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## **Gut Microbiome and Metabolic-associated Fatty Liver Disease: Current Status and Potential Applications**

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### **Abstract**

Metabolic-associated fatty liver disease (MAFLD) is one of the most common chronic liver diseases worldwide. In recent years, the occurrence rate of MAFLD has been on the rise, mainly due to lifestyle changes, high-calorie diets, and imbalanced dietary structures, thereby posing a threat to human health and creating heavy social and economic burdens. With the development of 16S sequencing and integrated multi-omics analysis, the role of the gut microbiota (GM) and its metabolites in MAFLD has been further recognized. The GM plays a role in digestion, energy metabolism, vitamin synthesis, the prevention of pathogenic bacteria colonisation, and immunoregulation. The gut-liver axis is one of the vital links between the GM and the liver. Toxic substances in the intestine can enter the liver through the portal vascular system when the intestinal barrier is severely damaged. The liver also influences the GM in various ways, such as bile acid circulation. The gut-liver axis is essential in maintaining the body's normal physiological state and plays a role in the onset and prognosis of many diseases, including MAFLD. This article reviews the status of the GM and MAFLD and summarizes the GM characteristics in MAFLD. The relationship between the GM and MAFLD is discussed in terms of bile acid circulation, energy metabolism, micronutrients, and signalling pathways. Current MAFLD treatments targeting the GM are also listed.

## INTRODUCTION

Metabolic-associated fatty liver disease (MAFLD), originally known as <sup>2</sup> non-alcoholic fatty liver disease (NAFLD), is one of the most common chronic liver diseases worldwide. Liver inflammation and fibrosis are the pathological processes implicated in MAFLD. MAFLD can develop into non-alcoholic steatohepatitis (NASH), which can then progress to liver cirrhosis, hepatic failure, and liver cancer<sup>[1]</sup>. The rate of MAFLD has been on the rise, with a global rate of 25%-30%, due to lifestyle changes, excessive calorie intake, and unbalanced diet structures. In certain groups, such as patients with Type 2 diabetes, the rate of MAFLD even exceeds 70%<sup>[2, 3]</sup>. MAFLD poses a threat to human health and leads to substantial social and economic burdens. The gut microbiota (GM) lives in the human intestinal tract. In the past 10 years, there has been an exponential growth of studies on the relationship between GM and human health and disease in databases such as PubMed. The GM consists of over  $10^{14}$  microorganisms<sup>[4]</sup>, and its genome comprises of over 3 million genes, whereas the human genome consists of approximately 23,000 genes<sup>[5]</sup>. Therefore, the GM is considered as one of the “new organs” in human beings. Steady-state GM plays a role in digestion, energy metabolism, vitamin synthesis, the prevention of pathogenic bacteria colonisation, and immunoregulation<sup>[6, 7]</sup>. <sup>3</sup> The gut-liver axis is one of the vital links between the GM and the liver. The intestine and liver both originate from the ventral foregut endoderm. When the intestinal barrier is severely damaged, toxic substances in the intestinal tract enter the portal vein through the superior and inferior mesenteric veins and then flow into the liver. Meanwhile, the liver influences the intestinal microecology in various ways. For example, the liver secretes bile acids (BA), which enter in the enterohepatic circulation to alter the intestinal microecology<sup>[8]</sup>. In recent years, the development of 16S sequencing and integrated multi-omics analysis has helped to further understand the role of the GM and its metabolites in MAFLD. It is reported that *Akkermansia muciniphila* can improve liver function, reduce oxidative stress, inhibit inflammation, and reverse the metabolic disorder caused by high-fat diets<sup>[9]</sup>. Some secondary bile acid-producing

bacteria, such as *Lactobacillaceae* and *Lachnospiraceae*, have cholesterol-reducing potential<sup>[10]</sup>. Specific bile acids produced by bacteria can regulate GM structure and restore GM balance<sup>[11]</sup>. Numerous studies have confirmed that GM metabolites play a significant role in the onset and progression of MAFLD when they are present in the intestine or enter the circulation. GM can mediate the fermentation of dietary fibres, leading to the production of short-chain fatty acids (SCFAs) as the primary metabolites of this process. Among the SCFAs, butyric acid can improve the MAFLD induced by high-fat diets *via* activating peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ) to inhibit liver inflammation and enhance the expression of glucagon-like peptide-1 receptor (GLP-1R)<sup>[12]</sup>. Furthermore, SCFAs can also activate G-protein-coupled receptor (GPCR) and induce <sup>13</sup> the release of glucagon-like peptide-1 (GLP-1) and peptide YY (PYY), resulting in metabolically balanced feedback regulation<sup>[13]</sup>. SCFAs can not only regulate glycolipid metabolism, inhibit fat synthesis, and reduce liver fat content but also escalate intestinal barrier function, thereby improving MAFLD<sup>[14]</sup>. Therefore, the main aims of this research field are to observe and characterize the GM in MAFLD, investigate the impact of GM and its metabolites on the onset and progression of MAFLD, and explore the potential of targeting the GM for MAFLD treatment.

This article primary focuses on elucidating the impact of the gut-liver axis on MAFLD. It provides an overview of the existing clinical MAFLD cases and commonly utilized animal models. The review involves the important aspects such as bile acid circulation, energy metabolism, microelement disorder, and other relevant factors such as signal pathways and GM metabolites implicated the development of MAFLD. Then, current MAFLD treatments utilizing GM as the target are presented. **Table 1** summarizes the key characteristics of GM in both clinical MAFLD cases and commonly used MAFLD animal models.

