Review Article

Gut microbiota and non-alcoholic fatty liver disease

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BACKGROUND: Non-alcoholic fatty liver disease (NAFLD) is a common disorder with poorly understood pathogenesis. Beyond environmental and genetic factors, cumulative data support the causative role of gut microbiota in disease development and progression.

DATA SOURCE: We performed a PubMed literature search with the following key words: "non-alcoholic fatty liver disease", "non-alcoholic steatohepatitis", "fatty liver", "gut microbiota" and "microbiome", to review the data implicating gut microbiota in NAFLD development and progression.

RESULTS: Recent metagenomic studies revealed differences in the phylum and genus levels between patients with fatty liver and healthy controls. While bacteroidetes and firmicutes remain the dominant phyla among NAFLD patients, their proportional abundance and genera detection vary among different studies. New techniques indicate a correlation between the *methanogenic archaeon* (*methanobrevibacter smithii*) and obesity, while the bacterium *akkermanshia municiphila* protects against metabolic syndrome. Among NAFLD patients, small intestinal bacterial overgrowth detected by breath tests might induce gut microbiota and host interactions, facilitating disease development.

CONCLUSIONS: There is evidence that gut microbiota participates in NAFLD development through, among others, obesity induction, endogenous ethanol production, inflammatory response triggering and alterations in choline metabolism. Further studies with emerging techniques are needed to further elucidate the microbiome and host crosstalk in NAFLD pathogenesis.

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KEY WORDS: non-alcoholic fatty liver disease; non-alcoholic steatohepatitis; gut microbiota; 16S rRNA sequencing; archaea

Introduction

Non-alcoholic fatty liver disease (NAFLD), the liver manifestation of metabolic syndrome, is the main cause of liver enzymes abnormalities in Western countries.[1, 2] NAFLD definition requires lack of ongoing or recent excessive alcohol consumption (>20 g/d for men and >10 g/d for women, respectively) and exclusion of other causes of liver steatosis.[3] Histologic evidence of steatosis or in the absence of histology, γ-GT and/or aminotransferases elevations as well as compatible sonographic findings suffice for diagnosis. The spectrum of the disease encompasses liver steatosis, non-alcoholic steatohepatitis (NASH), NASH-related cirrhosis and hepatocellular carcinoma.[4] Moreover, NAFLD patients are at greater risk to develop cardiovascular diseases[5] and their overall mortality is higher than that of a matched general population.[6]

Obesity, diabetes mellitus and hypertriglyceridemia are the main risk factors for the development and progression of NAFLD.[7] Given the mounting prevalence of overweighed and obese individuals (65% and 30%, respectively in the USA),[8] it is apparent that NAFLD and its consequences represent major public health issues. NAFLD prevalence ranges from 3% to 30%,[7, 9] depending on the used diagnostic methods (biochemical markers, radiology, histology). NAFLD and NASH prevalence among an urban USA population is estimated to 20%[10] and 4%,[2] respectively. However, epidemiological data vary in special populations: Hispanics show higher prevalence than non-Hispanics, whereas non-Hispanics black individuals[11] as well as populations from Alaska[12] and American-Indians,[13] exhibit significantly lower prevalence (0.6%-2%) of NAFLD.

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A "two hits" hypothesis about NAFLD pathogenesis has been proposed.[14] The first hit is increased triglycerides accumulation in the liver, whereas a second one (e.g. oxidative stress) induces liver parenchyma inflammation leading to NASH. Recently, investigators have proposed the "multiple parallel" hits hypothesis:[15, 16] inflammation may either precede or follow simple steatosis with multiple factors, namely lipotoxicity, increased oxidative stress, mitochondrial dysfunction and iron overload acting in parallel to promote NASH.[16] Moreover, a genetic predisposition to the disease is possible since a mutation in the patatin-like phospholipase domain-containing 3 gene has been recognized to strongly predict liver fat accumulation and disease progression.[17]

The aim of this review is to highlight key issues on gut microbiota-host crosstalk regarding the pathogenesis of NAFLD and NASH. We present herein the yield of a PubMed search from 1995 to 2014 using the key words "non-alcoholic fatty liver disease", "non-alcoholic steatohepatitis", "fatty liver", "gut microbiota" and "microbiome".

