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New insights and therapeutic implication of gut microbiota in non-alcoholic fatty liver disease and its associated liver cancer

Niama Ezzaidi[a,b,1], Xiang Zhang[a,1], Olabisi Oluwabukola Coker[a], Jun Yu[a,*]

[a] Institute of Digestive Disease and the Department of Medicine and Therapeutics, State Key Laboratory of Digestive Disease, The Chinese University of Hong Kong, Hong Kong

[b] MChem Chemistry and Drug Discovery, University of Sussex, UK

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ABSTRACT

The gastrointestinal tract represents one of the largest interfaces between the host and environmental factors. It contains a vast and complex community of microbes, forming what is collectively known as the microbiota. This gut microbiota plays a pivotal role in the maintenance of health, and 'dysbiosis' of the gut microbiota, commonly considered as perturbation of microbiota diversity and composition, has been associated with intestinal and extra-intestinal diseases, including non-alcoholic fatty liver disease (NAFLD) and its associated hepatocellular carcinoma (NAFLD-HCC).

In this review, we highlight microbiota dysbiosis and the microbiota-host interactions that link to the pathogenesis of NAFLD and NAFLD-HCC. We discuss the potential therapeutic implications of the gut microbiota in the progression of NAFLD-HCC.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a form of hepatic steatosis with the accumulation of fat in the liver, in the absence of alcohol use. NAFLD is a disease of increasing interest as its prevalence is on the rise; 30% of the adult population and 80% of obese individuals are affected by the disease, making it the most common chronic liver disease in developed countries [1,2]. NAFLD comprises a histological spectrum, ranging from simple steatosis to non-alcoholic steatohepatitis (NASH). The latter is the progressive form of NAFLD and currently constitutes the third most common indication for liver transplantation in countries like the United States [3]. Although, not all individuals with steatosis develop a more severe form of the disease, NASH can progress to cirrhosis and hepatocellular carcinoma (HCC). The number of NAFLD and its related HCC (NAFLD-HCC) has increased dramatically due to the rising incidence of obesity and metabolic syndrome [4].

The progression from NAFLD to NASH and its associated HCC is not fully understood. We have reported that the pro-inflammatory cytokine CXCL10 and its receptor CXCR3 play an essential role in the development of NASH in the context of NAFLD [5–7]. Autophagy impairment and mitochondrial dysfunction were found to participate in the CXCL10/CXCR3-induced NASH [8,9]. We also identified that O-GlcNAc transferase (OGT), Squalene epoxidase (SQLE), dysregulated metabolism and calcium signalling are implicated in NAFLD-HCC progression [10–12]. Recent studies have favoured the ‘multiple parallel hits’ hypothesis, based on the idea that many pathogenic events could lead to NASH, including insulin resistance, oxidative stress, adipose tissue dysfunction, endoplasmic reticulum (ER) stress, cytokine secretion, altered innate immunity regulation and intestinal microbiota dysbiosis [13].

One of the exciting advances in the past years has been the observation that there may be tight links between the intestinal

Abbreviations: NAFLD, non-alcoholic fatty liver disease; NAFLD-HCC, NAFLD-associated hepatocellular carcinoma; NASH, non-alcoholic steatohepatitis; HCC, hepatocellular carcinoma; OGT, O-GlcNAc transferase; SQLE, Squalene epoxidase; ER, endoplasmic reticulum stress; LPS, lipopolysaccharide; TLRs, Toll-like receptors; DCA, deoxycholic acid; SCFA, short-chain fatty acid; BCAA, branched chained amino acids; GCA, Glycocholic acid; TCA, Taurocholic acid; FXR, Farnesoid X Receptor; PAMPs, pathogen-associated molecular pattern molecules; DAMPs, Damage-associated molecular patterns; FMT, Fecal microbiota transplantation; MCD, methionine-choline-deficient; NAS, non-alcoholic fatty liver activity score; DG, Diammonium Glycyrrhizinate; NSBB, non-selective β blockers; PPI, Proton pump inhibitor

* Corresponding author. Institute of Digestive Disease and Department of Medicine and Therapeutics, Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, NT, Hong Kong.

E-mail address: junyu@cuhk.edu.hk (J. Yu).

[1] Equal contribution.

