The role of the gut microbiome and diet in the pathogenesis of NAFLD

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List of Abbreviations: AMP; adenosine monophosphate, AGEs; advanced glycated end products, BAs; bile acids, FLI; fatty liver index, FMO3; flavin-containing monooxygenase-3, FMT; faecal microbial transplant, FXR; farnesoid X receptor, GLP-2; Glucagon-Like-Peptide-2, GLUT2; glucose transporter-2, GM; gut microbiome, HCC; hepatocellular carcinoma, LPS; Lipopolysaccharides, NAFLD; non-alcoholic fatty liver disease, NASH; non-alcoholic steatohepatitis, NCS; noncaloric artificial sweeteners, ODMA; O-desmethylangolensin, PPARγ; peroxisome proliferator-activated receptor-γ, RAGE; receptor for advanced glycated end products, RCTs; randomised controlled trials, SCFAs; short chain fatty acids, STZ-HFD; streptozotocin-high fat diet, TLR; toll like receptor, TMA; trimethylamine, TNF; tumour necrosis factor, T2DM; type 2 diabetes mellitus.

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is the leading cause of chronic liver disease, with a prevalence that is increasing in parallel with the global rise in obesity and type 2 diabetes mellitus. The pathogenesis of NAFLD is complex and multifactorial, involving environmental, genetic and metabolic factors. The role of the diet and the gut microbiome is gaining interest as a significant factor in NAFLD pathogenesis. Dietary factors induce alterations in the composition of the gut microbiome (dysbiosis), commonly reflected by a reduction of the beneficial species and an increase in pathogenic microbiota. Due to the close relationship between the gut and liver, altering the gut microbiome can affect liver functions; promoting hepatic steatosis and inflammation. This review summarises the current evidence supporting an association between NAFLD and the gut microbiome and dietary factors. The review also explores potential underlying mechanisms underpinning these associations and whether manipulation of the gut microbiome is a potential therapeutic strategy to prevent or treat NAFLD.
**Introduction**

Non-alcoholic fatty liver disease (NAFLD) is a major cause of chronic liver disease worldwide.\(^1\),\(^2\) Recently it has become evident that NAFLD not only increases the risk of chronic liver disease and primary liver cancer, but NAFLD has important implications for the development of diseases beyond the liver, such as type 2 diabetes mellitus (T2DM), cardiovascular disease and chronic kidney disease.\(^3\)\(^,\)\(^4\) The prevalence of NAFLD is increasing in parallel with the global rise in obesity and T2DM.\(^6\) NAFLD represents a spectrum of liver disease severity, beginning with the accumulation of triglycerides in the liver (steatosis). Almost a quarter of individuals with steatosis develop liver inflammation and progress to non-alcoholic steatohepatitis (NASH). NASH is a potentially progressive liver condition and with ongoing liver injury and cell death can result in fibrosis and cirrhosis.

The pathogenesis of NAFLD is complex and multifactorial, involving environmental, genetic and metabolic factors. Given the well-known association between obesity and NAFLD, in recent years there has been a growing interest in the role of diet and the gut microbiome (GM) in the pathogenesis of NAFLD. The gut–liver axis is defined by the strong anatomical and functional interactions between the gastrointestinal tract and the liver. An important element of this axis is the GM, which is involved in host nutrient metabolism, maintenance of structural integrity of the gut mucosal barrier and immunomodulation.\(^7\) Disturbance of the GM has been implicated in many disease processes, particularly those in which the gut-liver axis plays an important role, such as NAFLD. Similarly, dietary antigens have a major influence on the gut-liver axis, including modification of the GM, and therefore are implicated in the pathogenesis of NAFLD. This review summarises the current evidence supporting an association between NAFLD and the GM and dietary factors. The review also explores potential underlying mechanisms underpinning these associations and whether manipulation of the gut microbiome is a potential therapeutic strategy to prevent or treat NAFLD.

**Gut microbiome**

The intestinal lumen is naturally colonized by trillions of microorganisms from more than 1000 species including bacteria, protozoa, archaea, fungi, and viruses.\(^7\) Advances in culture-independent microbiologic technology over the last decade have facilitated the characterisation of the composition and diversity of the bacterial component of the GM. Although the profound variability between individuals’ GM complicates attempts to define what is ‘normal’ microbiota, the most common bacterial phyla found in the faeces of healthy subjects are; Bacteroidetes (65.2%), Firmicutes (29.6%), Proteobacteria (2.9%) and Actinobacteria (0.5%).\(^8\) Early life plays an important role in establishing the GM, with both vaginal delivery (versus caesarean section) and breastmilk (versus formula milk) demonstrating beneficial effects on the GM composition.\(^9\),\(^10\) Early microbiota perturbation can lead to long-term deranged metabolic phenotypes, including NAFLD and obesity.\(^10\)\(^,\)\(^11\) Later in life, the GM composition is dependent on many factors including; genetics, age, diet and medications.\(^12\) Ageing is associated with alterations in the GM composition and reduced phylogenetic diversity, which has been postulated to partly underlie the pathogenesis and progression of various metabolic diseases that are prevalent in old people such as adiposity, insulin resistance, and NAFLD.\(^13\) Figure 1 shows the proportions of the phylum, class and genus of bacteria commonly found in the health gut.\(^8\),\(^12\),\(^14\)-\(^16\)
Evidence for a role of dysbiosis in the pathogenesis of NAFLD

The term dysbiosis refers to disruption of the normal GM that is associated with pathology within the host. Dysbiosis has been linked to several aspects of the metabolic syndrome, including NAFLD. In NAFLD, early evidence linking gut dysbiosis with liver injury came from human studies showing an association between NASH and small intestinal bacterial overgrowth. More recent evidence, from both animal and human studies, indicates that microbial populations are altered in patients with NAFLD.

Several animal studies have demonstrated that dysbiosis is associated with more severe hepatic steatosis and hepatic inflammation; these findings are summarised in Table 1. Even co-housing non-dysbiotic mice with dysbiotic mice can lead to transfer of gut microbes and a subsequent exacerbation of NAFLD. Although dysbiosis is harmful, the presence of a healthy GM in animals has been shown to be protective against the development of NAFLD. For example, Cano et al. demonstrated that inducing beneficial changes to the GM with the probiotic Bifidobacterium pseudocatenulatum, reduced the risk of developing NAFLD in mice, and Mazagova et al. found that germ-free mice developed more severe experimental liver fibrosis compared to conventional mice, demonstrating that the presence of commensal gut microbes is hepatoprotective.

Several studies have explored the composition of the GM among cohorts of human subjects with varying stages of NAFLD. Table 2 summarises the overall characteristics of these studies and provides details regarding the specific microbiologic results. Despite the variability in study design, methods, and clinical endpoints, these human studies demonstrate measurable differences in the GM between healthy controls and individuals with hepatic steatosis and NASH. The GM composition has also been found to vary according to the severity and stage of NAFLD. Loomba et al. found distinct changes in the GM composition between individuals with advanced fibrosis (stage 3 or 4) and those with mild fibrosis (stage 1 or 2). The authors went on to suggest that a faecal-microbiome-derived metagenomic signature could be used as an adjunct tool to current invasive approaches to determine the stage of liver disease in NAFLD.