Gut microbiome

Gut microbiota is a group of commensal microorganisms that live synergistically with the host. They process complex, otherwise indigestible, polysaccharides to short-chain fatty acids, thus providing extra energy for the host. They also participate in the synthesis of vitamins (e.g. vitamin K) and in the development and maintenance of the immunity at the intestinal lumen level.[18, 19]

The adult type gut microbiota is made up from bacteria, viruses, protozoa, archaea, eukaryotes, yeasts and parasites. It counts more than 10^{14} cells,[20] more than 100 different bacterial species;[18] their genome counts up to 300 000 genes,[21] 100 times the number of the human genome. Among them, bacteria predominate with the Gram-positive short-chain fatty acids-producing firmicutes and the Gram-negative hydrogen-producing bacteroidetes being the main phyla, followed by proteobacteria, actinobacteria, bifidobacteria, etc.[22, 23] Based on the abundant genera, two basic enterotypes are recognized:[24] Enterotype 1, where *bacteroides spp.* dominate and enterotype 2, with abundance of *prevotella spp.*. The existence of a third enterotype--enterotype H[25]--with abundance of both *bacteroides spp.* and *prevotella spp.* has also been proposed.

Evaluating gut microbiota in NAFLD

In order to reveal microbiota composition, culture-dependent and culture-independent techniques have been implicated.[26] Traditional culture allows detection and semi-quantification of many bacterial groups. Nevertheless, since a large amount of gut bacteria requires special conditions (e.g. anaerobic environment) to grow, a loss of 80% of the detectable bacteria is anticipated.[22] Trying to overcome this problem, culture-independent techniques have been developed. Apart from the widely used quantitative real-time polymerase chain reaction (qRT-PCR), additional techniques based on the diversity in the sequence of the bacterial 16S ribosomal RNA (16S rRNA) gene are now utilized: either the entire or conserved regions of the 16S rRNA gene are amplified, the results are compared with the sequence-containing libraries, thus accurate bacterial species identification or the partial 16S rRNA sequencing (pyrosequencing) provides information about the number, nature and abundance of the diverse bacterial species[27] without need of an *ex vivo* bacterial culture or DNA cloning.

Furthermore, the "-omics" studies are reliable methods to specify the functional species that interact with the host. Accordingly, the entire bacterial genome (metagenomics), the expressed mRNA (metatranscriptomics), the obtained proteins from the investigated microenvironment (metaproteomics), and the produced metabolites (metabolomics) are used to distinguish different phenotypes as well as, potential host-microbiome interactions.

Table 1 summarizes data from human studies investigating stool microbiota in NAFLD. Zhu et al[25] studied a pediatric population of 22 biopsy-proven NASH subjects, 25 obese children and 16 healthy controls using 16S rRNA sequencing in stool samples. Sequencing revealed a significant increase in bacteroidetes with decreased levels of firmicutes among obese and NASH individuals. Actinobacteria were significantly decreased in NASH patients, whereas proteobacteria counts showed a gradual increase from healthy to obese and NASH patients. Authors also provided evidence of increased ethanol levels in NASH children, postulating a possible role of abundant ethanol-producing bacteria (such as *Escherichia*) in NASH progression.