### **1. GM AND METABOLIC DISORDERS IN MAFLD**

### **2. THE INFLUENCE OF GM ON RELEVANT SIGNALING PATHWAYS**

### **3. THE BACTERIAL FLORA THERAPY OF MAFLD**

## 1. GM and metabolic disorders in MAFLD

### 1.1 Bile acid metabolism disorders

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Primary bile acids are synthesized in the liver before being secreted into the gall bladder and released into the duodenum after a meal. Bacteria metabolize primary bile acids in the intestinal tract into secondary bile acids, which are then reabsorbed into the portal vein. While most bile acid molecules are captured by the liver and undergo recirculation, a small fraction of them persists in the blood as signalling molecules. Bile acid synthesis in hepatocytes involves the oxidation of cholesterol mediated by cytochromes P450 enzymes. The synthesis mainly occurs through the classic and alternative pathways, producing cholic and chenodeoxycholic acids, which are subsequently conjugated to taurine and glycine, respectively, to form conjugated bile acids. Synthesized primary bile acids are deposited into the gallbladder *via* the bile salt export pump. Gall bladder contraction triggered by eating promotes bile acid secretion into the intestinal tract<sup>[15]</sup>. Primary bile acids increase the permeability of the intestinal mucosa, resulting in endotoxemia and aggravating MAFLD. Thus, the bile acid level is elevated in the liver tissue, serum, and urine of MAFLD patients. Meanwhile, there is a significantly higher proportion of hydrophobic and cytotoxic bile acids<sup>[16]</sup>. Patients with NASH exhibit increased synthesis of bile acids compared to other conditions. The ratio of primary bile acids to secondary bile acids is also higher than in healthy individuals<sup>[17]</sup>. Bile salt hydrolases produced by *Bacteroides*, *Clostridium*, *Lactobacillus*, *Bifidobacterium*, and *Listeria* in the GM can deconjugate conjugated bile acids to form free bile acids. *Clostridium*, *Fusobacterium*, *Peptococcus*, and *Pseudomonas* species have the ability to catalyze the desulfuration of bile acids. *Bacteroides*, *Eubacterium*, *Clostridium*, *Escherichia*, *Eggerthella*, *Peptostreptococcus*, and *Ruminococcus* are implicated in the dehydroxylation of primary bile acids to produce secondary bile acids<sup>[18]</sup>. Moreover, it was found that *Clostridium leptum* is positively related to taurocholic acid and negatively related to cholic acid and chenodeoxycholic acid. This indicates that *Clostridium leptum* may promote the transformation from primary bile acids to



secondary bile acids, thereby reducing the damage caused by primary bile acids to the liver<sup>[19]</sup>.

About 95% of primary and secondary bile acids can be reabsorbed in the intestine and transported back into the liver through the portal veins. However, lithocholic acids present in secondary bile acids are primarily excreted with the feces. Hepatocytes synthesize new bile acids to compensate for the bile acids lost in the enterohepatic circulation. After reabsorption, bile acids are conjugated to the farnesoid X receptor (FXR) and G protein-coupled bile acid receptor (GPBAR; also named TGR5), thereby promoting the secretion of fibroblast growth factor 19 (FGF19) by intestinal cells. FGF19 conjugates to fibroblast growth factor receptor 4 (FGFR4), activating c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK). These two signaling pathways decrease the genetic expression of cholesterol 7  $\alpha$ -hydroxylase (CYP7A1), which inhibits bile acid synthesis through negative feedback<sup>[20]</sup>. The effect of choline on lipid metabolism may be mediated by activating FXR to participate in liver lipid metabolism, thereby reducing the synthesis of cholesterol and triglycerides. The effect of bile acids on glucose metabolism has been established, as evidenced by the presence of insulin resistance and hyperglycemia in FXR gene-deficient mice, and the administration of oral dietary cholic acid to activate FXR can inhibit the expression of the gluconeogenesis gene in mice, reduce fasting glucose, and increase insulin sensitivity. Choline alleviates metabolic inflammation induced by TNF- $\alpha$  and LPS, while bile acids such as cholic acid (CA), deoxycholic acid (DCA), and chenodeoxycholic acid (CDCA) can inhibit the release of monocyte chemotactic protein-1 (MCP-1) induced by TNF and LPS, suggesting that bile acids have anti-inflammatory effects<sup>[21]</sup>. Choline is a pleiotropic hormone-like signaling molecule with both metabolic and endocrine functions. It plays an important role in regulating cholesterol and triglyceride metabolism, insulin resistance, metabolic inflammation, and liver steatosis *via* activation of choline-specific receptors that are widely distributed in the body.

GM can regulate the synthesis and reabsorption of bile acids. Germ-free animals exhibit reduced excretion of bile acids in feces, accompanied by a significant increase in

the total bile acid content in the gall bladder and small intestine<sup>[22, 23]</sup>. Probiotics supplements or fecal microbiota transplantation can distinctly reduce the total bile acid content in the liver, gall bladder, and cecum germ-free animals<sup>[24]</sup>. In aseptic conditions, tauro- $\beta$ -muricholic acid, a primary bile acid, accumulates due to its inability to undergo further metabolism. It can be used as an antagonist to inhibit intestinal FXR expression, thereby downregulating FXR expression. The expression of liver CYP7A1 promotes liver bile acid synthesis and is regulated via the enterohepatic circulation<sup>[23]</sup>. There is an increased expression of bile acid transporters (ASBT) in the ileum and colon of germ-free animals. This leads to decreased bile acid excretion in the feces, resulting in highly efficient bile acid reabsorption. The key enzymes in bile acid synthesis, such as CYP7A1, CYP7B1, and CYP27A1, can all be regulated by the GM, mainly via induction of the FXR signaling pathway<sup>[25]</sup>. Moreover, bile acids also have an effect on the GM. Amphipathic bile acids directly perform the anti-bacterial function by breaking the cell membrane of bacteria, which is critical to maintaining the steady state of the bacterial flora. Studies revealed that bile duct ligation could arouse bacterial translocation in rat's mesenteric lymph nodes as early as after one week. Three weeks after bile duct ligation, the translocation was found to be expanded to tissue such as the liver, spleen, and lung. In addition, gram-negative bacteria in the animal cecum and endotoxin levels in the blood were significantly elevated. The villi of the distal ileum were flattened, and Peyer's patches increased in size<sup>[26]</sup>. Bacteria overgrowth in the small intestine, bacterial translocation, and endotoxemia can be effectively inhibited by taking bile acids orally<sup>[27]</sup>. Secondary bile acids can inhibit the growth of *Clostridium difficile*<sup>[28]</sup>. In addition, bile acids can enrich the bacteria that utilize bile acids. For example, bacteria with bile salt hydrolase activity, such as *Lactobacillus reuteri*, can resist cytotoxicity resulting from bile salts<sup>[29]</sup>. An in vitro culture experiment revealed that bile acids were required for the growth of *Bilophila wadsworthia*<sup>[30]</sup>. Studies have shown that diets rich in milk fat altered the bile acid profile, mainly by increasing the total amount of bile acid, and the abundance of *Bilophila wadsworthia* also increased with the increase in bile acids.