The findings in both animal and human studies are inconsistent and sometimes conflicting, and it is still unclear which specific microorganisms are harmful or protective. This may in part be due to the heterogeneous cohorts used across studies in addition to the varying GM sequencing technologies that were used. More work is needed, particularly longitudinal human studies, to delve further into the significance of GM alterations in NAFLD, especially if analyses of the GM is to become part of clinical practice.

Potential mechanisms linking diet, dysbiosis and NAFLD
1. **Role of short chain fatty acids in metabolic and inflammatory pathways**

Short chain fatty acids (SCFAs), such as acetic, propionic and butyric acid, are generated through the fermentation of polysaccharides by gut microbes in the large bowel. The diet and composition of the GM impacts the quantity and type of SCFAs synthesized in the gut. Diets high in fibre, particularly from plant-based foods or a Mediterranean diet, are associated with increased levels of faecal SCFAs. Altering the GM with prebiotics and probiotics that promote the growth of beneficial microbiota can induce changes in SCFA production. SCFAs have a role in inflammation, lipid and glucose metabolism and regulation of energy harvested from the diet. As the effects of SCFAs are so diverse and widespread, elucidating the overall impact has been difficult. While animal studies have shown diets high in SCFAs to have favourable metabolic affects (reduced hepatic cholesterol and fatty acid synthesis and increased lipid oxidation), human studies have not been so clear.

SCFAs are a major source of energy, although most SCFAs are utilized in the gut, some are transported through the portal vein and channelled into the tricarboxylic acid (TCA) cycle or utilised for hepatic gluconeogenesis or lipogenesis. Thus, changes in the GM that favour SCFA production can increase energy delivery to the liver and reduce faecal energy loss. The first evidence to support this was provided by Turnbaugh et al. who found that, compared to their lean littermates, obese mice had more carbohydrate metabolising genes in their GM, increased concentration of SCFAs in their caecum, and less energy in their stool. Furthermore, faecal microbial transplant (FMT) from obese mice to germ free mice caused greater fat gain than FMT from lean animals. In humans, increased production of SCFAs by the GM was also observed in obese people, compared to lean subjects.

SCFAs can alter lipid and glucose metabolic pathways through activation of G-coupled receptors. Kimura et al. showed that through activation of G-protein receptor-43, SCFAs suppress insulin signalling in adipocytes, which inhibits fat accumulation in adipose tissue and promotes the metabolism of unincorporated lipids and glucose in the liver and other tissues. The intestinotrophic effects of SCFA were first proposed by Koruda et al. who found that SCFA supplementation to rats having parenteral nutrition prevented associated mucosal atrophy. Beneficial effects of SCFA on the intestinal mucosa are thought to be mediated through Glucagon-Like-Peptide-2 (GLP-2), as SCFA supplementation induces the expression of ileal proglucagon mRNA and plasma GLP-2. In mice, increasing GLP-2 levels, by microbial intervention or subcutaneous GLP-2 administration, reduces intestinal permeability, which leads to lower plasma lipopolysaccharide (LPS) and cytokines levels, and consequently reduced hepatic oxidative stress and inflammation.

SCFA supplementation has shown beneficial effects on several inflammatory conditions including asthma, arthritis and colitis. The anti-inflammatory effects of SCFA are thought to be mediated through activation of G-protein coupled receptor-43. Maslowski et al. induced colitis in mice and demonstrated that inflammation is more severe in germ-free mice than in those conventionally raised. The colitis was ameliorated by acetate supplementation, a finding that was absent in G-protein coupled receptor-43 knock out mice. By suppressing colitis, SCFAs can improve gut permeability and therefore reduce hepatic delivery of harmful microbial cell components and metabolites. Conversely, Rau et al. found that patients with NAFLD had higher concentrations of intestinal acetate and propionate, which correlated with peripheral levels of pro-inflammatory T-cells. Therefore the role on SCFAs in inflammatory pathways within NAFLD is still controversial.
2. **Bacteria-derived ethanol**

Histologically, NAFLD and alcohol-induced liver injury are remarkably similar, and are therefore likely to share common pathogenic pathways. Some microbiota harbour genes that can ferment dietary sugars into ethanol. The amount of alcohol produced depends on the availability of carbohydrates from the diet and the composition of the GM, particularly the presence of Proteobacteria (especially *Klebsiella pneumoniae* and *Escherichia coli*). Animal studies have demonstrated that obese mice with NASH have higher early-morning breath alcohol content compared with lean mice without NASH; which is eliminated by neomycin treatment. In human studies, patients with NAFLD, particularly children, have been shown to have increased blood ethanol levels. Yuan et al. found high-alcohol-producing *Klebsiella pneumoniae* in the GM of up to 60% of individuals with NAFLD in a Chinese cohort. The study went on to show that transfer of high-alcohol-producing *Klebsiella pneumoniae*, by oral gavage or FMT, into healthy mice induced NAFLD. Gut-derived ethanol is not only directly hepatotoxic but it may alter the gut-liver axis, by increasing intestinal permeability and endotoxemia, to compound hepatic damage.

3. **Role of choline deficiency in lipid metabolism and inflammation**

Choline is an essential nutrient sourced from foods such as meat and eggs. Choline deficiency induces many features of NAFLD and is often utilized in studies to create animal models of NAFLD. Choline deficiency leads to impaired synthesis of phosphatidylcholine resulting in diminished VLDL assembly and secretion and consequently reduced hepatic triglyceride clearance. In addition to hepatic steatosis, choline deficient mice also develop hepatic inflammation and fibrosis, which is thought to be caused by impaired mitochondrial β-oxidation and increased oxidative stress. The GM plays an important role in choline metabolism and therefore regulates the concentration that is delivered to the liver via the portal circulation. Dysbiosis, and in particular an excess of the class Erysipelotrichia, has been associated with choline depletion in both animal and human studies. Choline is metabolised by the GM to generate methylamines such as trimethylamine (TMA), which is converted in the liver to TMA-N-oxide (TMAO) by flavin-containing monooxygenase-3 (FMO3). TMAO has adverse effects on glucose homeostasis and is implicated in atherosclerosis. NAFLD is associated with lower levels of choline and higher levels of TMA in the blood, implicating the role of gut microbiota in the imbalance of choline metabolism. Around 10-15% of bacterial species require choline to synthesize phosphatidylcholine, a component of their membrane; therefore in the context of bacterial overgrowth, the demand for choline may increase and contribute to choline deficiency.

