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Role of Gut Bacterial and Non-bacterial Microbiota in Alcohol-associated Liver Disease: Molecular Mechanisms, Biomarkers, and Therapeutic Prospective

Nima Zafari[1,*], Mahla Velayati[1,*], Mostafa Fahim[1], Mina Maftooh[1], Ghazaleh Pourali[1], Majid Khazaei[1], Mohammadreza Nassiri[4], Seyed Mahdi Hassanian[1,2], Majid Ghayour-Mobarhan[1], Gordon A Ferns[5], Mohammad Ali Kiani[2,6], Amir Avan[1,2,3,#]

Affiliations:

1) *Metabolic Syndrome Research Center, Mashhad University of Medical Sciences, Mashhad, Iran.*
2) *Basic Sciences Research Institute, Mashhad University of Medical Sciences, Mashhad, Iran.*
3) *Medical Genetics Research Center, Mashhad University of Medical Sciences, Mashhad, Iran*
4) *Recombinant Proteins Research Group, The Research Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran.*
5) *Brighton & Sussex Medical School, Division of Medical Education, Falmer, Brighton, Sussex BN1 9PH, UK.*
6) *Department of Pediatrics, Ghaem Hospital, Mashhad University of Medical Sciences, Mashhad, Iran.*

#Corresponding Authors:

Amir Avan, PhD and Mohammad Ali Kiani *MD, Medical Genetics Research Center, Mashhad University of Medical Sciences, Mashhad, Iran and Metabolic syndrome Research center, Mashhad University of Medical Sciences, Mashhad, Iran, Tel:+985138002298, Fax:+985138002287; Email: avana@mums.ac.ir*

* Equally contributed as first author

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Abstract

Alcohol-associated liver disease (ALD) comprises a spectrum of liver diseases that include: steatosis to alcohol-associated hepatitis, cirrhosis, and ultimately hepatocellular carcinoma. The pathophysiology and potential underlying mechanisms for alcohol-associated liver disease are unclear. Moreover, the treatment of ALD remains a challenge. Intestinal microbiota include bacteria, fungi, and viruses, that are now known to be important in the development of ALD. Alcohol consumption can change the gut microbiota and function leading to liver disease. Given the importance of interactions between intestinal microbiota, alcohol, and liver injury, the gut microbiota has emerged as a potential biomarker and therapeutic target. This review focuses on the potential mechanisms by which the gut microbiota may be involved in the pathogenesis of ALD and explains how this can be translated into clinical management. We discuss the potential of utilizing the gut microbiota signature as a biomarker in ALD patients. Additionally, we present an overview of the prospect of modulating the intestinal microbiota for the management of ALD.

Keywords: Alcohol-associated liver disease, Gut microbiota, Fungi, Viruses, Biomarker, Gut-liver axis.

Introduction

Excessive alcohol consumption is one of the top ten risk factors for disease worldwide. There is a strong relationship between alcohol consumption and risk of several diseases including cancers and liver cirrhosis. About 6% of all global deaths are attributable to the alcohol-related injury. Alcohol consumption not only adversely affects the quantity and quality of life in consumers, but also has an impact on their family members (1).

Alcohol use disorder (AUD), sometimes called alcoholism, is a pattern of alcohol use including compulsive alcohol consumption and impaired ability to stop drinking despite its adverse consequences. The diagnosis of AUD may be established using specific diagnostic criteria (2). The prevalence of this multifaceted disorder has been increasing at an alarming rate. Alcohol causes damage to multiple end organs; however, liver injury and cirrhosis are the most common causes of death in patients with AUD (3).

Alcohol-associated liver disease (ALD) includes a wide spectrum of liver disease from steatosis to alcohol-associated hepatitis (AH), cirrhosis, and may culminate in hepatocellular carcinoma (4). Approximately 60–80% of liver-related deaths are caused by alcohol consumption. It has been estimated that one-third of liver cirrhosis cases are due to alcohol in Western Europe (5). Furthermore, ALD has been identified as the most common reason for liver transplantation in the United States after the advent of effective medical treatment for chronic hepatitis C infection (6). Despite recent progress, treatment of ALD remains a major challenge because there is no effective therapy for severe disease apart for liver transplantation. Based on data from a multicenter study, relapse of severe AUD after transplantation occurs in 20% of liver transplant recipients with prior ALD leading to allograft cirrhosis in 35% of these cases (7).

The pathogenesis of ALD is not fully understood. Progression of hepatic steatosis to advanced liver disease occurs in only a small portion of heavy drinkers highlights that the amount of alcohol consumed is not the only factor that contributes to ALD development. There is accumulating evidence that there is a close relationship between ALD and human gut microbiome (8). An ecosystem of various microbial communities such as bacteria, fungi, and viruses exists in the human intestine and these microorganisms may be a leading

factor in the development of ALD. This finding provides greater opportunities for identifying the cellular and molecular mechanisms of ALD and helps us to understand the association of not only bacterial but also fungal and viral microbiota with ALD. Given the interaction between alcohol, liver disease, and gut microbiota, further promising therapeutic targets are provided for the treatment of patients with ALD. Therefore, this review mainly focuses on how gut bacterial and non-bacterial communities affect the development of ALD. We propose the mechanisms by which gut microbiota dysbiosis contributes to ALD. Moreover, we explain microbiota-based therapeutic strategies and show how targeting gut microbiota could be an attractive new approach for managing ALD.

How Gut Microbiota Affects Alcohol-associated Liver Disease

Bacteria, fungi, archaea, and viruses are components of the human gut microbiota. Changes in the microbiota's relative abundance and the equilibrium that can have an unfavorable effect on the host are called microbiota dysbiosis. Previously, it has been shown that intestinal microbial dysbiosis and microbial-derived metabolites such as secondary bile acids and short-chain fatty acids (SCFAs) affect host health and can be related to a wide variety of human diseases (9, 10). Only 15–20% of patients with AUDs develop ALD and intestinal microbial dysbiosis has been proposed as the reason for this heterogeneity. Here we review the potential pathogenic mechanisms behind the relationship between gut microbiota, alcohol, and liver injury (Figure 1). Although previous publications have mainly focused on the role of bacterial microbiota, we also describe the emerging role of the non-bacterial microbiome in the pathogenesis of ALD.

