramide production in mice [68]. Similarly, the use of the antibiotics polymyxin B and neomycin in mice treated with a high-fructose diet led to reduced fat accumulation in hepatocytes [118, 119].

In short, the depletion or alteration of the gut microbiota caused by antibiotics appears to reduce liver disease development. However, the risk of antibiotic resistance prevents its use as a therapeutic strategy; thus, exploring new techniques to modulate the gut microbiota is needed to improve NAFLD.

Prebiotics

Prebiotics are poorly digested food ingredients that improve the growth of beneficial microorganisms in the intestines and, therefore, positively alter the gut microbiota [120]. They cause gut-mediated alterations in luminal and peripheral metabolism such as decreased bacterial hepatotoxins, enhanced intestinal epithelial barrier, decreased inflammation, reduced de novo lipogenesis, modified appetite and satiety, and enhanced glycaemic control, and all these effects potentially lead to NAFLD improvement. Prebiotics stimulate the bacterial production of SCFAs, favour the growth of indigenous *Bifidobacteria* and *Lactobacilli* as well as other beneficial bacterial species, and decrease luminal pH and thus prevent the growth of pathogens [121]. Prebiotics also stimulate GLP-2 (gut trophic hormone), which can control endotoxin translocation via augmented expression of epithelial TJ proteins and improved gut barrier function [24]. Accordingly, prebiotic treatment has been linked with reduced levels of serum endotoxin [122]. In humans, a pilot study on seven patients with NASH (confirmed by biopsy) showed that treatment with 16 g/day of oligofructose (inulin-type fructans) in the diet for 8 weeks significantly decreased hepatic inflammatory markers [123].

Numerous promising animal studies also consider prebiotics as an effective dietary treatment for NAFLD. In rodents, prebiotics reduced hepatic triglyceride concentration and plasma lipid levels [124–126]. These reductions might be due to the reduction of de novo fatty acid synthesis through decreased gene expression of enzymes in the lipogenesis pathway [125, 127–129]. Prebiotics were also shown to improve the metabolic and liver disorders induced

by HFD treatment. For instance, a HFD diet together with fungal chitin–glucan (CG) reduced TG deposition in the liver compared to HFD alone. CG treatment also remarkably decreased HFD-induced fat mass growth, body weight gain, and blood glucose and cholesterol level increases, regardless of caloric intake. These improvements were associated with Clostridia *cluster XIVa* gut bacteria (*Roseburia* spp.) and were not influenced by incretin GLP-1 hormone [130]. Similarly, in a NAFLD mouse model, adding fructooligosaccharides (FOS) to the diet decreased hepatic TG by altering microbiota structure. This effect was associated with stimulated fatty acid oxidation via peroxisome proliferator-activated receptor alpha (PPAR-alpha) and increased cholesterol deposition by inhibited sterol regulatory-element-binding proteins (SREBPs) [122].

Probiotics

Probiotics are live bacteria or yeast that are beneficial to the host when used in sufficient amounts. Although the molecular mechanisms by which the probiotics exert their effects are not yet fully revealed, many animal studies as well human trials have shown NAFLD improvement following probiotic administration. As an example, Vajro et al. [131] used *Lactobacillus rhamnosus strain GG* (LGG) supplementation or placebo in the diet of 20 obese children with NAFLD for 8 weeks. They observed a reduction in alanine aminotransferase and antipeptidoglycan–polysaccharide antibodies in the group treated with the probiotic compared with the group treated with the placebo only, regardless of BMI. This highlights the use of probiotics as a therapeutic tool in obese children with NAFLD. Similarly, Famouri et al. studied the effect of use of a probiotic capsule (consisting of 4 probiotic strains) for 12 weeks in children with NAFLD and found decreased liver enzymes, TG and cholesterol level and improved sonography grade after probiotic intervention compared to the placebo group [132]. Alisi et al. studied the effect of the VSL#3 probiotic supplement (a mixture of 8 different lactic acid-producing bacteria) on NAFLD children [133]. They showed that using VSL#3 supplementation for 4 months improves fatty liver and BMI via increasing GLP-1 levels. However, VSL#3 supplementation was recently found to increase adiposity in obese Latino adolescents with no improvement of liver fat [134], indicating that the efficacy of this probiotic cocktail may be highly variable. Aller et al. performed a 12-week double-blind experiment to assess the effects of daily administration of a probiotic tablet containing 500 million *Lactobacillus bulgaricus* and *Streptococcus thermophilus* on adult NAFLD patients. There were no changes in the anthropometric parameters and cardiovascular risk factors between the treated and control groups; however, probiotic administration resulted in a remarkable improvement in aminotransferase levels [135]. Finally, probiotics could be effective in the context of other liver diseases; an interesting meta-analysis revealed that patients who received probiotics prior to liver transplantation had substantially decreased rates of infections and hospital accommodations [136].