4. **Lipopolysaccharide-induced hepatic inflammation**

LPS, a constituent of gram negative bacteria, are found in the systemic circulation of individuals with NAFLD, and correlate with the severity of steatohepatitis in both human and animal studies. The concentration of LPS in the GM and plasma is increased by the consumption of a western diet, high in saturated fat and low in fibre. It has been hypothesised that a high-fat diet may facilitate LPS uptake through elevated chylomicron production in intestinal epithelial cells. In mice on a standard diet, continuous subcutaneous infusion of low-dose LPS results in hepatic steatosis and hepatic insulin resistance. LPS interact with toll-like receptors (TLR), particularly TLR-4, on hepatic Kupffer cells and stellate cells to stimulate pro-inflammatory and profibrotic pathways via a
range of cytokines, including interleukin-1, interleukin-6 and tumour necrosis factor (TNF).\textsuperscript{75-78} TLR-4-deficient mice display decreased liver injury, inflammation, and lipid accumulation in comparison with wild-type mice in NAFLD models.\textsuperscript{79,80} TLR signalling in the mucosa also lead to the production of inflammasomes (cytosolic multiprotein oligomers of the innate immune system) which initiate a variety of pathways to produce pro-inflammatory and pro-fibrotic mediators such as caspase-1, interleukin-1β and interleukin-18.\textsuperscript{81,82} In support of a role for inflammasomes in the development of more severe liver disease in NAFLD, Wree et al. found significantly higher levels of the inflammasome NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3) in the livers of individuals with NASH compared to those with simple steatosis.\textsuperscript{83} Conversely, other studies have shown that the absence of inflammasomes is associated with more aggressive disease. Pierantonelli et al. demonstrated that, in a Western lifestyle model, the combination between high-fat and high-carbohydrate diet and the lack of the NLRP3-inflammasome increased the degree of liver injury and was associated with an abundance of gram-negative Proteobacteria and Verrucomicrobia (mucus-degrading bacteria that promote bacterial translocation), higher intestinal bacterial translocation, increased TLR activation and a more severe degree of liver injury.\textsuperscript{84}

5. **The role of bile acids in lipid metabolism and gut microbiome modulation**

Bile acids (BAs) have been implicated in the pathogenesis of NAFLD through their role in lipid metabolism and as signalling molecules via the farnesoid X receptor (FXR). By binding to the FXR, BAs can increase insulin sensitivity and decrease hepatic gluconeogenesis and circulating triglyceride concentrations.\textsuperscript{85} Obeticholic acid, an FXR agonist, has been shown to improve hepatic histology in ‘The Farnesoid X nuclear receptor ligand obeticholic acid for non-cirrhotic, non-alcoholic steatohepatitis (FLINT)’ trial.\textsuperscript{86} However, results with this FXR agonist were less promising in a planned interim analysis of a further large trial in patients with NASH, where treatment with obeticholic acid failed to produce resolution of NASH (compared to placebo), although treatment with obeticholic acid 25 mg/day significantly improved liver fibrosis.\textsuperscript{87}

BAs and the GM have a bi-directional relationship and are both highly influenced by the diet. Dietary fat content regulates BA synthesis; in particular a high-fat diet increases production, while a low-fat diet reduces production.\textsuperscript{88,89} The GM regulates BA homeostasis through a number of mechanisms including dihydroxylation of primary BAs to secondary BAs. BAs reciprocally regulate the GM, both directly via antibacterial activity (particularly deoxycholic acid) and indirectly via FXR induced antimicrobial peptides. The GM and BAs indirectly interact through TMAO, a product of GM choline metabolism. TMAO inhibits two key enzymes involved in BA metabolism: CYP7A1 and CYP27A1, therefore reducing the overall BA pool size.\textsuperscript{90} Furthermore, FXR activation can regulate FMO3 activity and therefore TMAO production. Bennet et al. demonstrated that FXR ligands administered to wild-type mice could induce FMO3 expression and increase TMAO levels, but this effect was abrogated in FXR knock out mice.\textsuperscript{91}

Several studies have demonstrated that the interaction of BAs with the GM plays an important role in NAFLD pathogenesis. Parseus et al. fed germ-free and conventionally raised wild-type and FXR knock-out mice a high-fat diet for 10 weeks. The GM promoted weight gain and hepatic steatosis in an FXR-dependent manner, and the BA profiles and GM composition differed between FXR knock-out and wild-type mice.\textsuperscript{92} These findings were supported by Jiang et al. who found that
improvements in hepatic steatosis related to antibiotic treatment were dependent on FXR signalling.  

6. **Intestinal permeability induced hepatic inflammation**

The portal vein exposes the liver to potentially harmful substances derived from the gut, including translocated bacteria and toxic bacterial products such as LPS. Dysbiosis can disrupt the integrity of the mucosal wall, increasing mucosal permeability. Dietary factors have an important role in the maintenance of the intestinal mucosal barrier, and not just through their role in altering the GM composition. Dietary depletion of glutamine, tryptophan and zinc or excess ingestion of fat, alcohol and food additives, have been directly associated with increased intestinal permeability.

Animal studies have shown convincing evidence that increased intestinal permeability leads to translocation of bacteria and bacterial toxins, such as LPS, that worsen the severity of NASH. Miele et al. found that patients with NAFLD had significantly increased gut permeability (as measured by urinary excretion of 51chromium-radiolabeled EDTA) compared to healthy volunteers. Furthermore, in patients with NAFLD both gut permeability and the prevalence of small intestinal bacterial overgrowth correlated with severity of steatosis, although not with steatohepatitis. Verdam et al. found plasma Immunoglobulin G levels against endotoxin were increased in biopsy-proven human NASH and positively correlated with the severity of inflammation. Conversely, Yuan et al. found that in paediatric patients with NASH, serum endotoxin levels were not correlated with disease severity, however peripheral endotoxin levels may not represent the concentration in the portal system, and the pathogenesis of NAFLD in children may be different to adults.

7. **Adipose tissue metabolism and inflammation**

Adipose tissue expansion, dysfunction, and inflammation are hallmarks of obesity and play a critical role in the development of NAFLD. Through several mechanisms the composition of the GM is thought to alter the metabolism and function of adipose tissue. LPS and other GM derived TLR ligands have been shown to contribute to adipose tissue inflammation. Caesar et al. showed that mice fed a lard diet had increased TLR signalling and white adipose tissue inflammation compared with mice fed an isocaloric fish oil diet. In addition, mice genetically deficient in various components of the TLR signalling pathway were protected against white adipose tissue inflammation. De Groot et al. demonstrated that FMT from a donor after Roux-en-Y bariatric surgery, to a host with metabolic syndrome led to a reduction in the adipocyte inflammatory marker chemokine ligand-2 gene expression in adipose tissue and circulating levels in plasma.

SCFAs, in particular propionate, have been demonstrated to regulate adipose tissue metabolism, both directly through modulation of the transcription factor peroxisome proliferator-activated receptor-γ (PPARγ), and indirectly through activation of the sympathetic nervous system. The GM composition alters adipose tissue thermogenesis, including brown adipose tissue activity and browning of white adipose tissue. Schugar et al. demonstrated that in mice the genetic deletion of the TMAO-producing enzyme FMO3 protected against high-fat diet induced obesity, in part by stimulating the beiging and enhanced thermogenesis of white adipose tissue.
Diet and NAFLD

Dietary factors can impact the development of NAFLD via their critical role in the gut-liver axis. A consistent finding from studies that have examined the link between diet and NAFLD is that a hypercaloric diet, regardless of whether the excess calories have been provided either as fat, sugar, or both, increases liver fat content. \(^{106}\) Figure 2 summarises the effects of dietary factors on the GM and their effects on hepatic pathways leading to the development of hepatic steatosis, inflammation and fibrosis.

