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MICROBIOTA AND METABOLIC DYSFUNCTION ASSOCIATED WITH STEATOTIC LIVER DISEASE

Q. Zhu[1,2], S. Francque[1,2], W.J. Kwanten[1,2]

[1]Department of Gastroenterology Hepatology, Antwerp University Hospital, Antwerp, Belgium
[2]Laboratory of Experimental Medicine and Paediatrics, University of Antwerp, Antwerp, Belgium

Sven Francque and Wilhelmus J. Kwanten share senior authorship

Corresponding author: Prof. Dr. Sven Francque, MD; email: sven.francque@uza.be

Abstract – Disease progression in metabolic dysfunction-associated steatotic liver disease (MASLD) is driven by multiple mechanisms that act together in genetically predisposed subjects. Amongst others, the gut-liver axis plays a crucial role in the development and progression of MASLD. This inevitably involves the microbiome. The exact impact of the microbiome on MASLD is not yet fully understood, but the imbalance in gut microbiota (GM) can cause altered integrity of the intestinal epithelium, increased permeability of the intestinal barrier, and various harmful substances and metabolites secreted by the GM reaching the liver. The gut and liver interact bidirectionally through multiple molecules and products, such as nutrients, microbial antigens, metabolites, and bile acids (BAs), which affect both metabolism and immunity, thus controlling both gastrointestinal and liver health and disease. Understanding the factors that shape microbiome complexity may help improve prevention, diagnosis, and treatment in a precise and personalized manner. This review focuses on the recent data on the aetiopathogenesis of MASLD linked to the gastrointestinal microbiome and looks critically into the research methods that etiopathogenesis aim at comprehensively and accurately reflecting the true status of the GM.

Keywords: Microbiota, MASLD, Methodology, Treatment.

INTRODUCTION

The microbiome is the complete collection of microbiotas, their genes, and their microenvironment in a specific area. The microbiome inhabiting the human body includes bacteria and non-bacterial microorganisms (e.g., viruses, fungi, and archaea). Various disease states are associated with the disruption of microbial composition and function, known as dysbiosis. Microbiotas are primarily found in the digestive system. Understanding factors that shape the microbiota complexity may help improve the prevention, diagnosis, and treatment of certain diseases in a precise and personalized manner.

Metabolic dysfunction-associated steatotic liver disease (MASLD), formerly known as non-alcoholic fatty liver disease (NAFLD)[1], is defined as a pathological condition marked by the excessive accumulation of lipids within hepatocytes (steatosis) in the presence of cardiometabolic risk factors. MASLD can coexist with other causes of steatosis (e.g., alcohol or viral hepatitis) and chronic liver disease, but in isolation, it excludes the consumption of alcohol above the same thresholds that also define NAFLD. MASLD is associated with metabolic syndrome, including but not limited to obesity, type 2 diabetes mellitus, insulin resistance, and

dyslipidemia[2]. MASLD is driven by multiple mechanisms that act together in genetically predisposed subjects (e.g., lipotoxicity, alterations of immune cells, or the hepatic microvasculature). Increasing evidence shows that despite some differences in definition, MASLD and NAFLD have minimal discrepancies, so findings in patients with NAFLD remain valid[3]. Therefore, in this review, we will mainly use the new nomenclature and only use the former when scientifically relevant.

The gut and liver interact bidirectionally through multiple molecules and products, such as nutrients, microbial antigens, metabolites, and bile acids (BAs), which affect the metabolism and immunity of both, thus controlling the health and disease of both the gut and liver. Central to this interaction, and a key factor in how the microbiome affects MASLD, is the gut-liver axis. The intestinal barrier is composed of a complex and multi-layered defence system including the physical barrier formed by tightly arranged epithelial cells and mucus layers; the chemical barrier with gastric acid, digestive enzymes, bile, and antimicrobial peptides; the biological barrier constituted by the commensal gut microbiota; and the immunological barrier. This article summarises the most notable research published between March 2023 and March 2024 to review the latest knowledge about the development and treatment of MASLD linked to the gastrointestinal microbiome, and focusses on the shortcomings and room for improvement in current clinical research methodology.

