






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Review

Gut Microbiota and Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD): Emerging Pathogenic Mechanisms and Therapeutic Implications

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Abstract: Non-alcoholic fatty liver disease (NAFLD), recently redefined as metabolic dysfunction-associated steatotic liver disease (MASLD), is the most common cause of chronic liver disease worldwide. Characterized by excessive hepatic fat accumulation, this disease encompasses a spectrum from simple steatosis to more severe forms, including steatohepatitis, fibrosis, and cirrhosis. Emerging evidence highlights the pivotal role of gut dysbiosis in the pathogenesis of MASLD. Dysbiosis disrupts the gut–liver axis, an intricate communication network that regulates metabolic, immune, and barrier functions. Alterations in gut microbiota composition, increased gut permeability, and translocation of pro-inflammatory metabolites/factors have been shown to trigger liver inflammatory and fibrotic cascades, exacerbating hepatic inflammation and injury. Recent studies have identified microbiome signatures associated with MASLD, offering promise as non-invasive diagnostic biomarkers and paving the way for new potential therapeutic strategies targeting gut dysbiosis. This review explores the crucial role of the gut microbiota in MASLD pathogenesis and highlights the need for further targeted research in this field to validate

microbial biomarkers and optimize therapeutic strategies. Comprehensive understanding of the gut–liver axis may enable innovative diagnostic and therapeutic approaches, transforming the clinical management of MASLD.

Keywords: MASLD; NAFLD; steatosis; liver; gut microbiota; gut microbiome

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterized by the abnormal accumulation of fat within hepatocytes (>5%) in the absence of other common causes, such as significant alcohol consumption, autoimmune or viral hepatitis, amongst others [1]. It is currently the most common cause of chronic liver disease globally, affecting up to 25–30% of the general adult population and posing a major public health concern with a considerable clinical and economic burden [2,3]. Given the established relationship of NAFLD with cardio-metabolic diseases and risk factors, in 2023, a Delphi consensus statement introduced the term metabolic dysfunction-associated steatotic liver disease (MASLD) to underscore the association of NAFLD with metabolic dysfunction, which may co-exist with liver disease, and remove the term “fatty” from the relevant nomenclature due to its association with perceived stigma [4,5]. Notably, in contrast to the diagnosis of NAFLD, which is defined by the absence of other liver conditions, the proposed diagnostic criteria for MASLD are dependent on hepatic steatosis and the presence of at least one of five cardio-metabolic risk factors, namely elevated body mass index (BMI), fasting glucose, triglycerides, blood pressure, and low HDL cholesterol [4]. The proposal of the new MASLD nomenclature has also raised a debate regarding its interchangeable use with the term NAFLD, and, accordingly, the inter-application of related scientific evidence [6,7]. Although similar pathophysiological mechanisms are believed to likely underpin both of these entities, research specifically exploring MASLD and the impact of the new MASLD diagnostic criteria in comparison to NAFLD is now growing, but is still limited [6–8]. For the purposes of the present review in the following sections, the term MASLD is used to describe the disease and related pathophysiological mechanisms; however, the term NAFLD is used where the related primary research study was before the introduction of the MASLD nomenclature/definition and/or refers to a NAFLD diagnosis/model.

MASLD encompasses a disease spectrum ranging from simple steatosis to metabolic-associated steatohepatitis (MASH)—previously referred to as non-alcoholic steatohepatitis (NASH)—which may then progress to hepatic fibrosis, cirrhosis, and even hepatocellular carcinoma (HCC) [8]. The exact pathophysiology underlying the progression of this disease remains to be completely understood, as its course can vary significantly amongst individuals [9,10]. In contrast to previous assumptions that suggested a linear disease progression, recent insights reveal a more complex disease trajectory, where the disease may progress to MASH or cirrhosis, whereas others may see stabilization or even regression of their condition [9,10]. As such, the pathogenesis of MASLD is a complex interplay of genetic, metabolic, and environmental factors [8–10]. For example, genome wide association studies have identified the role of specific genes in association with MASLD, including *PNPLA3* (patatin-like phospholipase containing 3), *TM6SF2* (transmembrane 6 superfamily member 2), *MBOAT7* (membrane bound O acyltransferase 7), and *GCKR* (glucokinase regulator) [11]. Furthermore, lifestyle factors, such as high-fat/high-sugar diets, and a sedentary lifestyle with low levels of physical activity, have been implicated in promoting the development and progression of MASLD [12,13]. Relating to lifestyle factors, it is also noteworthy that the aforementioned 2023 Delphi consensus statement

introduced the term metabolic and alcohol related/associated liver disease (MetALD) to describe MASLD in those with a weekly intake of alcohol greater than 210–420 g and 140–350 g for males and females, respectively [4]. Indeed, alcohol consumption is linked to increased risk of multiple cancer types, including HCC, through various underlying mechanisms which appear to include increased inflammation, oxidative stress, reactive oxygen species, acetaldehyde toxicity and DNA damage [14,15].

Recent evidence has also suggested the role of gut dysbiosis in the pathogenesis of MASLD [16–18]. The human microbiome describes the entirety of microorganisms that inhabit the human body, and since these reside mostly in the gut, it is often referred to as the gut microbiome [16,17,19]. The adult gut bacteria belong to two main phyla, namely Gram-positive Firmicutes and Gram-negative Bacteroidetes [16,17,19]. Overall, the gut microbiome is responsible for the regulation of the micro-environment and immune-system reactions in the gut and is therefore involved in maintaining physiological homeostasis [18]. However, changes secondary to genetic and environmental factors may create an imbalance between the gut microbiota and the host, resulting in modulation of the structure or diversity of the gut microbiota, known as “gut dysbiosis” [20]. This dysbiosis may then trigger a cascade of metabolic derangements implicated in the pathogenesis of various disease states, including obesity, type 2 diabetes mellitus (T2DM), and MASLD [20]. Understanding the pathogenic mechanisms behind MASLD and the contribution of the microbiome may aid our understanding of the disease’s natural history and risk of progression, which may improve diagnostics and promote the identification of novel therapeutic targets.

Based on the increasing body of relevant translational and clinical evidence, the present review aims to summarize recent data on the role of gut microbiota in the pathogenesis of MASLD, including implicated microbial signatures and factors that promote dysbiosis. Insights are also highlighted regarding the potential of microbial-based diagnostic biomarkers and the alteration of gut microbiota through therapeutic interventions, such as the use of probiotics, prebiotics, synbiotics, phages, fecal microbiota transplantation (FMT) and novel targeted therapies.

2. Gut Microbiota Dysbiosis and Microbial Signatures Associated with MASLD

Recent research has evolved our understanding of the gut microbiome, revealing it to be a dynamic ecosystem of microorganisms which plays an active role in the digestion and absorption of nutrients, whilst interacting with the host through endocrine/immune-mediated mechanisms [20,21]. The gut microbiota modulates the host’s immune system through the release of microbial-derived metabolites which are transported via the portal circulation and ultimately systemic circulation [20]. These microbial-derived metabolites affect antigen recognition, immune cell recruitment and proliferation and induce both pro- and anti-inflammatory cascades [22]. The association between compositional and functional alterations of the gut microbiota and MASLD, including severe disease states, such as cirrhosis and HCC, have long been described in the literature [20,23]. Referred to as gut dysbiosis, this often entails either a reduction in commensal bacteria, complete loss of microbial species, or overgrowth of pathogenic commensals, which then results in immune-mediated changes contributing/causing metabolic derangements and disorders [21]. Of note, the outgrowth of pathobionts (pathogenic commensals), such as *Proteobacteria*, particularly the *Enterobacteriaceae* family, is observed in prevalent metabolic disorders (e.g., obesity, T2DM and MASLD), and is therefore considered a potential diagnostic marker of gut dysbiosis [24,25].

Gut dysbiosis is driven by the complex interactions between the genetic background of the host (including their health status and drug medications) and lifestyle habits (e.g., diet

and physical activity/exercise) [21]. Changes in diet have demonstrated significant shifts in the composition of the gut microbiota, with diets rich in fat and sugars exhibiting an association with intestinal inflammation and weakening of the gut barrier in both animal models and human studies [26,27]. Food additives and preservatives, which are now integral to most processed food items in Western-type diets, have also been shown to promote the overgrowth of *Proteobacteria* and directly alter the gut microbial composition, inducing inflammation of the gastrointestinal system [21,28,29].