## **1.2 Choline metabolism disorders**

Choline is a quaternary amine rich in methyl groups that exists in tissues in either free or esterified forms. In the liver, choline exists in the form of phosphatidylcholine (PC). Choline has been recognized as an essential nutrient by the National Academy of Sciences (NAS) since 1998. The biological functions of choline mainly include neurotransmitter synthesis, lipid metabolism, and cell membrane signal transduction. Choline can also serve as a methyl donor for the synthesis of PC in the liver<sup>[31]</sup>. PC, in turn, is indispensable for the synthesis and secretion of very low-density lipoprotein (VLDL). Moreover, choline also prevents abnormal lipid accumulation by mediating liver lipid transport. Therefore, the lack of choline may lead to hepatic steatosis<sup>[32]</sup>. For over 50 years, researchers have recognized the association between choline deficiency and accumulation of fat in the liver. Choline-deficient diets are often used in animal experiments to induce MAFLD. The administration of diets deficient in choline and vitamin B12 to weanling rats induces fatty liver and renal cortical necrosis, resulting in high deaths rate within 10 days<sup>[33, 34]</sup>. Research has demonstrated that patients with MAFLD exhibit varying degrees of decreased choline levels in their plasma, which is associated with the degree of liver damage<sup>[31, 35]</sup>.

Choline has two primary sources. Approximately 70% of choline is obtained from dietary sources, while the remaining 30% is synthesized by the GM. Among 79 gut microbiota strains screened from the human gastrointestinal tract, eight strains have been identified to significantly affect choline metabolism (5 *Anaerococcus hydrogenalis*, *Clostridium asparagiforme*, *Clostridium hathewayi*, *Clostridium sporogenes*, *Edwardsiella tarda*, *Escherichia fergusonii*, *Proteus penneri*, and *Providencia rettgeri*). However, genetic analysis has revealed that the gene set responsible for anaerobic choline metabolism is widely distributed among the three main bacterial groups present in the human gut, including *Proteobacteria*, *Firmicutes*, and *Actinobacteria*. This metabolic pathway contributes to the bioavailability of choline in the human body and subsequently affects serum choline concentration. On the other hand, choline levels in the diet may also affect the gut microbiota. In patients with choline deficiency, choline supplementation has been shown to decrease the abundance of *Gammaproteobacteria*. This reduction in



*Gammaproteobacteria* can alleviate the inhibition of key enzymes in choline metabolism, thereby reducing the occurrence of MAFLD [36-38]. As a result, GM disruption can alter choline metabolism and reduce the host's capacity to efficiently utilize choline, leading to a relative deficiency of choline and increased production of substances such as trimethylamine N-oxide (TMAO). In turn, this can lead to the occurrence of hepatic steatosis[32, 39, 40]. Bacterial species such as *Escherichia coli* and *Desulfovibrio desulfuricans* within in GM have the capability to utilize choline and convert it into methylamine. When there is a disruption in the GM involved in the metabolism of choline, it can lead to a deficiency of choline and potentially contribute to the development of MAFLD[40]. In the presence of abundant MAFLD-associated intestinal bacteria, there is an increase demand for choline by these bacteria. This leads to choline deficiency in the host, exacerbating the risk of MAFLD and potentially progressing to NASH[41]. Reduced choline utilization may lead to decreased PC synthesis in the body, thereby inducing fatty acid synthesis and increasing TG production. Meanwhile, it decreases the surface activity of lipid droplets lacking PC. Large lipid droplets are easier to form. Therefore, it is difficult for LPL to decompose lipid droplets[9, 42]. GM disruption can alter the body's reservoir of choline, thereby inducing choline deficiency and decreasing VLDL secretion, which leads to the accumulation of fat in the liver[43]. Moreover, TMAO and GM are closely associated with choline metabolism. GM can produce enzymes that catalyze the transformation of dietary choline into methylamine. After metabolism, methylamine is transformed into TMAO by GM-produced trimethylamine-lyase. TMAO can regulate protein activity and stability, increase foam cell production, inhibit cholesterol transport, aggravate liver fat deposition, and even induce liver inflammation[31, 32, 44]. Trimethylamine lyases in GM can decompose dietary choline into TMA, which enters the liver through the portal vein and is oxidated into TMAO. TMAO can upregulate the expression of sterol regulatory element-binding protein-1c (SREBP-1c). SREBP-1c is a critical transcription factor in the regulation of liver lipid metabolism, which promotes TG synthesis, aggravating hepatic steatosis[45]. TMAO also upregulates glucose metabolism and increases serum inflammatory factors for IR promotion[46, 47]. It

affects lipid metabolism and cholesterol's steady state by reducing the transformation of cholesterol into bile acids<sup>[35]</sup>. TMAO contributes to the progression of MAFLD through various mechanisms. GM metabolites also include secondary bile acids and ethanol, which have been discussed in the previous section.