1. **Fat**

Increased dietary fat intake, particularly saturated fat, is associated with the development of hepatic steatosis. \(^{107-109}\) A high fat diet can alter the GM rapidly, decreasing the microbial diversity and favouring gut bacteria associated with the development of NAFLD. \(^{110}\) Changes associated with a high fat diet include reduced levels of the *Bifidobacterium* genus and an increase in the ratio of Firmicutes to Bacteroidetes phyla. \(^{73}\) Ingestion of long-chained saturated fats, compared to short-chained unsaturated fats, are associated with a more pronounced reduction in phylogenetic diversity and beneficial bacteria. \(^{102}\) The mechanisms by which dietary fatty acids affect the GM are not well defined. Only a minority of fatty acids will pass through the gastrointestinal tract and directly modulate GM composition. Fatty acids have a broad spectrum of antibacterial activity including lysis of bacterial cell membranes and inhibition of bacterial adenosine triphosphate (ATP) production, \(^{111,112}\) and the antibacterial action of fatty acids is affected by carbon chain length, saturation and double bond position. \(^{113}\)

Changes in the GM caused by a high fat diet are associated with; increased energy harvest from the gut, upregulation of genes related to lipid metabolism in the distal small bowel and the production of SCFAs favouring the development of NASH. \(^{73,102}\) High fat diets in animals, particularly those involving long-chained saturated fatty acids, are accompanied by increased intestinal permeability, resulting in bacterial translocation and elevated LPS levels. \(^{73}\) Inflammatory processes are also upregulated, with increased hepatic TLR-4 activation and a rise in inflammatory mediators including TNF-\(\alpha\), interleukin-1, and plasminogen activator inhibitor-1. \(^{73,102}\)

Despite the association between a high fat diet and the development of NAFLD and dysbiosis, some fatty acids have demonstrated favourable effects on the GM and the development of hepatic steatosis. In a randomised controlled trial (RCT), Scorletti et al. demonstrated that consumption of the omega-3 polyunsaturated fatty acid, docosahexaenoic acid, was independently associated with a decrease in liver fat percentage in patients with NAFLD. \(^{114}\) The ingestion of omega-3 polyunsaturated fatty acids and oleic acid have been shown to protect against high-fat diet induced dysbiosis and improve the composition of the GM; including increased abundance of butyrate-producing bacteria and the *Bifidobacterium* genus. \(^{115-117}\)

2. **Fructose**

Dietary fructose has been strongly implicated in the pathogenesis of NAFLD. A large association study demonstrated an increased risk of NAFLD in those consuming regular sugary drinks, especially
if overweight. Fructose is both a substrate and an inducer of hepatic de novo lipogenesis. In individuals with NAFLD, 26% of hepatic triglycerides are produced by de novo lipogenesis using fructose and other dietary sugars as substrates. Fructose increases hepatic lipogenesis by activating several key transcription factors such as sterol response element-binding protein-1c and carbohydrate-responsive element-binding protein. Dietary fructose can induce rapid and harmful changes in the GM composition, including a reduction in the phylogenetic diversity and a lower concentration of *Bifidobacterium* genus. Fructose induced dysbiosis has been demonstrated to increase intestinal macrophage counts and lower tight junction occludin protein expression, associated with worse endotoxaemia, more bacterial translocation and increased hepatic TLR expression.

During hepatic fructose metabolism by fructokinase, ATP is rapidly consumed, which results in the breakdown of adenosine monophosphate (AMP) to inosine monophosphate and the generation of uric acid. Uric acid therefore increases in the plasma and liver with fructose consumption, and may mediate some of the adverse effects associated with fructose. Uric acid can exacerbate hepatic insulin resistance through activation of retinol binding protein-4, impair hepatic fatty acid oxidation through inhibition of AMP-activated protein kinase, and induce hepatic oxidative stress through NADPH oxidase activation. Xanthine oxidase inhibitors that block uric acid generation, have been shown to inhibit fructose induced hepatic steatosis, and serum uric acid concentrations and a high dietary fructose consumption are both independently associated with NASH in children.

### 3. Advanced glycated end products

Advanced glycated end products (AGEs) are formed in food when reducing sugars react non-enzymatically with the amino groups on proteins. The concentration of AGEs is high in western diets and contributes to tissue injury via activation of RAGEs (receptor for AGEs) and generation of reactive oxygen species. RAGEs have been found on hepatic stellate cells and stimulation has been shown to exacerbated liver inflammation and increase hepatic proliferation and expression of collagen in animal models of NAFLD. The absorption of dietary AGEs is limited, the majority pass through the gastrointestinal tract to the colon, where they can act as substrates for the GM. Several animal and human studies have shown that the consumption of AGEs is associated with a compositional change in the GM and altered production of SCFAs, however there is a lack of agreement between studies on the specific microbial changes, which may be due to the different glycated substrates used.

### 4. Mediterranean diet

A Mediterranean diet is characterised by increased consumption of vegetables, legumes, fruits, nuts, olive oil and fish and low consumption of red meat, dairy products and saturated fats. A weight neutral Mediterranean diet can reduce liver steatosis and may improve insulin sensitivity. A Mediterranean diet contains a high concentration of monounsaturated fatty acids and a balanced polyunsaturated fatty acid omega-6 to omega-3 ratio, which has favourable effects on hepatic lipid metabolism. The high levels of polyphenols, carotenoids, vitamin E and vitamin D are protective against the development of NAFLD through their antioxidant and immunomodulatory properties. Some of the hepatoprotective effects of a Mediterranean diet may be mediated through beneficial changes in the GM composition, including; increased levels of the *Bifidobacterium* genus,
reduced levels of gram-negative LPS containing bacteria, altered SCFA production and reduced toxic metabolites such as TMAO.37, 136

5. Noncaloric artificial sweeteners

Noncaloric artificial sweeteners (NCS) are widely used by patients with the metabolic syndrome; however their safety and side effect profile remains a topic of controversy. The use of NCS have been linked to the pathogenesis of obesity, T2DM and NAFLD through a number of postulated mechanisms. In an animal study the use of NCS weakened the ability of sweet taste to predict energy and evoe autonomic and endocrine learned responses, such as the cephalic response, that prepares the digestive tract to optimally deal with ingested food.137 Other animal studies have shown that NCS interact with sweet taste receptors expressed in enteroendocrine cells and increase intestinal glucose absorption (through sodium-dependent glucose transporter isoform 1 and GLUT-2) leading to obesity, hyperinsulinemia and insulin resistance.138, 139

Many NCS are associated with changes in the composition of the GM, while others have shown no effect.140 Suez et al. found that saccharin intake in mice was associated with reductions in intestinal Akkermansia muciniphila, a commensal bacterium that exhibits probiotic properties.141 These mice developed impaired glucose tolerance, an effect that was abrogated by antibiotic treatment, and fully transferrable by FMT. Trocho et al. found that aspartame accumulates in the liver of both healthy and cirrhotic rats and might increase the risk of NAFLD via mitochondrial dysfunction and ATP depletion in the liver.142 Conversely, other animal studies have shown that NCS are not associated with hepatic steatosis, and some, such as xylooligosaccaride, may actually be protective.143