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microbiota and host metabolism. The gut microbiota is a multispecies community of microorganisms that includes a wide variety of bacteria, fungi, viruses as well as archaea, residing in the gut [14]. Studies have shown that this previously neglected organ carries out fundamental physiological functions such as energy harvest [15], immunity regulation [16], nutrient metabolism and mucosal defence [17]. Moreover, it exerts a protective effect on several host tissues and plays an important role in the development of diseases both within and beyond the gastrointestinal tract, either directly via translocation or indirectly through microbial metabolism [18]. Multiple studies from our group have indicated the involvement of gut microbiota in gastrointestinal cancers [19–22]. Others have reported that gut microbiota alteration is associated with NAFLD and HCC progression [23,24]. Several mechanisms by which the intestinal microbiome can affect NAFLD pathogenesis and maintenance have been identified. Increased intestinal permeability, intestinal bacterial overgrowth and elevated serum endotoxin, have been reported in NAFLD and NAFLD-HCC studies [25,26].

In this review, we focus on the relationship between the gut microbiota and NAFLD and NAFLD-HCC. We provide an overview of gut microbiota dysbiosis and microbial metabolites alteration in NAFLD and NAFLD-HCC. Moreover, we discuss the therapeutic application of gut microbiota in this important disease.

2. GUT microbiota dysbiosis and NAFLD

The gastrointestinal tract represents one of the largest interfaces between the host and environmental factors in human. It contains a vast and complex community of trillions of microbes forming what is collectively known as the microbiome. The gut microbiome refers to the ensemble of all genomes of microbes in the gut and encodes pathways that produce a wide array of bioactive small molecules [14]. Although the overall interrelationship of human with the gut microbiota can be considered a mutualistic symbiosis, its disruption ("dysbiosis") can lead to the development of various chronic diseases with an underlying inflammatory condition. Microbiome related pathologies have dramatically increased over the past century, suggesting that a change in lifestyle might disrupt gut microbiota symbiosis due to the loss of beneficial and protective microbes.

Several lines of evidence suggest a role for gut microbes in the etiology and progression of NAFLD [27,28]. We have analyzed the gut microbiota from 16 histology-proven NASH patients and 22 healthy controls by 16s rRNA sequencing [29]. Our results showed that NASH patients have gut dysbiosis with lower abundance of *Faecalibacterium* and *Anaerosporobacter* but higher abundance of *Parabacteroides* and *Allisonella* [29]. The reduced *Firmicutes* and increased *Bacteroidetes* were associated with the improvement in hepatic steatosis [29]. In a separate report, NASH patients showed a higher abundance of *Bacteroides*, while NASH-fibrosis patients were related with higher *Ruminococcus abundance* [30]. In a paediatric study, *Actinobacteria*, *Ruminococcus, Blautia,* and *Dorea* were increased in NASH patients, while *Bacteroidetes* were reduced in NAFLD [31].

A recent study added the direct evidence that gut microbiota may play a causative role in the development of NAFLD by showing that the gut microbiota, from a genetically obese human, promoted the onset of liver steatosis by impacting hepatic transcriptional profile of lipid metabolism in germ-free mice [32]. Germ-free mice were transplanted with gut microbiota from a genetically obese human donor before (PreM) and after a dietary weight loss program (PostM). Mice in the PreM group but not PostM group developed hepatic steatosis with distinct gut microbiota in the stool and transcriptional alteration in the liver [32]. The intestinal microbiome could contribute to all the histological components of NAFLD: hepatic steatosis, inflammation, and fibrosis by faecal transplantation [32]. The diagnostic value of gut microbiota in NAFLD was presented by Loomba and colleagues [33]. In this study, which included 86 NAFLD patients diagnosed by liver biopsy, 37 intestinal bacterial species distinguished advanced from simple fibrosis. Advanced fibrosis was associated with an increased abundance of *Proteobacteria* and *Escherichia coli* and a decrease in *Firmicutes*, suggesting that a panel of microbiome markers could be used to diagnose advanced fibrosis in NAFLD [33].