Strong evidence for NAFLD and increasing evidence for MASLD show that this disease spectrum occurs in the context of metabolic dysfunction often in tandem with obesity and poor dietary habits. Alterations in the gut microbiota have been implicated in NAFLD/MASLD, including NASH/MASH, as demonstrated in animal models and human studies [18,19,21]. Of note, Rabot et al. described that germ-free mice showed lower lipid levels in the liver compared to conventional mice [30]. This was further reinforced by the transfer of gut microbiome into germ-free mice, which resulted in the development of fasting hyperglycemia, and NAFLD when compared to healthy mice [31]. Certain bacterial species, such as *Lachnospiraceae* spp. and *Barnesiella intestinihominis*, were positively associated with NAFLD in mice compared to controls, while *Bacteroides vulgatus* was negatively under-represented in the NAFLD animal model [31]. In most human studies of NAFLD or MASLD, gut dysbiosis is hypothesized to occur through the malfunction of the defense mechanisms of the gut–liver axis where invading pathogens and toxins induce systemic inflammation [21]. The most accepted microbial signature amongst NAFLD patients includes an overgrowth of *Bacteroidetes* spp. and differences in the composition of *Firmicutes* spp., resulting in a decreased *Firmicutes* spp. to *Bacteroidetes* spp. ratio [32,33]. An important consideration is that this ratio only represents a crude estimate given the huge diversity of microorganisms within these phyla [19]. Additionally, differences in the molecular methods used to identify these species may yield significantly different results. Species that have demonstrated a positive association with NAFLD patients include the *Enterobacteriaceae* family, *Escherichia* spp., *Clostridium* spp., *Anaerobacter* spp., *Streptococcus* spp., and *Lactobacillus* spp., whereas *Rikenellaceae*, *Ruminococcaceae*, *Faecalibacterium* spp., *Coprococcus* spp., *Anaerospobacter* spp., and *Eubacterium* spp. have become less prominent [21,34]. Amongst NASH patients when compared to healthy controls *Proteobacteria*, *Enterobacteriaceae*, and *Escherichia* demonstrated increased abundance, while *Faecalibacterium prausnitzii* (*F. prausnitzii*) and *Akkermansia muciniphila* were comparatively reduced [19,35,36]. Functionally, there is a shift from beneficial to harmful microbes leading to the promotion of a pro-inflammatory state which is the result of gut barrier dysfunction, exposing the liver to microbiota-derived factors and culminating in disease progression [36]. Unsurprisingly, the NAFLD associated microbial signatures also overlap with other metabolic diseases, where reduced levels of *F. prausnitzii* were observed in cirrhosis, obesity, T2DM, and inflammatory bowel disease [37–40]. *F. prausnitzii* is considered a beneficial microbe given its role in promoting anti-inflammatory pathways [38]. Conversely, increased levels of *B. vulgatus* were associated with advanced fibrosis, insulin resistance, and T2DM, promoting pro-inflammatory cascades [32].

Differences in microbiome composition between patients with NAFLD or MASLD based on their BMI have also been documented [2,41]. In one cohort lower levels of *Ruminococcaceae* and increased levels of *Veillonellaceae* with lower species diversity were noted amongst NAFLD patients with obesity compared to those that were lean [42]. These differences are not limited to bacterial species alone, but also viruses and fungal species [43,44]. Furthermore, Lang et al. demonstrated that patients with NAFLD and an increased disease activity score exhibited decreased bacteriophage diversity when compared to patients with lower disease activity scores [43]. It should be noted that, given the recent intro-

duction of the MASLD nomenclature, most of the relevant human studies have been conducted under the NAFLD diagnostic criteria. Studies in this field which adopt the MASLD nomenclature/criteria are now emerging, with one such study showing a different microbial signature for MASLD [45], which was characterized by a higher abundance of *Ruminococcaceae*, *Bilophila* spp., and *Sellimonas* spp., and a lower presence of *Deftuviitaleaceae*, *Lachnospiraceae* and *Coprobacter* spp. This highlights the need for further targeted studies exploring the impact of the newly defined MASLD diagnostic criteria on the gut microbiome to build a robust body of evidence specifically for MASLD which will supplement and may even show differences from that for NAFLD.

Collectively, most of the evidence discussed originates from association studies, which have demonstrated that correlations between gut microbial compositions and NAFLD or MASLD exist. However, current evidence is significantly limited by the lack of studies on potential underlying causal links, and by the marked heterogeneity between the relevant studies due to differences in geographical location, diet and co-morbidities which limit the reproducibility of results between cohorts [22]. Thus, potential underlying causal links remain to be determined, exploring whether gut dysbiosis is a direct cause rather than a disease-associated effect resulting from the host's immune and endocrine/metabolic systems.

3. Mechanisms of Microbiome Modulation

The gut–liver axis refers to the bi-directional interplay between the gut epithelium, the biliary tract, immunological barriers, and the hepatic circulation (portal and systemic circulation). Gut-derived factors (e.g., microbial and by-products of dietary intake) regulate bile acid synthesis and liver metabolism, whilst liver-derived factors (e.g., bile acids) also affect gut microbial composition and function [21]. Any disruption of this regulated symbiotic relationship of the gut–liver axis (e.g., through gut dysbiosis or increased intestinal permeability) can result in failure to regulate the gut microbiota and promote hepatic pro-inflammatory changes [18,21]. Figure 1 summarizes key aspects of the relationship of the gut–liver axis with certain factors which relate to gut dysbiosis and have been implicated in the pathogenesis of NAFLD/MASLD.

The gut barrier is composed of a mucus layer and an epithelial cell layer, which are held together via tight junction proteins, such as claudins and occludins [46]. These proteins play a significant role in maintaining the integrity of the gut barrier by hindering the translocation of microbial pathogens to the intestinal epithelial cells [18,46]. The gut barrier also consists of commensal bacteria, mucins secreted by goblet cells, and an immunological barrier composed of components of both cellular and humoral immunity [18,46]. Antimicrobial peptides and immunoglobulins, such as IgA, control the load and composition of microorganisms in the lumen, by binding to microbial antigens and toxins and transporting infiltrated bacteria to mesenteric lymph nodes to induce the priming of the adaptive immune response in the gut [21]. Emerging evidence has demonstrated that disruption of the gut barrier function (e.g., through disturbance of tight junctions) promotes intestinal permeability and excessive para-cellular leakage, which can lead to the development of NASH/MASH in patients [47–49]. A meta-analysis by Luther et al. has shown that NAFLD patients had altered gut permeability compared to healthy controls, with a stronger association in patients with NASH [49]. This is reinforced on a molecular level, where patients with NAFLD exhibited lower levels of expression of tight junction proteins such as zonula occludens-1 and junctional adhesions molecules [47,50]. An unhealthy diet composed of low fiber, high sugar and food additive content, as well as excessive alcohol consumption, and excessive exposure to antibiotics and non-steroidal anti-inflammatory drugs (NSAIDs) have also been associated with tight junction disruption

and increased intestinal permeability [51–53]. However, underlying causal links between gut barrier dysfunction and NAFLD/MASLD still remain unclear, with some evidence demonstrating that disrupted gut permeability is a consequence rather than a cause of the disease [22]. Indeed, in one animal model study alterations in tight junction proteins were only shown after initial hepatic injury [49]. Despite the inconsistency of evidence regarding the initial triggering pathogenetic event, there is consensus that impaired gut barrier function further exacerbates steatohepatitis [21,22]. According to Gabele et al., the induction of inflamed and disrupted gut permeability led to the translocation of microbial derived factors, such as lipopolysaccharide (LPS; or endotoxin), to systemic circulation, which resulted in worsening hepatic inflammation and fibrosis [54].

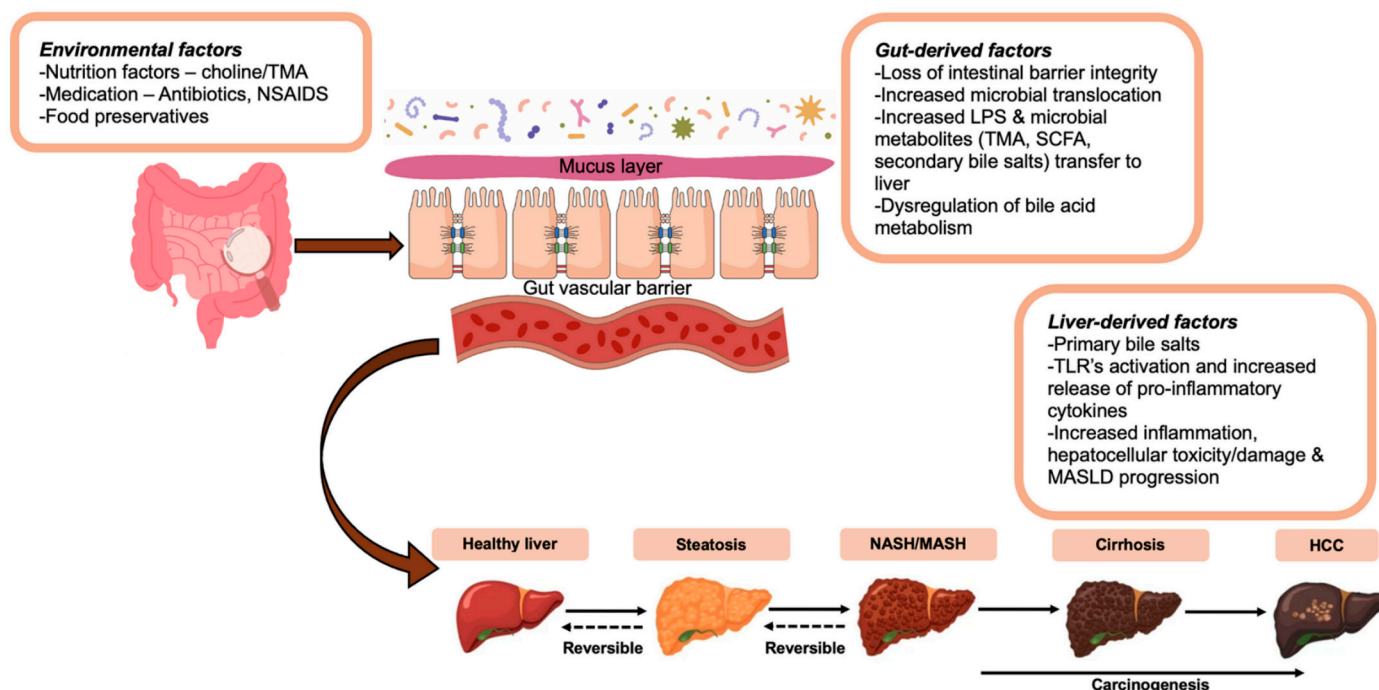


Figure 1. The gut–liver axis and key factors contributing to gut dysbiosis in the pathogenesis of NAFLD/MASLD. These include factors relating to the environment, diet and medications (e.g., the use of food preservatives and antibiotics), gut-derived factors (e.g., loss of the intestinal barrier integrity) and liver-derived factors (e.g., increased hepatic inflammation) which are either directly or indirectly implicated in gut dysbiosis and the development and/or progression of NAFLD/MASLD. Abbreviations: LPS, lipopolysaccharide (endotoxin); MASLD, metabolic associated steatotic liver disease; NAFLD, non-alcoholic fatty liver disease; NSAIDS: Non-steroidal anti-inflammatory drugs; SCFA, short-chain fatty acids; TMA, trimethylamine; TLR, Toll-like receptors.