Bacterial dysbiosis and gut barrier dysfunction

The gut-liver axis is implicated in the development and progression of ALD. This axis has a major role in intestinal barrier function, intestinal immunity, as well as hepatic and systemic inflammation. The liver interacts with the gut microbiota through several pathways including its responses to gut bacterial products received via the portal vein,

enterohepatic circulation, and bile acid production. On the other hand, the intestinal barrier comprises the mucous layer (gut microbiota, secretory immunoglobulin A (IgA), and antimicrobial peptides), the epithelial intestinal layer, and the lamina propria with its resident immune cells. It is noteworthy that the liver is the first organ to encounter intestinal products following the breech of the intestinal barrier. Additionally, gut-vascular barrier controls the translocation of microbiota and microbial-derived products to the systemic circulation. In summary, a normal gut-liver axis depends on the intact intestinal barrier, normal liver function as well as the healthy gut microbiota. This gut–liver axis is disrupted in ALD.

Numerous animal and human studies have revealed that alcohol consumption can alter gut microbial features. Chronic ethanol administration in mice not only changes the diversity in the ileum and the liver but also changes the composition of bacteria, especially in the ileum. These compositional alterations include an increase of gram-negative endotoxin-producing bacteria. Interestingly, gram-negative *Prevotella* increases in both the mucus layer of the ileum and the liver suggesting the relationship between intestinal dysbiosis and bacterial translocation to the liver (11). Moreover, loss of alpha-diversity in addition to higher levels of *Firmicutes* were observed in alcohol-treated rhesus macaques. Alteration in glycolysis metabolism in the alcohol-consumption period and differences in fatty acid metabolism in the abstinence period have been observed (12). Kosnicki *et al.* (13) investigated gut microbial changes in response to moderate levels of alcohol consumption in the rat and compared the findings to human fecal microbiome data collected from citizen science American Gut Project. Ethanol-consuming rats exhibit dramatic shifts in the overall diversity of the gut microbiota and significant changes in the relative abundance of several bacteria, such as the *Lactobacilli*. Gut microbial biodiversity was higher in human alcohol consumers in comparison to non-drinkers, however, differences in the relative abundance of bacteria between the two groups of human follow similar trends in the rat model. In both rat and human ethanol-treated groups, the abundance of *Peptococcus*, *Clostridiaceae*, and *Lactobacillus* was lower. Additionally, the abundance of *Oxalobacter*, *Adlercreutzia*, *Ruminococcaceae*, *Clostridiales*, *Barnesiellaceae*, *Paraprevotella*, *Phascolarctobacterium*, *Butyricimonas*, and *Sutterella* was higher in these groups.

Endotoxemia is defined as the elevation of plasma levels of lipopolysaccharides (LPS) that may be due to increased gut permeability, high levels of intestinal LPS-containing bacteria, or both. It is known that alcohol consumption can elevate serum levels of LPS by disruption of intestinal epithelial integrity and inducing microbial dysbiosis (14, 15). Sturm et al. have proposed that alcohol could damage the intestinal barrier integrity and enhance circulating LPS levels (16). Moreover, alcohol consumption increases *Actinobacteria* and decreases *Verrucomicrobia,* driven completely by a reduction in *Akkermansia* in mice. On the other hand, antibiotic therapy of these mice can reduce circulating LPS, suggesting a central role of gut dysbiosis in alcohol-induced endotoxemia (17). Bacterial-derived LPS is absorbed by the intestine and travels through the liver. Subsequently, LPS interacts with Toll-like receptor 4 (TLR4) which is expressed in all cell types of the liver, especially Kupffer cells. In response to this interaction, Tumor necrosis factor-alpha (TNF-α) is released by Kupffer cells which then causes inflammation and liver fibrosis by activating the nuclear factor Kappa-B pathway (18). According to this process, LPS-TLR4-TNF-α pathway acts as an important factor in ALD pathogenesis.

The study of Maccioni *et al.* (19) in 106 patients with AUD and 24 healthy participants demonstrated that microbial dysbiosis in duodenal mucosa and increased translocation of either microbial products or microbes were associated with early stage of progressive ALD. In AUD patients, duodenal mucosa-associated microbiota undergoes some changes. *Nubsella*, *Shuttleworthia, Rothia*, and *Streptococcus* are increased in the AUD group whereas *Mycobacterium*, *Alcaligenes*, *Lachnoclostridium*, *Ralstonia*, *Rarobacter*, *Ethanoligenens*, and *Dolosigranulum* are higher in healthy individuals. Intriguingly, elevated intestinal permeability is not associated with microbial translocation and duodenal dysbiosis, but there is a linkage between alterations in fecal microbiota and increased intestinal permeability. This study indicates that microbial translocation does not necessarily require high intestinal permeability and might occur via other mechanisms. Moreover, intestinal permeability and fecal microbiota can become normal following short time of abstinence but cannot prevent microbial translocation and liver damage.

Intestinal virome

The majority of viruses in the human body are in the gastrointestinal tract. Human intestinal viral microbiome (virome) is specific to each individual. The human virome mainly consists of bacteriophages (phages). Bacteriophages infect bacteria which can either be a specific bacterial strain or a broader range of strains. *Caudovirales* including *Siphoviridae*, *Myoviridae*, and *Podoviridae* families are the most predominant bacteriophages in human virome. Some evidence indicates that phages can increase intestinal permeability by infecting gut bacteria. In this context, a close relationship between human virome and many human diseases such as inflammatory bowel disease, diabetes, and colorectal cancer have been observed (20). However, less is known about the role of human virome in ALD. Recently, the virome signature in alcohol-associated hepatitis patients has been defined for the first time. A multicenter observational study on 36 patients with alcohol use disorder and 17 individuals as the control group revealed that *Escherichia*, *Enterobacteria*, and *Enterococcus* phages become over-represented during alcohol-associated hepatitis and mammalian viruses such as *Parvoviridae* and *Herpesviridae* become increased (21). Another study conducted by Hsu *et al.* reported that ALD was associated with altered fecal virome. A study done by Hsu *et al.* on 62 patients with alcohol use disorder showed that *Propionibacterium*, *Lactobacillus*, and *Leuconostoc* phages decreased in these patients compared to the control group however, these changes were reversible after 2 weeks of alcohol abstinence (22). Although the exact mechanisms behind the role of gut virome in the pathogenesis of ALD have not been clarified, intestinal virome may aggravate ALD through intervention with the symbiotic bacteria. Phages regulate the abundance of gut bacteria by modulating bacterial cell lysis. Additionally, phages are able to transfer additional genomes such as bacterial virulence factors to intestinal bacteria (23). The investigations of gut virome are just starting. The association of gut virome with ALD is important in fully understanding the pathogenesis of this disorder; therefore, further studies are necessary.

