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PREBIOTICS TARGETING GUT-LIVER AXIS TO TREAT NON-ALCOHOLIC FATTY LIVER DISEASE

Running title: Use of prebiotics in NAFLD management

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Abstract

Non-alcoholic steatohepatitis (NASH) is a high-prevalence, rapidly growing form of non-alcoholic fatty liver disease (NAFLD), which is closely linked to obesity and metabolic disorders. Gut microbiota has been increasingly recognized as a key factor the onset of NAFLD in recent years. The liver can be strongly influenced by changes in the gut microbiota through the portal vein, giving the gut-liver axis a very important role in understanding the pathophysiology of liver diseases. A healthy intestinal barrier is characterized by selective permeability to nutrients, metabolites, water and bacterial

products and its impairment may be a predisposing or aggravating condition for the progression of NAFLD. In most cases, NAFLD patients follow a Western diet pattern, which is closely linked to obesity and associated metabolic diseases, promoting inflammation, structural and behavioural changes in the gut microbiota. In fact, factors such as age, gender, genetic or environmental factors may induce a dysbiotic microbiota that promotes epithelial barrier dysfunction and increased intestinal permeability, favouring the progression of NAFLD. In this context, new dietary approaches, such as prebiotics, are emerging to prevent disease and maintain health. In this review, we reported the role of the gut-liver axis in the pathogenesis of NAFLD and investigated the potential therapeutic effect of prebiotics on the enhancement of intestinal barrier dysfunction, hepatic steatosis and, consequently, the progression of NAFLD.

Key words: NAFLD, gut-liver axis, prebiotics, intestinal permeability

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a term that encompasses a continuum of liver abnormalities characterized by excessive hepatic fat accumulation[1]. Histologically, NAFLD is typically classified into two categories: non-alcoholic fatty liver (NAFL), also referred to as simple steatosis, and non-alcoholic steatohepatitis (NASH)[2], a condition characterized by lobular inflammation and ballooning degeneration with or without the presence of hepatic fibrosis. The pathogenic drivers involved in the progression from NAFL to NASH and fibrosis are not likely to be identical in all the individuals with NAFLD and a high heterogeneity has been found in the mechanism that leads to the disease and the accompanying clinical manifestations[3]. Increasing research suggests that

the gut microbiota, through the gut-liver axis, may contribute to the development and differentiated progression of NAFLD[4].

Lifestyle-based therapies involving dietary improvement and regular exercise remain the first therapeutic approach in patients with NAFLD[5]. In this sense, the increased intake of energy-dense foods, rich in fat and added sugar, the so-called Western-diet, is strongly associated with obesity and related metabolic diseases[6]. Animal and human studies have revealed that the adherence to a western pattern influences the intestinal dysbiosis leading to an impairment that in turn is linked to the development of metabolic disorders[7]. A dysbiotic microbiota could promote epithelial barrier dysfunction and increased intestinal permeability, which facilitates the translocation of bacteria and bacterial components, such as lipopolysaccharide (LPS) endotoxin, into the bloodstream, promoting the progression of NAFLD[8]. In recent years, several approaches have emerged targeting the gut microbiota according to its role in the pathogenesis of NAFLD[9]. One dietary strategy for modulating gut microbiota is prebiotic consumption, which has been related to the modulation of liver function and reduction of dysbiosis and liver fat[10].

Therefore, in this review, we summarized the role of the gut-liver axis in the pathogenesis of NAFLD. Furthermore, we evaluated the potential therapeutic effects of prebiotics in ameliorating intestinal barrier dysfunction and hepatic steatosis and, consequently, NAFLD.

2. Gut-liver axis in the physiopathology of NAFLD

The liver can be strongly influenced by changes in the gut microbiota through the portal vein, giving the gut-liver axis a very important role in understanding the pathophysiology of various liver diseases[11]. Indeed, liver damage has been associated with small intestinal

bacterial overgrowth (SIBO) and gut microbiota dysbiosis[12,13]. For example, patients with NAFLD, compared to healthy individuals, showed differences in the observed bacterial signature at the level of phylum (increased Proteobacteria), family (increased Enterobacteriaceae and decreased Rikenellaceae and Ruminococcaceae), and genera (increase of Escherichia, Dorea, Peptoniphilus and decrease of Anaerosporobacter, Coprococcus, Eubacterium, Faecalibacterium and Prevotella)[14]. Moreover, faecal transfer from morbid obese women, with histologically-proven liver steatosis, to chow diet fed mice induced an increase in liver triglyceride content[15]. Conversely, in mice and rat models, Akkermansia and Bifidobacterium species have been shown to reinforce the gut barrier and reduce liver inflammation[16,17]. Alteration of the gut-liver axis due to factors such as age, gender, genetic factors, drug intake and lifestyle factors, including dietary intake, can therefore induce dysbiosis and/or increased intestinal permeability[18]. The study of their function and composition is important to reduce the consequent proinflammatory changes and to limit disease progression.