The increased bacteroidetes/firmicutes ratio detected by Zhu et al[25] was not confirmed by Raman et al,[28] who studied 30 obese adult patients with clinical, biochemical and radiological suspicion of NAFLD and 30 healthy controls using multi-tag pyrosequencing in a single stool sample per patient. Although differences in family and genera level were detected, the authors could not demonstrate a significant difference in the phylum level between the two groups. Similarly, using qRT-PCR in stool samples of 33 patients with biopsy-proven NAFLD (22 with NASH) and 17 healthy controls, Mouzaki et al[29]revealed significantly lower counts of Bacteroides in NASH patients than patients with simple steatosis and healthy controls. Firmicutes, proteobacteria and actino-

Table 1. Studies on stool microbiota in NAFLD

Studies	Population	Method for NAFLD/ NASH diagnosis	Patient composition	Method for stool microbiota identification	Results (NAFLD/NASH vs HC)	
					Bacteroidetes/ Firmicutes ratio	Genus
Zhu et al[25]	Children and adolescents	Biopsy	25 Obese/ 22 NASH/ 16 controls	16S rRNA pyrosequencing	⇑	⇑: *Prevotella, Escherichia* ⇓: *Alistia, Blautia, Coprococcus, Eubacterium, Oscillospira, Bifidobacterium*
Raman et al[28]	Adults (obese)	Radiological/ Clinical/ Biochemical	30 NAFLD/ 30 controls	Multi-tag pyrosequencing	⇔	⇑: *Lactobacillus, Robinsoniella, Roseburia, Dorea* ⇓: *Oscillibacter*
Mouzaki et al[29]	Adults (transaminasemia)	Biopsy	11 NAFLD/ 22 NASH/ 17 controls	qRT-PCR	⇔	⇔ : *Escherichia coli*
Wong et al[30]	Adults	Biopsy	16 NASH/ 22 controls	16S rRNA pyrosequencing	⇑	⇑: *Parabacteroides, Allisonella* ⇓: *Faecalibacterium, Anaerosporobacter*

NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis; HC: healthy controls; qRT-PCR: quantitative real-time polymerase chain reaction; 16S rRNA: 16S ribosomal ribonucleic acid; ⇑: increased in NAFLD/NASH; ⇔: no difference; ⇓: decreased in NAFLD/NASH.

bacteria counts were not different significantly among the groups, whereas archaea were detectable only in 9/50 subjects (2 with simple steatosis, 2 with NASH and 5 healthy controls).

Trying to clarify the impact of pro- and prebiotics on gut microbiota composition, Wong et al[30] evaluated biopsy-proven NASH patients and controls. Participants provided stool samples for microbial counts measurements using 16S rRNA sequencing. Bacteroidetes were the dominant phyla in both groups (NASH and healthy volunteers), followed by firmicutes, which showed a notable presence in healthy controls.

Finally, the effect of choline-deficient diet, a mechanism implicated in NAFLD pathogenesis, was studied by Spencer et al.[31] Stool samples were collected in predefined time points and 16S rRNA pyrosequencing was used for microbiota specification. The authors did not find any common change in the taxons' abundance in the different time points of the study. However, the dramatically fall to zero in *Gammaproteobacteria* (phylum Proteobacteria) counts and the strong correlation of baseline levels of *Gammaproteobacteria* to liver fat enrichment during the choline-deficient diet postulated that *Gammaproteobacteria* levels may predict the possibility of an individual to develop fatty liver when exposed to choline-deficient diet.

Taken together, the above studies provide different evidence regarding NAFLD/NASH and gut microbiota composition. Bacteroidetes/firmicutes ratio is increased in NASH children, while no change is revealed in NAFLD/NASH adults compared to healthy controls. This discrepancy at the phylum level raises questions about a potential role of microbiota in NAFLD/NASH development. When focusing at the genera, a wide diversity regarding flora composition can be detected. This finding can be attributed to different methods used or even different studied populations; however, it is unlikely to extract safe conclusions regarding a potential causative role of special phyla and genera in NAFLD/NASH development.

Potential mechanisms that implicate gut microbiota in the pathogenesis of NAFLD

Accumulating data from preclinical and clinical studies suggest a critical role of gut microbiota in NAFLD pathogenesis, mainly through predisposition to obesity, metabolism alterations (insulin resistance) and promoted liver inflammation. Moreover, many bacterial byproducts, such as ethanol, might demonstrate hepatotoxicity via stimulation of Kupffer cells to produce and exude nitric acid and cytokines[32] (Fig.).