Intestinal mycobiome

Although only a small proportion of the human microbiota are fungi, recent studies have shed light on the importance of these micro-organisms in many human diseases. *Ascomycetes*, *Basidiomycetes*, and *Zygomycetes* are the most prominent phyla in human adults. ALD patients have a lower fungal diversity with an overgrowth of *Candida* and a decrease in *Epicoccum*, *Debaryomyces*, *Galactomyces*, and unclassified fungi (24, 25). Further studies are required for understanding the accurate mechanisms by which fungal dysbiosis is involved in the pathogenesis of ALD. However, according to previous research, the gut mycobiome is involved in ALD pathogenesis via two main pathways. The first pathway is the overgrowth of fungi in response to chronic alcohol consumption. Increased mycobiota populations produce more fungal products such as β-glucan that can be translocated easily into the liver through the already disrupted intestinal barrier. In the liver, β-glucan binds to the C-type lectin-like receptor CLEC7A on Kupffer cells and induces IL-1β expression and secretion contributing to hepatocyte damage and ALD (24). The second mechanism is fungi-derived metabolites. Commensal gut *Candida albicans* could secrete a type of peptide toxin called Candidalysin which has the ability to recruit immune cells and induce hepatocyte death (26). In alcohol-associated hepatitis patients, not only does the number of Candidalysin-producing C. albicans increase but there is also a significant increase in the expression of the Candidalysin encoding gene extent of cell elongation 1 (ECE1) (27). Further compelling investigations should explore the correlation between gut mycobiome and the pathogenesis of ALD.

Factors Contributing to Intestinal Dysbiosis

Fatty acid metabolism and histone deacetylase

Not only do fatty acids play a protective role in gut barrier function, but they also prevent bacterial translocation as well as microbial toxin, preventing subsequent liver injuries. Butyrate is a SCFA produced by gut bacteria during the fermentation of non-digestible polysaccharides. Butyrate can ameliorate ALD by stabilizing the intestinal barrier and reducing alcohol-induced endotoxemia. Additionally, butyrate down-regulates gasdermin D (GSDMD)-mediated pyroptosis, which is a form of programmed cell death initiated by inflammation (28). It has been shown that the lack of butyrate-producing microbiota is a

characteristic feature of ethanol-induced microbial dysbiosis and ALD (29). Moreover, it is identified that histone deacetylase (HDAC) activity in the intestine exacerbates ALD. HDAC11 mediates the response of Kupffer cells to LPS following alcohol consumption (30). Ethanol-induced HDAC3 leads to alcohol-associated liver injury (31). In addition, HDAC8 overexpression exacerbates alcohol-associated hepatitis in mice by activating pro-inflammatory responses and miR-451a ameliorates ALD via repressing HDAC8 (32). It is known that intestinal microbiota-derived SCFAs are the major regulators of HDACs (33) and their protective role against ALD may be due to their inhibitory effects on them. However, further research is needed for identifying the exact mechanisms behind microbiota-derived fatty acid effects on ALD which provides opportunities for treating this disease.

MicroRNAs

MicroRNAs (miRNAs) have recently emerged as mediators of intestinal permeability. Therefore, their roles in ALD are currently under investigation. miRNAs belong to a group of non-protein-coding RNAs (ncRNAs) and regulate gene expression. Experimental studies demonstrated the crucial role of miRNAs in the hepatic response to LPS. Data from studies revealed that miR-155 is a major factor in ALD. miR-155 enhances the TNF-α secretion from Kupffer cells and its inhibition prevents LPS-induced ALD (34). Moreover, miR-212 and miR-122a regulate intestinal permeability through the ZO-1 protein which is involved in intercellular tight junctions (35). It is known that during alcohol consumption, hepatocyte-derived miR-122 transfers via exosomes to reprogram monocytes and macrophages which leads to sensitization of these cells to LPS and increased inflammation (36). Another study showed that ethanol administration enhanced LPS-induced up-regulation of miR-217 in Kupffer cells and subsequent hepatic inflammation. miR-217 further mediates ethanol and LP-induced sirtuin 1 inhibition that leads to activation of nuclear factor kappaB (NF-κB) and the nuclear factor of activated T cells c4 (NFATc4) as inflammatory regulators (37). Altogether, variant miRNAs can be considered principal players in the pathogenesis of LPS-mediated ALD. Therefore, clarifying the roles of these

miRNAs in ALD would be of importance to understand its pathogenesis and to develop effective treatment strategies.

Bile acid metabolism and FXR signaling

It has been shown that bile acid metabolism is altered following alcohol consumption. FGF19 (an important regulator of bile acid synthesis) and both forms of bile acids, conjugated and total serum are elevated in patients with alcohol-associated hepatitis. Taurine-conjugated bile acids (taurocholic acid, aurochenodeoxycholic acid, and tauroursodeoxycholic acid) show more elevations than glycine-conjugated forms (glycocholic acid, glycochenodeoxycholic acid, and glycoursodeoxycholic acid) (38, 39). Ciocan *et al.* (40) studied cirrhotic patients with severe alcohol-associated hepatitis showed that the bile acid pool shifts towards more hydrophobic bile acids during alcohol-associated hepatitis. These changes may be the reason for microbial dysbiosis. On the other hand, altered gut microbiota may change features of the bile acid pool by transforming primary bile acid into its secondary form. Gut microbial dysbiosis in these patients was characterized by increased *Actinobacteria* and decreased *Bacteroidetes*. Furthermore, elevated LPS-producing gram-negative bacteria such as *Gammaproteobacteria* and reduced gram-positive primary-to-secondary bile acid transforming bacteria could be observed in these patients. In addition, high glutathione and low biotin metabolisms following dysbiosis take part in alcohol-associated hepatitis initiation and progression by means of interfering with Ursodeoxycholic acid (UDCA)-protective effect on mitochondrial metabolism. The bile-acid receptor TGR5 (or GPBAR1) plays a central role in biliary homeostasis. Deficient TGR5 increases steatosis and inflammation in the liver of alcohol-fed mice. Results indicate that the lack of TGR5 leads to decreased secondary bile-acid levels due to low abundance of bile-acid transforming bacteria. Moreover, TGR5 deficiency changes the gut microbiota characterized by an increase in the *Deferribacteres* phylum and the *Mucispirillum*, *Enterococcus*, *Prevotella*, and *Bilophila* genera. These changes were independent of alcohol consumption. It was also suggested that intestinal microbiota transplantation from TGR5-deficient mice to wild-type mice deteriorated alcohol-induced liver injury (41).