### **1.3 Lipid metabolism disorders**

The GM has the ability to generate energy from indigestible substances (e.g., *Firmicutes* can ferment resistant starch to provide energy for intestinal epithelial cells)<sup>[48]</sup>. Therefore, GM is critical in the development of obesity, and its disruption can lead to obesity-related MAFLD<sup>[49]</sup>. Obesity can increase the level of proinflammatory cytokines secreted by macrophages and promote adipose tissue infiltration, leading to the development of hepatic steatosis<sup>[50]</sup>. The intestines of obese people are rich in *Firmicutes*. *Firmicutes* can ferment indigestible dietary fiber (polysaccharide) and produce additional energy from the intestine content, which promotes the progression of obesity and MAFLD. The fecal microbiota of obese mice caused by high-fat fodder was transplanted to mice fed with regular fodder, and it was found that mice transplanted with fecal microbiota on a high-fat diet had more fat deposition than mice transplanted with fecal microbiota on a regular diet. A further study found that structural changes in the GM could lead to greater lipid absorption by the body, promoting the biosynthesis of fatty acids. However, it was proven that some probiotics, such as *Lactobacillus*, could reduce liver fat deposition by reducing fatty acid absorption in the host intestine<sup>[51, 52]</sup>.

Fasting-induced adipocyte factor (FIAF) is a lipoprotein lipase inhibitor, and inhibition of the FIAF gene can significantly reduce body fat deposition. Lipoprotein lipase (LPL) is the key regulatory factor of fatty acid released from lipoprotein in skeletal muscles, the heart, and adipocytes. Under physiological conditions, GM can inhibit FIAF gene expression, promote LPL expression, and decrease triglyceride (TG) accumulation in the cells<sup>[50, 53, 54]</sup>. AMP (adenosine monophosphate)-activated protein kinase (AMPK) is a regulatory factor that serves in maintaining energy balance in the cells, playing a vital part in energy balance. AMPK can be directly phosphorylated by

acetyl-CoA carboxylase, which promotes fatty acid oxidation in the tissue and further reduces fat deposition. GM disruption can lead to a reduction in the levels of AMPK in skeletal muscles and the liver, which subsequently leads to inhibition of fatty acid oxidation and excessive accumulation of fat in the liver<sup>[50]</sup>. Besides, the GM can induce or inhibit angiopoietin-like protein 4 (AGTPL-4) through bile acids to influence LPL activity, thereby affecting fat deposition inside the liver and steatosis outside the liver<sup>[42]</sup>. A study<sup>[55]</sup> revealed that giving mice feeders rich in saturated fatty acids, cholesterol, and sugar could increase lipid accumulation in their livers and cause a significant rise in the relative abundance of *Firmicutes* in mice's intestines. *Firmicutes* are important in the fermentation of resistant starch and dietary fibers and for energy use. The fermentation products are present in the form of SCFAs. On the one hand, SCFAs are essential energy substances of intestinal epithelial cells, which can enhance energy production by promoting sugar and fat synthesis<sup>[56]</sup>. On the other, SCFAs can alter fatty acid oxidation by inhibiting AMPK, leading to the accumulation of fatty acids in the liver<sup>[57]</sup>. Moreover, SCFAs are the ligands of G protein-coupled receptors 41 (GPR41). After conjugating to GPR41, SCFAs can mediate leptin production by stimulating GPR41 in mouse adipocytes to regulate energy metabolism<sup>[58]</sup>.

#### **1.4 Insulin resistance (IR)**

Insulin is an important hormone that regulates the steady state of glucose levels in the body. The activation of insulin receptors through phosphorylation initiates the body's biological response, including increased glucose transport in skeletal muscles and adipose tissues, glycogen synthesis, and lipogenesis. IR reduces the biological effect of insulin, causing hyperinsulinemia (HIS). In turn, HIS leads to abnormal glucose transport and increased glycogenolysis and fatty acid synthesis, promoting MAFLD. Therefore, IR is one of the vital causes of MAFLD<sup>[59]</sup>. The composition and relative abundance of GM bacteria differ between obese individuals and those who are slim. The GM of obese individuals is mainly composed of *Firmicutes* and *Actinobacteriota*, while *Bacteroidete* are also present but less predominant. As mentioned above, *Firmicutes* with high relative abundance can enhance energy consumption by utilizing more

indigestible substances in the intestinal content<sup>[60]</sup>. An imbalanced ratio of gut bacteria can also disrupt intestinal permeability, leading to increased absorption of LPS. Administration of antibiotics in high-fat diet (HFD) mice has been shown to decrease blood LPS concentration. Furthermore, this reduction in blood LPS concentration contributes to a decrease in adipose tissue inflammation and oxidative stress, which helps prevent adipose tissue hypertrophy and improves glycolipid metabolism parameters of HFD mice<sup>[61]</sup>.

LPS are cell wall components found in gram-negative bacteria and are considered to be a key trigger of IR. Studies<sup>[62, 63]</sup> have shown that IR caused by intestinal endotoxins is mainly mediated by Toll-like receptor 4 (TLR4). In other words, LPS can activate the TLR4 located on the surface of insulin target cells. TLR4 can stimulate hepatocytes to produce inflammatory factors. Stimulating the production of proinflammatory kinase (e.g., JNK) can inhibit the phosphorylation of insulin receptor substrates, thereby inhibiting the insulin signal transduction pathway. Moreover, GM disruption can accelerate the above processes to trigger IR. Reduced insulin sensitivity leads to a decrease in the rate of blood glucose utilization. HIS occurs when islet  $\beta$  cells are in the compensatory hypersecretory state. Further, HIS can disrupt the islet signaling pathway in the liver, forming a vicious circle. IR can alter the regulation of fat by insulin, enhancing steatosis and increasing free fatty acids in the serum. Fatty acids can cause hepatotoxicity. On one side, they can trigger MAFLD via various mechanisms, such as causing mitochondrion swelling to increase their permeability, inflammatory invasion, hepatocyte degeneration and necrosis, and induction of cell apoptosis<sup>[54]</sup>. On the other hand, the liver can transform free fatty acids into TG. Excessive fat will be deposited in the liver when the fat synthesized in the liver exceeds the hepatocyte's ability for oxidative utilization and synthetic lipoprotein transport, thereby promoting the development of MAFLD<sup>[61]</sup>.