2.1 Intestinal permeability

Trillions of microbes colonizing the human body are spread along the gastrointestinal (GI) tract[11]. The intestinal barrier is composed of an epithelium that absorbs nutrients and, at the same time, protects against pathogens and viruses thanks to the mucus layer and the tight junctions, which control substances passing through the epithelial cells[19]. Intestinal permeability (IP) can be considered as a measurable characteristic of the intestinal barrier[19]. Altered or increased IP has been shown to contribute to the development of NASH due to the passage of bacterial-derived compounds into the systemic circulation[20], which may trigger tissue and systemic inflammation[14] and activate Toll-like inflammatory receptors (TLRs) in hepatocytes[21–23]. An altered intestinal barrier may favour the

translocation LPS, one of the constituents of the outer membrane of gram-negative bacteria, which promotes endotoxemia and liver inflammation[24,25]. LPS, absorbed from the gut, is transported through the portal vein to the liver, where stimulates immune cells and the inflammatory cytokine pathways, is uptaken by hepatocytes and Kupffer cells and later excreted in the bile[25] (**Figure 1**).

Figure 1. Intestinal permeability in the pathogenesis of NAFLD. Gut liver dysbiosis and small intestinal bacterial overgrowth can cause increased intestinal permeability, which facilitates the translocation of bacteria and bacterial components, such as the endotoxin lipopolysaccharide, into the bloodstream, reaching the liver, where they activate Kupffer cells by binding to Toll-like receptors. This pathogenic mechanism leads to processes such as liver inflammation and damage, fibrogenesis and systemic inflammation, favouring the progression of NAFLD. Abbreviations: LPS, Lipopolysaccharides; NAFLD, Non-alcoholic fatty liver disease; NASH, Non-alcoholic steatohepatitis; SIBO, Small Intestinal Bacterial Overgrowth; TJ, Tight Junction; TLRs, Toll-like inflammatory Receptors.

Importantly, in a cohort of 6,727 individuals serum LPS levels were associated with hospitalization, cancer or liver related death in the general population, with the highest tertile potentially accounting for 30% of the risk of liver disease[26]. Also, in a study performed in patients with biopsy-proven NAFLD, higher mean serum LPS values were observed in patients with NAFLD (n= 211, 61.6 pg/mL [IQR 42.4-100.0]) compared to patients without NAFLD (n= 65, 43.9 pg/mL [31.6-56.0] ($p < 0.001$). However, no significant differences were found between patients with NAFL and NASH (n = 25, 108.0 pg/mL [IQR 48.5-140.5] vs. n = 25, 70 pg/mL [IQR 48.1-119,2], p = 0.337)[8]. According to this, a study in male C57bl/6 mice found that the induction of intestinal inflammation in experimental NASH promotes the translocation of LPS and may increase the levels of microbial-derived LPS, promoting the progression of NASH itself[27]. Moreover, LPS induces the activation of monocytes and macrophages, which promote the secretion of a

number of proinflammatory cytokines such as tumor necrosis factor (TNFα) and interleukin 6 (IL-6)[24].

Additionally, a study by Bischoff et al. revealed that fructose plays an important role on the impairment of intestinal barrier[19]. In TLR-4 mutant mice, the occurrence of NAFLD induced by fructose was related to an increase in intestinal permeability, leading to an endotoxin-dependent activation of hepatic Kupffer cells[28]. Indeed, it has been shown that dietary fat consumption increases intestinal permeability through the production of proinflammatory cytokines such as TNFα, IL6 and interferon gamma (IFN-γ) as well as negative changes in the composition of the intestinal mucosa and microflora, leading to a disruption of the barrier[29].

2.2. Nutrients and bacterial metabolites in NAFLD

Under normal conditions, many nutrients and beneficial microbial products can reach the liver via the portal system. The gut microbiome contributes to nutrient processing and signalling and produces metabolites with essential functions[30]. For example, dietary soluble fibers are fermented by gut microbiota into short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate, which can be absorbed and enter the liver via the portal system[31]. SCFAs, the main source of nutrition and well-being of the intestinal epithelium (colocytes), have been shown to have a beneficial anti-inflammatory effect on microbial metabolism[32]. However, dysregulated fermentation of soluble dietary fibres in an inulin-containing diet induces cholestasis, liver inflammation and promotes the development of liver cancer in mice, in contrast to that of insoluble fibres[33]. Thus, despite their benefits, the use of these compounds should be approached with caution.