Although research has been more traditionally focused on bacterial component of the gut microbiota, investigation of enteric fungal and viral species has increased due to advancement in sequencing and analysis technologies. This has provided opportunities to recognise their implication in liver disease aetiologies. A recent study showed that chronic alcohol consumption was associated with altered intestinal fungi and translocation of fungal β-glucan into systemic translocation, suggesting the manipulation of intestinal fungi as a new possible strategy for attenuating alcohol-related liver disease [34]. Moreover, despite that certain viruses including hepatitis B and C are implicated in liver cancer, the potential correlation between enteric virus and liver diseases have not been established. A clinical case was however reported of concomitant norovirus-induced gastroenteritis and acute liver dysfunction [35]. Whether disruption of enteric virus is associated with liver diseases as shown for colorectal cancer [36] warrants investigation.

Although the exact mechanism linking the gut microbiota with NAFLD development and progression remains unknown, potential explanations include bacterial overgrowth, bacteria translocation, increased endotoxin absorption and impaired enterohepatic bile acids circulation (Fig. 1) [37,38]. Studies in germ-free and conventionalized mice revealed that the gut microbiota promotes absorption of monosaccharides from the gut lumen, resulting in the induction of de novo hepatic lipogenesis [18]. Bacterial products derived from gut microbes, including LPS, peptidoglycan and bacterial DNA can travel up the portal vein to activate toll-like receptors (TLRs) on Kupffer cells, leading to an inflammatory response that promotes NASH. Because the gut microbiome can affect host metabolic processes such as energy extraction from food, the composition of the gut microbiota is now considered a major environmental factor contributing to NAFLD [18].

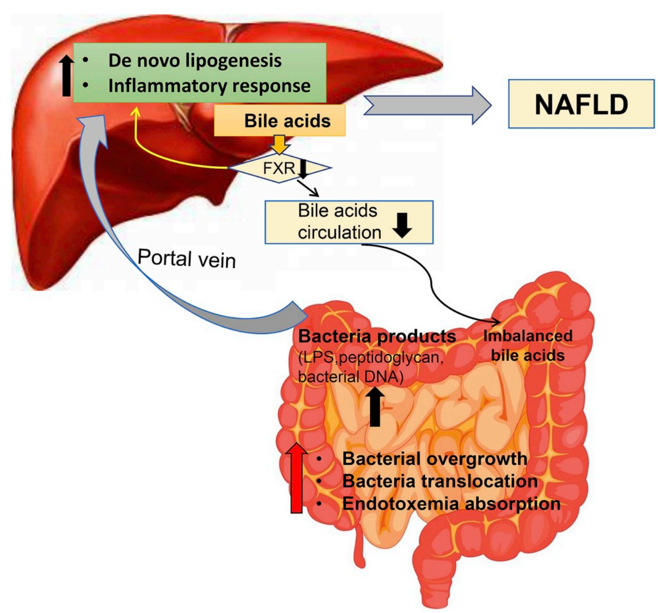

Fig. 1. Gut Microbiota-liver axis: The mechanisms linking the gut microbiota with NAFLD development and progression. Bacterial overgrowth, bacteria translocation and increased endotoxin absorption in the intestine will generate bacteria products (LPS, peptidoglycan, bacterial DNA), which could transport to the liver through portal vein, thereby inducing de novo lipogenesis and inflammatory response, leading to NAFLD development. On the other hand, the increased FXR antagonistic bile acid in NAFLD patients impair FXR signalling pathway, thereby inducing lipogenesis in the liver and imbalanced bile acids in the intestine.

Dynamic shifts in the gut-liver axis, as a result of alterations to the integrity of the intestinal epithelium or microbial composition have been implicated in NAFLD manifestation [16,17,39,40] (Fig. 1). The gut microbiota have the potential to increase intrahepatic fat through mechanisms such as altered appetite signalling, increased energy extraction from diet and altered expression of genes involved in de novo lipogenesis [18] or β-oxidation [41]. Integrative multi-omics analysis combining metagenomic, liver transcriptomics and metabolomics in steatosis patients revealed that gut microbiota and its metabolites influence host gene pathways involved in immune system and metabolic disorders [15,28].

3. GUT microbiota dysbiosis in NAFLD-HCC

The dysbiosis of gut microbiota is emerging as a new factor promoting the development of NAFLD-HCC. HCC has been reported to be linked to gut microbiota profile and inflammation in NAFLD patients with increased *Bacteroides* and *Ruminococcaceae* but decreased *Bifidobacterium* [42]. Administration of antibiotics and gut sterilization could reduce the prevalence of HCCs in obese mice, suggesting that microbiota dysbiosis plays a crucial role in the pathogenesis of HCC [43]. Microbiota research in HCC is at a preclinical stage [44]. How the microbiota contribute to NAFLD-HCC is largely unknown.