### **1.5 Increased endogenous ethanol**

The GM can produce and metabolize ethanol. In the relatively hypoxic environment of the intestine, pyruvic acids produced through carbohydrate

decomposition can be metabolized by the GM into acetaldehyde, which is further reduced into ethanol<sup>[64]</sup>. When there is intestinal bacteria overgrowth (Small intestinal bacteria overgrowth often exists in MAFLD) or excessive carbohydrate intake, ethanol metabolism mediated by the GM becomes active<sup>[65]</sup>. A current study<sup>[66]</sup> suggested that the primary product of *Enterobacteriaceae* (e.g. *Escherichia*) metabolism is ethanol. Other GM, such as *Bacteroides*, *Bifidobacterium*, and *Clostridium*, may also produce ethanol. The ethanol metabolized by GM is also called endogenous ethanol. Under normal conditions, the liver efficiently eliminates endogenous ethanol from the bloodstream of the portal vein through the action of liver alcohol dehydrogenase, catalase, and the ethanol oxidation system. However, in the intestines of MAFLD patients, the abnormal increase of ethanol-producing bacteria promotes and increased production of ethanol. Some patients with MAFLD have a preference for carbohydrates. Due to these two factors, reactive oxygen species are constantly provided to the liver, inducing liver oxidation and triggering inflammation, which is the “second strike” to the liver<sup>[66]</sup>. A study on children with MAFLD<sup>[67]</sup> revealed that the relative abundances of *Gammaproteobacteria* and *Prevotella* in these children were significantly higher than in healthy children. For this reason, the production of endogenous ethanol was also distinctly enhanced. Besides, an animal experiment<sup>[68]</sup> proved that administering antibiotics to alter the GM could reduce the ethanol concentration of the air exhaled by obese mice. A similar conclusion was also confirmed in NASH patients<sup>[69]</sup>, as the air exhaled by NASH patients also had higher ethanol concentrations than healthy individuals. Besides, it was observed that the relative abundance of *Escherichia* in the GM increased significantly, which also confirmed that the increase in endogenous ethanol caused by GM disruption is related to MAFLD.

GM disruption leads to an increase in the relative abundance of bacteria producing ethanol, thereby increasing the ethanol content in the intestines. Ethanol activates various cytokines in the intestinal epithelial cells to increase intestinal wall permeability. Meanwhile, ethanol and acetaldehyde, which are metabolized products, enter the liver through the portal vein. These products can either directly stimulate



hepatocytes or activate liver TLR to produce multiple cytokines and inflammatory mediators, resulting in inflammatory liver injury. In addition, the acetaldehyde produced by ethanol through intestinal metabolism can damage the expression of tight-junction proteins between intestinal epithelial cells to alter the intestinal barrier function, leading to bacterial translocation and endotoxemia. (**Figure 1**)

## **2. The influence of GM on relevant signaling pathways**

### **FXR signaling pathway**

The FXR signaling pathway is one of the members of the nuclear receptor superfamily, and FXR's primary function is to regulate bile acid metabolism and enterohepatic circulation. The synthesis, metabolism, and reabsorption of bile acids are regulated by the negative feedback of FXR-relevant signaling pathways in the liver and ileum. Activating FXR can adjust the metabolic state of blood fat, blood glucose, and cholesterol and improve IR<sup>[70, 71]</sup>. Obeticholic acid is an FXR agonist, which inhibits bile acid synthesis and enhances bile salt excretion through the FXR/FGF15/19 signaling pathways, thereby reducing bile acid reabsorption by the liver. Meanwhile, obeticholic acid can regulate the GM, improve intestinal mucosa barrier function, reduce inflammation, decrease the production and translocation of intestinal endotoxin, maintain gut-liver axis balance, and alleviate liver inflammation<sup>[70]</sup>. GM can also activate FXR in various ways (e.g., via increasing fatty acid oxidation). Activated FXR improves glucose metabolism by inhibiting gluconeogenesis and glycogenolysis, reducing fat synthesis, and enhancing skeletal muscle insulin sensitivity. GM disruption can inhibit the transduction of the FXR signaling pathway, leading to an escalation of fatty acid synthesis and the generation of lipid toxicity. This, in turn, further deteriorates hepatic steatosis and promotes the occurrence and progression of MAFLD<sup>[72-74]</sup>.

### **GPBAR signaling pathway**

The mechanism of the GPBAR signaling pathway is similar that of FXR, and these two pathways are closely related to each other<sup>[75]</sup>. After reabsorption, bile acids induce ileal

cells to secrete FGF19 by conjugating to the FXR and GPBAR of ileal cells. FGF19 further conjugates to FGFR4, which reduces CYP7A1 gene expression by activating the JNK and ERK signaling pathways. Therefore, bile acid synthesis is inhibited in negative feedback<sup>[23]</sup>. GM disruption can alter FXR expression and the transduction of the GPBAR signaling pathway, leading to the production of proinflammatory factors. For example, GPBAR can activate cyclic adenosine monophosphate (cAMP) and epidermal growth factor receptor kinase (EGFR) pathways, resulting in the activation of protein kinase C (PKC), which leads to the activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B). Activated NF- $\kappa$ B enhances the expression of numerous proinflammatory factors, such as IL-1 $\beta$ , IL-6, and TNF- $\alpha$ . As a result, this activates the inflammatory immune response of the liver, promoting the occurrence and progression of MAFLD<sup>[76, 77]</sup>.