Bile acids (BAs), synthesized from cholesterol in the liver, are one of the main components of bile juice and play an important role in the absorption of dietary fats and certain vitamins[34]. In this regard, a high fat diet has been shown to increase BA synthesis and bile excretion by the gallbladder and to induce intestinal hyper-permeability, promoting hydrophobic transition of BAs that alters the barrier by inducing oxidative stress and apoptosis of intestinal epithelial cells[29]. Furthermore, a high-fat-diet (HFD) modulates the composition of bile, favoring the presence of hydrophobic BAs including lithocholic acid (LCA), deoxycholic acid (DCA), and chenodeoxycholic acid (CDCA). Among BAs, one can distinguish primary BAs, produced by the liver, and secondary BAs, converted by the gut microbiota[35]. The conversion from primary to secondary BAs correlates with NAFLD and the progression of NASH, depending on the amount and type of secondary BAs produced[36]. In this regard, it has been shown that dehydroxylation of CDCA leads to the formation of LCA, a substance toxic to liver cells[36]. Furthermore, a reduction in BAs could be associated with NAFLD through the production of trimethylamine N-oxide (TMAO), which would induce a decrease in the total pool of BAs by inhibiting the cholesterol 7 alpha-hydroxylase (CYP7A1) and cytochrome P450 family 27 subfamily A member 1 (CYP27A1), enzymes involved in BAs metabolism[14]. In this regard, a systematic review and meta-analysis showed that patients with NAFLD had higher levels of circulating TMAO (SMD: 0.66, 95% CI -0.12 to 1.21, p = 0.02, I2 : 94%)[37]. In this sense, foods rich in trimethylamine (TMA) precursors, such as animal proteins, are metabolised by the digestive system into choline, L-carnitine and betaine which, when present in excess, are metabolized into trimethylamine (TMA), which in turn is metabolized in the liver to TMAO[37].

In subjects with NAFLD, those with increasing disease severity have a lower ratio of betaine to choline, as patients with NAFLD have a higher amount of TMA and TMAO,

due to the reduction of choline caused by the microbiota's switch to methylamine production[14,38]. Furthermore, as previously reported, increased TMAO levels induce a decrease in BAs levels via inhibition of CYP7A1 and CYP27A1 enzymes[39].

Lastly, ethanol also plays an important role in NAFLD: adults who do not consume ethanol have been found to have higher respiratory ethanol concentrations. This apparent contradiction may be attributed to the production of ethanol by the bacteria of the gut microbiota in patients with NAFLD[14]. In this regard, it has been demonstrated that certain bacteria such as *Klebsiella pneumoniae* are able to produce ethanol from glucose in the absence of alcohol[40] both in mice and humans.

3. Use of prebiotics for the treatment of NAFLD

In most cases, patients with NAFLD follow a Western diet pattern, characterized by energy-dense and ultra-processed foods. This type of diet, in addition to worsening the liver's condition, has been associated with an increase in Gram-negative bacteria, LPS and altered intestinal permeability[41]. In this regard, prebiotics, may be a valuable ally in modulating the microbiota following their metabolization in the gastrointestinal tract[42].

Currently, a prebiotic is defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit"[43]. Notably, the effect of prebiotics in the intestinal bacterial population have been studied, as reported elsewhere[44]. In recent years, research has focused on the use of dietary fiber and prebiotics, as many of these polysaccharides can be metabolized by the gut microbiota leading to the production of SCFAs[45]. Metabolites derived from the fermentation of prebiotics also show anti-inflammatory and immunomodulatory effects, suggesting an interesting role in the treatment of various pathological conditions, which has consequently led to a growing

interest in dietary strategies aimed at modulating the gut microbiota[45]. Therefore, in this review we report some human and animal studies that have shown substantial benefit regarding prebiotics' use in individuals with NAFLD (**Table I**).