Gut microbiota produce a large array of bioactive molecules from mainly dietary compounds, establishing an intense microbiota-host transgenomic metabolism with a major impact on physiological and pathological conditions. Gut microbial dysbiosis is closely associated with hepatic inflammatory disease and HCC through the gut-liver axis. Dysbiosis causes an increase in inflammatory cytokine secretion, activation of TLR-4 and TLR-9, and modification of bile acids metabolism, hence promoting carcinogenesis [45]. The altered microbiota could increase the levels of deoxycholic acid (DCA), which is a BA generated by the gut microbiota. Enterohepatic circulation of DCA causes inflammatory and tumour-promoting factors in the liver, thereby facilitating HCC development in mice [43]. However, further studies are required to investigate the complex relationship between the microbiota and HCC. With the better understanding of the interactions between gut microbiota and liver through the gut-liver axis, new treatment strategies for HCC are being proposed and developed [44].

4. Metabolome in NAFLD and NAFLD-HCC

Small molecule metabolites generated by the gut microbiota has attracted great scientific attention, helping to understand the metabolic alterations that play role in the progression of NAFLD and NAFLD-HCC [46]. Lipids (short chain fatty acid, SCFA), glucose, amino acid, and bile acids metabolisms are now being studied to understand the complex pathogenesis of NAFLD and NAFLD-HCC [47]. Kalhan et al. reported that lipids (carnitines), amino acids (leucine, isoleucine, valine, and phenylalanine) and bile acids (glycocholate, taurocholate, and glycochenodeoxycholate) were elevated in NAFLD patients [48]. Metabolomics signatures from 78 NAFLD patients and 67 healthy controls showed that branched chained amino acids (BCAA), Glycocholic acid (GCA), Taurocholic acid (TCA), and Phenylanine associated positively with the development of simple steatosis to NASH, NASH-cirrhosis and HCC, while Glutathione was inversely associated [49]. Metabolomic profiling of NAFLD patients could be a useful tool for non-invasive diagnos is of NAFLD [50]. Knowledge gained on genetic signatures associated with NAFLD and NASH, as well as the role of plasma metabolites [49], should be applied in the clinical setting to identify patients at risk of NAFLD, NASH and HCC.

SCFAs including formate, acetate, propionate and butyrate can enter the liver through portal vein and cause lipid accumulation and glucogenesis [51]. On the other hand, metabolites derived from aromatic amino acids can have anti-inflammatory effects in host cells [52]. *Venkatesh* et al. reported that tryptophan derived metabolites could protect NAFLD through improving intestinal epithelial barrier integrity [53]. Moreover, NASH and HCC patients could be differentiated by metabolites including 2-methylguanosine, gluconic acid, indoxylsulfuric acid, cAMP, indolelactic acid, and acetyl-DL-leucine.

Bile acids and their receptor Farnesoid X receptor (FXR) play a critical role in NAFLD development (Fig. 1). As FXR showed protective effect on lipid and glucose metabolism, its modulators that influence endogenous bile acids levels could have beneficial therapeutic effects in NASH [54]. FXR/TGR5 agonist INT-767 significantly alleviates high-fat-induced liver steatosis and inflammation with restored lipid and glucose metabolism and attenuated insulin resistance through upregulating FXR level [54]. On the other hand, increased FXR antagonistic bile acid in NAFLD patients impair FXR signalling pathway in the liver [55], thereby inducing lipogenesis and damage fatty acid oxidation [56]. Therapeutic targets on FXR signalling and the microbiome related with bile acid production may provide option for NAFLD treatment. Indeed, FXR agonist obeticholic acid is in Phase 2/3 clinical trial for NAFLD therapy [57]. However, intestinal FXR promote NAFLD progression and thus the side effect should be considered [57].

