The role of intestinal microbiota in the pathogenesis of NAFLD: starting points for intervention

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Abstract
In recent years, close links between intestinal microbiota and host metabolism have been recognized. Intestinal bacteria can participate in the extraction of calories from food, and circulation of bacterial products, in particular lipopolysaccharides (LPS), is responsible for the “metabolic endotoxemia”, which contributes to insulin resistance and its complications, such as non-alcoholic fatty liver disease (NAFLD). Indeed, qualitative and quantitative intestinal dysbiotic changes have been clearly documented in NAFLD patients, and several mechanisms by which the intestinal microbiota can directly promote liver fat deposition, inflammation and fibrosis have also been described. Consistently, although with some differences concerning type and proportion of results, experimental and clinical studies are quite concordant in demonstrating beneficial effects of probiotic and/or prebiotic therapy in NAFLD. Although some physiopathological bases have been produced, major doubts still remain concerning how and when to intervene. Indeed, most of the available works were performed with mixtures of probiotics and/or prebiotics, and a baseline assessment of dysbiosis aimed at selecting the best candidates for treatment and predicting response has not been performed in any of the clinical studies in NAFLD. While future research is expected to solve these issues, the particularly favorable safety profile suggests that probiotic/prebiotic therapy could already be “tested” in NAFLD patients on an individual basis, at least once all the measures recommended by the latest guidelines have failed.

Key words: intestinal microbiota, non-alcoholic fatty liver disease, dysbiosis, small intestinal bacterial overgrowth, probiotics, prebiotics, lipopolysaccharides, lipopolysaccharide binding protein.

Alterations of the intestinal microbiota during non-alcoholic fatty liver disease
One important advance in the past years has been the recognition that there are close links between intestinal microbiota and host metabolism, and that the microbiota is a major environmental factor contributing to obesity and its complications, such as insulin resistance, type 2 diabetes, cardiovascular disease and non-alcoholic fatty liver disease (NAFLD) [1, 2]. Indeed, it has been demonstrated that intestinal bacteria can participate in the digestion of otherwise indigestible dietary polysaccharides, thereby influencing the amount of calories extracted from food [3]. Moreover, the consumption of a high fat diet is associated with loosening of intestinal tight junctions, increased intestinal permeability and elevated systemic levels of lipopolysaccharides (LPS) [4, 5]. This
“metabolic endotoxemia” determines low-grade systemic inflammation, affecting insulin signaling and contributing to insulin-resistance and its complications [6].

Qualitative and quantitative intestinal dysbiotic changes have been clearly documented during NAFLD, both in patients with simple fatty liver and in those with non-alcoholic steatohepatitis (NASH) [7–14]. Microbiota samples from patients with NAFLD have a lower proportion of members of the Ruminococcaceae family than those from healthy subjects [7], and NASH patients show a significantly higher percentage of Clostridium coccoides than patients with simple steatosis [8]. Altogether, while some conflicting results have emerged between available studies addressing qualitative changes of intestinal microbiota in NAFLD patients, data concerning quantitative changes are quite concordant. Indeed, a number of works pointed to a high prevalence of small-intestinal bacterial overgrowth (SIBO) in patients with NAFLD [12–14].

There are several mechanisms by which the intestinal microbiota has been proven or suggested to contribute to liver fat deposition, inflammation and fibrosis. First, hepatotoxic bacterial products, i.e., pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), reaching the liver via the portal circulation, can activate specific toll-like receptors (TLRs) on different hepatic cells. In this context, the better recognized among bacterial constituents is LPS, which can activate TLR4 on Kupffer cells and hepatocytes, but also on cholangiocytes and on hepatic progenitor cells [15, 16], triggering the signaling cascade and the consequent secretion of several inflammatory cytokines. Recently, in patients with NAFLD, we demonstrated that TLR4 expression on bile duct/ductules and hepatic progenitor cells was significantly associated with inflammation, activation of fibrogenic cells and fibrosis, indicating the biliary clearance of excess LPS as a possible trigger of the inflammatory cascade in these cellular elements [16, 17]. Consistently, LPS binding protein (LBP), a sensitive marker of LPS activity, was found to be elevated in NAFLD patients and to correlate with the stage of fibrosis [16]. Furthermore, LPS is not the only driver of systemic inflammation, and other bacterial products derived from gut microbiota can regulate insulin sensitivity and produce inflammation also by reducing anti-inflammatory strategies [18].

Second, the intestinal microflora of obese mice was found to be responsible for increased production of endogenous ethanol [19], which reaches the liver via portal blood, leads to hepatocyte triglyceride accumulation, and contributes to the production of reactive oxygen species and to inflammation. In humans, the specific composition of the gut microbiome appears to be responsible for the abundance of alcohol-producing bacteria only among NASH patients, and not in healthy or obese non-NASH subjects [9].

Moreover, a high-fat diet was demonstrated to promote the formation of the intestinal microbiota, which converts dietary choline into methylamines, reducing plasma phosphatidylcholine and producing effects similar to those of the choline-deficient diet, a well-validated experimental model of NASH [20, 21].