Table I. Selection of articles studying the effect of prebiotics on NAFLD/NASH

Reference	Study design	Aim	Intervention	Outcome
Human studies				
Daubioul C. et al, (2005)[52]	Double-blind, crossover, placebo-controlled design 7 participants, 48-66 y.o. with liver-biopsy-confirmed NASH	To investigate the effect of daily ingestion of OFS in patients with NASH	Patients received 16 g/day of OFS or maltodextrin (placebo) for 8 weeks, and biological parameters were analyzed after 4 and 8 weeks of dietary supplementation	Compared with placebo, OFS significantly reduced serum aminotransferases and AST, after 8 weeks, and insulin level after 4 weeks
Chambers E. et al, (2019)[51]	Double-blind, RCT trial 18 participants, 18-65 y.o. with NAFLD and BMI 20-40 kg/m^2	To investigate the impact of raising colonic propionate production on hepatic steatosis.	Subjects received 20g/d of control inulin or IPE for 42 days.	The change in IHCL after the supplementation period was not different between groups (p=0.082). However, IHCL was significantly increased within the inulin-control group
Bomhof M. et al, (2019)[46]	Multicenter RCT trial 14 participants ≥ 18 y.o. with liver-biopsy-confirmed NASH (NAS score ≥ 5) and BMI>25 kg/m^2 (or BMI >23 kg/m^2 if Asians)	To investigate the potential therapeutic effect of a prebiotic supplement on the improvement of the histological parameters of NASH.	Participants were randomized into either a treatment group, receiving 8g prebiotic oligofructose daily for 12 weeks, followed by 16g daily for 24 weeks, or into an isocaloric maltodextrin control group.	Prebiotic improved hepatic steatosis compared with placebo and overall NAS score (p=0.016). Oligofructose enhances Bifidobacterium, while reducing Clostridium cluster XI and I bacteria (p<0.05).
Chong C. et al, (2020)[48]	Three-arm, double-blind, single-center RCT trial 62 participants, 18-75 y.o. with histological evidence of NAFLD, BMI>27 kg/m^2 and T2DM.	To determine whether inulin supplementation after brief metronidazole therapy is effective in reducing ALT and maintaining weight loss achieved through a VLCD.	Subjects underwent VLCD for 4 weeks and then (1) metronidazole (400 mg, 2 x d/ 7d) and then inulin (4 g, 2 x d/12w) (2) placebo (2 x d/ 7d) and then inulin (4 g, 2 x d/12w) or (3) placebo (2 x d/ 7d) - placebo (4 g, 2 x d/12w)	BMI and ALT decreased after VLCD by 2.4 kg/m^2 and 11 U/L, respectively. ALT further decreased after metronidazole-inulin compared with placebo-placebo (p =0.026). VLCD treatment after 4 weeks reduced Firmicutes/Bacteroidetes bacteria (p =0.002), Roseburia and Streptococcus (p =0.005), and Dialister (p =0.03)
Animal model				

Jian-Gao Fan et al, (2005)[53]	RCT 42 male sprague-sawley rats with NASH after high fat diet	To explore the relationship between changes of intestinal environment and pathogenesis of NASH	Treatment period: 8w. Model group, control group, and treatment group (45 mL/d of lactulose syrup instead of water).	Significant decrease in serum levels of AST, ALT and indices of liver inflammation.
Qiu S. et al, (2022)[50]	RCT in 10 healthy C57BL/6 male mice (7 weeks old) and 5 mice with NAFLD following a high-fat and high-sugar diet	To investigate the mechanism and the therapeutic effect of GOS on NAFLD	Treatment period: 12w. Control and model NAFLD group (double distilled water). Positive control group (50 mg/kg metformin), high-dose (2730 mg/kg GOS), medium-dose (1365 mg/kg GOS) and low-dose GOS groups (681.5 mg/kg GOS)	GOS improved HFHSD-induced dyslipidemia, reduced ALT and AST levels ($p<0,001$), significantly reduced TNF-α and IL-6 content in serum of mice ($p<0,001$ and $p<0,05$), and significantly increased IL-10 level($p<0,001$). GOS also improved oxidative damage and significantly reduced lipid droplets($p<0,001$) in mouse liver. In addition, GOS may reverse the changes in gut flora structure induced by HFHSD

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, Body Mass Index; GOS, Galactose oligosaccharide; IHCL, intrahepatocellular lipids; IPE inulin-propionate ester; HFHSD, High-Fat and High-Sugar Diet; IL-6, Interleukin 6 ; IL-10, Interleukin 6; NAFLD, Non-alcoholic fatty liver disease; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; OFS, oligofructose; RCT, randomized controlled trial; T2DM, Type 2 Diabetes Mellitus; TNF-α, tumor necrosis factor; VLCD, Very-Low-Calorie Diet.