Obesity

More than a decade ago, investigators showed that germ-free mice gained 42% less weight in comparison with animals that hosted gut microbiota, despite the fact that they consumed more calories.[33] Moreover, when cecal microbiota was transplanted from normal to germ-free mice, the animals gained 57% more weight without any change in consumed calories. The same group emphasized their results by showing that germ-free mice were unable to gain weight while maintaining a high-

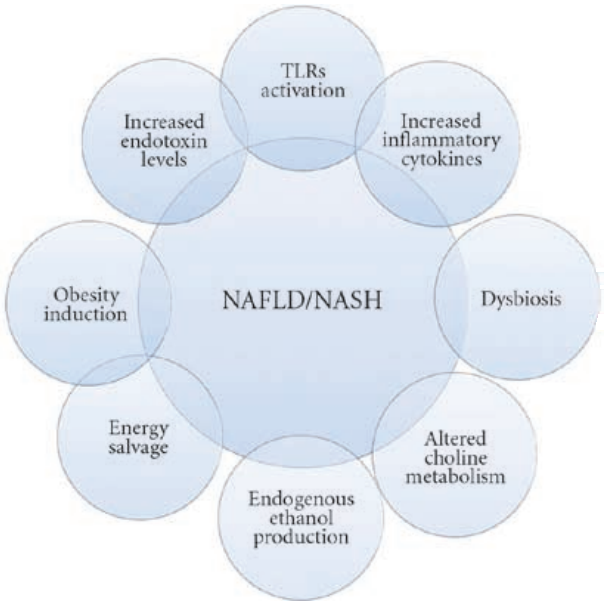

Fig. Potential mechanisms through which gut microbiota contribute to NAFLD/NASH pathogenesis. Overlapping external circles indicate common pathogenic mechanisms. NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis; TLRs: Toll-like receptors.

calorie diet.[34]

Turnbaugh et al[35] transplanted gut microbiota from obese ob/ob mice and lean ob/+ or +/+ mice to lean germ-free mice, respectively. Animals that received microbiota from obese donors showed a higher fat gain than those that received microbiota from lean donors. Obesity-associated gut microbiota was able to extract more energy from the diet via digesting otherwise indigestible polysaccharides into short-chain fatty acids. Further supporting the aforementioned hypothesis, the total fecal short-chain fatty acids concentration from 33 obese human individuals was 20% higher than that from 30 lean volunteers.[36]

A potential role of specific gut microbiota in obesity and subsequent NAFLD development has been proposed. Obese mice hosted 50% less bacteroidetes and more firmicutes as compared with lean controls, whereas archaea counts were significantly increased in the obese animals.[37]

The decreased bacteroidetes/firmicutes ratio was also detected in obese human studies. Overweighed and obese children at the age of 7 had less bacteroidetes and more *S. aureus* in stools collected at the age of 6 and 12 months of age.[38] A study of 12 obese individuals revealed more bacteroidetes and less firmicutes, but this ratio was reversed when they followed a fat or carbohydrate restricted diet for 1 year.[39] On the contrary, firmicutes and archaea showed lower counts in obese patients after bariatric surgery.[40] Enterotype 1 has been associated with the long-term consumption of animal proteins and saturated fat, whereas diet based on carbohydrates predisposes to Enterotype 2.[41] These data underline that the role of gut microbiota diversity pends to be further clarified in obesity.

In contrast, a recently described bacterium[42] *Akkermansia muniphila* has been associated with non-obese phenotype. Pregnant women who gained excess weight during pregnancy[43] as well as obese and overweight preschool children[44] had low fecal concentrations of *A. muniphila*. Similarly, *A. muniphila* counts were markedly increased in obese mice that underwent Roux-Y gastric bypass surgery.[45]

Experimental models provided evidence for a modulating role of *A. muniphila* in weight gain, type 2 diabetes mellitus and eventually NAFLD. "Treatment" of mice under high-fat-diet with *A. muniphila* led to induction of Tregs cells in the visceral adipose tissue. This resulted in the attenuation of adipose tissue inflammation and increased glucose tolerance, effects that resemble the antidiabetic function of metformin.[46] Similarly, Everard et al[47] showed that *A. muniphila* administration in type 2 diabetes mellitus mice led to a gradual reversion of insulin resistance, adipose tissue inflammation, fat gain and endotoxemia.