The immunological tolerance of the liver is also important in the understanding of the pathogenic mechanisms underpinning the modulation of the gut microbiome. The liver is capable of processing low-level exposure to innocuous molecules, such as microbial antigens, by hepatic antigen presenting cells [55]. Antigen presentation by this collection of cells results in the suppression of T-cell responses and induces anti-inflammatory cytokines (e.g., transforming growth factor-beta and interleukin-10) [55]. This mechanism protects the liver from immune-mediated injury and induces hepatic tolerance. However, when there is a dysfunction of the gut barrier function, the liver is overwhelmed with gut-mediated antigens, resulting in the loss of immunological tolerance and the development of a pro-inflammatory state [56]. Activation of pattern recognition receptors on hepatic macrophages by microbial antigens results in increased production of inflammatory and fibrogenic cytokines (e.g., tumor necrosis factor-alpha, interleukin-1, interleukin-6), and

activation of CD8+ cytotoxic T-cells, which contributes to inflammatory-mediated hepatic injury [55,56]. The activation and maintenance of hepatic inflammation may then lead to fibrosis, cirrhosis, or even HCC [56].

Various microbiome-derived compounds have been implicated in the pathogenesis of NAFLD/MASLD, including short-chain fatty acids (SCFAs), LPS, choline, and choline metabolites. SCFAs are fatty acids with fewer than six carbons, and commonly include acetate, propionate and butyrate [21]. Produced via anaerobic fermentation by gut microbiota from non-digestible starch and fiber, these SCFAs play an important role in promoting intestinal integrity, supporting glucose (gluconeogenesis) and lipid (lipogenesis) metabolism, as well as immune-modulation [57]. Notably, increased fecal levels of SCFAs (acetate and propionate) were found to be associated with NAFLD and hepatic fibrosis [32]. This impact is mediated through the functioning of these SCFAs as signaling molecules via the activation of G-protein coupled receptors (GPR41 and GPR43), triggering the secretion of peptide-YY from gut entero-endocrine cells which in turn slows gastric emptying, thus increasing nutrient absorption and hepatic lipid accumulation and gluconeogenesis [58]. However, some SCFAs, such as butyrate, have demonstrated anti-inflammatory properties and may play a protective role against diet-induced obesity, hepatic steatosis, and insulin resistance [59,60]. Therefore, the metabolic and immune-modulatory effects of SCFAs have the potential for both harmful and protective impact; although the majority of published data favor the induction of anti-inflammatory properties, but under certain conditions pro-inflammatory cascades are also induced [61,62]. Human studies have also reflected this, with circulating butyrate measured in patients with cirrhosis being inversely correlated to inflammatory makers in one study, whereas in another study increased levels of butyrate were associated with mild or moderate NAFLD [63,64]. Animal models of NAFLD have shown that supplementation of SCFAs (butyrate) in mice fed a high-fat diet resulted in reductions in hepatic and adipose tissue inflammation, and reduced endotoxin-releasing bacteria in the gut microbiome [57,65]. This may suggest that SCFA supplementation may have beneficial metabolic effects, although large-scale human studies of SCFA supplementation amongst patients with NAFLD or MASLD are lacking to support this. Variations in diet, and other factors, as well as in SCFA sample processing, are likely to have contributed to the observed differences amongst clinical studies, highlighting the need for further, well-designed research to determine the potential contribution of SCFAs in the pathogenesis of MASLD and discriminate whether the net impact is positive or negative.

Another important gut-derived factor to consider is choline, which is mainly obtained from dietary sources such as eggs, cheese, and red meat [66]. Notably, choline is required for the synthesis of phosphatidylcholine, a component of cell membranes, whilst it is also necessary for the production of both the neurotransmitter acetylcholine and very low-density lipoproteins (VLDLs) [21]. Given the latter, choline deficiency results in reduced production of VLDLs and the consequent accumulation of triglycerides in the liver [67]. As such, choline-deficient diets have been used as a method to induce NASH/MASH in animal models, with choline supplementation reversing the disease process [68]. However, under certain conditions choline may play a role in the development of NAFLD/MASLD. Specific gut microbiota can convert choline into trimethylamine (TMA), which is then oxidized by hepatic monooxygenases to trimethylamine N-oxide (TMAO) that is considered a harmful metabolite [67,68]. Choline conversion by the gut microbiota is considered to play a role in NAFLD/MASLD pathogenesis through direct and indirect mechanisms [21]. Animal models have shown that higher rates of conversion of choline to TMA by the gut microbiota leads to reduced bioavailability of choline, resulting in hepatic inflammation and lipid accumulation mediated by lower VLDL levels [66,69]. Additionally, TMAO may act directly on the liver contributing to impaired glucose metabolism through the reduced

activity of CYP7A1 and CYP27A1 enzymes, decreasing the conversion of cholesterol into bile acids and promoting hepatic steatosis as demonstrated in both preclinical and clinical studies [70,71]. Compared to healthy controls, patients with NAFLD have exhibited higher levels of TMAO, and this was also correlated with disease severity [72]. Similar associations have been shown between TMAO and cardio-metabolic diseases, such as T2DM and cardiovascular disease [73]. TMAO promotes macrophage migration and their transformation into foam cells and has been associated with endothelial dysfunction and thrombus formation, increasing the risk of atherosclerosis and cardiovascular disease [74]. Notably, TMAO has been established as a prognostic marker for both short- and long-term cardiovascular events in patients with acute coronary syndrome [75]. However, the potential of serum TMAO levels as a biomarker of MASLD progression and prognosis or even as a therapeutic target for atherosclerosis remains to be elucidated in future studies.

Bile acids have also been implicated in the pathogenesis of NAFLD/MASLD in relation to gut microbiome modulation [76]. Bile acids are synthesized in the liver from cholesterol and are secreted in the form of conjugated bile salts via the bile duct [76]. They are classified as primary (e.g., cholic acid and chenodeoxycholic acid) and secondary bile acids (e.g., deoxycholic and lithocholic acid) [76]. Primary bile acids are conjugated in the gall bladder before being secreted into the intestines where they aid in the absorption of lipids and lipid-soluble vitamins, whilst they also prevent bacterial overgrowth and maintain the composition of the microbiome [77]. Gut microbiota deconjugate primary bile acids into secondary bile acids, which are then reabsorbed in the distal ileum and are recycled to the liver through the portal circulation [76,77]. In the presence of dietary triggers, changes in the secretion of bile acids may result in alterations of the gut microbiome, which has been associated with steatosis/steatohepatitis [77]. According to Chen et al., increased levels of primary bile acids (chenodeoxycholic acid) were noted in NASH patients and correlated with the underlying histological severity and fibrosis grade [78]. This is likely mediated through the signaling capabilities of bile acids, where they bind to cellular receptors, such as the nuclear farnesoid X receptor (FXR) and TGR5 (G protein-coupled bile acid receptor 1; GPBAR-1) [79]. A plausible hypothesis is that gut dysbiosis results in modification of the primary and secondary bile acid balance, resulting in disruption of FXR signaling and the promotion of metabolic and immune modulatory responses, including lipid and glucose dysregulation [17,79]. However, due to the dearth of animal and human studies regarding the role of bile acids in the pathogenesis of NAFLD/MASLD, it is difficult to draw robust conclusions. Notably, bile acids can be influenced by diet (e.g., by dietary protein and fiber content) [80]; hence, more translational and clinical research is required to elucidate the potential pathophysiologic links between bile acids and MASLD and how these may be addressed by therapeutic interventions, such as certain dietary changes. A detailed description of the molecular pathways (e.g., bile acids signaling, FXR regulation and TLR4 signaling) which are implicated in the pathophysiologic mechanisms that appear to mediate the development of obesity, MASDL and other obesity-related diseases is beyond the scope of the present review as these have been discussed in detail in other reviews [81–88].

4. Microbiota-Based Biomarkers and Therapeutic Implications

Our increasing understanding of the gut microbiome has resulted in its emergence as a potential diagnostic and therapeutic target for MASLD. Currently, liver biopsy is the gold standard for diagnosis, but given its invasive nature there is a growing drive for reliable and non-invasive markers [8]. The gut microbiome represents a rich source of metabolite biomarkers, which has prompted clinical exploration into the utility of gut, serum and fecal microbiota and metabolite biomarkers. According to Loomba et al. a 37-bacterial strain panel was able to accurately predict the presence of fibrosis in patients with NAFLD [32].

Progression of NAFLD may also be monitored by the integration of multiple measurements of blood serum metabolites, as highlighted in the study by Hoyles et al., where data from metagenomics, liver transcriptomics and metabolomics were integrated as a potential diagnostic tool to determine disease stages [89]. Additionally, associations have been identified between the presence of *Veillonellaceae* bacteria and metabolites produced by NAFLD-associated microbes, such as *Bacteroidaceae* and *Prevotella* spp. [42,90]. These associations were found to be elevated in fecal samples from patients, suggesting their potential utility as diagnostic markers for MASLD [42,90]. However, the use of fecal samples as a biomarker source for MASLD is challenged by the confounding effects of age, sex, diet, medication, hormonal and lifestyle factors on the gut microbiota and the associated high cost of sample processing [18]. Moreover, the feasibility of using the gut microbiota as a potential therapeutic target is gaining substantial attention given its role in modulating host metabolism and immunity. Emerging research has explored the potential in mitigating hepatic inflammation and metabolic dysfunction through restoration of microbial balance by interventions like targeted therapies, probiotics, prebiotics, synbiotics, bacteriophages and FMT.