Finally, more recently, also bile acids have been claimed as a possible contributor to the link between gut microbiota composition, dysmetabolism and NAFLD. Indeed, in murine models, it has been observed that bile acids not only play a key role in fat digestion and absorption, but also function as signaling molecules, binding to cellular receptors such as the bile-acid synthesis controlling nuclear receptor farnesoid X receptor (FXR) and the G-protein coupled bile salt receptor TGR5 [22, 23]. Since FXR and TGR5 are involved in the regulation of glucose homeostasis [24, 25], the gut microbiota might regulate metabolism also by impacting the composition of the bile-acid pool [26].

Further evidence is required before the exact nature of the relationship between dysbiosis and NAFLD can be definitely solved. Indeed, it remains unclear whether only dysbiosis contributes to NAFLD or also NAFLD can favor dysbiosis, and whether dysbiosis is associated specifically with NAFLD or, more generally, with the metabolic disorders which subent NAFLD development. The most consistent hypothesis is that an unbalanced (high-fat) diet both is directly responsible for liver fat accumulation and contributes to intestinal dysbiosis, which further promotes NAFLD and its progression. However, although further clarifications are certainly needed, a first message should pass: dysbiosis is associated with NAFLD and this relationship is harmful for the liver.

**Acting on intestinal microbiota may prove effective in NAFLD patients**

Experimental and clinical studies with probiotics and prebiotics support the role of intestinal microbiota in the pathogenesis of dysmetabolism and liver disease, and launch the modulation of dysbiosis as a possible therapeutic target in NAFLD [27].

Prebiotics are basically food for probiotics. Taking prebiotics helps probiotics work better and more efficiently. They are defined as a “non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon” [28]. Common prebiotics are...
inulin and carbohydrate fibers called oligosaccharides. On the other hand, probiotics are defined as preparations "containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host exerting beneficial health effects" [29]. Finally, synbiotics are supplements that contain both probiotics and prebiotics. Probiotics are available commercially in many products but primarily as foods and dietary supplements. As with any health-related product, it is important for probiotics to be safe and effective.

The agency charged with regulation of probiotic foods and food supplements, i.e., the European Food Safety Authority (EFSA), releases periodically the list of safe microbial cultures.

In the field of NAFLD, the most robust experience is that with VSL#3, a mixture of probiotic bacteria including *Lactobacillus*, which was proven to attenuate liver inflammation, and sometimes also to reduce fibrosis, in different animal models [30–32]. Evidence also comes from human studies. In NAFLD and alcoholic cirrhosis patients, VSL#3 improves plasma levels of lipid peroxidation markers [33], while *Lactobacillus* spp., *Streptococcus thermophilus* and *Bifidobacterium bifidum* decrease hepatic enzymes and liver fat content [34, 35]. Furthermore, in a recently published randomized controlled trial, 4-month supplementation with VSL#3 in obese children was found to significantly improve fatty liver severity, as determined by ultrasonography, and the effect was likely mediated by increased plasma levels of glucagon-like peptide 1 [36].

In mouse models, *Lactobacilli*, alone or mixed with other probiotics/prebiotics, were also shown to have direct metabolic effects, as demonstrated by their ability to increase hepatic peroxisome proliferator-activated receptor-α (PPAR-α) activity [37], and to modulate insulin sensitivity [38]. Interestingly, a butyrate-producing probiotic (MIYAIRI 588) was recently found to reduce hepatic lipid deposition and inflammation, as well as serum endotoxin levels and insulin resistance, in a rat model of NAFLD [39]. Of note, patients with NAFLD have significantly increased gut permeability, as well as a higher prevalence of SIBO, compared to healthy subjects [12], and butyrate has been strongly implicated in the maintenance of intestinal barrier integrity [40].

After the 2007 Cochrane meta-analysis was not conclusive [41], mainly due to the limited number of available randomized controlled trials, other studies have been produced, and a more recent meta-analysis found probiotic treatment to reduce alanine aminotransferase (ALT) and improve insulin resistance in NAFLD patients [42]. A recent study in 32 NAFLD patients found a significant effect of probiotics on the reduction of ALT, cholesterol, triglyceride and body mass index (BMI) levels and, even more interestingly, their ability to potentiate weight loss induced by metformin [43]. However, the effect of probiotic/prebiotic treatment on body weight in humans certainly needs to be further explored, and these results should be considered preliminary.

Finally, also rifaximin, which is a virtually unabsorbable antibiotic with broad spectrum antimicrobial activity and an excellent safety profile, will likely carve itself a role in this field. It has already been shown that rifaximin significantly reduces plasma LPS levels and improves liver function tests in liver transplant candidates with alcoholic cirrhosis, and, currently, there is an ongoing randomized trial aimed at assessing the efficacy of rifaximin in NAFLD including the measurement of proinflammatory cytokine and endotoxin levels [44]. If this trial gives positive results, we will have proof of concept that intestinal decontamination, which has already been demonstrated to improve liver function and disease severity in patients with decompensated cirrhosis [45], could be a feasible and safe approach to prevent LPS-induced liver injury also in the context of NAFLD. In any case, before long-term efficacy and safety results with rifaximin, as well as with other unabsorbable antibiotics, are obtained, it seems imprudent to consider this approach in NAFLD patients, even on an individual basis.