METHODOLOGY

We conducted an extensive literature search using PubMed to identify relevant studies published from March 2023 to March 2024. Our search strategy employed Boolean operators 'AND' and 'OR' in combination with following keywords "Microbiota", "NAFLD", "NASH", "MASLD", "MASH", "pathogenesis", "treatment", and "methodology". The full texts of potentially relevant articles were then obtained for in-depth review. After thorough evaluation, data from these studies was extracted and qualitatively summarised under relevant headings, aiming to provide a comprehensive narrative overview of the latest insights into the role of microbiota in the pathogenesis and treatment of MASLD.

UPDATED PATHOGENESIS

Dysbiosis of the gut microbiota (GM) can result from various factors, including dietary habits, medication effects, lifestyle factors, health conditions (chronic diseases like diabetes and obesity, and infections), environmental influences (pollutants, toxins, and dietary changes), genetic susceptibility, and the immune system. Since GM is crucial for maintaining the integrity of the intestinal barrier, all the factors mentioned above will indirectly destroy the integrity of the intestinal barrier with subsequent increased permeability and/or will lead to the release of various harmful substances and metabolites (such as endotoxins) secreted by the GM, which hence can induce MASLD when reaching the liver. The research over the past year has mainly expanded our understanding of the above-mentioned mechanisms, the upstream causes of GM dysbiosis, and the downstream destruction of the intestinal barrier and its impact on the gut-liver axis. The summary of the results of this section is shown in Table 1.

Various factors that cause changes in intestinal pH and osmotic pressure can subsequently lead to changes in GM, thereby damaging the intestinal microbial barrier and inducing MASLD. It was shown that the tolerable pH and osmotic pressure of different intestinal bacteria are heterogeneous, explaining why changes in these factors can affect the GM composition and products[4]. Quantifying the taxon-specific response of the intestinal microbiome to environmental perturbations aids in understanding and predicting the dynamic changes in the microbiota during health and disease[4]. This potentially offers a novel approach through which we can proactively adjust the pH and osmotic pressure to conditions unfavorable for diseases such as MASLD. In addition, GM imbalance can also lead to chemically modified BAs that affect glucose and lipid metabolism in the liver by dysregulating BA signaling pathways[5]. High-fat-diet (HFD) can significantly increase the abundance of GM like *Firmic-*

TABLE 1. SUMMARY OF SELECTED STUDIES RELATED TO MASLD-PATHOGENESIS.

First author	Key points	Diet/model used	Sample type	Type of microbiome sequencing
Zhou et al[6]	Tight junction proteins, key enzymes of lipogenesis	HFD	Fecal samples from mice	16S rRNA
Zeng et al[10]	ROS	HFD	Fecal samples from mice	16S rRNA
Huang et al[8]	Desulfovibrionaceae, LPS	HFD	Fecal samples from mice	16S rRNA
Ng et al[4]	Intestinal pH, osmotic pressure	Guar gum diet	Fecal samples from mice	16S rRNA
Caballano-Infantes et al[9]	CDI, ROS, CPT1A, glycolysis, Warburg effect	CDI	Fecal samples from human	16S rRNA

HFD: high-fat-diet; ROS: reactive oxygen species; LPS: lipopolysaccharide; CDI: *Clostridioides difficile* infection; CPT1A: carnitine palmitoyltransferase 1A.

utes, *Clostridia*, *Lachnospirales* and *Rikenellaceae*, while *Muribaculaceae*, *Bacteroidales*, *Prevotellaceae*, and *Proteobacteria* are notably decreased[6]. This GM dysbiosis is followed by an increase in reactive oxygen species (ROS) in the mouse intestinal cells. Intriguingly, the ability to produce higher amounts of ROS can be transferred through fecal microbiota transplantation. As a consequence, some downstream changes show up: the tight junctions between the intestinal epithelial cells loosen, characterised by reduced tight junction proteins such as zonula occludens-1, with subsequent activation of inflammatory responses, mitochondrial dysfunction and intestinal epithelial cell apoptosis[5,6]. All these factors have been shown to exacerbate steatosis by promoting the generation of ROS and the excessive production of free fatty acids[7]. Another downstream change is the increased expression levels of fatty acid synthase and acetyl-CoA carboxylase 1 in the liver, the key enzymes of lipogenesis, ultimately inducing steatosis.