5. GUT microbiota and immune response

Emerging evidences have suggested that gut microbiota induced inflammation in NAFLD and NAFLD-HCC through the immunomodulatory properities of the metabolites they produced [46]. The reciprocal interactions of the intestinal microbiota and immune system has been discussed [58]. Liver is central in immune regulation [59]. Gut microbiota could alter the immunological profile of the liver, including adaptive immunity (Treg, Th1, Th2, Th17, NKT cells and B cells) and innate immunity (Neutrophils, Kupffer cells, dendritic cells and NK cells) [24] (Fig. 2). Altered gut permeability can contribute to the activation of local immune responses, result in tissue inflammation

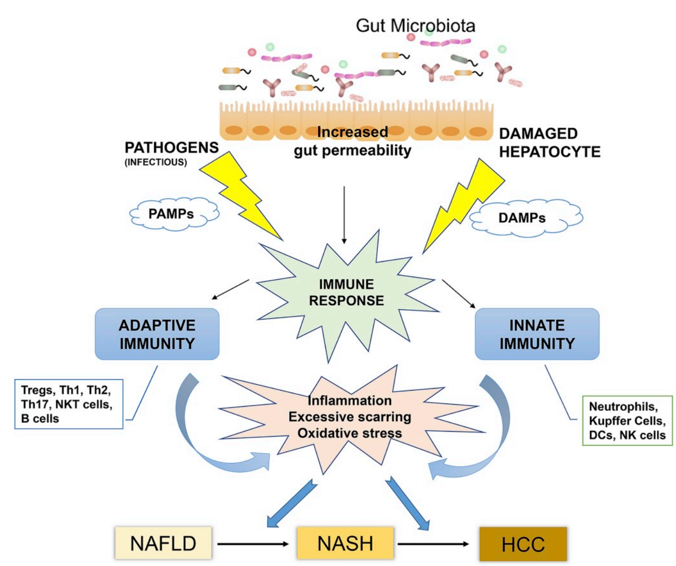

Fig. 2. The association of Gut Microbiota and Immunity in NAFLD. Gut microbiota could alter the immunological profile of the liver, including adaptive immunity (Treg, Th1, Th2, Th17, NKT cells and B cells) and innate immunity (Neutrophils, Kupffer cells, dendritic cells and NK cells). Microbe pathogen-associated molecular pattern molecules (PAMPs) could translocate to the liver through increased gut permeability and induce immune response and inflammation. Damage-associated molecular patterns (DAMPs) from damaged hepatocytes might act on various immune cells and contribute to chronic inflammation and proinflammatory cytokines/chemokines induction. Altered gut permeability can contribute to the activation of local immune responses, resulting in tissue inflammation, excessive scarring and oxidative stress, thus promoting fatty liver progression from steatosis to steatohepatitis and subsequently to cirrhosis or ultimately to HCC.

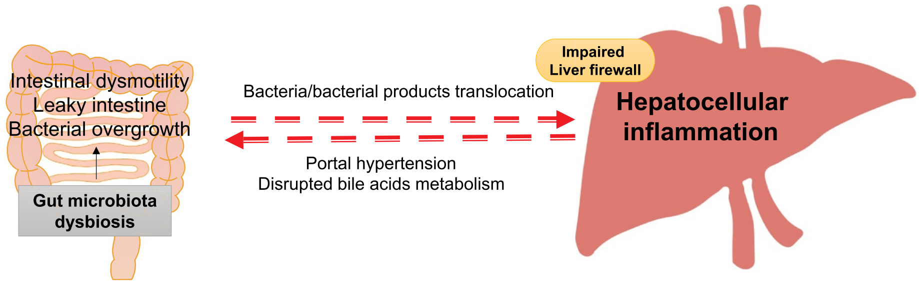

Fig. 3. The potential causal link between hepatocellular inflammation and gut microbiota dysbiosis. Dysbiotic microbiota, altered intestinal barrier, and bacteria overgrowth promote the translocation of several bacterial products into the portal circulation and/or disrupted bile acid metabolism. Hepatocellular Inflammation may be secondary to altered intestinal dysmotility and translocation of either intact bacteria or microbial cell components with impaied liver firewall. Liver disease may directly contribute to the alteration of intestinal permeability and microbiome dysbiosis through portal hypertension and/or disruption of bile acid metabolism.