6. Green tea

Green tea and its polyphenols, such as epigallocatechin gallate, have demonstrated protective effects against the development of NAFLD. The consumption of green tea in mouse models of NAFLD has led to reductions in; hepatic steatosis, hepatic inflammation, hepatic fibrosis and insulin resistance.144-147 There are various mechanisms by which green tea may exert these beneficial effects. Consumption of green tea has been found to restore the changes in GM composition, such as the Firmicutes to Bacteroidetes ratio, which are associated with the development of obesity, hepatic steatosis and insulin resistance.148 Several studies have also demonstrated that green tea has anti-inflammatory effects, possibly through interaction with the 67-kDa laminin receptor.149 Green tea can also act as an anti-oxidant, directly by scavenging reactive oxygen and nitrogen species,150 and indirectly by upregulating the transcription of genes related to the cellular antioxidant defence.151 In a placebo-controlled RCT, green tea consumption was associated with significantly reduced alanine transaminase and aspartate transaminase levels after 12 weeks, in individuals with NAFLD.152 In contrast, a cross-sectional study of 1,024 Japanese men did not find an association between green tea consumption (≥ three cups of green tea a day) and hepatic steatosis diagnosed by ultrasonography.153

7. Caffeine and coffee

There is evidence suggesting that caffeine and coffee may be protective against the development of several elements of the metabolic syndrome, including NAFLD.154, 155 A large meta-analysis found
both caffeinated and decaffeinated coffee consumption to be inversely associated with the risk of T2DM in a dose-response manner.\textsuperscript{156} The mechanism of action is thought to be largely mediated by an ester of caffeic acid named chlorogenic acid, which improves glucose metabolism by inhibiting gut absorption of glucose and hepatic gluconeogenesis.\textsuperscript{157} Although chlorogenic acid is not specific to coffee and occurs in other food stuffs another meta-analysis found a significantly decreased risk of both developing NAFLD and it progressing to fibrosis in individuals who drank coffee on a regular basis.\textsuperscript{158} Caffeine is thought to have antifibrotic properties, through antagonism of adenosine receptor A2a, which inhibits hepatic stellate cells.\textsuperscript{159} Caffeine also leads to more rapid gut transit and decreased energy harvest from the diet, in part through reducing the expression of aquaporin-8, a water channel protein expressed in the intestinal mucosa.\textsuperscript{160} Consumption of coffee can alter the GM composition, in healthy volunteers and consumption of three cups of instant coffee per day was associated with an increase in the quantity and metabolic activity of \textit{Bifidobacterium}, a genus of bacteria thought to be protective against the development of NALFD.\textsuperscript{161}

**Linking sex differences in NAFLD to the gut microbiome**

The prevalence and severity of NAFLD is higher in men than in women during the reproductive age. After the menopause, NAFLD occurs at a higher rate in women, suggesting that oestrogen is protective.\textsuperscript{162-166} Several possible mechanisms for sexual dimorphism in NAFLD have been proposed, including; altered distribution of fat, differences in mitochondria functioning and variation of gene expression in key organs that determine insulin sensitivity.\textsuperscript{167, 168} Variation in several aspects of the gut-liver axis between men and women may also be responsible. The physiological differences in the GM composition between sexes are modest, but some researchers have found variations in GM metabolic activity and in several of the pathways by which the GM is thought to influence NAFLD pathogenesis.

Oestrogen has an influence on the composition of the GM. Dietary administration of oestrogen-like compounds can promote the proliferation and growth of certain types of gut bacteria, for example the consumption of soy phytoestrogen can increase the concentration of \textit{Bifidobacterium}.\textsuperscript{169, 170} Some bacteria can metabolise oestrogen-like compounds to produce more biologically active forms that have high affinity for human oestrogen receptors.\textsuperscript{171} For example, some gut bacteria can metabolize phytoestrogens into O-desmethylangolensin (ODMA) and equol, which are structurally similar to mammalian oestrogen.\textsuperscript{172} These metabolites have beneficial metabolic effects; ODMA producing individuals are leaner, and Equol supplementation improves glycaemic control and lowers low-density lipoprotein cholesterol.\textsuperscript{170, 172, 173}

Sex hormones strongly affect BA profiles and significant gender-specific differences become more prominent in response to a high-fat high-sugar diet.\textsuperscript{174} Jena et al. demonstrated that male mice fed a high-fat western diet, develop more severe hepatic inflammation, hepatic steatosis and insulin resistance compared to female mice, in an FXR dependant manner.\textsuperscript{175} Xi et al. investigated the mechanistic link between the GM and hepatocellular carcinoma (HCC) in NAFLD using a streptozotocin-high fat diet (STZ-HFD) induced NASH-HCC murine model and compared results for both sexes.\textsuperscript{176} STZ-HFD feeding induced a much higher incidence of HCC in male mice with substantially increased intrahepatic retention of hydrophobic BAs and decreased hepatic expression of tumour-suppressive microRNAs. Metagenomic analysis showed differences in the GM involved in BA metabolism between normal male and female mice, and such differences were amplified when
mice of both sexes were exposed to STZ-HFD. Treating STZ-HFD male mice with 2% cholestyramine led to significant improvement of hepatic BA retention, tumour-suppressive microRNA expressions, microbial gut communities, and prevention of HCC.

**The gut microbiome as a therapeutic target in NAFLD**

Despite the significant rising epidemic of NAFLD, no pharmacological interventions are currently specifically licensed for its treatment. Modifications in lifestyle such as a low-calorie, low-fat, low-glycaemic index diet and increased physical activity, are the only reliable treatment options shown to reverse the early histologic damage caused by NAFLD. Pharmacologic options, such as metformin, vitamin E, omega-3 fatty acids, ursodeoxycholic acid and lipid lowering drugs, have all been studied in patients with various stages of NAFLD, with variable results. Due to poor patient compliance with lifestyle interventions, and given the importance of the gut-liver axis in the pathogenesis of NAFLD, the effect of manipulating the GM has attracted considerable recent research interest. Through altering the GM composition, this therapeutic strategy has been postulated to; improve intestinal barrier function, prevent bacterial translocation, decrease the prevalence of LPS containing bacteria, decrease the production of harmful bacterial products, and reduce overall inflammation.177

Mechanisms for altering the GM include antibiotics, FMT or probiotics, pre-biotics and synbiotics (a combined pro-and pre-biotic). The use of antibiotics is limited due to their side effects and the emergence and prevalence of bacterial resistance. The therapeutic benefit of FMT has been investigated for several aspects of the metabolic syndrome, with variable results.178 Two studies reported improved peripheral insulin sensitivity at 6 weeks in patients with the metabolic syndrome receiving FMTs from lean donors, versus patients receiving the placebo control.179, 180 One study went on to show that the improvement in insulin sensitivity was dependent on the baseline composition of the hosts GM.179 FMT from lean donors does not appear to reduce the BMI of overweight recipients.181-183 Very limited human data is currently available regarding the impact of FMT on NAFLD. Craven et al. found that FMT from slim healthy donors to individuals with NAFLD did not affect hepatic steatosis or insulin sensitivity but did reduce gut permeability.184 More work is needed before the therapeutic role of FMT in NAFLD can be established.