### **TLR signaling pathway**

TLR is essential in the gut-liver axis, especially in maintaining the intestinal mucosa barrier. A damaged intestinal mucosa barrier leads to increased permeability, which induces GM transposition. Therefore, a growing number of bacterial metabolites, bacterial substances, and other compounds can enter the liver through the portal vein, causing inflammation, oxidative stress, and lipid deposition. This eventually leads to fat liver injury, which can progress rapidly to liver fibrosis, also called “intestinal leakage”<sup>[78]</sup>. Bacterial flora translocation increases the endotoxin level in the portal vein or the liver. Pathogen-associated molecular patterns accumulate in the portal vein circulation, promoting the development of liver inflammation<sup>[79]</sup>. Besides, the increased abundance of pathogenic bacteria caused by GM disruption (or distinct abnormal relative abundance of opportunistic pathogens) lead to the excessive production of LPS. Subsequently, LPS stimulates endothelial cell TLR4 and dendritic cell TLR9 and induces the production of inflammasomes (e.g., NLRP3) and proinflammatory factors (e.g., IL-1 $\beta$ ). This further damages intestinal mucosa permeability and reduces liver insulin sensitivity, thereby increasing visceral and subcutaneous fat and promoting the occurrence and progression of MAFLD<sup>[80]</sup>.

### **Immunoregulation**

Liver inflammation is the critical driving factor of MAFLD development, and the gut-liver immune axis plays a vital role in the process. LPS, peptidoglycan (PGN), and bacterial DNA can be translocated into the liver through the injured intestinal barrier, causing immune cell hyperactivation. LPS can take advantage of the injured intestinal barrier to enter the liver *via* portal blood flow and induce the activation of inflammasomes<sup>[81]</sup>. PGN is one of the components of the bacterial cell wall. It is a macromolecule polymerized by acetylglucosamine, acetylmuramic acid, and amino acid short-chain peptide, which plays a role in insulin tolerance<sup>[82]</sup>. PGN and TLR2 can activate relevant NF- $\kappa$ B and TNF- $\alpha$  signaling pathways after conjugating to nucleotide oligomerization domain (NOD) 1 or NOD2, resulting in liver inflammation. NOD1 can also detect nutrient overload by sensing changing in bacterial microorganisms and promoting the translocation of PGN to regulate the energy metabolism in the gut-liver axis<sup>[83]</sup>. Bacterial DNA can directly activate immune cells such as macrophages, natural killer cells, B lymphocytes, and dendritic cells. It can also conjugate to TLR9 inside lysosomes *via* endocytosis, activating the NF- $\kappa$ B pathway and secreting inflammatory factors, such as IL-1 $\beta$ , IL-6, and TNF- $\alpha$ <sup>[84]</sup>.

### 3. The bacterial flora therapy of MAFLD

**Antibiotics** Research<sup>[85]</sup> has shown that short-term use of antibiotics can reduce circulating endotoxins and serum transaminases, improving the clinical symptoms of MAFLD patients. Among the antibiotics, the application of rifaximin has received the greatest attention. After rifaximin treatment, the BMI index, transaminase level, and hepatic steatosis degree of MAFLD patients decreased significantly. Even clinical research revealed that rifaximin could reduce the fermentation of carbohydrates and sterols by altering the GM structure, lowering serum inflammatory factors, and improving IR. Antibiotics cocktail (ampicillin, neomycin, metronidazole and vancomycin) can regulate free and conjugated secondary bile acid levels to decrease liver inflammation<sup>[86]</sup>, inhibit intestinal FXR to reduce hepatic steatosis<sup>[73]</sup>, and inhibit the activation of liver macrophage to lower liver inflammation<sup>[87]</sup>. However, antibiotics

play a dual role. Short-term antibiotic treatment can exert therapeutic effects, while long-term application may result in bacterial drug resistance and increase the risk of secondary infection.

Probiotics clinical experiments on MAFLD patients<sup>[88]</sup> demonstrated that *Lactobacillus*, *Streptococcus*, and *Bifidobacterium* could significantly reduce the patient's serum transaminase level. After taking *Lactobacillus bulgaricus* and *Streptococcus thermophilus* for three months, the transaminase level of MAFLD patients improved<sup>[89]</sup>. The application of *Clostridium butyricum* in clinic settings and MAFLD animal models has shown promising potential in preventing hepatic steatosis<sup>[90, 91]</sup>. In addition, multiple probiotic formulations present better therapeutic effects than one specific bacterial strain. For example, VSL3 (consisting of eight probiotic bacterial strains: *Streptococcus thermophilus*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, and *Lactobacillus bulgaricus*) has better therapeutic effects than any single bacterial strain<sup>[90, 92, 93]</sup>. Studies targeting children with MAFLD<sup>[68, 94, 95]</sup> revealed that after VSL3 treatment, patients' fatty liver disease condition and BMI were distinctly improved. Follow-up research showed that the total quantity and activity of GLP-1 increased after VSL3 treatment. Meanwhile, VSL3 has been found to regulate plasmic peroxide, such as malondialdehyde and 4-hydroxynonenal, leading to therapeutic effects and relieving chronic liver disease. It achieves this by protecting the intestinal barrier and reducing endotoxemia and oxidative/nitroso stress. An animal experiment also verified<sup>[96]</sup> that probiotics can lower the weight of mice and improve GM disruption. Probiotic intervention can increase the abundance of intestinal anaerobic bacteria (e.g., *Actobacillus* and *Bifidobacterium*). However, this decreases the abundance of *Escherichia* and *Enterococcus*, enhancing the integrity of the intestinal mucosa barrier. Highly expressed TLR4 in the liver improves serum inflammatory factors, liver histology, serum liver enzyme, metabolic index, and glucose metabolism. Evidence indicates that probiotics can decrease liver and systematic inflammation by inhibiting the LPS/TLR4 signal transduction inflammatory cascade.