Recently, Huang et al[8] pointed out that *Desulfovibrionaceae*, a type of sulfate-reducing bacteria, exacerbates high-fructose diet-induced overweight, glucose dysregulation, and lipid metabolism disorders by promoting the translocation of lipopolysaccharide (LPS) to systemic and hepatic sites. This results in alterations in glucose homeostasis and lipid metabolism, culminating in the development of MASLD. In addition, a study on *Clostridioides difficile* infection (CDI) found that purified, toxigenic *Clostridium difficile*-secreted membrane vesicles (MV) lead to an increase in carnitine palmitoyltransferase 1A (CPT1A) in hepatocytes, which positively correlates with both fibrosis and disease activity scores[9]. The MVs also promote a shift in the metabolic balance of pyruvate kinase M2 (PKM2) towards glycolysis, enhancing the Warburg effect. While the study did not measure the MV content in the blood, it suggests that the relationship between MVs secreted by CDI and MASLD is worth further exploration.

CURRENT METHODOLOGICAL CONSIDERATIONS AND IMPROVEMENTS

Clinical data on the role of GM in MASLD are conflicting, which is partly due to the methodological limitations of clinical GM research. In the field of microbiome research, from sample collection methods, research model selection, and sequencing technology to downstream data processing procedures, each step can lead to differences in relative and absolute abundance, thus affecting the accuracy, authenticity, and interpretability of the research results. Therefore, here we discuss the challenges of research in this field and some new technological improvements that have been underutilized in relation to MASLD. The summary of the results of this section is shown in Table 2.

TABLE 2. SUMMARY OF SELECTED STUDIES RELATED TO CURRENT METHODOLOGICAL SHORTCOMINGS AND IMPROVEMENTS.

First Author	Topic of investigation	Methodological improvement	Methodological shortcomings	Sample type	Type of microbiome sequencing
Shalon et al[11]	Ingestible capsule	Reflect a more actual intestinal environment	Neglect eating and the microbiota growth in the capsule	Intestinal fluid, stool and saliva samples from human	16S rRNA and Metagenomic sequencing
Maghini et al[12]	Preservatives	Take preservatives into consideration	Need examination of more types of preservatives	Fecal samples from human	16S rRNA and Metagenomic sequencing
Schirmer et al[13]	Integration of microbiota and microbial metabolites	Systematically identify the microbial effector molecules	Additional gut environmental factors may still be neglected	Fecal samples from human	Metagenomic sequencing integrated with Metabolomics and Culturomics
Cumeras et al[14]	Application of human fecal reference materials	Reduce the influence outside the samples	Lack of further confirmation of reliability	NIST human fecal research grade reference materials	Mass Spectrometry
Lee et al[15]	GMoC	Simulate physiologically relevant intestinal environment	Unable to reproduce the complex interaction of multiple microbes in the intestine	Caco-2 cells co-cultured with bacteria	N/A
Li et al[5]	Simple co-culture method	Transwell with divided spaces of different oxygen conditions	Unable to simulate the physiological conditions of bacteria directly attached to epithelial cells	Fecal samples from human	Metagenomic sequencing

NIST: National Institute of Standards and Technologies; GMoC: Gut Microbiome-on-a-Chip.

Fecal samples are the primary means for conducting microbiome research, but they cannot fully and accurately reflect the *in situ* condition of the GM. Shalon et al[11] used an ingestible capsule that can be activated by different pH values in the intestinal tract to collect liquid samples from the intestine for microbiome analysis. They revealed differences compared to studies using fecal samples and found a correlation between the concentration gradient of BAs and microbial abundance. Even though this substantially improves our understanding of the local microbiome's effects, one must be aware that microbiota composition can change due to continued growth in the capsule after sample collection. Moreover, the effects of meals also need further investigation, as changes in osmolality and pH but also substrates for microbiota can change the temporal composition and hence the interpretation of the results. Further technology and experimental design improvement is needed, but this provides a first promising step to improve understanding of the local microbiome. Different preservatives for stool samples have been shown to have varying effects on microbial cell lysis and nucleic acid degradation, thereby affecting the total microbial load and microbial transcription levels[12]. This implies that attention needs to be paid to the influence of preservative selection for interpretation and further research. Schirmer et al[13] established an approach in ulcerative colitis to link disease-associated microbiota to microbial metabolites by integrating paired metagenomics, stool and plasma metabolomics, and culturomics. This more holistic approach contributes to a systematic identification of microbial effector molecules, and although it may still neglect some additional gut environmental factors, we have reason to assume that this integrative method improves our understanding of the interactions of the microbiome with, e.g., the liver and thus MASLD. In addition, human fecal reference materials, so-called standardized stool samples, have been used in GM research to reduce variability due to differences between laboratories, detection machines or sample preparation and processing. Cumeras et al[14] conducted metabolomic analyzes on four types of homogenized human fecal reference materials, either lyophilized or in an aqueous form, derived from vegan and omnivorous subjects. The different homogenized fecal samples exhibited substantial distinctiveness, providing strong evidence for using standardized fecal samples as controls to ensure consistency across analytical methods in various laboratories. However, further research is needed to evaluate the reliability of different types of fecal reference materials and their appropriate application scenarios.