The farnesoid X receptor (FXR) is a bile acid-sensing nuclear receptor that is highly expressed in the liver and the intestines. Bile acid homeostasis depends on FXR both in the liver and the intestine. FXR reduces bile acid synthesis by modulating the activity of CYP7A1 which is the rate-limiting enzyme in bile acid synthesis. The expression of small heterodimer partner (SHP) nuclear receptor mainly relies on the activity of hepatic FXR. Then follows, the interaction of SHP with liver receptor homolog-1 (LRH-1) which represses cytochrome P450 enzyme (Cyp)7A1 and CYP8B1. In the intestines, bile acids induce activation of FXR leading to the secretion of fibroblast growth factor 15/19 (FGF15/19) inside the portal vein. Subsequently, down-regulation of hepatic CYP7A1 occurs following FGF15/19- FGF receptor 4 (FGFR4) interaction in the liver (42). The animal study of Huang *et al.* (43) reported that intestinal FXR is essential for preventing ALD. Results revealed that intestinal FXR deficiency disrupts mucosal integrity and increases intestinal permeability by reducing E-cadherin levels and Mucin 2 secretion. Schneider and collaborators (44) investigated primary sclerosing cholangitis in an animal model revealed that bile acid-FXR dependent negative feedback of gut microbiota on bile acid synthesis was essential for liver health. It demonstrated that the disruption of this pathway increased hepatic bile acid concentrations, leading to liver injury. Additionally, deoxycholic acid (DCA)-treated mice presented gut microbiota dysbiosis and lower FXR activity. These changes were accompanied by upregulation of hepatic bile acid synthesis and intestinal inflammation (45, 46). Data from these studies suggest a close relationship between gut microbiota-FXR-bile acid axis and liver function. Numerous studies confirmed the association of this axis with ALD. It has been shown that ethanol administration increases the expression of hepatic CYP7A1, increasing both intestinal bile acid content and circulating bile acid levels by lowering FXR activity in enterocytes. These alterations can be reversed by commensal microbiota depletion with non-absorbable antibiotics. Therefore, ALD following high levels of bile acid synthesis is dependent on gut microbiota (47). A recent animal study conducted by Helsley *et al.* published in the year 2022 (48) suggested that gut microbial metabolite trimethylamine (TMA) elevated in the circulation during alcohol-associated hepatitis. In addition, inhibition of TMA pathway improves ethanol-induced liver injury. It is identified that choline TMA lyase inhibition upregulates CYP7A1, resulting in increased hepatic bile acid synthesis and decreased hepatic feedback

regulation of bile acid metabolism (49). However, the exact underlying molecular mechanisms behind the relationship between TMA pathway and bile acid metabolism have not been identified and further studies are needed. Overall it has been seen that gut microbiota plays a vital role in the pathogenesis of ALD by modulating bile acid pool and FXR activity. Therefore, bile acid pool and FXR are promising areas of therapy development focus.

Nod-like receptor pyrin domain-containing proteins (NLRPs) inflammasome

Recent studies have shown that the NLRP3 inflammasome may mediate inflammatory and pro-fibrogenic stress signals in the liver during ALD. Pyroptosis is a unique form of hepatocellular death driven by translocated gut bacteria, endotoxemia, or PMN inflammation (50, 51). During pyroptosis, the NLRP3 inflammasome is released from hepatocytes into the extracellular space where it can be taken up by other cells. This process may trigger inflammation and fibrogenesis in the liver (52). Therefore, increased translocation of bacteria and bacteria-derived particles such as LPS into the liver through the portal vein activates the NLRP3 inflammasome which contributes to liver injury (53, 54). In addition, dying cells release an endogenous ligand called spliceosome-associated protein 130 (SAP130) that can interact with Macrophage-inducible C-type lectin (Mincle) receptor on the surface of Kupffer cells. Subsequent release of the NLRP3 Inflammasome and IL-1β from Kupffer cells aggravates ALD and leads to infiltration of invariant natural killer T cells into the liver (55). Moreover, a previous study done by *Han et al.* (56) has shown that hepatic FXR activity is inversely correlated with NLRP3 inflammasome levels. FXR down-regulates NLRP3 inflammasome by preventing endoplasmic reticulum stress.

NLRP6 inflammasome plays a key role in regulating gut microbiota and intestinal epithelial integrity. NLRP6 inflammasome is a vital factor for exocytosis of mucin granule from goblet cells. Since mucus production acts as antimicrobial protection, NLRP6 inflammasome is a crucial regulator of the intestinal ecosystem (57). Lack of NLRP6 inflammasome leads to increased intestinal inflammation and altered fecal microbiota characterized by expanded bacterial phyla *Bacteroidetes* (*Prevotellaceae*) (58). *Mao et al.* (59) demonstrated that *Faecalibacterium prausnitzii* enhanced the production of the NLRP6 inflammasome and

antimicrobial peptides that inhibit *Candida albicans*' growth, pathogenicity, as well as intestinal inflammation. Furthermore, the role of several microbiota-associated metabolites such as taurine, histamine, and spermine in modulating NLRP6 inflammasome and anti-microbial peptides suggests them as therapeutic candidates for restoring normal intestinal microenvironment (60). Intriguingly, recent research by *Mainz et al.* shown that NLRP6 aggravated ALD and its inhibition reduced hepatic immune cell infiltration (61). Given the role of NLRP6 inflammasome in the development of ALD, targeting this inflammasome to alleviate ALD is another promising area of research that requires dedicated investigation.

Mucosa-associated invariant T cells

Mucosa-associated invariant T (MAIT) cells, defined as CD3+, Vα7.2+, and CD161+ T lymphocytes, are found in liver, blood, and intestinal mucosa. MAIT cells express invariant T-cell receptors that recognize bacteria-derived riboflavin (vitamin B2) metabolites presented by antigen-presenting cells (APCs) such as dendritic cells and B cells. This leads to the activation of MAIT cells and initiates subsequent inflammatory responses that play a key role in controlling the infection. Viruses can also be recognized by MAIT cells through the interleukin receptors IL12R and IL18R on their cell surface. Therefore, MAIT cells are a key component of the host immune system against pathogens. Intriguingly, normal intestinal microbiota cannot be recognized by MAIT cells (62). Gut dysbiosis including abnormal bacteria, fungi, and viruses can stimulate MAIT cells. The number of MAIT cells are found to be reduced in the blood during ALD presumably because of their migration to the liver (63, 64). Moreover, the remaining population of MAIT cells is dysfunctional. The disrupted microbial ecosystem and impaired intestinal mucosal barrier during ALD lead to chronic exposure of MAIT cells to gut-derived bacterial products. This interaction may be a basis for hyper-activated MAIT cells. Interestingly, hyper-activated human MAIT cells can stimulate the proliferation of hepatic myofibroblasts and result in alcohol-related cirrhosis (65). Finally, hyperactivation of MAIT cells makes them exhausted and functionally deficient, thus losing their antimicrobial properties (66). Therefore, approaches that can

reduce the extent of ALD, potentially through targeting the mucosa-associated invariant T, might be promising.