Therapeutic drugs proposed for NAFLD/MASLD include drugs targeting bile acid regulation, such as FXR agonists and antagonists, as well as fibroblast growth factor (FGF) mimetics [22,91]. Other innovative treatments, including peroxisome proliferator-activated receptor (PPAR) agonists, glucagon like peptide-1 (GLP-1) agonists, and thyroid hormone receptor beta (THR- β) agonists, are also being actively explored [22,92]. These therapies are at various stages of clinical development, with several showing encouraging results in terms of improving hepatic steatosis, reducing fibrosis, and addressing systemic metabolic dysfunction [92,93]. Table 1 summarizes these pertinent therapeutic agents for the treatment of NAFLD/MASLD (under investigation/development or approved) which should also be explored regarding their impact on gut microbiota. These drugs and their targets have been discussed extensively in other reviews [92–97].

Table 1. Selected pertinent therapeutic agents for the treatment of NAFLD/MASLD (Abbreviations: ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; FGF, fibroblast growth factor, FXR, farnesoid X receptor; GGT, gamma-glutamyl transferase; GLP-1, glucagon-like peptide 1; MASLD, metabolic associated steatotic liver disease; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PPAR, peroxisome proliferator-activated receptor; THR- β , thyroid hormone receptor beta).

Drug [Ref.]	Selected Key Clinical Evidence to Date		Drug Target and Mechanism of Action
	Cohort	Key Results	
Nidufexor [98]	NASH patients	Improved NAFLD activity score and lower triglyceride levels	FXR agonists. FXR is a nuclear receptor primarily expressed in the liver. FXR activation regulates bile acid synthesis and improves lipid metabolism. This modulation of bile acids and cholesterol homeostasis has been shown to reduce hepatic inflammation and injury
PX-104 [99]	Non-diabetic NAFLD patients	Improved insulin sensitivity and liver enzymes (ALT and GGT) levels	
Cilofexor [100]	NASH patients	Reduction in elevated liver enzyme (GGT) levels, hepatic steatosis and serum bile acids	
Tropifexor [101]	NASH patients	Reduction in elevated liver enzyme (ALT and AST) levels, and hepatic steatosis	
Obeticholic acid [102,103]	NASH and NASH-related fibrosis patients	Improved NASH activity scores and fibrosis severity	
Ursodeoxycholic acid [104]	NASH patients	Reduction in elevated liver enzyme (ALT and GGT) levels	
Semaglutide [105]	NASH patients	Significant NASH resolution in patients on semaglutide compared to placebo	
Aldafermin [106]	NASH and NASH-related fibrosis patients	Dose-dependent reductions in serum bile acids and improvements in fibrogenesis markers	
Efruxifermin [107]	NASH-related fibrosis	Improvements in fibrosis stage and fibrogenesis markers	
Saroglitazar [108]	NAFLD patients	Reduction in elevated liver enzyme (ALT) levels and improved hepatic stiffness and metabolic parameters	
Resmetirom [109]	NASH-related fibrosis	Significant NASH resolution and improvement in fibrosis stage compared to placebo	THR- β agonist. THR- β agonists activate the beta thyroid hormone receptors predominantly expressed in the liver. This enhances hepatic fatty acid oxidation, reduces lipogenesis, and lowers circulating triglycerides, resulting in reduced hepatic steatosis and improved glucose and lipid metabolic profiles

Given the contribution of gut dysbiosis to disease pathogenesis, probiotics (defined as live microorganisms that confer health benefits when consumed in adequate amounts) have been proposed for the treatment of NAFLD/MASLD [110,111]. According to the International Scientific Association for Probiotics and Prebiotics consensus statement, probiotics have demonstrated potential in improving hepatic function and lipid profiles in patients with NAFLD [110,111]. However, the evidence remains inconsistent, with most studies conducted on animal models [18,110]. The most widely used probiotics include *Bifidobacterium* and *Lactobacillus* species or a combination of them, but next generation probiotics, such as *F. prausnitzii*, *A. muciniphila*, or *Clostridium* strains, have also demonstrated beneficial results [3,41]. Notably, combination probiotics, such as the high concentration probiotic mixture VSL#3 with eight probiotic strains of the genera *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* [112], have proven superior to single probiotics [18]. Animal models have demonstrated that VSL#3 has a protective effect against NAFLD by inhibiting inflammatory pathways (e.g., c-Jun N-terminal kinase and nuclear factor-kappa B signaling), and by maintaining the integrity of the gut-barrier (therefore improving gut dysbiosis through these effects) [113,114]. However, in another animal study, VSL#3 did not alter hepatic inflammation, although some improvement in hepatic fibrosis was observed [115]. According to a randomized controlled trial (RCT) patients with NAFLD receiving VSL#3 exhibited improved triglyceride levels and inflammatory markers when compared with controls [116]. Similarly, another RCT demonstrated that the administration of a 16-strain probiotic mixture to patients with NAFLD resulted in amelioration of histological findings, such as reduced hepatocellular ballooning and fibrosis along with improved hepatic function, when compared to placebo [117]. In contrast, a further RCT, which evaluated a probiotic mixture containing strains of *Lactobacillus* and *Bifidobacterium*, did not demonstrate any significant improvement in hepatic steatosis or function amongst patients with NAFLD [118]. This was also echoed by the findings of other RCTs where no improvement in hepatic function, steatosis or fibrosis was observed amongst patients receiving combination probiotics [119,120].

Contrary to probiotics, which include live organisms, prebiotics constitute non-digestible compounds, predominantly carbohydrates, which may lead to beneficial changes to the host through influencing the composition and/or activity of the gut microbiota [111]. Thus, prebiotics are defined as substrates which are selectively utilized by host microorganisms and confer a health benefit, working primarily by promoting the growth of beneficial bacteria via their degradation by enzymes in the gut, and this definition has been expanded to include non-carbohydrate substances as well [111]. For example, they increase the production of *Lactobacillus* and *Bifidobacterium*, which confers positive alterations in the composition and function of the gut microbiota [21]. Prebiotics have also been associated with increased production of SCFAs, which as aforementioned have anti-inflammatory properties and may promote the intestinal barrier integrity [121]. Overall it is considered that, by modulating the composition of the gut microbiota, prebiotics modify the concentrations of SCFAs, bile acids, and other microbial-derived molecules transported to the liver to alleviate hepatic inflammation and steatosis associated with NAFLD/MASLD [121]. The exact potential of prebiotics in NAFLD/MASLD treatment is limited by the scarcity of clinical studies, with the need for further research to fully elucidate their role as an impactful therapeutic modality.

Synbiotics are a combination of probiotics and prebiotics which have also demonstrated therapeutic potential for NAFLD/MASLD, by way of reducing de novo lipogenesis and stimulating fatty acid beta-oxidation [17]. Although not definite, there are data indicating that synbiotics supplementation in patients with NAFLD have a positive impact on BMI and hepatic function [122]. Other studies have also described the role of synbiotics

in improving liver stiffness and promoting anti-inflammatory and antioxidant properties [122,123]. However, similar to probiotics and prebiotics, not all results have been consistent, and further, large-scale clinical research studies are needed to investigate their therapeutic potential.

The utilization of bacteriophages and FMT in NAFLD/MASLD is considered to be in its early stages when compared with the use of pro-/pre-/syn-biotics as relevant therapeutic options. Bacteriophages are viruses that infect and kill bacteria, which have been proposed as a tool for targeting dysbiotic parts of the gut microbiota in patients with NAFLD/MASLD [18]. However, so far, these have been found effective only in alcoholic liver disease, targeting *E. faecalis*, which is overrepresented in such patients, resulting in improved hepatic function and inflammation [124]. Concerns regarding the safety and cost of phage therapy may also represent a challenge for their future application [17]. Similarly, FMT has only been used widely in the context of *Clostridium Difficile* infections and to a lesser extent in hepatic encephalopathy [125,126]. Animal studies have demonstrated the beneficial effects of FMT on hepatic lipid accumulation and its success in patients with cirrhosis and alcoholic hepatitis [127,128]. An RCT by Xue et al. in patients with NAFLD, showed that FMT from healthy donors resulted in reductions in hepatic lipid accumulation and improved lipid profiles through amelioration of gut dysbiosis [129]. Another study by Stols-Goncalves et al. also confirmed alterations in the gut microbiome following FMT [130]; however, two patients in the study developed extended-spectrum beta-lactamase infections, raising safety concerns particularly amongst immunocompromised patients and highlighting the importance of 'healthy' donor selection. Rigorous experimental protocols and well-designed RCTs are still required to study the potential efficacy (including long-term effects) of FMT in the treatment of NAFLD/MASLD, as has also been shown by a recent systematic review [131,132]. Indeed, that systematic review of RCTs showed that supplementation with probiotics and synbiotics appears promising for improving hepatic steatosis, though additional RCTs are required to study the efficacy of FMT approaches in patients with MASLD [132]. For such microbiome-targeted therapies, and particularly for FMT, challenges relating to potential adverse effect risks (e.g., potential pathogen transmission associated with FMT) and variability in patient responses, as well as the scalability of these therapeutic interventions should be further explored. Table 2 presents RCTs with interventions targeting the gut microbiota in the management of hepatic steatosis/steatohepatitis, such as probiotics, prebiotics, synbiotics and FMT. As a common limitation of these existing RCTs is their relatively small sample size and/or short duration, large scale and longer-term RCTs are still required before these interventions can be introduced in routine clinical practice.