Ethanol

Endogenous ethanol is a metabolite of many gut microbiota species. After its absorption, it reaches the liver via the portal vein.[48] Alcohol dehydrogenase catalyzes its oxidation in the liver, leading to acetate and acetaldehyde formation.[49] The first is a substrate for fatty acids synthesis, and the second produces reactive oxygen species. Therefore, ethanol metabolism induces triglycerides accumulation in the liver[50] and hepatic oxidative stress, thus fulfilling both steps of the "two hits" hypothesis.

Increased ethanol levels have been detected in obese patients[51] as well as, in non-alcohol consuming children with NASH,[25] indicating its causative role in NAFLD/NASH development. Moreover, significantly increased expression of the ethanol-metabolizing enzymes alcohol dehydrogenase, catalase and aldehyde dehydrogenase has been detected in NASH livers.[52] Ethanol may also promote NAFLD by increasing gut mucosal permeability[53] that induces endotoxemia:[54] five days ciprofloxacin administration in NASH patients with ethanol detected in their blood resulted in immeasurable levels of ethanol,[55] pointing to a close relationship of endogenous ethanol production and gut microbiota.

Lipopolysaccharide-endotoxemia-Toll-like receptors

Endotoxin is part of the Gram-negative bacterial cell

membrane. Lipopolysaccharide (LPS), the active component of endotoxin, binds to the LPS-binding protein and its CD14 receptor[56] to form a complex that interacts with Toll-like receptors (TLRs) and activates inflammatory cascade.[57]

Genetically obese mice develop steatohepatitis after infusion of low doses of LPS.[58] Furthermore, LPS injected in NAFLD mice further promotes liver injury by enhancement of produced proinflammatory cytokines,[59] whereas high-fat-diet induces increased plasma circulating LPS.[60] Diet-induced NAFLD in rodents is also associated with increased levels of LPS.[60, 61] In human studies, subjects with NAFLD experience significantly higher levels of circulating endotoxin,[62, 63] with marked increases in early fibrosis, when compared with healthy controls.

TLRs activation leads to the translocation of NF-κB in the nucleus and induction of proinflammatory genes transcription, such as TNF-α, IL-1β, IL-6 and IL-12.[64] IL-1β augments triglycerides accumulation in the hepatocytes by enhancement of diacyloglycerol transferase that converts diglycerides into triglycerides.[65] Moreover, TNF-α inhibits insulin receptors as well as insulin-receptors-substrate-1, leading to increased levels of circulating insulin and therefore to insulin resistance.[62] Consequently, influx of fatty acids derived from adipose tissue is facilitated.[66]

Recently, inflammasomes deficiencies have been implicated in NAFLD development. Inflammasomes are cytoplasmic multiprotein complexes that regulate the cleavage of pro-IL-1β and pro-IL-18 into the respective cytokines, through activation of caspase-1.[48] Genetically deficient for components of the inflammasome (NLRP3 and NLRP6) mice had higher levels of LPS and bacterial DNA in the portal circulation. The former binds to TLR-4 and the latter to TLR-9, leading to increased expression of TNF-α in the liver, thus promoting liver inflammation and steatosis.[67]

To summarize, NAFLD and NASH are characterized by increased levels of endotoxin. Endotoxin, one among other components of the second hit, in the "two hits" hypothesis triggers a cascade to increased levels of proinflammatory cytokines, insulin resistance and triglycerides production, thus facilitating NAFLD development and progression. Moreover, TLRs- and inflammasome-deficient mice showed either NAFLD or steatosis worsening, strengthening the role of the endotoxin-path in NAFLD pathogenesis.