**Table 3** lists the RCTs addressing the potential benefit of GM manipulation with probiotics, prebiotics and synbiotics in patients with NAFLD.185-202 Most of these clinical trials use probiotics containing bacteria of the genus *Lactobacillus, Bifidobacterium* and *Streptococcus* with or without a prebiotic such as fructo-oligosaccharide or inulin. Although studies differ in their design and intervention, the outcome of treatment appears positive, with many of the studies showing that in patients with NAFLD, probiotics can significantly improve hepatic steatosis and fibrosis as well as other metabolic parameters including glucose tolerance and obesity. It is uncertain from the study design of these trials whether the probiotics acted to favourably change the GM. Furthermore, other studies have shown that probiotics/prebiotics/synbiotics have no effect on biochemical or radiological end points in NAFLD. Thus, several questions remain unanswered. For example, what is the mechanism by which probiotics improve NAFLD; what is the most effective probiotic-prebiotic combination; what is the optimal duration of treatment and which aspect of the disease process in NAFLD benefits from treatment. Although promising results along with minimal cost and side effects make probiotics an exciting treatment option for NAFLD that could be extensively used as a health-
food supplement to treat early disease, further RCTs with larger sample sizes, longer follow-up, and assessments of efficacy based on liver histology (or acceptable alternative diagnostic surrogate markers) are urgently needed.

Conclusion

Abundant evidence from animal and human studies show that the diet and the GM play a role in the pathogenesis of NAFLD. The consumption of long-chained saturated fatty acids, fructose and AGEs, all plentiful within the western diet, can cause dysbiosis and potentially contribute to the development of hepatic steatosis and inflammation. Dysbiosis alters metabolic pathways and inflammatory processes through; altered production of SCFAs, altered choline and BA metabolism, increased production of bacteria-derived ethanol, higher abundance of LPS containing gram negative bacteria and increased intestinal permeability. The clinical significance of specific gut microbial alterations associated with NAFLD still remains unclear and therefore there is currently no diagnostic or therapeutic role for analysing the GM or modulating its composition in NAFLD. Further work is required to investigate the significance of GM alterations in NAFLD, and to clarify the therapeutic role of probiotics, prebiotics and synbiotics in the management of patients at different stages of the disease process.
References


H., Microbiome Signatures Associated With Steatohepatitis and Moderate to Severe Fibrosis in Children With Nonalcoholic Fatty Liver Disease. *Gastroenterology* 2019, **157**(4), 1109-1122.


168. Skubic, C.; Drakulić, Ž.; Rozman, D., Personalized therapy when tackling nonalcoholic fatty liver disease: a focus on sex, genes, and drugs. *Expert Opin Drug Metab Toxicol* 2018, 14 (8), 831-841.


175. Jena, P. K.; Sheng, L.; Liu, H. X.; Kalanetra, K. M.; Mirsoian, A.; Murphy, W. J.; French, S. W.; Krishnan, V. V.; Mills, D. A.; Wan, Y. Y., Western Diet-Induced Dysbiosis in Farnesoid X Receptor


Figure 1. Represents the proportions of the phylum, class and genus of bacteria commonly found in the health gut. Bacteroidetes comprise the majority phylum of the GM, of which the majority genus is *Bacteroides*. Firmicutes consists of predominant genera such as *Faecalibacterium*, *Roseburia* and *Oscillibacter*. The Proteobacteria phylum is proportionally less abundant and mainly represented by the *Parasutterella* genus. Actinobacteria, such as *Propionibacterium* and *Bifidobacterium*, are found in small numbers in the healthy gut.
Table 1. The design and main findings of animal studies investigating the role of the gut microbiome in the pathogenesis of NAFLD.

<table>
<thead>
<tr>
<th>Author</th>
<th>Study design</th>
<th>Main findings</th>
<th>Bacteria in the gut microbiota of mice with NAFLD</th>
</tr>
</thead>
</table>
| Le Roy et al.\(^20\)   | Germ-free mice received a faecal transplant from 2 different groups of mice; either mice that demonstrated weight gain, systemic inflammation & insulin resistance on a high fat diet, or mice that demonstrated weight gain but no inflammation or insulin resistance on a high fat diet. | Germ-free mice took on the phenotype of their faecal donors. The mice that developed the inflammatory & insulin resistance phenotype also developed hepatic steatosis. | Genus: **Allobaculum**  
Species: **Bacteroides vulgatus**  
Phylum: Firmicutes  
Genus: Barnesiella, Roseburia  
Species: Lachnospiraceae bacterium, Barnesiella intestinihominis |
| Henao-Mejia et al.\(^21\) | NAFLD mouse models were used in dysbiotic (inflammasome deficient) & non-dysbiotic (wild type) mice to examine the effect of inflammasome deficient changes in the GM (increased Bacteroidetes) on the development of NAFLD. NAFLD mouse models used were; methionine choline-deficient diet model, leptin receptor deficiency steatosis model, & the high fat diet model. | Inflammasome deficiency changes in the GM were associated with:  
• ↑ hepatic steatosis  
• ↑ hepatic inflammation.  
• Co-housing dysbiotic & non dysbiotic mice exacerbated NAFLD in both groups of mice. | Genus: **Lactobacillus**  
Phylum: Bacteroidetes  
Family: Prevotellaceae |
| Zeng et al.\(^22\)      | Obese mice (C57BL/6 model) were fed a high fat (45% energy) or low-fat (10% energy) diet for 10 weeks. | Mice on a high fat diet had:  
• ↑ body weight (by 34%)  
• ↑ hepatic fat & inflammation  
• ↑ levels of lactobacillus in faeces which correlated positively with the severity of hepatic steatosis. | Species: Lactobacillus gasseri, Lactobacillus taiwanensis |
| Cano et al.\(^23\)      | Obese (high fat diet-induced) & lean mice were given either placebo or a probiotic consisting of Bifidobacterium pseudocatenulatum for 7 weeks. | Obese mice taking probiotic showed:  
• ↓ food intake & body weight  
• ↓ insulin resistance  
• ↓ hepatic fat  
• ↓ serum inflammatory markers | Genus: **Bifidobacteria**  
Family: Enterobacteriaceae |

\(^{20}\) Reference: Le Roy et al.  
\(^{21}\) Reference: Henao-Mejia et al.  
\(^{22}\) Reference: Zeng et al.  
\(^{23}\) Reference: Cano et al.
Table 2. The design and main findings of human studies investigating the role of the gut microbiome in the pathogenesis of NAFLD.