Table 2. Selected randomized controlled trials on interventions targeting (directly or indirectly) the gut microbiota in the management of hepatic steatosis/steatohepatitis (Abbreviations: ALT, Alanine Aminotransferase; APRI, Aspartate Aminotransferase to Platelet Ratio Index; AST, Aspartate Aminotransferase; BMI, body mass index; CFU: colony-forming units; CK-18, cytokeratin-18; CRP, C-reactive protein; FGF19, fibroblast growth factor 19; FMT, fecal microbiota transplantation; GGT, gamma glutamine transferase; HDL, High-Density Lipoprotein; HOMA-IR, Homeostasis Model Assessment of Insulin Resistance; IL-6, interleukin-6; IR, insulin resistance; LDL, low-density lipoprotein; MASLD, metabolic associated steatotic liver disease; MRI, Magnetic Resonance Imaging; NAFLD, non-alcoholic fatty liver disease; NAS: non-alcoholic fatty liver activity score; NASH, non-alcoholic steatohepatitis; RCT: randomized controlled trial; TLR-4, Toll-like receptor 4; TNF- α , tumor necrosis factor-alpha).

Study [Ref.]	Design and Duration	Study Intervention	Study Groups	Key Study Outcomes	Key Study Limitations
Derosa et al., 2022 [116]	Double-blind, placebo-controlled, RCT; 12 weeks	Probiotic group: 2 sachets daily in the morning of VSL#3 [a high-concentration multi-strain probiotic mix containing eight different strains: one strain of <i>Streptococcus thermophilus</i> BT01, three strains of <i>Bifidobacteria</i> (<i>B. breve</i> BB02; <i>B. animalis</i> subspecies [subsp.] <i>lactis</i> BL03, previously identified as <i>B. longum</i> BL03; and <i>B. animalis</i> subsp. <i>lactis</i> BI04, previously identified as <i>B. infantis</i> BI04), and four strains of <i>Lactobacilli</i> (<i>L. acidophilus</i> BA05, <i>L. plantarum</i> BP06, <i>L. paracasei</i> BP07, and <i>L. helveticus</i> BD08, previously identified as <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> BD08)] Control group: placebo	Probiotic (VSL#3) group: 30 patients with NAFLD (mean age \pm standard deviation: $55.7.0 \pm 6.7$ and 55.9 ± 7.5 years for males and females, respectively) Placebo control group: 30 patients with NAFLD (mean age: 55.8 ± 7.7 and 57.5 ± 7.9 years for males and females, respectively)	The VSL#3 group had significantly improved hepatic parameters, echography grading, inflammatory biomarkers and triglyceride levels, compared to the placebo	Small sample size. Short duration.
Chong et al., 2021 [119]	Double-blind, placebo-controlled, proof-of-concept RCT; 10 weeks	Probiotic group: VSL#3 Control group: placebo	VSL#3 group: 19 patients with NAFLD (mean age: 57.0 ± 8.0 years) Placebo control group: 16 patients with NAFLD (mean age: 58.0 ± 7.0 years)	No significant differences in biomarkers of cardiovascular risk and liver injury between the VSL#3 and placebo groups.	Small sample size. Short duration.

Table 2. Cont.

Study [Ref.]	Design and Duration	Study Intervention	Study Groups	Key Study Outcomes	Key Study Limitations
Duseja et al., 2019 [117]	Double-blind, placebo-controlled, RCT; 12 months	Probiotic group: 2 capsules three times daily (each capsule containing 112.5 billion live, lyophilised, lactic acid bacteria and bifidobacteria: <i>Lactobacillus paracasei</i> DSM 24733, <i>L. plantarum</i> DSM 24730, <i>L. acidophilus</i> DSM 24735, <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> DSM 24734, <i>Bifidobacterium longum</i> DSM 24736, <i>B. infantis</i> DSM 24737, <i>B. breve</i> DSM 24732, <i>Streptococcus thermophilus</i> DSM 24731) Control group: placebo	Probiotic group: 19 patients with NASH (mean age: 38.0 ± 10.0 years) Placebo control group: 20 patients with NASH (mean age: 33.0 ± 6.0 years)	A significant improvement in liver histology, ALT, and cytokine profiles in the probiotic group compared to the placebo group.	Small initial sample size, with a further reduction in patients at follow-up for a repeat liver biopsy. At baseline, most patients had mild hepatic histology. Histopathology interpretation by a single pathologist at respective study centers.
Mohamad Nor et al., 2021 [118]	Double-blind, placebo-controlled, RCT; 6 months	Probiotic group: One 3 g sachet twice daily of HEXBIO [®] microbial cell preparation (B-Crobes Laboratory Sdn. Bhd; each sachet consists of 30 billion CFU with six probiotic strains: <i>Lactobacillus acidophilus</i> BCMC [®] 12,130 (107 mg), <i>L. casei</i> subsp. BCMC [®] 12,313 (107 mg), <i>L. lactis</i> BCMC [®] 12,451 (107 mg), <i>Bifidobacterium bifidum</i> BCMC [®] 02290 (107 mg), <i>B. infantis</i> BCMC [®] 02129 (107 mg) and <i>B. longum</i> BCMC [®] 02120 (107 mg)] Control group: placebo	Probiotic group: 17 patients with NAFLD (mean age: 54.70 ± 10.19 years) Placebo control group: 22 patients with NAFLD (mean age: 52.47 ± 16.73 years)	Probiotics did not significantly improve clinical outcomes in NAFLD patients compared to placebo, but may help to stabilize the mucosal immune function and protect against increased intestinal permeability.	Small sample size. The unanticipated reduction in total fat intake in the probiotics group may have influenced the study outcomes.
Ayob et al., 2023 [133]	Double-blind, placebo-controlled, RCT; 6 months	Probiotic group: one 3 g sachet twice daily of HEXBIO [®] microbial cell preparation (B-Crobes Laboratory Sdn. Bhd) Control group: placebo	Probiotic group: 18 patients with NAFLD (mean age: 55.00 ± 11.07 years) Placebo control group: 22 patients with NAFLD (mean age: 49.95 ± 14.05 years)	Compared to placebo, probiotic supplementation ameliorated dysbiosis in patients with NAFLD, leading to stabilization of pro-inflammatory cytokine expression and modulation of mucosal immune function.	Small sample size. Due to the COVID-19 pandemic, some study patients experienced interruptions in receiving probiotics.

Table 2. Cont.

Study [Ref.]	Design and Duration	Study Intervention	Study Groups	Key Study Outcomes	Key Study Limitations
Wong et al., 2013 [134]	Open-label RCT; 6 months	Probiotic group: one 10 g sachet of Lepicol probiotic (200 million probiotic cultures) formula (Healthy Bowels Company Ltd., UK) twice daily. The Lepicol probiotic formula contains: <i>Lactobacillus plantarum</i> , <i>L. deslbrueckii</i> , <i>L. acidophilus</i> , <i>L. rhamnosus</i> , and <i>Bifidobacterium bifidum</i> , as well as 3 g of fructo-oligo-saccharides (prebiotics). Control group: usual care	Probiotic group: 10 patients with biopsy-proven NASH (mean age: 42.0 ± 9.0 years) Control group: 10 patients with biopsy-proven NASH (mean age: 55.0 ± 9.0 years)	Probiotic treatment may be more effective than usual care in reducing liver fat and AST levels in patients with NASH.	Small sample size. Open-label RCT. Results cannot be extrapolated directly to other probiotic formulae [the Lepicol probiotic formula contains also 3 g of fructo-oligo-saccharides (prebiotics)].
Sepideh et al., 2016 [135]	Double-blind, placebo-controlled, RCT; 8 weeks	Probiotic group: 1 g daily of Lactocare (Zist-takhmir Co., Tehran, Iran) which contains 7 strains of naturally occurring bacteria (<i>Lactobacillus casei</i> , <i>L. acidophilus</i> , <i>L. rhamnosus</i> , <i>L. bulgaricus</i> , <i>Bifidobacterium breve</i> , <i>Bifidobacterium longum</i> , <i>Streptococcus thermophiles</i>) Control group: placebo	Probiotic group: 11 patients with NAFLD (mean age: 42.10 ± 1.99 years) Placebo control group: 11 patients with NAFLD (mean age: 47.33 ± 2.53 years)	The probiotic group exhibited a reduction in insulin resistance, glycemic and inflammatory markers compared to the placebo group.	Small sample size. Short duration. Significant dropout rate.
Aller et al., 2011 [136]	Double-blind, placebo-controlled, RCT; 12 weeks	Probiotic group: one tablet daily (500 million of <i>Lactobacillus bulgaricus</i> and <i>Streptococcus thermophiles</i> ; Nutricion Medica, SL, Spain) Control group: placebo (one placebo tablet daily with 120 mg of starch)	Probiotic group: 14 patients with biopsy-diagnosed NAFLD (mean age: 49.4 ± 10.9 years) Placebo control group: 14 patients with biopsy-diagnosed NAFLD (mean age: 44.3 ± 15.1 years)	Significantly reduced liver enzymes in the probiotic group compared to the placebo group.	Small sample size. Short duration.

Table 2. Cont.