Extracellular vesicles

Extracellular vesicles (EVs) provide cell-to-cell communication, and contain biomaterials such as proteins and microRNAs which transfer specific cargo from the cell of origin to the target cell. EVs have been identified as a novel mechanism responsible for ALD. EVs released by intestinal epithelial cells increase intestinal permeability by reducing the expression of zonula occludens-1 (ZO-1) and MUCIN-2. Epithelial cell-derived EVs have harmful effects on hepatocyte viability and lipid accumulation by infiltration of CD11b-positive immune cells and inducing pro-inflammatory cytokines (67). The number of circulating EVs is elevated in ALD mice. Circulating EVs containing heat shock protein 90 induce macrophage activation (68). Hepatocyte-derived EVs are enriched in organelle proteins, miRNAs, and mitochondrial DNA. These EVs stimulate hepatic macrophages to produce profibrogenic IL-1β and IL-17 in a TLR9-dependent manner (69). Furthermore, chronic-plus-binge ethanol intake induces the release of proinflammatory mitochondrial DNA-enriched EVs by hepatocytes (70). Together, understanding the molecular mechanism of EVs in the pathogenesis of ALD can open novel avenues for therapy.

Glucocorticoid Receptors

Data from a recent animal study (71) showed that the glucocorticoid receptor (GR) is a key contributor in alcohol-associated tissue injury and can be a potential therapeutic target for ALD therapy. Ethanol and corticosterone increase the relative abundance of *Enterobacteriaceae* and *Escherichia coli* while decreasing the abundance of *Lactobacillus* in Hepatocyte-specific GR-deficient mice in a synergic manner. Additionally, GR is associated with gut barrier dysfunction, endotoxemia, and systemic inflammation.

Gut Microbiota Signature as a Biomarker in Alcohol-associated Liver Disease

Previous studies have shown that the microbial signature can be used for identifying AUD patients. 36 AUD patients enrolled in the study done by Addolorato *et al.* (72) and the results indicated the decreased microbial alpha diversity in these patients. Data from this study showed that an elevation of *Bacteroides* and a reduction of *Akkermansia* could be used to identify AUD patients with an accuracy of 93.4%. A study conducted by Gurwara and collaborators (73) on 34 polyp-free individuals demonstrated that heavy drinkers exhibit the lowest relative abundance of *Subdoligranulum*, *Roseburia*, and *Lachnospiraceaeunc,* but the highest relative abundance of *Lachnospiraceaeunc*. Bjørkhaug *et al.* (74) enrolled 24 alcohol over-consumer patients and 18 control patients. Data from this study showed that over-consumers had higher levels of *Proteobacteria*, *Sutterella*, *Holdemania,* and *Clostridium*, but a lower relative abundance of *Faecalibacterium*. A lower concentration of butyric acid has also been found in this group.

The diagnosis of ALD is based on history, clinical manifestations, and laboratory data. Unfortunately, there is no single test for confirming this diagnosis, making the diagnosis of ALD challenging. Although liver biopsy may be used to confirm the diagnosis of ALD, it is an invasive and expensive procedure. ALD is reversible in nature thus regular screening and early detection are beneficial. Recent studies have focused on novel diagnostic and prognostic biomarkers for ALD. Clinical application of biomarkers such as microbial dysbiosis, alterations in microRNA expression, and cytokine dysregulation is under investigation (75).

Numerous studies have been conducted to evaluate the gut microbiome as a diagnostic and prognostic biomarker for ALD. According to data from these studies, gut dysbiosis has emerged as a biomarker for ALD. Specific microbial signatures have the capability to differentiate distinct complications of alcohol consumption in alcoholic patients. For instance, a human study found that patients with severe alcohol-associated hepatitis display higher levels of *Haemophilus* (76). Lower alpha diversity and higher beta diversity have been observed in both gut bacteria and extracellular vesicles of these patients. *Bacilli*,

Lactobacillales, and *Veillonella* were remarkably increased in the gut bacteria of patients with severe alcohol-associated hepatitis while *Eubacterium*, *Oscillibacter,* and *Clostridiales* were decreased (77). A study on fecal samples from 74 patients revealed the increased relative abundance of *Veillonella* and decreased relative abundances of *Akkermansia* in alcohol-associated hepatitis patients with more severe disease. Therefore, gut microbiota signature can predict the severity of the disease in these patients (78). According to the results from a study in the year 2019, cytolysin-positive *Enterococcus faecalis* is closely associated with the prognosis of alcohol-associated hepatitis patients. Cytolysin is a bacterial exotoxin produced that can lyse eukaryotic cells. In addition, they confirmed the results in an animal study and found that cytolytic *E. faecalis* can induce hepatocyte death independent of alcohol; however, alcohol consumption facilitates the entrance of cytolytic *E. faecalis* to the liver by destroying gut barrier, thus alcohol exacerbates alcohol-induced hepatitis. Altogether, cytolysin-positive *E. faecalis* is correlated with the severity and mortality of AH patients (79). Fecal microbial evaluation in 78 participants indicated that fecal enrichment with *Actinomycetaceae*, *Coriobacteriaceae Atopobium*, *Fusobacteriaceae*, *Saccharibacteria incertaesedis*, and *Veillonellaceae* families represents the severe alcohol-associated hepatitis. On the other hand, *Closteridiales*, *Lachnospiraceae,* and *Ruminococcaeae* families are enriched in heavy drinkers. This microbiome signature shows whether heavy drinkers progress into alcohol-associated hepatitis (80). Furthermore, a recent study indicated that gut microbial dysbiosis in alcohol-associated fatty liver disease is different from metabolic associated fatty liver disease in mice. *Enterococcaceae* at the family level and *Enterococcus* and *Streptococcus* at the genus level were the most abundant bacteria in the alcohol-associated fatty liver disease. The metabolic-associated fatty liver disease was characterized by high *Lachnospiraceae* at the family level, high *Erysipelatoclostridium*, *Gordonibacter,* and *Streptococcus* at the genus level, and low *Bifidobacterium* at the genus level (81). Likewise, microbial signature has been used for detecting alcohol-related cirrhosis. Previous studies showed that rectal mucosal microbiome can distinguish the alcohol-related cirrhosis from non-alcohol related cirrhosis. Reduced abundances of *E. coli* and *Enterobacteriaceae* in rectal mucosa may be used as a marker for alcohol-related cirrhosis (82). It has been demonstrated that as the ALD progresses, the degree of gut microbiota imbalance becomes more severe. Moreover,

Streptococcus has been identified as a microbial marker of alcohol-associated liver cirrhosis. Therefore, this marker may be used to evaluate the severity of liver injury in ALD patients (83). In addition to the microbial signature, some alterations in bile acid metabolism are identified as a biomarker for ALD. For instance, taurocholic acid, taurochenodeoxycholic acid, glycocholic acid, and glycochenodeoxycholic acid are predictors of ALD progression.