Altogether, a significant amount of results from experimental studies, and accumulating evidence from human studies, imply that manipulation of intestinal microbiota by probiotics and/or prebiotics, and/or rifaximin might exert beneficial effects in NAFLD and in the frequently associated metabolic disorders. Consistent with these data, the latest work demonstrated intestinal decontamination, with or without probiotics, is a potential treatment option for NAFLD and NAFLD-related metabolic syndrome [43].

**Starting points for intervention on intestinal microbiota in patients with NAFLD**

As pointed out above, qualitative and quantitative changes of the gut microbial community have been clearly documented in patients with NAFLD [7–14], and different possible mechanisms by which the intestinal microbiota can contribute to NAFLD pathogenesis have been demonstrated or suggested [9, 16, 19–21]. Although some physiological and pathological bases have been produced, major doubts still remain as to how and when to intervene. Indeed, most of the available studies were performed with a mixture of probiotics, frequently combined with one or more prebiotics, rendering the recognition of the specific agent responsible...
for the observed beneficial effects quite impossible. While waiting for further research aimed at discerning which effect should be attributed to each probiotic/prebiotic, we could already try to modulate intestinal microbiota using the mixtures of agents which have demonstrated beneficial effects in the better-designed available studies in NAFLD patients. However, even trying to simplify things by this approach, we would still face a significant unsolved question: is probiotic/prebiotic therapy effective in all NAFLD patients, or do we need a baseline screening test for prediction of the response? The truth is that we don’t have an answer to this question, since a baseline assessment of dysbiosis has not been performed in any interventional study with this type of treatment in NAFLD patients. It is therefore desirable for future studies to be designed considering also a baseline assessment of dysbiosis, aiming to evaluate if and how the response to treatment can be predicted on an individual basis. However, how should we assess dysbiosis in an easy, reliable and cost-effective, i.e., clinically applicable, way? Pyrosequencing of 16S ribosomal RNA, which has been essential for acquiring most of the knowledge concerning the gut bacterial composition in NAFLD, does not seem to be the most appropriate tool. In the same way, all the available methods to test for small intestinal bacterial overgrowth (SIBO), including direct aspiration and culture of the duodenal fluid and glucose/lactulose breath test, have substantial limitations, and no “gold standard” diagnostic test for the condition exists [46, 47]. When considering possible serum markers, it should be noted that various toxins produced by members of gut microbiota may enter the bloodstream via the enterohepatic circulation or the impaired gut barrier. Lipopolysaccharide is a major component of the Gram-negative bacterial wall, which can be detected and measured in the blood by the Limulus lysate assay. However, this test has a limited utility in the routine clinical setting, since LPS has a short half-life and high susceptibility to interfering substances [48]. LPS binding protein is an acute phase protein mainly produced by the liver in response to bacteraemia or endotoxemia. It circulates in the blood, and its serum levels, which can be conveniently detected by commercial ELISA, indicate the amount of effective LPS load and the induced innate immune response [49]. Circulating LBP levels are increased in NAFLD patients [16, 50] and associated with the stage of fibrosis, as we recently demonstrated [16]. One limitation of LBP is that it only reflects Gram-negative bacteria and not Gram-positive translocation. However, its relatively long half-life, and the reliability and limited cost of the test, launch LBP as an attractive candidate to be further tested in the clinical ground.

In conclusion, to date, although some convincing knowledge on the implication of dysbiosis in the pathogenesis of NAFLD has been acquired, we still lack sufficient evidence to suggest when and how this knowledge should be applied. Future research is expected to further explore the physiopathological links between intestinal microbiota and NAFLD, to identify clinically relevant markers of dysbiosis and predictors of response, as well as to indicate the best agent, or combination of agents, to be used. Treatment of NAFLD should be first aimed at antagonizing insulin resistance, which is also central to all associated metabolic disturbances [51]: proper diet [52] and physical activity are the first keys to success. Currently, taking advantage of the optimal safety profile of probiotic/prebiotic therapy, we can only suggest that an approach with these agents in NAFLD could be explored in selected cases, after all the measures recommended by the latest guidelines [53], i.e., lifestyle interventions and eventually vitamin E in non-diabetics or pioglitazone in diabetics, have failed.

Conclusions

Different pathogenic pathways are involved in the development of NAFLD, and, in recent years, growing evidence has indicated the participation of intestinal dysbiosis. While waiting for further research in order to understand when and how we should better modulate the gut intestinal microbiota in NAFLD patients, the very favorable risk profile already permits us to “test” probiotics/prebiotics once the currently recommended therapeutic measures have failed.

Conflict of interest

The authors declare no conflict of interest.

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