Study [Ref.]	Design and Duration	Study Intervention	Study Groups	Key Study Outcomes	Key Study Limitations
Ahn et al., 2019 [137]	Double-blind, placebo-controlled, RCT; 12 weeks	Probiotic group: mixture (109 CFU/1.4 g) of six probiotics [<i>L. acidophilus</i> CBT LA1, <i>L. rhamnosus</i> CBT LR5 isolated from Korean human feces, <i>L. paracasei</i> CBT LPC5 isolated from Korean fermented food (jeotgal), <i>P. pentosaceus</i> CBT SL4 isolated from a Korean fermented vegetable product (kimchi), <i>B. lactis</i> CBT BL3, and <i>B. breve</i> CBT BR3 isolated from Korean infant feces] Control group: placebo	Probiotic group: 30 patients with NAFLD (mean age: 41.7 ± 12.49 years) Placebo control group: 35 patients with NAFLD (mean age: 44.71 ± 13.31 years)	Significantly reduced levels of triglycerides and intrahepatic fat in the probiotic group compared to the placebo group.	Small sample size. Short duration. The controlled attenuation parameter was measured with the M FibroScan probe, while patients with BMI ≥ 28 kg/m ² should be assessed with an XL probe to increase the reliability of steatosis measurements and to reduce scan failures. Eight study patients were excluded due to unreliable measurement results.
Escouto et al., 2023 [138]	Double-blind, placebo-controlled, RCT; 24 weeks	Probiotic group: one capsule daily (1 × 10 ⁹ CFU <i>Lactobacillus acidophilus</i> ATCC SD5221 and 1 × 10 ⁹ CFU <i>Bifidobacterium lactis</i> HN019; Beneflora Caps; Biolab Sanus Farmaceutica Ltd.a.) Control group: placebo	Probiotic group: 23 patients with NASH (median age: 58.0 years) Placebo control group: 25 patients with NASH (median age: 57.0 years)	No statistically significant differences for liver fibrosis, steatosis, and inflammatory activity between the RCT groups. Significantly improved APRI score with probiotic supplementation compared to placebo.	Small sample size. Short duration. Inclusion of patients with liver cirrhosis. Most study participants were on statins and/or metformin treatment, which may have influenced relevant metabolic markers.
Bomhof et al., 2019 [121]	Single-blinded (participants blinded to treatment), placebo-controlled, RCT; 36 weeks	Prebiotic group: oligofructose (Orafti P95, Beneo-Orafti Inc., Tienen, Belgium) 8 g once daily for 12 weeks followed by 16 g daily for 24 weeks Control group: placebo	Prebiotic group: 8 patients with biopsy-confirmed NASH (mean age: 45.3 ± 5.6 years) Placebo control group: 6 patients with biopsy-confirmed NASH (mean age: 53.3 ± 4.8 years)	Significantly improved NAS score and steatosis in the probiotic group compared to the placebo group.	Small sample size. Single-blind RCT. The placebo group had poorer glycemic control compared to the prebiotic group. Following the NASH diagnosis, several study participants made lifestyle changes resulting in significant weight loss prior to randomization.

Table 2. Cont.

Study [Ref.]	Design and Duration	Study Intervention	Study Groups	Key Study Outcomes	Key Study Limitations
Reshef et al., 2024 [139]	Double-blind, placebo-controlled, RCT; 12 weeks	Prebiotic group: 8 g twice daily of inulin-type fructans (ITF) supplementation with inulin/oligofructose (75/25) Control group: placebo	Prebiotic group: 8 patients with NASH (median age: 47.8 years) Placebo control group: 11 patients with NASH (median age: 50.0 years)	The prebiotic group had a significant increase in the relative abundance of fecal <i>Bifidobacterium</i> ; however, no significant improvement was observed in liver fat content or inflammatory biomarkers.	Small sample size. Short duration. Potential confounders, such as the use of metformin.
Scorletti et al., 2020 [140]	Double-blind, placebo-controlled, RCT; ≈12 months (10 months minimum to 14 months maximum)	Synbiotic group: synbiotic consisting of fructo-oligosaccharides with a polymerization degree < 10 (4 g twice daily; two sachets daily) plus <i>Bifidobacterium animalis</i> subsp. <i>lactis</i> BB-12 (10 billion CFU daily minimum; one capsule daily). Actilight®950P was the prebiotic component of the synbiotic Control group: placebo (4 g twice daily of maltodextrin; one capsule daily, plus two sachets daily)	Synbiotic group: 45 patients with NAFLD (mean age: 50.2 ± 12.4 years) Placebo control group: 44 patients with NAFLD (mean age: 51.6 ± 13.1 years)	Compared to placebo, the synbiotic supplementation induced changes in the fecal microbiome (growth of <i>Faecalibacterium</i> and <i>Bifidobacterium</i> at the expense of <i>Oscillibacter</i> and <i>Alistipes</i>). The gut microbiota composition changes were not associated with any primary outcome. No significant difference in hepatic fat reduction between the two RCT groups.	Small sample size. Selection of one strain of bacteria for the synbiotic.
Mofidi et al., 2017 [141]	Double-blind, placebo-controlled, RCT; 28 weeks	Synbiotic group: synbiotic supplementation capsule twice daily [Protexin; Probiotics International Ltd.; each capsule contained: 200 million bacteria (seven strains: <i>Lactobacillus casei</i> , <i>L. rhamnosus</i> , <i>L. acidophilus</i> , <i>L. bulgaricus</i> , <i>Streptococcus thermophilus</i> , <i>Bifidobacterium breve</i> , and <i>B. longum</i>); prebiotic (125 mg fructo-oligosaccharide; probiotic cultures (magnesium stearate); and hydroxyl-propylmethyl cellulose] Control group: placebo	Synbiotic group: 21 patients with NAFLD (mean age: 40.09 ± 11.44 years) Placebo control group: 21 patients with NAFLD (mean age: 44.61 ± 10.12 years)	Both RCT groups exhibited reductions in hepatic steatosis and fibrosis with the mean reduction being significantly greater in the synbiotic group compared to the placebo.	Small sample size. Only participants with BMI ≤ 25 kg/m ² were included in this RCT, thus these findings cannot be directly extrapolated for patients with NAFLD and overweightness or obesity.

Table 2. Cont.

Study [Ref.]	Design and Duration	Study Intervention	Study Groups	Key Study Outcomes	Key Study Limitations
Malaguarnera et al., 2012 [142]	Double-blind, placebo-controlled, RCT; 24 weeks	Synbiotic group: sachet of <i>Bifidobacterium longum</i> and fructo-oligosaccharides (Fos) (Zirfos, Alfa Wassermann, Bologna, Italy) 2.5 g, vitamin B1 (1.4 mg), vitamin B2 (1.6 mg), vitamin B6 (2.0 mg), and vitamin B12 (1.0 mg) plus lifestyle modification (diet and exercise) Control group: placebo plus lifestyle modification (diet and exercise)	Synbiotic group: 34 patients with NASH (mean age: 46.9 ± 5.4 years) Placebo control group: 32 patients with NASH (mean age: 46.7 ± 5.7 years)	Significantly reduced NASH activity index and steatosis, as well as serum endotoxin, AST, CRP, TNF- α levels and HOMA-IR, in the synbiotic group compared to the placebo group.	Small sample size. Short duration. Potential diet-related confounding.
Asgharian et al., 2016 [143]	Double-blind, placebo-controlled, RCT; 8 weeks	Synbiotic group: 500 mg capsule (Familact by Zisttakhmir) once daily containing seven probiotic bacteria species (<i>Lactobacillus casei</i> , <i>L. acidophilus</i> , <i>L. rhamnosus</i> , <i>L. bulgaricus</i> , <i>Bifidobacterium breve</i> , <i>B. longum</i> , and <i>Streptococcus thermophilus</i>) and fructo-oligosaccharides Control group: placebo	Synbiotic group: 38 patients with NAFLD (mean age: 46.57 ± 1.7 years) Placebo control group: 36 patients with NAFLD (mean age: 47.78 ± 1.7 years)	The synbiotic group demonstrated significantly reduced steatosis grade on ultrasound compared to baseline. No association of synbiotic supplementation with changes in ALT, AST and CRP levels.	Small sample size. Short duration.
Abhari et al., 2020 [144]	Double-blind, placebo-controlled, RCT; 12 weeks	Synbiotic group: one synbiotic capsule daily containing 10^9 spore of <i>Bacillus coagulans</i> (GBI-30) plus 0.4 g inulin Control group: placebo	Synbiotic group: 23 patients with NAFLD (mean age: 47.7 ± 11.4 years) Placebo control group: 22 patients with NAFLD (mean age: 46.7 ± 12.4 years)	Hepatic steatosis measured by the controlled attenuation parameter score in Fibroscan, serum ALT and GGT levels decreased significantly more in the synbiotic group compared to the placebo. Synbiotic supplementation significantly reduced serum TNF- α levels and nuclear factor-kB activity.	Small sample size. Short duration.

Table 2. Cont.