## Prebiotics

Fructo-oligosaccharides (FOS) is an indigestible fermentable dietary fiber compound that lowers liver oxidative stress and inflammation by improving intestinal permeability and the integrity of close junctions<sup>[97]</sup>. Lactulose is another prebiotic that enhances the growth of *Bifidobacteria* and *Lactobacillus* and inhibits endotoxic gram-negative bacteria. After taking lactulose for six weeks, the inflammation and liver injury of HFD obese mice was reduced, which was related to lowered LPS level in the circulation<sup>[98]</sup>. Clinical research<sup>[99]</sup> has shown that the serum ALT and AST levels of NASH patients decreased significantly after receiving *Bifidobacteria* and FOS treatments. Combining multiple probiotic bacterial strains (*Lactobacillus casei*, *Lactobacillus bulgaricus*, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *Bifidobacteria breve*, *Bifidobacteria longum*, and *Streptococcus thermophilus*) and FOS in combination with lifestyle interventions were more beneficial than lifestyle changes alone for MAFLD patients<sup>[100]</sup>. Combining *Bifidobacteria longum* and FOS with lifestyle interventions can significantly decrease NASH activity index and liver fat accumulation<sup>[101]</sup>. The result of a meta-analysis revealed that combining prebiotics could distinctly lower hepatic steatosis and the levels of ALT, AST, LDL, TG, and TC. Moreover, it was also helpful for reducing levels of inflammatory factors such as TNF- $\alpha$  and IR<sup>[102]</sup>. However, the administration of inulin diet, a soluble fiber, to mice with TLR5 gene knock-out led to an increase in the mice's bilirubin level, indicating that excessive inulin intake may cause liver injury and even liver cancer<sup>[103]</sup>. Research has also highlighted that acetate, the fermentation product of inulin in the colon, can provide excess substrate for fat synthesis in the liver, escalating the production of lipids in the liver<sup>[104]</sup>.

## Fecal microbiota transplantation (FMT)

The earliest FMT treatment can be traced back to the book called *A Handbook of Formulas for Emergencies (Zhou Hou Bei Ji Fang)* written by Ge Hong from the Eastern Jin Dynasty (266 A.D. - 317 A.D.) in China. In the book, FMT treatment, also called "Huang Long Soup," for food poisoning and severe diarrhea was first recorded. Later, Li Shizheng also recorded in *the Compendium of Materia Medica (Bencao Gangmu)* that FMT by oral



administration can treat severe diarrhea, fever, vomiting, and constipation<sup>[105]</sup>. FMT in modern medicine started in the year 1985. Ben Eiseman performed FMT by enema for patients with severe pseudomembranous colitis using feces from the patient's family member, and three out of the four patients were cured<sup>[106]</sup>. FMT was officially written into the clinical guidance for recurrent *Clostridium difficile* infection treatment in 2013<sup>[107]</sup>. As research progresses, numerous pieces of evidence support the potential efficacy of FMT in treating GM-related liver disease and metabolic disorders such as MAFLD. Studies have shown<sup>[108, 109]</sup> that transplanting the GM of slim or obese mice can induce the recipient to have a phenotype similar to that of the host. The bacterial flora from slim mice can make obese mice lose weight. Six weeks after overweight patients with metabolic syndrome received bacterial flora from the slim individuals, the sensitivity of their liver and peripheral insulin was significantly enhanced<sup>[110]</sup>. Several studies have demonstrated<sup>[111-114]</sup> that the therapeutic effects of FMT on patients with T2DM and ulcerative colitis were related to GM steady state, normal blood fat level, and IR improvement. Feces from HFD-responsive and non-responsive mice were transplanted into germ-free mice. Mice receiving bacterial flora from the responsive group developed steatosis and exhibited increased relative abundance of *Barnesiella* and *Roseburia*. In contrast, the non-responsive group showed an increased relative abundance of *Allobaculum* in their bacterial flora<sup>[55]</sup>. In addition, FMT could significantly restore the GM disruption in NASH mice models induced by HFD by increasing the relative abundance of probiotics (e.g., *Christensen* and *Lactobacillus*) and mitigate endotoxemia, hepatic steatosis, and inflammation<sup>[115]</sup>. He *et al* conducted a retrospective analysis on 237 patients receiving FMT treatment (among which 42 were patients with metabolic syndrome). The analysis revealed that FMT could distinctly improve the fasting blood glucose, TG, LDL-c, HDL-c, and non-HDL-c of patients with metabolic syndrome and downgrade the ASCVD risk in high-risk groups. In another RCT admitting 75 MAFLD patients, He *et al*<sup>[116]</sup> divided the patients into an FMT group (47 individuals) and a non-FMT group (28 individuals). The patients from the non-FMT group took oral probiotics, while the FMT group received three FMT enemas within

three days. Both groups received a healthy diet and conducted exercise regularly for over 40 minutes. After treatment for one month, it was found that FMT lead to a reduction in liver fat deposition by improving the GM disruption, lowering the incidence of fatty liver disease. Moreover, the GM reconstruction effect of FMT on thin-type MAFLD patients was better compared to obese MAFLD patients. FMT can be administered through various methods to meet the requirements of different patients, including via nasogastric tubes, nasojejunal feeding tubes, gastroscopes, colonoscopes, colonic catheters, retention enema, and capsules. However, FMT may pose certain risks. For example, the GM condition of different providers may affect the therapeutic effect, infection may occur during the transplantation, and it is uncertain how the GM can be effectively colonized in the patient's intestine. All these problems require further exploration.