Given the increased levels of EVs during ALD and their effects on gut barrier function, E+Vs have emerged as biomarkers for ALD. According to a human study, extracellular vesicles carrying sphingolipid cargo show a good diagnostic performance and predict 90-day survival in alcohol-associated hepatitis patients (84). Furthermore, urinary extracellular vesicles can be used as new biomarkers for cirrhosis in ALD (85). Damaged hepatocytes-derived EVs with a specific three miRNAs cargo including let7f, miR-29a, and miR-340 are considered a potentially novel diagnostic biomarker for alcohol-associated steatohepatitis (86).

The gut virome has also been recently identified as a prognostic biomarker in ALD patients. Jiang et al. demonstrated that Staphylococcus phages and *Herpesviridae* are associated with the severity of alcohol-associated hepatitis and can predict mortality in ALD patients (21). Furthermore, a relationship between the progression of ALD and bacteriophage-bacteria interactions has been observed. For instance, an increased abundance of phages targeting *Enterobacteria* and *Lactococcus* species predicts progressive ALD (22).

In the last few years, several mycobiome-related noninvasive indicators have been found for ALD patients. Candidalysin can predict the severity of alcohol-associated hepatitis and is positively associated with the mortality of these patients (27). Additionally, a relationship between the level of serum anti-Saccharomyces cerevisiae antibodies (ASCA) and mortality in alcohol-associated hepatitis patients has been confirmed (25). *Lang et al.* showed that circulating levels of ASCA are higher in alcohol-associated hepatitis patients compared with non-alcoholic and even alcohol-use disorder patients. Table 1 summarizes the potential prognostic biomarkers in patients with alcohol-associated liver disease.

In recent years, the advances in machine learning tools for biomarker discovery have attracted ample attention. Nowadays, the model of end-stage liver disease (MELD) score is used for the prediction of mortality in alcohol-associated hepatitis patients. However, analysis of gut microbiota for predicting mortality in patients with alcohol-associated hepatitis showed promising results. Comparing four popular machine learning models including gradient boosting, random forest, support vector machine, and logistic regression models by *Gao et al.* revealed that Gradient boosting has a better performance than MELD score for both a 30-day mortality prediction using the fecal bacteria and metabolic pathways dataset, as well as 90-day mortality prediction using the fungi dataset (87).

How gut microbiota modification can influence alcohol-associated liver disease treatment

For many years, abstinence and the management of subsequent alcohol withdrawal syndrome have remained the first line intervention in the treatment of ALD. Although the cornerstone of the treatment of ALD is still alcohol abstinence and nutritional support, some other therapeutic options may be beneficial for these patients. The application of glucocorticosteroids showed mixed outcomes in various clinical trials and approximately 40% of patients with ALD do not respond to corticosteroid treatment. In addition, Pentoxifylline possesses anti-fibrotic potential and is considered a substitute for corticosteroid treatment in some cases of severe alcohol-associated hepatitis. Although numerous studies have indicated the efficacy of Anti-Tumor necrosis factor (TNF) therapy, the results have not been confirmed in larger clinical trials. Eventually, liver transplantation is the definitive therapy for patients who progress to end-stage liver disease. Liver transplantation in ALD patients is controversial. Moreover, we have very few options for ALD patients who do not respond to steroids. Therefore, searching for new therapeutic options is necessary.

Recently, manipulation of the gut microbiome has emerged as a potentially novel therapeutic strategy for the management of ALD. Probiotics, prebiotics, fecal microbiota

transplantation (FMT), bacteriophages, and other microbiota-based treatments can help these patients by modulating intestinal microbiota. In this review, we discuss microbial-based therapies for the treatment of ALD patients.

Probiotics

Probiotics are live, nonpathogenic microorganisms that benefit the host provided that they are used in appropriate quantity. Many experimental studies conducted in animal models have confirmed the efficacy of probiotics as an option for controlling ALD. Probiotics exert their effects through a variety of mechanisms such as promoting gut barrier integrity, reducing endotoxemia, modulating intestinal microbiota composition, increasing intestinal SCFA content, production of antimicrobial peptides, improving the immune system, and decreasing hepatic inflammation as well as oxidative stress. Table 2 summarized studies that evaluate the efficacy of probiotics in the treatment of patients with ALD.

Lactobacillus products are one of the most popular products among commercially available probiotics. Additionally, *Lactobacillus*-based probiotics have been widely studied in ALD models. Efficacy of *Lactobacillus rhamnosus GG* (LGG) culture supernatant has been evaluated using chronic-alcohol-induced hepatic steatosis model of mice. Results showed that hepatic AMPK activity can be controlled by LGGs. Moreover, this probiotic prevents alcohol-induced hepatic apoptosis by up-regulation of Bcl-2 and down-regulation of Bax (88). LGG granules can overcome chronic ALD in a dose-dependent manner. Alcohol consumption for 8 weeks decreased *Lactobacillus* and *Bifidobacterium* in mice. In addition, elevation of *Clostridium perfringens* numbers in ileum and proportional increase in the number of several gram-negative bacteria such as *Proteobacteria*, *Campylobacterales*, and *Helicobacter* in cecum have been observed. LGG also reduces circulating level of LPS and TNF-α (89). As mentioned above, miR122a has a central role in regulating intestinal permeability. Given that upregulation of miR122a leads to increased intestinal permeability by suppressing occludin protein levels, LGG improves gut barrier function by inhibition of miR122a expression (90).