Study [Ref.]	Design and Duration	Study Intervention	Study Groups	Key Study Outcomes	Key Study Limitations
Sayari et al., 2018 [145]	Double-blind, placebo-controlled, RCT; 16 weeks	Sitagliptin plus Synbiotic group: Sitagliptin (50 mg once daily) plus synbiotic [500 mg once daily; each capsule (Familakt) contained 109 CFU of <i>Lactobacillus casei</i> , <i>L. rhamnosus</i> , <i>L. acidophilus</i> , <i>L. bulgaricus</i> , <i>Bifidobacterium breve</i> , <i>B. longum</i> , <i>Streptococcus thermophilus</i> ; prebiotic (fructo-oligosaccharide); probiotic cultures (magnesium stearate); and hydroxyl-propyl methylcellulose] Sitagliptin plus placebo group: Sitagliptin (50 mg once daily) plus placebo (maltodextrin; once daily)	Sitagliptin plus Synbiotic group: 70 patients with NAFLD and BMI from 25 to 29.9 kg/m ² who had impaired fasting blood glucose and/or impaired glucose tolerance (mean age: 42.48 ± 11.41 years) Sitagliptin plus Placebo group: 70 patients with NAFLD and BMI from 25 to 29.9 kg/m ² and who had impaired fasting blood glucose and/or impaired oral glucose tolerance test (mean age: 43.42 ± 11.65 years)	The Sitagliptin [a dipeptidyl peptidase-4 (DPP-4) inhibitor] plus Synbiotic group had significantly greater improvements in AST, fasting glucose, cholesterol and LDL levels compared to the Sitagliptin plus Placebo group.	Small sample size. Short duration.
Xue et al., 2022 [129]	RCT; 4 weeks (RCT participants returned for re-examination 1 month post-treatment)	FMT group: allogenic FMT (heterologous from healthy donors via colonoscopy followed by three enemas over 3 days) Non-FMT control group: oral probiotics (a <i>Bifidobacterium</i> viable preparation and <i>Lactobacillus acidophilus</i> capsules)	FMT group: 47 patients with NAFLD (mean age: 57.3 ± 13.4 years) Non-FMT group: 28 patients with NAFLD (mean age: 60.2 ± 8.5 years) Healthy control group: 10 healthy undergraduate students (control group that received no intervention)	The mean hepatic fat attenuation values decreased from 278.3 to 263.9 dB/m in the FMT group ($p = 0.049$), while these increased from 265.5 to 282.5 dB/m in the Non-FMT group ($p < 0.01$). Compared to healthy controls, patients with NAFLD before the FMT had lower Chaol indices ($p < 0.05$), suggesting lower abundances of gut microbiota. Post-FMT, there was no statistical difference in the Chaol indices between the FMT NAFLD patients and healthy controls. In the FMT group, better effects on the gut microbiota reconstruction were noted in lean patients with NAFLD, compared to those with obesity.	Small sample size. Short duration. Patients with NAFLD were randomly divided into the FMT and non-FMT RCT group based on the wishes of the participants. Age difference between the FMT/non-FMT groups and the healthy control group. Potential confounders relating to exercise/diet.

Table 2. Cont.

Study [Ref.]	Design and Duration	Study Intervention	Study Groups	Key Study Outcomes	Key Study Limitations
Witjes et al., 2020 [146]	Double-blind (all study participants, i.e., vegan donors and patients with NAFLD and all trial physicians/authors were blinded for the treatment until trial completion), proof-of-principle RCT; 24 weeks (FMT performed three times at 8-week intervals)	Allogenic FMT group: FMT with feces from healthy, lean, vegan donors three times at 8-week intervals (same donor for the three FMTs in each participant) Autologous FMT group: 8-week intervals Allogenic/Autologous FMT via gastroduodenoscopy at baseline, and via a duodenal tube via a CORTRAK enteral access (Medline Industries, Northfield, IL), at 8 weeks and 16 weeks	Allogenic FMT group: 10 omnivorous adults with treatment-naïve biopsy-proven NAFLD (hepatic steatosis on ultrasound) and obesity (mean age: 51.2 ± 6.6 years) Autologous FMT group: 11 omnivorous adults with treatment-naïve biopsy-proven NAFLD (hepatic steatosis on ultrasound) and obesity (mean age: 48.5 ± 10.2 years)	No statistically significant changes in the overall NAFLD activity score, steatosis grade and fibrosis scores. In the allogenic FMT group a trend for an improved necro-inflammation score was noted. Significant changes in the expression of hepatic genes involved in inflammation and lipid metabolism were also noted in the allogenic FMT group, compared with the autologous FMT. Intestinal microbial community structure changed following allogenic FMT, and was associated with changes in plasma metabolites and markers of steatohepatitis.	Underpowered study due to slow recruitment. Differences in baseline AST and ALT levels between the allogenic and autologous FMT groups. Study participants were relatively healthy, and, thus, the study sample was not representative of the entire spectrum of patients with NAFLD.
Craven et al., 2020 [147]	Double-blind, RCT (3:1 ratio to allogenic or autologous FMT); 24 weeks (follow-up appointments for 6 months post-FMT)	Allogenic FMT group: FMT with feces from healthy lean donors Autologous FMT group Allogenic/autologous FMT was delivered to the duodenum via endoscopy	Allogenic FMT group: 15 patients with NAFLD (mean age: 47.6 ± 14.9 years) Autologous FMT group: 6 patients with NAFLD (mean age: 57.5 ± 13.0 years)	No significant changes in hepatic proton density fat fraction or HOMA-IR in the allogenic or autologous FMT group. NAFLD patients (n = 7) with elevated small intestinal permeability at baseline had a significant reduction 6 weeks post allogenic FMT.	Small sample size. The two FMT groups differed regarding biochemical data, intestinal permeability, and fibrosis staging.

Table 2. Cont.

Study [Ref.]	Design and Duration	Study Intervention	Study Groups	Key Study Outcomes	Key Study Limitations
Abdel-Razik et al., 2018 [148]	Double-blind, placebo-controlled, RCT; 6 months	Rifaximin group: rifaximin 1100 mg daily (550 mg tablets 1 × 2 before meals) Control group: placebo	Rifaximin group: 25 patients with biopsy-proven NASH (mean age: 40.2 ± 9.88 years) Placebo control group: 25 patients with biopsy-proven NASH (mean age: 38.4 ± 9.21 years)	Significantly reduced NAFLD-liver fat score, HOMA-IR and toll-like receptor-4 expression, as well as ALT, AST, GGT, TNF- α , IL-6, CK-18 and endotoxin levels, were noted in the Rifaximin group, but not in the placebo group.	Small sample size. A second liver biopsy at 6 months post-treatment was not performed for any participants. The effect of rifaximin treatment on the gut flora was not assessed.
Neuschwander-Tetri et al., 2015 [149]	Double-blind, placebo-controlled, RCT; 72 weeks	Obeticholic acid group: 25 mg of 6-ethylchenodeoxycholic acid (obeticholic acid) once daily, per os (orally) Control group: placebo	Obeticholic acid group: 141 patients with biopsy-proven non-cirrhotic NASH (mean age: 52.0 ± 11.0 years) Placebo control group: 142 patients with biopsy-proven non-cirrhotic NASH (mean age: 51.0 ± 12.0 years)	More patients in the obeticholic acid group compared with placebo had improvement in the NAFLD activity score, fibrosis, hepatocellular ballooning, steatosis, and lobular inflammation. The mean change in the NAFLD activity score was also greater in the obeticholic acid group compared with placebo. The proportion of patients with resolution of NASH (change from baseline NASH diagnosis to non-NASH) did not differ between the two RCT groups. Compared with placebo, obeticholic acid treatment was associated more with pruritus and a temporary increase in total and LDL cholesterol with a modest decrease in HDL cholesterol.	Missing detailed tracking of interventions for the treatment of hyperlipidemia during the RCT, including dosing information. Smoking history was not captured until midway of the RCT.

Table 2. Cont.

Study [Ref.]	Design and Duration	Study Intervention	Study Groups	Key Study Outcomes	Key Study Limitations
Harrison et al., 2018 [150]	Double-blind, placebo-controlled, RCT; 12 weeks	<p>NGM282 groups: (a) 3 mg NGM282 (an engineered FGF19 analogue) subcutaneously once daily (b) 6 mg NGM282 subcutaneously once daily</p> <p>Control group: Placebo</p>	<p>3 mg NGM282 group: 27 patients with biopsy-proven NASH (mean age: 52.0 ± 7.1 years)</p> <p>6 mg NGM282 group: 28 patients with biopsy-proven NASH (mean age: 56.4 ± 7.8 years)</p> <p>Placebo control group: 27 patients with biopsy-proven NASH (mean age: 52.8 ± 11.3 years)</p>	<p>Between baseline and 12 weeks, at least a 5% reduction in absolute hepatic fat content was noted for 74% and 79% of the participants in the 3 mg and 6 mg dose group, respectively (only for 7% in the placebo group).</p> <p>Adverse events were more frequent in the NGM282 groups compared to placebo, with injection site reactions, diarrhea, abdominal pain and nausea being the most commonly ($\geq 10\%$) reported adverse events.</p>	<p>Small sample size. Short duration. Inclusion of few non-white participants and numerically more men (non-significantly) in the NGM282 groups, although, in post hoc analysis, sex and race had no effect on reductions in hepatic fat content.</p>

5. Future Perspectives and Research Needs to Exploit/Target the Gut Microbiota in the Context of MASLD

In recent decades, remarkable progress has been achieved in our understanding of the MASLD pathophysiology which has rightly attracted the intense focus of translational and clinical research given its increasing prevalence and the significant related burden on healthcare systems globally [151]. Elucidating the complex interplay of the underlying pathophysiology of MASLD, particularly regarding the role of novel mediators such as that of the gut microbiota and the gut–liver axis, can further advance more effective and tailored diagnostic and management strategies for this chronic liver condition. Thus, further research focus is currently required on how insight into the role that the gut microbiota plays in maintaining or impairing liver health can support more personalized approaches for the diagnosis and treatment of MASLD, and, especially, of MASH [152,153]. In this context, future research should also address various barriers and constraints which presently prohibit or delay the integration of gut microbiome-based diagnostic approaches and microbiome-targeted therapies to routine clinical practice. Such constraints relate to both the validation/standardization (e.g., issues relating to sample collection, as well as to the duration and method of testing) and the feasibility and availability/scalability (e.g., the clinical applicability, safety, and scalability of FMT) of the relevant diagnostic methods and treatments.

Overall, progress in the detection/analysis of gut microbiome-based biomarkers is expected to aid in the early diagnosis and effective management of MASLD in patients at high risk for MASH and related complications. Indeed, establishing validated and readily available gut microbiome-based biomarkers, together with omics technologies (e.g., metabolomics, lipidomics and transcriptomics) [154], could lead to novel algorithms for prompt diagnosis of MASLD/MASH in routine clinical practice. These, for example, could take into account the bi-directional links within the adipose tissue–liver–gut axis (e.g., adipokines and adipose tissue biomarkers which are implicated in hepatic steatosis and/or inflammation and the development and/or progression of MASLD) [155], as well as the interplay between the gut microbiome and specific MASLD phenotypes, thus helping the early identification of the individuals at high risk for MASH and disease progression within the MASLD spectrum based on their metabolic and gut-microbiome profiles [152,153]. It should also be highlighted that MAFLD is linked to markedly increased risk for a number of additional cardio-metabolic conditions, including T2DM and cardiovascular disease, with overlapping pathophysiologic processes [156]. Thus, omics and gut microbiome-based biomarkers in combination with polygenic risk scores may offer valuable novel screening and diagnostic tools for the prompt and effective monitoring of the broader cardiovascular-liver-metabolic health [156].