In mouse models of NAFLD, a larger panel of probiotic strains has been assessed. As an example, it was revealed that the use of *Lactobacillus casei* strain (LcS) as a supplement suppressed the methionine–choline-deficient (MCD) diet-induced development of NASH by reducing serum LPS concentrations [137]. Thus, the modulation of the gut microbiome using LcS administration may be beneficial to normalizing TJ proteins, protecting gut barrier integrity, and thus improving hepatic inflammation. Similarly, several studies have shown that VSL#3 diminished fat deposits and inflammatory and oxidative liver damage and decreased serum levels of alanine aminotransferase [102, 138, 139]. In another study performed by Cano et al. [140], mice treated with *Bifidobacterium pseudocatenulatum* CECT 7765 showed improvements in the immunological and metabolic dysfunctions associated with HFD-induced obesity. Moreover, *Bifidobacteria* supplementation caused decreased IR, reduced fat accumulation, and reduced serum inflammatory markers compared with the levels of the mice fed a HFD lacking probiotics. However, mice fed a normal chow diet with or without probiotics did not show any differences in metabolic and liver parameters. Likewise, enhanced immune defence mechanisms in macrophages and dendritic cells and reduced gut inflammatory signals were observed after oral consumption of *Bacteroides uniformis* CECT 7771 in HFD-fed mice, which showed less hepatic fat deposition than did control mice [141]. In addition, *Akkermansia muciniphila*, a bacterial species with anti-obesity properties, was shown to improve immune-mediated liver injury in C57BL/6 mice by alleviating inflammation and hepatocellular death [142]. In a fructose-enriched diet mouse model of NAFLD, LGG treatment modulated the gut microbiota resulting in decreased hepatic expression of the genes that function in the lipogenesis pathway and ameliorated liver steatosis. LGG treatment also caused reduced expression of the pro-inflammatory cytokines including TNF-α, IL-1β and IL-8R in the liver [143].

Altogether, these studies confirm that probiotic administration can exert beneficial effects on NAFLD development/progression. However, only a few strains/bacterial cocktails have proved to be effective and to slightly ameliorate some of the parameters associated with the disease.

Synbiotics

Mixing probiotics and prebiotics, termed synbiotics, has been shown to improve the survival and establishment of diet derived-microbial communities in the intestine by

stimulating the growth or metabolism of specific health-promoting bacteria. There are few human studies evaluating the effects of synbiotics on NAFLD patients. However, it was shown that synbiotic supplementation (FOS and probiotic strains) for 28 weeks together with lifestyle modifications is more beneficial to NAFLD treatment than lifestyle modifications alone. In addition, it was observed that this synbiotic supplementation attenuates inflammatory signals and reduces BMI as well as waist-to-hip ratio. These effects were evident at week 14 and continued until the end of the treatment [59]. Accordingly, Malaguarnera et al. [144] indicated that administration of *Bifidobacterium longum* with FOS along with lifestyle modification for 24 weeks significantly decreased hepatic fat accumulation and the NASH activity index when compared to lifestyle modification alone. Safavi et al. [145] observed that synbiotic supplementation in obese children resulted in a considerable improvement in all blood lipid parameters after 8 weeks of treatment. Conversely, the synbiotic supplementation used by Ipar et al. [146] showed remarkable improvement in total and LDL (low-density lipoprotein) cholesterol levels but not in triglyceride levels.