TLR 4 is considered to be a key target in the treatment of ALD because of its vital role in the gut-liver axis. TLR 4 can be modulated by *Lactobacillus rhamnosus R0011* and *acidophilus R0052* resulting in the regulation of gut-liver axis and improvement of ALD (91). Interestingly, the therapeutic efficacy of LGG could be improved by adding inosine to the treatment. Combination of inosine and LGG ameliorates hepatic inflammation during ALD by blocking the phosphorylation of p38 and JNK. Furthermore, combined therapy improves intestinal villi and tight junction proteins more significantly as opposed to LGG alone. LGG and inosine combination has also immunomodulatory effects characterized by increasing Tregs population as well as inducing inhibitory effects on Th1(92). A fermentation broth which fermented the mixture of *Pueraria lobata*, *Lonicera japonica*, and *Crataegus pinnatifida* by *Lactobacillus rhamnosus 217-1* suppresses inflammation and oxidative stress in the liver of patients with ALD. The fermentation broth regulates gut-liver axis through improving gut integrity and reducing endotoxemia (93). In summary, *Lactobacillus rhamnosus* improves ALD; however, further research regarding the underlying mechanisms is necessary. Other types of *Lactobacillus* such as *Lactobacillus plantarum* have been evaluated for their anti-ALD potentials (94, 95). However, therapeutic use of these probiotics in clinical practice depends on evaluating their efficacy in future clinical trials. A study on 410 fecal samples from 212 Korean twins has shed light on the vital role of butyrate-producing genus *Roseburia* in human gut ecosystem and ALD pathogenesis. Enrollment of twins limits the variability in host genetics. Data from this study indicates that there is strong relationship between low Alcohol Use Disorders Identification Test (AUDIT) scores and the abundance of the butyrate-producing genus *Roseburia*. Administration of *Roseburia* to ALD murine models recovers gut barrier integrity and restores the gut microbiota. Occludin which is a protein involved in tight junctions, REG3γ as an antimicrobial peptide, and the expression of IL-22 could be increased by *R. intestinalis* (96).

Bifidobacterium animalis ssp. lactis has been observed to have beneficial effects on gut microbiota. The probiotic containing these bacteria can mitigate liver damages in ALD by suppressing liver inflammation and oxidative stress (97). As a probiotic, *Komagataeibacter hansenii CGMCC 3917* regulates gut microbiome and reduces endotoxemia as a probiotic. *K.*

hansenii CGMCC 3917 administration to alcohol-treated mice follows an increase in *Bacteroidetes* and a decrease in *Actinobacteria*, *Proteobacteria*, and *Firmicutes*. This probiotic regulates fatty acid metabolism by controlling the activity of related enzymes. In addition to elevation of SCFA contents in the faeces, colon and cecum; this probiotic can decrease hepatic and circulating levels of long chain fatty acids (98). *Pediococcus pentosaceus CGMCC 7049* is a new ethanol-resistant strain isolated from healthy human adults. *P. pentosaceus* administration reverses alcohol-induced dysbiosis by increasing the microbial diversity, promoting SCFA-producing bacteria, and elevating the relative abundance of *Lactobacillus*, *Pediococcus*, *Prevotella*, *Clostridium* and *Akkermansia* in mice. this probiotic supplementation can improve intestinal barrier integrity characterized by increase in ZO-1, mucin proteins, and Reg3β peptide (99). It is shown that SCFA luminal contents as well as the activity of SCFA transporters in the proximal colon and liver can be influenced by synbiotic regiment consisting of *Faecalibacterium prausnitzii* and potato starch. This regiment results in attenuation of alcohol-induced hepatic inflammation and oxidative stress and improvement in tight junction protein expression (100). Moreover, this synbiotic alleviates reduced expression of adherens junction proteins in hepatocytes (101). Additional bacteria such as *Lactococcus lactis* (102) and *Bacillus subtilis* (103) have been studied for their therapeutic effects in ALD. Further studies are required in this field for the purpose of finding more effective probiotics against ALD. Moreover, clinical studies should evaluate the efficacy of these probiotics in humans.

Li *et al.* reported in a study of 158 patients, that *Lactobacillus casei* supplementation increases the intestinal amount of *Lactobacillus* and *Bifidobacterium* in ALD patients when compared with the control group indicating that this probiotic can regulate intestinal flora disorders in patients with ALD (104). It is known that endotoxemia leads to neutrophil dysfunction and subsequent increased infection risk and mortality. *Lactobacillus casei Shirota* supplementation 3 times daily for 4 weeks restores neutrophil phagocytic capacity in alcohol-associated cirrhosis patients possibly by changing IL10 secretion and TLR4 expression (105). A reduction in microbial-derived LPS in patients with alcohol-associated hepatitis after 7 days of oral supplementation with cultured *Lactobacillus subtilis/Streptococcus faecium* represents the ability of this probiotic in restoration of

bowel flora in these patients (106). Short-term oral supplementation with *Bifidobacterium bifidum* and *Lactobacillus plantarum 8PA3* restoration of the bowel flora. Additionally, this supplementation is associated with greater improvement in ALD than abstinence plus vitamins treatment (107).

Prebiotics

Prebiotics are natural or synthetic substances utilized by host microbial communities that modulate the intestinal microbiota, thus resulting in beneficial effects on the host. There are numerous prebiotics with potentially beneficial effects in the treatment of ALD. Table 3 summarizes prebiotics possessing anti-ALD potential in animal studies.

Human beta defensin-2 (hBD-2) is a small anti-microbial peptide with protective effects against ALD as determined by decreased plasma ALT activity. This peptide modifies the gut microbiota composition in ethanol-treated mice characterized by reduction in multiple genera including *Barnesiella*, *Parabacteroides*, *Akkermansia*, and *Alistipes.* Two independent cohorts of mice with different baseline gut microbiota investigate the effects of hBD-2 on ALD and revealed that the degree of improvement in liver injury and potential mechanisms are different between these cohorts. T regulatory cell abundance increases in the intestine and mesenteric lymph nodes in Cohort 1 mice, while elevation in hepatic and small intestinal IL-17A and IL-22 levels is observed in Cohort 2 group. The distinction between Cohort 1 and Cohort 2 mice suggests dependency of the beneficial effects of hBD-2 on intestinal microbiota (108).

It has been shown that inhibition of gut microbial choline TMA lyase by small molecule inhibitors such as iodomethylcholine (IMC) and fluoromethylcholine (FMC) protects mice from ALD. IMC and FMC treatment effectively blunt ethanol-induced ALT, TMA and TMAO elevations as well as protection against hepatic steatosis. These two choline TMA lyase inhibitors exert their effects at least partly by reorganization of gut microbiota. IMC reverses the remarkable increase in *Faecalibaculum* and *Escherichica/Shigella*, and decrease in *Bacteroidales_S24-7.* On the other hand, *Turicibacter*, *Oscillibacter*, and *Lachnospiraceae* are the most altered bacteria following FMC treatment (48).