### **Phage therapy**

Phages are viruses that specifically infect and kill bacteria. They have the ability to adapt and evolve, enabling them to overcome the developing defensive mechanism of bacteria. Phages do not have the same mechanism as antibiotics. Thus, antibiotic resistance does not affect phages, and bacteria with high antibiotic resistance can still be inhibited by phages<sup>[117]</sup>. By studying the changes in bacterial composition and relative abundance, bacteria can be targeted for eradication using phages specific to that bacteria, after determining the mechanism by which a specific bacterium affects the onset or progression of MAFLD or whether the two are causally related. Its adverse effects on MAFLD can be eliminated without affecting the normal function of other bacteria<sup>[118]</sup>. For example, using phages for the targeted eradication of high-alcohol-producing *Klebsiella pneumoniae* (HiAlc-Kpn, found in over 60% of MAFLD patients, can produce enormous amounts of alcohol and is the leading cause of the bacterial auto-brewery syndrome) can effectively mitigate the bacterial auto-brewery syndrome of MAFLD model mice<sup>[119]</sup>. An analysis was conducted on feces samples of NASH patients and healthy people. The *Enterococcus faecalis* level in the feces samples of alcoholic hepatitis patients is 300 higher than in healthy subjects. The relative abundance of

*Enterococcus faecalis* significantly increased in approximately 80% of feces samples from patients with alcoholic hepatitis. Further analysis showed that a gene which can encode cytolysin existed in approximately 30% of *Enterococcus faecalis* species<sup>[118]</sup>. Alcoholic hepatitis mouse models were built using a high-alcohol diet. After being transplanted with a feces sample containing cytolysin, these alcoholic hepatitis mice developed specific hepatocyte injury and died. However, alcoholic hepatitis mice transplanted with samples without cytolysin did not develop liver injury. Targeting the phages specific to *Enterococcus faecalis* can effectively reduce the abundance of this bacterium, especially strains producing cytolysin. This targeted approach has shown promising results in lowering the degree of liver injury in alcoholic hepatitis mice, serving as a protective measure<sup>[118]</sup>. This is a critical attempt at phage therapy in the gut-liver axis, indicating the potential application value of phage therapy in MAFLD treatment. (Figure 3)

In addition to the antibiotics mentioned above, probiotics, FMT, and other therapies that directly target GM, there is growing evidence that modifying dietary habits and increasing physical exercise both improve MAFLD and the GM disorder in MAFLD. High-fat and high-sugar diets can change the GM structure in different ways<sup>[120]</sup>. HFD mice have a greater abundance of *Firmicutes*, while mice with a high-sugar diet have a lower relative abundance of *Firmicutes* and *Bacteroides*, which is closely related to the onset and development of MAFLD. High-fructose intake will up-regulate the re-synthesis of fat and inhibit the oxidation of fatty acid  $\beta$ . It can cause hepatic steatosis, induce inflammation through the TLR signaling pathway, and release inflammatory factors. High-fructose intake can also reduce insulin sensitivity. HFD decreases the number of intestinal probiotics *Bifidobacteria* and bacteria that produce butyric acid. It also enhances intestinal permeability, LPS translocation, and chronic systemic inflammation. High saturated fatty acid intake can lower the GM diversity and increase the ratio of *Firmicutes* to *Bacteroides*, result in weight gain, and increase plasmic insulin and TG content<sup>[121]</sup>. In MAFLD mice induced by HFD, six-week HFD increases *Firmicutes* abundance and decreases *Bacteroides* abundance. The ratio of *Firmicutes* to

*Bacteroides* was significantly enhanced, and the ratio was maintained till the end of the experiment. However, exercise can improve GM disorder resulting from a HFD. This is achieved by changing the ratio of the two bacteria via reducing *Firmicutes* abundance and increasing *Bacteroides* abundance<sup>[122]</sup>. Exercise distinctly alleviates GM disruption caused by HFD and restores the intestinal mucosa barrier function to a certain extent. The relative abundance of some GM reaches a level similar to normal rats. Meanwhile, exercise also significantly downregulated the expression of FXR and CD36 in the liver, indicating improvements in liver lipid metabolism<sup>[123]</sup>. Research combining exercise and lycium barbarum polysaccharide also found that aerobic exercise restores the close junction of the colon and ileum and improves intestinal mucosa permeability by enhancing ZO-1 expression. Relevant indicators such as intestinal LPS, liver LPS binding protein, and inflammatory factors are also downregulated<sup>[123]</sup>. However, research on the effects of exercise on GM in MAFLD patients is relatively sparse. Although animal experiments have demonstrated that exercise can improve MAFLD symptoms by restoring GM, more clinical experiments are needed.

## **CONCLUSION**

Generally, with the increase in metabolic diseases such as obesity, the incidence of MADLF also increases yearly. Many clinical studies and animal experiments have demonstrated that the GM is vital in the onset and development of MAFLD, but most studies remain at a phenomenal level. There is no definitive conclusion regarding the “cause” and the “effect” of GM disorder and MAFLD. However, the efficacy of treatments targeting GM, such as probiotics and FMT, has been confirmed in both clinical applications and basic research without reporting severe adverse events. There is also a wide range of opinions on how many bacterial flora therapies are used to improve MAFLD. A healthy lifestyle and good dietary habits are still the foundation of MAFLD treatments. Comprehensive therapies combining bacterial flora therapy certainly have good prospects, but continuous efforts are still needed to design high-quality long-term clinical experiments. Meanwhile, histological studies combining multi-omics, such

as metagenome, metabolome, and proteome, are needed. In the future, it is expected that GM can better help in the diagnosis, treatment, and prognosis evaluation.



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