and excessive scarring and provide a source of oxidative stress, hence promoting fatty liver progression from steatosis to steatohepatitis and subsequently to cirrhosis or ultimately to HCC [60] (Fig. 2). Microbe pathogen-associated molecular pattern molecules (PAMPs) could translocate to the liver through increased gut permeability and induce immune response and inflammation. Damage-associated molecular patterns (DAMPs) from damaged hepatocytes might act on various immune cells and contribute to chronic necroinflammation, proinflammatory cytokines induction and subsequent NAFLD and NAFLD-HCC development [61] (Fig. 2). A very recent study isolated a consortium of 11 bacterial strains from healthy human donor and found that these bacteria could promote systematic anti-cancer effect by inducing interferon-γ-producing CD8 T cells in the intestine [62]. Indeed, the contribution of the immune response to NAFLD progression by gut microbiota alteration is critical as deficiency or inhibition of innate or adaptive immune cells in mice results in less severe or no disease [63] (Fig. 2).

Dysbiotic microbiota and altered intestinal barrier through tight junction disruption promote the translocation of several bacterial products such as LPS into the portal circulation (Fig. 3) [64]. Small intestinal dysmotility and bacteria overgrowth are associated with the severity of NAFLD in patients with leaky intestine (Fig. 3) [65]. Bacterial translocation induces inflammation through inflammatory factors and causes hemodynamic derangement [66]. However, the contribution of the intestinal microbiome to liver disease goes beyond simple bacterial translocation that promote hepatic injury and inflammation as the host factors are also important in the disease progression [65]. Gut microbiota may also contribute to hepatic fibrosis by direct activation of hepatic stellate cells by LPS or by stimulation of profibrotic pathways through TLR9-dependent recognition of certain bacteria by Kupffer cells in the liver [67].

Although studies seem to suggest that hepatocellular inflammation may be secondary to altered intestinal permeability and translocation of either intact bacteria or microbial cell components into circulation [68] (Fig. 3), the causal link between them is disputable. The liver acts as a functional vascular firewall that clears commensals that have penetrated either intestinal or systemic vascular circuits [69]. Increased intestinal permeability and translocation of bacteria or their components may occur when the liver firewall is severely impaired. As such, liver disease may directly contribute to the alteration of intestinal permeability and microbiome dysbiosis through portal hypertension and/or disruption of bile acid metabolism [65,70]. It has been reported that the decrease in total fecal bile acids in cirrhosis patients could result in intestinal bacterial overgrowth as bile acids have direct bacteriostatic effects [65].

6. GUT microbiota-targeted therapeutics in NAFLD

Gut microbiota plays a vital role not only in the digestion and absorption of nutrients, but also in homeostatic maintenance of host immunity, metabolism and the gut barrier. As the therapeutic option for NAFLD is limited, interventions on the gut microbiota have been explored. Several attempts to target gut microbiota with probiotics, antibiotics, synbiotic or fecal microbiota transplantation (FMT) have been pursued.

6.1. Probiotics

Probiotics are live micro-organisms which are beneficial for host's health and wellbeing. Probiotics could modulate the intestinal microbiota and may be beneficial in NAFLD treatment through immunoregulatory effects [71]. These probiotic organisms are usually present in considerable numbers in the intestinal tract. However, their levels can decrease significantly in pathological circumstances. These microorganisms promote mucosal barrier function and modulate the gut microbiota, suppressing pathogenic microbial growth. Thus, administration of probiotics will benefit the host metabolism by improving the intestinal balance of these microbes. Commercialised probiotics include lactic acid and spore-forming bacteria. Most of them include combination of *Bifidobacteria* and *Lactobacilli* which have been tested in several studies [72]. Okubo et al. found that the administration of *Lactobacillus casei* orally to mice suppressed methionine-choline-deficient (MCD) diet–induced NASH markedly, with a reduction of serum LPS levels, inflammation and fibrosis in the liver [73]. Similarly, *Wagnerberger* reported that the administration of *Lactobacillus casei* protected against high fructose diet-induced NASH in mice through the suppression of TLR-4 signalling cascade in the liver [74].

One promising probiotic, with strong anti-inflammatory properties, currently being studied is *Faecalibacterium prausnitzii*. A recent animal study showed that *Faecalibacterium prausnitzii* can decrease inflammation in the adipose tissue and improve liver functions in mice with NASH [75]. *Akkermansia muciniphila* is also being researched as it has been shown to reduce fat mass, improve insulin sensitivity and dyslipidemia in mice [76].