In summary, the mentioned studies show the beneficial effect of prebiotics, probiotics and synbiotics on fatty liver symptoms, one of the main mechanisms involved in the improvement of gut barrier function.

Diet

Diet composition is an important driver of the structure of the gut microbiota [122, 143, 147–150], and the role of diet in shaping the gut microbiota is even stronger than that of genetic factors [148]. Therefore, we hypothesize that the effects of diet on NAFLD development are due, at least partially, to changes in the composition of the gut microbiota.

Zeng et al. [150] fed C57BL/6 mice with obesity-related inflammatory fatty liver a HFD for 10 weeks to evaluate whether the NAFLD phenotype is correlated with microbiome alterations [150]. They found a higher amount of DNA from *L. gasseri* and/or *L. taiwanensis* (from the *Lactobacillus acidophilus* species group) in the high-fat diet than in the low-fat diet groups. Most of these bacteria are bile acid resistant; thus, the authors suggested that the increase in *Lactobacillus* species due to the HFD could influence lipid metabolism through the modulation of bile acid metabolism, and thus contribute to NAFLD development. Conversely, diet can be used to re-establish a healthy microbiota to improve NAFLD. A study showed that supplementation of a Chinese herbal formula (CHF) in HFD-induced NAFLD rat models improved NAFLD and led to a decrease in the levels of *Escherichia/Shigella* and other LPS-containing bacteria that may damage the gut barrier and activate a low-grade chronic inflammatory state [151]. The CHF supplementation also increased *Collinsella* abundance [151], which may affect human epithelial cell proliferation, and improve intestinal barrier integrity through SCFA production [152]. Finally, the Mediterranean diet, rich in polyunsaturated fats, polyphenols, carotenoids and vitamins, all of which have anti-inflammatory and antioxidant effects, was shown to be effective in reducing the risk of metabolic syndrome through the reinforcement of the gut barrier and the reduction of endotoxaemia [153].

Therefore, the gut microbiota may be linked to the deleterious effect of HFD on NAFLD and the modulation of gut microbiota composition through diet could be an effective strategy to improve liver pathology.

Faecal microbiota transplantation (FMT)

FMT consists of the transfer of faecal material containing bacteria from a healthy donor to a diseased patient to re-establish a balanced gut microbiota composition. FMT has been proven to be effective to cure *Clostridium difficile* infection, and its application in a broad range of non-gastrointestinal disorders including metabolic disorders has been envisaged [154, 155]. It has been recently shown that after 8 weeks of FMT, mice had significantly decreased intrahepatic lipid accumulation and levels of transaminases in the serum, together with a diminished degree of lobular inflammation and hepatocyte ballooning. This suggests a positive effect of FMT in HFD-induced metabolic disorders [156]. To our knowledge, no FMT studies have been published so far in the context of NAFLD. Nevertheless, the enormous interest in FMT and its potential in liver diseases is reflected in several ongoing trials [157, 158].

Conclusion

Intestinal host–microbiome communications play various roles in the development and progression of NAFLD and NASH. With the rapidly growing incidence of NAFLD, the need for new preventative or therapeutic strategies is important. Microbiota-based solutions, including protective bacterial species or bacterial products, should be developed in the future to improve NAFLD management. Manipulation of the microbiome, mainly through the diet, towards a healthy state that is protective against NAFLD may also be considered. Finally, clinical trials are in progress to use faecal microbiota transplants in the context of NAFLD. The future will tell if FMT will become a new therapeutic option for NAFLD, as it has for *Clostridium difficile* infection.

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