Choline

Choline, a phospholipid component of cell membrane, plays a crucial role in lipid transport from the liver.[68] Gut microbiota are implicated in choline metabolism by producing enzymes that catalyze choline into methylamines, which potentially induce inflammation when absorbed by the liver.[69] Metabolomics analysis in 129S6 mouse strain animals with high-fat-diet induced steatosis revealed a significant decrease in circulating phosphatidylcholine and increased urine excretion of its metabolites, thus supporting the presence of a gut microbiota phenotype that induces choline deficiency-mediated liver injury.[70] Apart from that, choline deficiency contributes to triglycerides accumulation in the liver and decreased liver secretion of very-low-density lipoprotein,[48] whereas choline deficient diet resulted in liver steatosis that reversed after choline substitution in animals.[71] Acknowledging the fact that the data derive from a deficient disease and therefore the model might not be suitable enough to explain NAFLD development, decreased choline levels and increased levels of toxic choline metabolites might represent the gut microbiota choline-deficiency-mediated mechanism in NAFLD development.

Dysbiosis

Investigators compared metabolic and histologic changes in mice receiving microbiota from donors with different metabolic phenotypes.[72] Mice receiving intestinal microbiota from donors with hyperglycemia and increased levels of proinflamamtory cytokines developed hyperglycemia, increased levels of insulin and macrovesicular steatosis, whereas mice receiving microbiota from normoglycemic donors did not show similar metabolic and histologic changes. Dysbiosis, namely increased counts of firmicutes in the former and increased population of *Bacteroides vugatus* in the latter receivers, was detected by means of 16S rRNA pyrosequencing techniques.

Small intestinal bacterial overgrowth - intestinal permeability

Small intestinal bacterial overgrowth (SIBO) is the presence of increased number of colonic type anaerobic and/or aerobic bacteria in the small intestine.[23] In combination with intestinal permeability, SIBO has been proposed as a pathogenetic mechanism of NAFLD/NASH development. Ectopic microbiota in SIBO may cause loose junctions in the small intestinal epithelium facilitating the flow of bacterial byproducts in the portal vein system and their absorption from the liver, where they manifest their toxic activities. There is no gold standard technique to diagnose SIBO yet, since even aspirate culture suffers from certain methodological caveats. Breath

testing is the current diagnostic test for SIBO, whereas culture independent techniques like qRT-PCR and deep sequencing have also been used in this setting.[23]

The correlation of NAFLD and SIBO has been investigated in experimental[73] and human studies[53, 55, 74-79] (Table 2). Increased prevalence of SIBO in NAFLD/NASH humans[74, 78] and in a rat NASH model[73] has been indirectly documented by measuring the orocecal transit time.

In obese bariatric surgery patients, SIBO detected using hydrogen breath testing has been revealed as an independent factor for severe steatosis in liver biopsy.[76] Wigg et al[79] detected SIBO in 50% of NASH patients, in whom higher levels of TNF-α were also identified and lactulose breath test detected SIBO in 78% of NASH patients in association with TLR-4 expression and plasma levels of IL-8.[77] In contrast to the aforementioned data, among 20 NAFLD patients SIBO was identified in only 3 using glucose breath test.[53] Using the lactulose/mannitol test that assesses intestinal permeability, a higher index of intestinal permeability as calculated by the lactulose/mannitol urine excretion ratio was demonstrated in NAFLD patients.[53] Similarly, Miele et al[75] detected SIBO in 60% of 35 biopsy-proven NAFLD patients and a significant correlation between the severity of steatosis and increased intestinal permeability was evident. Moreover, patients with NAFLD and increased intestinal permeability had a 2-fold higher prevalence of SIBO in comparison with patients with normal intestinal permeability. Authors could not support a causative role of SIBO in the progression of NAFLD, since they did not reveal correlation between increased intestinal permeability and/or SIBO with steatohepatitis.