<table>
<thead>
<tr>
<th>Author</th>
<th>Study design</th>
<th>Main findings</th>
<th>Bacteria in the gut microbiota of patients with NAFLD</th>
</tr>
</thead>
</table>
| **Spencer et al.**<sup>25</sup> | The GM of 14 adults before & during a 42 day period on a choline-depleted diet was analysed. Hepatic steatosis, associated with a choline deplete diet, was measured by MRI. | The risk of developing hepatic steatosis correlated with:  
• ↑ baseline levels of Erysipelotrichia  
• ↓ baseline levels of Gammaproteobacteria | Class: Gammaproteobacteria  
Class: Erysipelotrichia                                                                                                                               |
| **Wong et al.**<sup>26</sup>     | The GM composition was analysed in a group of 42 adults: 20 with biopsy proven NASH & 22 healthy controls. | Individuals with NASH (compared to healthy controls) had:  
• ↓ Faecalibacterium & Anaerosporobacter  
• ↑ Parabacteroides & Allisonella | Genus: Faecalibacterium, Anaerosporobacter  
Genus: Parabacteroides, Allisonella                                                                                                                     |
| **Mouzaki et al.**<sup>27</sup>  | The GM composition was analysed in a group of 50 adults: 11 with biopsy proven simple steatosis, 22 with biopsy proven NASH, & 17 healthy controls. | Individuals with NASH (compared to those with steatosis & healthy controls) had:  
• ↓ Bacteroidetes  
• ↑ Clostridium cocoides | Phylum: Bacteroidetes  
Species: Clostridium cocoides                                                                                                                        |
| **Zhu et al.**<sup>28</sup>      | The GM composition was analysed in 63 children; 22 with biopsy proven NASH, 25 obese children without NASH (clinically), & 16 healthy normal weight children. | The GM of children with NASH (compared to healthy controls) had:  
• ↓ Firmicutes & Actinobacteria  
• ↑ Bacteroidetes  
• ↓ Bifidobacterium | Phylum: Firmicutes, Actinobacteria  
Genus: Blautia, Faecalibacterium, Bifidobacterium                                                                                                    |
| **Raman et al.**<sup>29</sup>    | The GM composition was analysed in a group of 60 adults: 30 obese with clinically defined NAFLD (no biopsy) & 30 non-obese controls. | The GM of individuals with NAFLD (compared with non-obese controls) had:  
• ↑ Firmicutes (specifically Lactobacillus) | Phylum Firmicutes  
Genus: Lactobacillus                                                                                                                                   |
| **Loomba et al.**<sup>30</sup>   | The GM composition was analysed in a group of 86 adults with biopsy proven NAFLD, 72 with mild hepatic fibrosis (stage 1 or 2), 14 with advanced hepatic fibrosis (stage 3 or 4). | The GM in individuals with advanced hepatic fibrosis (compared with mild hepatic fibrosis) had:  
• ↑ Proteobacteria  
• ↓ Firmicutes  
• ↑ Escherichia coli & Bacteroides vulgatus | Phylum: Firmicutes  
Phylum: Proteobacteria  
Species: Escherichia coli, Bacteroides vulgatus                                                                                                    |
| **Schwimmer et al.**<sup>31</sup> | The GM composition was analysed in a group of 87 children with biopsy proven NAFLD & 37 obese children without NAFLD. | The GM in children with NAFLD (compared to obese children without NAFLD) had:  
• ↓ α Diversity  
• ↑ Prevotella copri | Species: Prevotella copri                                                                                                                               |
| **Tsai et al.**<sup>32</sup>     | The GM composition was analysed in a group of 75 adults; 25 with biopsy proven steatosis, 25 with biopsy proven NASH & 25 healthy controls. | The GM in individuals with NAFLD (compared to individuals without NAFLD) had:  
• ↓ Diversity  
• ↑ Bacteroidetes  
• ↓ Firmicutes  
• ↓ Clostridia | Phyla: Firmicutes  
Class: Clostridia  
Phyla: Bacteroidetes                                                                                                                                   |
| **Del Chierico et al.**<sup>33</sup> | The GM composition was analysed in a group of 61 children with NAFLD or obesity & 54 healthy controls. | The GM in children with NAFLD (compared to healthy controls) had:  
• ↓ α & β Diversity  
• ↑ Actinobacteria  
• ↓ Bacteroidetes | Phyla: Bacteroidetes  
Family: Rikenellaceae  
Genus: Oscillospira  
Phyla: Actinobacteria  
Genus: Bradyrhizobium, Anaerococcus, Peptoniphilus, Dorea, Ruminococcus  
Species: Propionibacterium acnes<sup>31</sup>                                                                                                           |
| **Wang et al.**<sup>34</sup>     | The GM composition was analysed in a group of 126 nonobese adults; | The GM in individuals with NAFLD (compared to individuals without NAFLD) had:  
• ↓ Diversity  
• ↑ Bacteroidetes  
• ↓ Firmicutes  
• ↓ Clostridia | Phyla: Firmicutes  
Phyla: Bacteroidetes                                                                                                                                   |
43 with NAFLD on ultrasound & 83 healthy controls.

<table>
<thead>
<tr>
<th>Diversity</th>
<th>Bacteroidetes</th>
<th>Firmicutes</th>
<th>Gram negative species</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Shen et al.35</th>
<th>The GM composition was analysed in a group of 47 adults; 25 with NAFLD &amp; 22 healthy controls.</th>
<th>The GM in individuals with NAFLD (compared to individuals without NAFLD) had:</th>
<th>Genus: Prevotella</th>
<th>Phyla: Proteobacteria, Fusobacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>↓ Diversity</td>
<td>↓ Diversity</td>
<td></td>
<td>Family:</td>
</tr>
<tr>
<td></td>
<td>↑ Proteobacteria</td>
<td>↑ Proteobacteria</td>
<td>Prevotella</td>
<td>Lachnospiraceae</td>
</tr>
<tr>
<td></td>
<td>↑ Fusobacteria</td>
<td>↑ Fusobacteria</td>
<td></td>
<td>Enterobacteriaceae</td>
</tr>
<tr>
<td></td>
<td>↓ Prevotella</td>
<td>↓ Prevotella</td>
<td></td>
<td>Erysipelotrichaceae</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Streptococcaceae</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Genus: Shigella</td>
</tr>
</tbody>
</table>

Figure 2. A summary of the effect of dietary factors on the gut microbiota and their effects on hepatic pathways leading to the development of hepatic steatosis, inflammation and fibrosis. Green tea, caffeine, coffee, a Mediterranean diet and some polyunsaturated fatty acids, such as omega-3, have demonstrated favourable effects on the composition of the GM. Consumption of saturated fatty acids, fructose and advanced glycated end products cause harmful changes to the GM composition. Dysbiosis is associated with altered production of SCFA, altered choline and bile acid metabolism, higher abundance of LPS containing bacteria, increased bacterial derived ethanol, increased intestinal permeability and upregulation of inflammatory processes. The harmful consequences of dysbiosis affect normal liver physiology, particularly given the close relationship between the gut and liver. Hepatic lipogenesis and triglyceride storage are upregulated whilst lipid oxidation is reduced, leading to hepatic steatosis. Activation of hepatic toll like receptors and the generation of reactive oxygen species drives hepatic inflammation and fibrosis. SCFA; short chain fatty acid, LPS; lipopolysaccharides, TMA; trimethylamine, TG; triglycerides, TLR; toll like receptors, ROS; reactive oxygen species.
Table 3. Randomised controlled trials addressing the therapeutic modulation of the GM in NAFLD patients.