6.2. Prebiotics

Prebiotics are selectively fermented ingredient that induces particular changes in the composition and/or activity of the gastrointestinal microbiota and confers health benefits on the host [77]. Increasing evidence has demonstrated that modulation of intestinal bacteria by prebiotics is beneficial to NAFLD patients. A recent pilot clinical trial studied the changes in liver biopsy non-alcoholic fatty liver activity score (NAS), body weight, glucose tolerance, inflammatory markers, and gut microbiota in 14 individuals with liver-biopsy-confirmed NASH (NAS ≥ 5) who received oligofructose (a mixture of fermentable dietary fibers) (8 g/day for 12 weeks followed by 16 g/day for 24 weeks) or isocaloric placebo for 9 months. Oligofructose attenuated hepatic steatosis and improved overall NAS score independent of weight loss accompanied with increased *Bifidobacterium* and decreased *Clostridium cluster XI* and I. Therefore, prebiotic supplementation reduced steatosis in NASH patients.

6.3. Synbiotics

Synbiotics, the synergistic combination of probiotics and prebiotics, have also been shown to have many potential effects in NASH models by regulating the gut microbiota, reducing the degree of fibrosis, and decreasing endotoxemia. In a study which involved 50 NASH patients [77], the administration of synbiotics presented a reduction in steatosis; however, it did not improve intestinal permeability or LPS levels.

6.4. FMT

FMT is a known concept involving the re-balance of the gut microbiota by transplanting fecal bacteria from healthy to diseased subjects. This operation has been reported in clinical use for various gastrointestinal and extra-intestinal diseases [78]. Promrat et al. applied FMT by transplanting gut bacteria from lean donors to NASH patients, which proved useful for declining the pathogenesis of NAFLD. In addition, a recent animal study [79] demonstrated the positive effects of FMT on lipid accumulation in the liver and hepatic histology. More studies and trials are however necessary to evaluate its long-term efficacy and safety.

6.5. Antibiotics

Antibiotics have been investigated as a possible therapeutic option. Combined administration of vancomycin and bacitracin decreased the abundance *Firmicutes* and *Bacteroidetes* in the gut, improving insulin resistance and secretion of glucagon-like peptide-1 [80]. Use of norfloxacin, neomycin together with cisapride has been shown to improve liver function in cirrhotic patients by altering bacterial overgrowth [81]. However, other studies showed no effect on liver function in NAFLD, following the administration of norfloxacin. Therefore, the role of antibiotics as a medical intervention for NAFLD is still being investigated and it is not established yet, due to the side effects and drug resistance that a long-term use can induce.

6.6. Others

A recent study showed that Diammonium Glycyrrhizinate (DG), a natural bioactive pentacyclic triterpenoid glycoside, protected against NAFLD in mice through modulation of gut microbiota and restoration of intestinal barrier [82]. The richness of gut microbiota was significantly increased by DG. DG augmented the levels of *Ruminococcaceae* and *Lachnospiraceae* which promoted SCFA production and elevated the abundance of probiotics such as *Lactobacillus*. In addition, DG supplementation dramatically alleviated intestinal low-grade inflammation, improved expression of tight junction proteins, increased goblet cell number and mucin secretion, and sequentially enhanced intestinal barrier function [82].

Non-selective beta-blockers (NSBB) have also been shown to be beneficial by reducing bacterial translocation [83]. Proton pump inhibitors (PPI), used by half of cirrhotic patients on a regular bases have been shown to change microbiota composition [84]. Omeprazole increases oral-origin *Streptococcaceae* in the stools, similar to the effect of the use of broad antibiotic therapy. However, the use of PPI was identified as a risk factor for hepatic encephalopathy and spontaneous bacterial peritonitis in patients with cirrhosis with ascites in a dose-dependent manner [85].

7. Concluding remarks

NAFLD and its associated HCC are increasingly important. Increasing evidence shows that the gut microbiota plays a key role in its pathophysiology. In this review, we have summarised the relationship between the gut microbiota and NAFLD and its associated HCC, discussed metabolites alteration in the disease and highlighted current perspectives and therapeutic implication of the gut microbiota.

Conflicts of interest

There are no conflicts of interest to disclose.

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