Direct observation of interactions between microbiome and intestinal cells will enhance understanding of the homeostatic and pathogenic mechanisms. The application of intestinal organoids and chip technology has emerged in an attempt to advance these studies. Nevertheless, these techniques remain costly and require further technical refinements to mimic the intestinal environment better. Recently, Lee et al[15] proposed a scalable Gut Microbiome-on-a-Chip (GMoC) with a reproducible 3D layered intestinal epithelium that can simulate key intestinal structures, functions, and cellular complexity, providing a physiologically relevant environment for microorganisms in the intestine. This chip has been used to culture the carcinogenic microorganism enterotoxigenic *Bacteroides fragilis* to observe various intestinal responses, ranging from morphological changes to the activation of carcinogenic signals. However, the chip still does not replicate the complex interactions among multiple microbes in the intestine or account for potential extra-intestinal interactions. In addition to the relatively complex devices mentioned above, Li et al[5] recently developed a simple and practical transwell co-culture method. This system is divided into spaces with different oxygen conditions, thus resolving the contradiction between aerobic and anaerobic culture conditions required for culturing intestinal organoids and facilitating the evaluation of bacterial metabolites. The study also elucidated the impact of the interaction between the GM and BA on the intestinal barrier. However, the device also has limitations, as it cannot replicate the physiological conditions of bacteria directly attached to epithelial cells.

THERAPEUTIC POTENTIAL

High-fat diet, antibiotic use, and hormone use can cause intestinal dysbiosis, thereby inducing MASLD through a series of the abovementioned mechanisms. It is imperative to prevent or select treatment targets precisely.

Moon et al[16] have demonstrated that ID119031166 (ID166), a novel selective non-bile acid farnesoid X receptor (FXR) agonist, can significantly reduce steatohepatitis activity as well as liver fibrosis in a free-choice diet-induced steatohepatitis hamster model. Furthermore, ID166 regulates the relative abundance of obesity-associated gut microbiota, such as *Prevotella* and *Bacteroides*, and normalizes the elevated plasma total BA levels in metabolic dysfunction-associated steatohepatitis (MASH) hamsters. Notably, some well-known FXR agonists, such as obeticholic acid, have been limited in clinical use due to their safety risks outweighing their moderate benefits[17]. In this preclinical study, ID166 has shown fewer side effects, including liver injury and pruritus. Currently, ID166 has entered a Phase I clinical trial and is anticipated to be further validated in subsequent phases.

7-keto-lithocholic acid (7-keto-LCA), a BA metabolite of the intestinal bacteria *Parabacteroides goldsteinii*, has been found to act as an intestinal FXR antagonist, promoting the self-renewal of intestinal stem cells *via* the Wnt-signaling pathway, which seems to alleviate MASLD by promoting intestinal barrier integrity[5]. This is inconsistent with the effects of FXR activation on reducing intestinal lipid absorption and selectively inhibiting hepatic fatty acid synthesis. Therefore, the complex mechanism of BA and FXR in both the intestines and the liver requires further study.

Many natural substances in food have previously been shown to improve liver steatosis. Recently, tomato pectin has been shown to increase the abundance of beneficial bacteria, such as *Akkermansia*, *Bacteroides*, and *Alloprevotella*, and regulate the production of BAs by inhibiting the ileal FXR/Fibroblast Growth Factor (FGF) 15/19 signaling pathway. This inhibition is followed by the stimulation of hepatic BAs production and excretion, inhibition of BA reabsorption, promotion of cholesterol efflux, and subsequent alleviation of HFD-induced hyperlipidemia[18]. This finding further supplements the bidirectional regulation between the GM and BAs. Resistant starch as a dietary supplement has been shown to benefit the treatment of MASLD after four months of continuous intake[19]. It can reduce serum levels of branched-chain amino acids (BCAA) by altering the GM represented by *Bacteroides stercoris*, ultimately alleviating MASLD.