Moreover, non-invasive gut microbiome profiling holds strong potential for the development of personalized dietary/nutritional interventions and pharmacological treatments for MASLD [152,153]. Of note, although MASLD is associated with increasing morbidity and mortality [151], progress in specific pharmacotherapy for MASLD/MASH faces various challenges, whilst drugs specifically for steatohepatitis are now being approved/developed [157–159]. As such, the primary management recommendations of the current MASLD clinical guidelines rely on dietary interventions/modifications to manage weight loss and hepatic inflammation [160–162]. In this context, insight into the complex crosstalk between dietary factors and the gut-microbiota may help the development of personalized nutrition approaches for patients with MASLD/MASH which would be supported in practice by gut microbiome profiling/biomarkers [152,153]. For example, large-scale and long-term trials are required to provide evidence on whether the Mediterranean diet, which has established cardio-metabolic benefits compared to Western dietary

patterns [153,163], could be further tailored based on gut microbiome-based biomarkers to better meet the needs of patients with MASLD/MASH or those with both MASLD and T2DM. Similar clinical trials can also explore how the profiling of the gut microbiome could help to tailor the optimum diet for such patients (e.g., in the context of very low-calorie, very low carbohydrate and ketogenic diets, as well as in relationship to intermittent fasting) [163,164], potentially combined with supplementation with probiotics, prebiotics, or synbiotics. The evidence from such studies could be further combined with genomic data to better predict MASLD phenotypes and their responses to specific dietary interventions and pharmacological treatments as part of a more holistic approach to precision nutrition/medicine for the management of MASLD (Figure 2) [163–165]. As such, there is a need for more studies in this field that can support evidence-based recommendations for a shift in routine clinical practice from generalized approaches to personalized management strategies, which would incorporate genetic, multi-omics and gut microbiome-based biomarkers, for the treatment of MASLD/MASH. This type of personalized/precision management may effectively decrease the development of MASLD and/or its progression to MASH, thus improving the overall cardiovascular-liver-metabolic health of these patients.

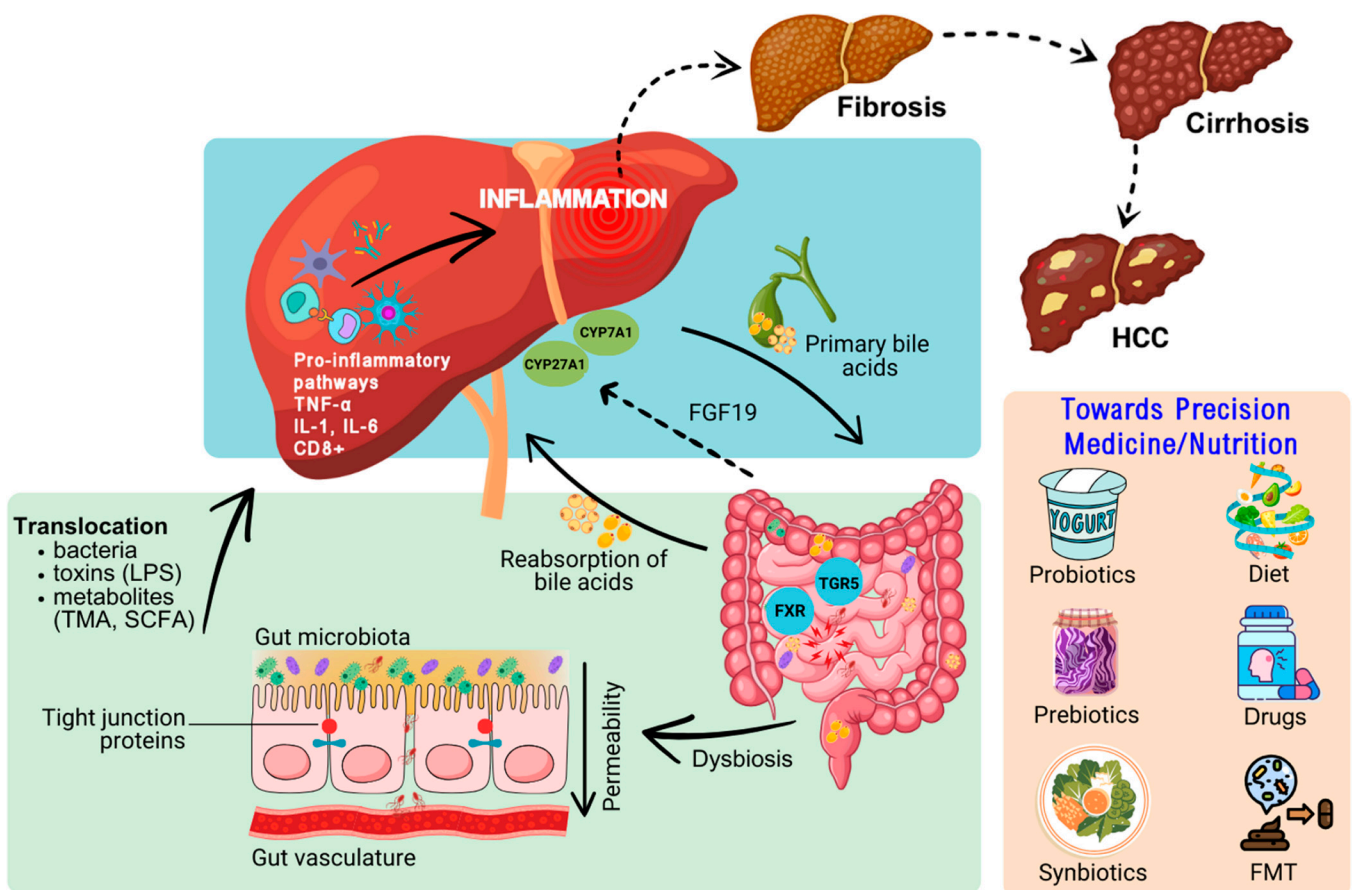


Figure 2. Schematic representation of underlying mechanisms/links which implicate the gut microbiota and the gut–liver axis in the pathophysiology of steatosis/steatohepatitis and which could be leveraged for the management of these hepatic pathologies in the context of personalized medicine/nutrition therapeutic approaches. Dysbiosis of the microbiome leads to damage and permeability of the gut barrier which enables translocation of bacteria, toxins, and metabolites into the liver via the portal vein. This may trigger pro-inflammatory pathways/cascades which can lead to fibrosis, cirrhosis, and even HCC. Conjugated primary bile acids are secreted from the liver into the small intestine. In the gut, bile acids interact with microbiota and cellular receptors, including the

TGR5 and FXR. The interaction of bile acids with FXR induces FGF19 secretion which serves as a negative feedback loop for bile acid synthesis in hepatocytes. Dietary patterns (e.g., the Mediterranean diet), supplementation with probiotics, prebiotics and synbiotics, as well as interventions such as FMT and novel drugs, are examples of precision medicine/nutrition approaches which may target gut dysbiosis for the treatment of MASLD. Abbreviations used include the following: CYP27A1, Cytochrome P450, Family 27, Subfamily A, Polypeptide 1; CYP7A1, Cytochrome P450, Family 7, Subfamily A, Polypeptide 1; FGF19, fibroblast growth factor 19; FMT; fecal microbiota transplantation; FXR, farnesoid X receptor; HCC, hepatocellular carcinoma; MASLD, metabolic associated steatotic liver disease; LPS, lipopolysaccharide (endotoxin); SCFA, short-chain fatty acids; TGR5, takeda G-protein-coupled receptor 5; TMA, trimethylamine.

6. Conclusions

A growing body of recent scientific evidence has enhanced our understanding of the gut microbiota's role in the pathogenesis of MASLD. The dynamic gut–liver axis facilitates the bi-directional communication and transport of both beneficial and harmful metabolites from the gut to the liver and vice versa. Within these interactions, when the liver is overwhelmed with harmful and pro-inflammatory molecules (e.g., due to unhealthy dietary habits), as well as gut dysbiosis, or increased gut permeability, hepatic function becomes impaired and MASLD pathogenetic mechanisms are triggered. Such hepatic dysfunction may also result in an inability to effectively regulate the gut microbiota through bile acids and other factors, further promoting gut dysbiosis and gut barrier dysfunction. This self-perpetuating vicious cycle may have far-reaching implications, exacerbating hepatic damage and overall health.

Recent clinical studies have uncovered promising NAFLD/MASLD-associated microbiome signatures which could potentially be used for non-invasive diagnosis and monitoring of disease progression. However, inconsistencies arising from variability between populations, disease stages, and confounding factors (e.g., ethnic background, dietary habits and comorbidities such as T2DM and obesity) complicate the establishment of definitive gut microbial signatures. Additionally, despite the growing prevalence of MASLD, current diagnostic methods remain sub-optimal. Therefore, there is an imperative need to explore new diagnostic, and therapeutic strategies which consider the interplay between the gut microbiota, its metabolites, and MASLD. Accordingly, robust validation through large, well-designed cohort studies and RCTs is required to fully evaluate the diagnostic potential of microbial signatures and biomarkers, as well as the therapeutic potential of probiotics, prebiotics, synbiotics, and other microbiota-based interventions. Future studies should also consider the dosing, feasibility and long-term safety of these interventions.

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