<table>
<thead>
<tr>
<th>Author &amp; year</th>
<th>Study Design</th>
<th>Bacterial Species</th>
<th>Condition</th>
<th>Main Outcome Related to NAFLD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malaguarnera et al.185 2012</td>
<td>RCT, N = 66 24 weeks</td>
<td>Bifidobacterium + fructo-oligosaccharides</td>
<td>NASH</td>
<td>Biochemistry (↓) AST, endotoxins (-) ALT, glucose, BMI Imaging/Biopsy Biopsy: steatosis &amp; NASH improved</td>
</tr>
<tr>
<td>Shavakhi et al.186 2013</td>
<td>Double-blind RCT, N = 64 6 months</td>
<td>Lactobacillus, Bifidobacterium, Streptococcus</td>
<td>NASH on metformin (↓) ALT, AST</td>
<td>US: hepatic steatosis improved</td>
</tr>
<tr>
<td>Wong et al.187 2013</td>
<td>RCT, N = 20 6 months</td>
<td>Lactobacillus, Bifidobacterium</td>
<td>NASH</td>
<td>(↓) ALT, AST, BMI, glucose MRS: hepatic steatosis improved</td>
</tr>
<tr>
<td>Alisi et al.188 2014</td>
<td>Double-blind RCT, N = 44 4 months</td>
<td>Lactobacillus, Bifidobacterium, Streptococcus</td>
<td>NAFLD children (↓) BMI (-) ALT, TG</td>
<td>US: hepatic steatosis improved</td>
</tr>
<tr>
<td>Eslamparast et al.189 2014</td>
<td>Double-blind RCT, N = 52 28 weeks</td>
<td>Lactobacillus, Bifidobacterium, Streptococcus + fructo-oligosaccharide</td>
<td>NAFLD</td>
<td>(↓) ALT, AST Transient elastography: liver stiffness improved</td>
</tr>
<tr>
<td>Agharian et al.190 2016</td>
<td>Double-blind RCT, N = 80 8 weeks</td>
<td>Lactobacillus, Bifidobacterium, Streptococcus + fructo-oligosaccharide</td>
<td>NAFLD</td>
<td>(-) AST, ALT US: hepatic steatosis improved</td>
</tr>
<tr>
<td>Ferolla et al.191 2016</td>
<td>RCT, N = 50 3 months</td>
<td>Lactobacillus + inulin</td>
<td>NASH</td>
<td>(↓) BMI (-) ALT, AST, LPS, intestinal permeability MRI-PDFF:-steatosis improved but no change in liver fibrosis</td>
</tr>
<tr>
<td>Famouri et al.192 2017</td>
<td>Triple-Blind RCT, N = 64 12 weeks</td>
<td>Lactobacillus, Bifidobacterium</td>
<td>NAFLD obese children (↓) ALT, AST, cholesterol, TG (-) BMI</td>
<td>US: hepatic steatosis improved</td>
</tr>
<tr>
<td>Manzhalii et al.193 2017</td>
<td>RCT, N = 75 12 weeks</td>
<td>Lactobacilli, Bifidobacteria, Streptococcus</td>
<td>NASH on a ↓ fat diet (↓) ALT, BMI, cholesterol</td>
<td>Transient elastography: liver stiffness improved</td>
</tr>
<tr>
<td>Mofidi et al.194 2017</td>
<td>Double-blind RCT, N = 50 28 weeks</td>
<td>Lactobacillus, Bifidobacterium, Streptococcus + fructo-oligosaccharide</td>
<td>NAFLD</td>
<td>(↓) AST, ALT, glucose, TG, cholesterol Transient elastography: hepatic steatosis &amp; liver stiffness improved</td>
</tr>
<tr>
<td>Bakhshi-moghaddam et al.195 2018</td>
<td>RCT, N = 102 24 weeks</td>
<td>Bifidobacterium + Inulin</td>
<td>NAFLD</td>
<td>(↓) ALT, ALT, GGT, TG, cholesterol US: hepatic steatosis improved</td>
</tr>
<tr>
<td>Kobyliak et al.196 2018</td>
<td>Double-blind RCT, N = 48 8 weeks</td>
<td>Bifidobacterium, Lactobacillus, Lactococcus, Propionibacterium, Acetobacter + omega-3 fatty acids</td>
<td>NAFLD with T2DM (↓) TG, cholesterol, FLI (-) AST, ALT SWE: No significant change in liver stiffness</td>
<td></td>
</tr>
<tr>
<td>Kobyliak et al.197 2018</td>
<td>Double-blind RCT, N = 58 8 weeks</td>
<td>Bifidobacterium, Lactobacillus, Lactococcus, Propionibacterium, Acetobacter</td>
<td>NAFLD with T2DM (↓) AST, FLI (-) ALT, TG, cholesterol SWE: No significant change in liver stiffness</td>
<td></td>
</tr>
<tr>
<td>Sayari et al.198 2018</td>
<td>RCT, N = 138 16 weeks</td>
<td>Lactobacillus, Bifidobacterium, Streptococcus + fructo-oligosaccharide</td>
<td>NAFLD taking sitagliptin (↓) Glucose, AST, cholesterol (-) ALT, TG, BMI US: no significant change in hepatic steatosis</td>
<td></td>
</tr>
<tr>
<td>Wang et al.199 2018</td>
<td>Double-blind RCT, N = 200 1 month</td>
<td>Lactobacillus, Lactobacillus, Enterococcus, Bacillus</td>
<td>NAFLD</td>
<td>(↓) AST, ALT, TG, cholesterol US: no significant change in hepatic steatosis</td>
</tr>
<tr>
<td>Ahn et al.200 2019</td>
<td>Double-blind RCT, N = 68 12 weeks</td>
<td>Lactobacillus, Pediococcus, Bifidobacterium</td>
<td>NAFLD with obesity (↓) TG (-) AST, ALT, LPS, cholesterol, glucose MRI-PDFF: hepatic steatosis improved Transient elastography: no significant change in liver stiffness</td>
<td></td>
</tr>
<tr>
<td>Duseja et al.201 2019</td>
<td>Double-blind RCT, N = 30 1 year</td>
<td>Lactobacillus Bifidobacterium Streptococcus</td>
<td>NAFLD</td>
<td>(↓) ALT, LPS (-) AST Biopsy: improved NAS score, hepatocyte ballooning &amp; fibrosis</td>
</tr>
<tr>
<td>Scorletti et al.202 2020</td>
<td>Double-blind RCT, N = 104 10-14 months</td>
<td>Bifidobacterium + fructo-oligosaccharide</td>
<td>NAFLD</td>
<td>(-) ELF score MRS: no significant change in hepatic steatosis</td>
</tr>
</tbody>
</table>
RCT; randomised control trial, NAFLD; non-alcoholic liver disease, NASH; non-alcoholic steatohepatitis, ALT; alanine transaminase, AST; Aspartate transaminase, LPS; lipopolysaccharide, TG; triglyceride, ELF; enhanced liver fibrosis, US; ultrasound, FLI; fatty liver index, SWE; shear wave elastography, MRS; magnetic resonance spectroscopy, NAS; NAFLD activity score.