It is reported that iron homeostasis is altered in obesity and MASLD[20]. Anaerobic bacteria, exemplified by *Clostridioides difficile*, would normally be inhibited by iron dysregulation but can continue to thrive in both iron-depleted and iron-repleted environments[21]. They achieve this by storing iron as iron phosphate minerals inside membrane-bound ferrosome organelles and releasing it during times of iron deficiency. Hence, it is imperative for future research to explore whether anaerobic bacteria implicated in MASLD, such as *Anaerorhabdus*[22], can also survive *via* ferrosome formation, thereby further clarifying the potential therapeutic link between the anaerobic microbiome and iron dysmetabolism in MASLD. When it comes to anaerobic bacteria, due to their sensitivity to oxygen, it is a challenge to culture them *in vitro* for study or as probiotics for human consumption. Another article reported that by utilizing the existing synergy between GM and improved oxygen tolerance, the anaerobic bacteria *Faecalibacterium prausnitzii* was successfully developed into a probiotic for human consumption[23]. Therefore, as this technology matures, more anaerobic bacteria associated with lower levels and/or severity of MASLD may be produced as probiotics. The era of being able to supplement, both aerobic and anaerobic, what we lack might be just around the corner. The summary of the results of this section is shown in Table 3.

CONCLUSIONS

Microbiota potentially plays an important role in the occurrence and development of MASLD, but the current research on them mainly relies on fecal samples, which cannot fully and accurately reflect their actual interactions in the intestine. This review provides an update on the most recent data regarding the role of microbiota in the etiopathogenesis of MASLD and their potential application as a treatment modality. Environmental factors, high-fat diet, antibiotics, and hormones can cause gut dysbiosis, disrupting the microbial barrier and inducing MASLD. Specific bacteria have been found to increase ROS, inflammation, and lipogenesis, thereby exacerbating steatotic liver disease.

TABLE 3. SUMMARY OF SELECTED STUDIES RELATED TO THERAPEUTIC POTENTIAL.

First author	Investigated substance and target	Function	Sample type	Type of microbiome sequencing
Moon et al[16]	ID166, FXR agonist	Attenuate MASLD activity and liver fibrosis, reduce BAs levels in MASH	Fecal samples from hamster	16S rRNA
Li et al[5]	7-keto-LCA, FXA agonist	Promote the self-renewal of intestinal stem cells	Fecal samples from human	Metagenomic sequencing
Wang et al[18]	Tomato pectin, natural substance	Alleviating hyperlipidemia by regulating BAs	Fecal samples from mice	16S rRNA
Ni et al[19]	Resistant starch, prebiotic of nondigestible fibers	Alleviating NAFLD through reducing BCAA	Fecal samples from mice	Metagenomic sequencing
Pi et al[21]	Ferrosome, organelle	Respond to changes in element levels in the host	Bacterial pellet, human stool sample and mouse fecal or cecal sample	ICP-MS
Khan et al[23]	Anaerobic probiotics, (anaerobic) microbiome	Improve oxygen tolerance through the synergy between intestinal microorganisms	Fecal samples from human and mice	Metagenomic sequencing

ID 166: ID119031166; MASLD: Metabolic Dysfunction Associated Steatotic Liver Disease; BAs: bile acids; MASH: metabolic dysfunction-associated steatohepatitis; 7-keto-LCA: 7-keto-lithocholic acid; FXR: farnesoid X receptor; HFD: high-fat-diet; BCAA: branched-chain amino acids; ICP-MS: Inductively coupled plasma mass spectrometry.

In the current era of improved technology with spatial omics, higher-resolution imaging, and sequencing, more detail of the heterogeneity of microbial composition can be revealed. The concept of biogeography of GM was proposed many years ago. Clarifying not only the spatial relationship but also the temporal alterations with changes in luminal factors (e.g., a meal) of microbiota in the intestine will help us to have a deeper understanding of their function. It is evident that we need to go beyond the analysis of fecal samples alone to make the next steps in understanding the gut-liver axis in health and disease, with hopefully improved treatment of MASLD.

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Author's Contributions

QZ conceptualization, literature search, and writing of the manuscript; WK & SF conceptualization and critical review of the manuscript.

ORCID ID

QZ: 0009-0006-9693-2495
WK: 0000-0001-9474-6695
SF: 0000-0002-7527-4714

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Not applicable.

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