In this regard, in a study of 14 individuals with histological NASH, participants were randomly assigned to receive oligofructose (8 g/day for 12 weeks followed by 16 g/day for 24 weeks) or isocaloric placebo for 9 months[46]. Importantly, participants receiving oligofructose significantly improved hepatic steatosis and NAFLD activity score (NAS) compared to placebo group ($p = 0.016$)[46]. In addition, oligofructose increased Bifidobacterium, while reducing bacteria within Clostridium cluster XI and I[46]. Another study of 62 patients adhering to a four-week low-calorie diet (VLCD) led to a decrease in the Firmicutes/Bacteroidetes (F/B) ratio ($p = 0.002$). Specifically, an increased abundance of Firmicutes and/or a decrease in Bacteroidetes was positively associated with body fat[47]. Interestingly, after the VLCD and a short treatment with metronidazole, these patients were subsequently supplemented with 4 g of inulin, showing a reduction of alanine aminotransferase (ALT) levels[48].

Alteration of the gut microbiota has also been observed in animal models following the use of prebiotics such as galactose oligosaccharides (GOS)[49]. GOS are present in commercially available products, and their dietary supplementation is a measure to maintain the balance of gut flora, to prevent and treat diseases and to promote host health[49]. In a study of 10 healthy male C57BL/6 mice with NAFLD induced by a high-fat, high-sugar diet, GOS-treated mice, compared with controls, had significantly higher levels of Bacteroidetes and Actinobacteria ($p < 0.0001$), while Firmicutes and Proteobacteria levels were significantly reduced ($p < 0.0001$). In addition, GOS intervention significantly reduced the F/B ratio ($p < 0.05$), indicating that GOS can reverse the changes in the structure of the HFHSD-induced gut flora[50]. Accordingly, the intake of GOS in NAFLD mice was associated with an improvement in dyslipidemia, a decrease in ALT, aspartate aminotransferase (AST), TNFα and IL-6 levels, and a reduction in liver lipid droplets[50].

Additionally, GOS may also improve insulin resistance in mice by effectively alleviating glucose tolerance in NAFLD model mice[50].

Furthermore, recent investigations suggest that diet, gut microbiota and liver fat storage could be linked through a mechanism involving SCFAs, the main products of dietary fiber fermentation in the colon. Particularly, propionate has been shown to alter hepatic metabolic processes that reduce lipid storage[51]. In this regard, in a parallel randomized trial, 18 adults were randomized to receive either 20 g/day of an inulin-propionate ester (IPE) or an inulin control for 42 days, showing that the change in intra-hepatocellular lipids (IHCL), after the supplementation period, did not differ between groups[51]. However, IHCL increased significantly in the inulin control group ($p = 0,012$), which was not observed in the IPE group ($p = 0,082$). As a result, the increased colonic propionate supply by IPE appears to attenuate the acetate-mediated increase in IHCL[51].

The beneficial effect of prebiotics has also been observed in patients with NASH[52]. In fact, it was found that 16 g/day of oligofructose administered for 8 weeks, in 7 participants with NASH confirmed by liver biopsies, compared with placebo, significantly reduced serum aminotransferase and insulin levels[52].
Similarly, in animal models, a study investigated the pathogenesis of NASH in a group of 42 rats divided into a model group ($n = 24$), which received a high-fat diet, a treatment group ($n = 12$), which received a high-fat diet and 45 mL/d of lactulose after the diet, and a control group ($n = 6$), which received a standard diet. After 8 weeks of diet and 8 weeks of treatment, serum levels of AST ($p < 0.01$) and ALT ($p < 0.05$) decreased significantly in the treated animals compared with the control group. Interestingly, lactulose improved liver inflammation in rats with NASH induced by a high-fat diet[53].

In summary, all these studies showed that prebiotics such as inulin[48,51], oligofructose[46,52], GOS[50] and lactulose[53] improved liver fat or inflammation in mice and in patients with NAFLD or NASH. In some cases, these substances have been combined with other therapies[48,50,51], but interventions focused on the gut microbiota using prebiotics could offer a safe and sustainable means of managing this disease. In addition, the use of prebiotics is sometimes combined with the use of probiotics, live microorganisms that when ingested provide benefits to human health. Therefore, some studies have focused on manipulating enteric flora through the consumption of synbiotic, a combination of probiotics and prebiotics that act synergistically to provide a health benefit to the host[54], in order to act as a new adjunctive therapeutic strategy in patients with NAFLD[55,56].

4. Conclusions

NAFLD is the most common liver disorder in Western countries and the prevalence is increasing worldwide. In recent years, the role of the gut microbiota in maintaining the homeostasis of the gut-liver axis has received major attention in chronic liver disease.

Thereby, the development of new strategies aimed at modulating the microbiota to improve NAFLD outcomes is crucial for the comprehensive management of this liver disease. However, due to certain limitations such as the heterogeneity of the populations or of the studied products, further clinical and/or laboratory research is needed to understand the exact mechanism and efficacy of these dietary factors in the treatment of NAFLD.

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