The role of archaea

Methanogenic archaea scavenge hydrogen (H) and ammonia (NH_4) to produce methane (CH_4). Hydrogen utilization optimizes microenvironmental intestinal lumen conditions that facilitate short-chain fatty acids production and salvage energy for the host by gut flora.[80] *Methanobrevibacter smithii* (*M. smithii*) is the predominant methanogenic archaeon detected in 70% of healthy individuals.[81] A causative role of *M. smithii* for obesity development has been proposed in a mouse model. Colonization either with *M. smithii* and *Bacteroides thetaiotaomicron* or *B. thetaiotaomicron* alone led to increased weight gain only in subjects colonized with *M. smithii*.[82]

Methane detection in breath test is associated with increased levels of *M. smithii* in stools,[83] setting this cheap and non-invasive method as a guide for further studies. Indeed, when methane-positive obese subjects were compared with obese methane-negative controls, they showed a 6.7 kg/m^2 body-mass-index (BMI) increase.[84] Moreover, when methane and hydrogen were

Table 2. Small intestinal bacterial overgrowth in NAFLD

Studies	Population	Method for NAFLD/NASH diagnosis	Patient composition (NAFLD/ NASH/HC)	Method for SIBO diagnosis	Results (NAFLD/NASH vs HC)
	Animal				
Wu et al[73]	Rats	Biopsy	0/8/8	Small intestinal motility using semi-solid colored marker	0.39±0.11 vs 0.58±0.06 (P<0.01)
	Human				
Volynets et al[53]	Adults (non- diabetics)	Radiological/Clinical/ Biochemical/Biopsy	17/3/10	GBT	15% vs 20% (P=NS)
Sajjad et al[55]	Adults	Biopsy	0/12/11	GBT	50% vs 9.1% (P=NA)
Fu and Jiang[74]	Adults	Radiological/Clinical/Biochemical	0/10/10	LBT (OCTT)	95±17 vs 59±18 min (P<0.001)
Miele et al[75]	Adults	Biopsy	18/17/24	GBT	60% vs 20.8% (P<0.001)
Sabaté et al[76]	Adults, obese, bariatric surgery	Biopsy	103/34/40	GBT	17.1% vs 2.5% (P=0.031)
Shanab et al[77]	Adults	Biopsy	0/18/16	LBT	77.78% vs 31.25% (P<0.0001)
Soza et al[78]	Adults	Radiological/Clinical/ Biochemical/Biopsy	5/5/10	LBT (OCTT)	127±61 vs 57±23 min (P=0.0037)
Wigg et al[79]	Adults	Radiological/Clinical/ Biochemical/Biopsy	3/19/23	LBT+^{14}C-D-XBT	50% vs 22% (P=0.048)

NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis; HC: healthy controls; SIBO: small intestinal bacterial overgrowth; GBT: glucose breath test; LBT: lactulose breath test; ^{14}C-D-XBT: ^{14}C-D-xylose breath test; OCTT: orocecal transit time; NA: not available; NS: not significant.

measured in 792 subjects using lactulose breath test, methane-positive and hydrogen-positive (M+/H+) individuals showed increased BMI and increased percent body fat compared with the other groups (M-/H-, M+/H-, M-/H+).[85] It was therefore proposed that hydrogen derived from increased microbiota fermentation acts as a tank providing fuel to hydrogen-required metabolism of methanogens. In order to strengthen the hypothesis that *M. smithii* influences obesity development, Mathur et al[86] correlated the location and extent of *M. smithii* colonization with weight gain in rats. Using qRT-PCR, investigators showed that *M. smithii* counts were higher in the small intestine (highest in the ileum), whereas total bacterial numbers were the lowest in the small intestine and the highest in the left colon and cecum. Of interest, mice fed high-fat-diet had a significantly increased *M. smithii* concentration in the duodenum, ileum and cecum, whereas no difference was found in the total bacterial counts. Furthermore, animals with all five intestinal parts colonized with *M. smithii* had higher body weight than those with limited colonization.

In conclusion, these studies provide evidence for the first time that methanogenic archaea counts, especially *M. smithii* that colonize not only the colon but also the small intestine correlate with diet-induced weight gain.

Probiotics as a novel therapeutic approach

Probiotics are "live microorganisms that, when administered in adequate amounts confer a health benefit on the host".[87] Studies in mice and rodents revealed a potential protective role of two Lactobacillus strains: *Lactobacillus rhamnosus GG*[88] and *Lactobacillus casei Shirota*,[89] in diet-induced NAFLD/NASH by demonstrating biochemical and histological attenuation of liver steatosis. Moreover, probiotics altered gut microbiota and prevented NAFLD progression in animals.[90] In humans, few low-quality randomized clinical trials investigated the potential therapeutic role for probiotics in patients with fatty liver. Wong et al[30] randomized 20 biopsy-proven NASH patients to receive either a combination of prebiotic and probiotic formula (containing *Lactobacillus spp.* and *Bifidobacterium spp.*) or prebiotic alone. Participants provided stool samples for microbial counts measurements using 16S rRNA sequencing, at baseline and at month six. Measurement of intrahepatic triglyceride content by spectroscopy was used to assess liver steatosis. Decreased counts of firmicutes and increased counts of Bacteroidetes were observed at 6 months in NASH patients experiencing lower levels of intrahepatic triglycerides content.

Moreover, despite different probiotics mixtures (*Lactobacillus bulgaricus* and *Streptococcus thermophilus* vs placebo,[91] *Lactobacillus GG* vs placebo,[92] *Bifidobacterium longum* with fructooligosaccharides vs placebo,[93] lepicol probiotic formula vs nothing[94]) and a variety of administration periods, a meta-analysis[95] provided evidence that probiotics might have a positive effect on biochemical markers in patients with NAFLD and NASH. More specifically, the use of probiotics was associated with a significant reduction of ALT, AST, cholesterol, TNF-α and homeostasis model assessment of insulin resistance. The amelioration of these parameters using probiotics creates promises for further scientific evaluation.

Perspectives

Gut microbiota is currently studied to elucidate its role in the pathophysiology of NAFLD. Excessive energy harvest, ethanol production, LPS-TLRs interaction and choline metabolism alterations are the main principal mechanisms implicated so far. Progress in molecular DNA-based techniques allows a more detailed characterization of this microbiota. While the gold standard for bacterial enumeration remains the qRT-PCR,[23] one of its disadvantages is that unknown species cannot be identified. In these terms, development of metagenomic techniques (16S rRNA, multi-tag sequencing) opens a promising window to investigate the microbiome. The aforementioned studies aimed to reveal a potential correlation between gut microbiota composition and NAFLD development or progression to NASH. Despite interesting findings regarding abundant phyla and genera, the results show wide discordance and heterogeneity. Factors that could explain these differences include selection criteria for the study populations, variable methodologies in defining NAFLD, unadjusted diet-induced manipulation of microbiota, medications intake that may alter gut microbiota, such as proton pump inhibitors,[96] as well as the DNA-based techniques' inability to distinguish whether the genetic material comes from alive and active or dead and inactive microorganisms.

Trying to temper these difficulties, RNA-, mRNA- and protein-based techniques (metagenomics, metatranscriptomics and metaproteomics) have been developed. Despite their limited availability, a future combination and standardization of these techniques might lead to deeper comprehension of the microbiota role in NAFLD.

Another issue that should be highlighted is that in the vast majority of the studies, only stool samples were examined. Isolate stool sample analysis may underestimate or even cloak the contribution of small intestinal flora and small bacterial overgrowth in NAFLD pathophysiology.[97] Advanced, culture-independent techniques applied directly on duodenal and small intestinal content

and biofilm may uncover a potential region-dependent interaction between liver steatosis and gut microbiota. Moreover, in both experimental models and in humans, the role of archaea, yeasts and viruses in NAFLD pathogenesis remains largely uninvestigated.

Finally, there is limited evidence for microbiota manipulation with probiotics as prevention or therapy of NAFLD.

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