One of the most important groups of prebiotics is soluble fiber such as pectin. Pectin improves ALD by modifying the enterohepatic cycle of bile acids and changing the intestinal microbiota. This fiber alters the overall composition of bile acids towards hydrophilic forms which is less toxic. Moreover, pectin lowers the level of bile acids in the plasma and liver, whereas it increases primary unconjugated bile acid level in the caecum. Gut bacteria harboring genes involved in encoding bile acid-metabolizing enzymes undergo alterations following pectin treatment. In addition to reduced abundance of *Lactobacillus* and *Enterococcus*, pectin treatment leads to an increase in abundance of *Bacteroides* and *Enterobacteriacae*. In response to bile acid alterations in the ileum, FXR signaling becomes inhibited and Cyp7a1 becomes upregulated subsequently. Although the synthesis of bile acids is increased, pectin reduces bile acid serum levels by enhancing their intestinal excretion (109).

It is known that alcohol administration to mice increases the levels of triglyceride, low density lipoprotein, free fatty acid, total cholesterol, as well as serum alanine aminotransferase and serum aspartate aminotransferase. Additionally, it reduces serum high-density lipoprotein. These alterations can be reversed by Ellagic acid supplementation. Ellagic acid is a natural compound mostly found in vegetables, fruits, and nuts. This natural compound improves alcohol-induced gut dysbiosis, promotes alcohol-induced loss of gut tight and adherent junction proteins, and prevents gut leakiness and endotoxemia. Moreover, ellagic acid alleviates oxidative stress, inflammatory response, steatosis, and histopathological features in ALD model of mice. Together, ellagic acid could be a good candidate for the treatment of ALD and further clinical assessments are warranted (110, 111).

Chu et al. indicated that Seladelpar (MBX-8025), a peroxisome proliferator-activated receptor-delta (PPARδ) agonists, improves ALD in mice. Bile acid metabolism disrupts after chronic ethanol intake which is characterized by increased total bile acid pool and serum bile acids. MBX-8025 restores bile acid homeostasis via reducing the total bile acid pool and secondary bile acids as well as increasing intestinal excretion of bile acids. PPAR expression is associated with the production of antibacterial peptides that can change microbiota composition. The reduction in hydrogen–producing bacteria, *Rikenellaceae* can

be reversed by MBX-8025 in ethanol-fed mice. Moreover, MBX-8025 decreases pathogenic family *Coriobacteriaceae* involved in cholesterol absorption. Improved gut barrier function and hepatic lipid metabolism are also associated with MBX-8025 treatment (112).

Polysaccharides are one of the major groups of prebiotics. Polysaccharides from *Crassostrea gigas* (steamed oyster) attenuates ALD in mice by increasing *Lactobacillus reuteri* and *Roseburia spp.* and decreasing Escherichia. This treatment follows an increase in SCFAs such as propionate and butyrate as well as an elevation in the expression of tight-junction proteins (113). Furthermore, polysaccharides from *Wolfporia cocos* ameliorates ALD in mice by modulating gut microbiota in mice. Treatment with these polysaccharides increases the *Firmictues* to *Proteobacteria* ratio, elevates the abundance of *Lachnospiraceae* including *Ruminoclostridum* and *unidentified_clostridials*. In addition, they generate Prostaglandin E2 (PGE2) which prevents the overgrowth of harmful gut fungi especially *Meyerozyma guilliermondii* (114). Data from previous studies evaluating the therapeutic potential of several herbs such as *Curcuma longa* and *Cnidium monnieri* have shown promising results (115).

Numerous studies have shown that altering the intestinal microbiota may be one of the major underlying mechanism by which herbal medicines improve ALD. An animal study by Eom *et al.* has shown hepatoprotective effects of *Dendropanax morbifera* leaf extracts against ALD. These extracts regulate gut microbial composition and metabolic activities characterized by an increase in *Bacteroides* and *Allobaculum* as well as an enhanced generation of beneficial monounsaturated fatty acids such as oleate and palmitoleate (116). A study by Xiang *et al.* revealed that *Schisandra chinensis* extract might be considered an effective preventive and therapeutic prebiotic against ALD. In addition to inhibiting growth of *Escherichia* and *Shigella*, this extract enhances SCFA-producing bacteria such as *Lactobacillus* and *Bifidobacterium* (117).

Antibiotics

Rifaximin is a non-absorbable antibiotic that has been studied for its therapeutic effects on ALD. An animal study by Kitagawa *et al.* reported that rifaximin reversed the alcohol-

induced increase in *Erysipelotrichales*. On the other hand, it increases *Bacteroidales* and prevents the LPS translocation into the portal vein. Interestingly, rifaximin involves in microbiota-related innate immune response characterized by regulating hepatic TLR2 and TLR4 mRNA levels (118). Rifaximin in combination with zinc acetate counteracts ALD-related fibrosis by maintaining intestinal integrity. It prevents the activation of Kupffer cells with the restoration of tight junction proteins and decreases the interaction of TLR4 and LPS (119).

Previously, some clinical trials confirmed the beneficial effects of rifaximin in the treatment of ALD patients. A study on 23 patients with alcohol-related decompensated cirrhosis and 46 control participants revealed that long-term administration reduces the complications of portal hypertension such as variceal bleeding, hepatic encephalopathy, spontaneous bacterial peritonitis, and hepatorenal syndrome (120). A clinical trial done by Kimer *et al.* on 32 patients with alcohol-associated hepatitis showed no significant difference in inflammation or metabolism between standard medical therapy and SMT plus rifaximin groups (121). Jiménez *et al.* conducted a trial evaluating the addition of rifaximin (1200 mg/day/90 days) to the standard treatment in alcohol-associated hepatitis patients. They enrolled 21 patients as rifaximin group and 42 patients as control group. Results from this study revealed that rifaximin was safe in severe alcohol-associated hepatitis. Furthermore, infections and acute-on-chronic liver failure were lower in rifaximin group. Collectively, mortality was lower in the rifaximin groups versus the control group (14.2% vs. 30.9) (122). However, larger clinical studies are required to confirm the efficacy of rifaximin for the treatment of ALD particularly alcohol-associated hepatitis.

Fecal microbial transplantation (FMT)

liver inflammation and necrosis as well as intestinal permeability are increased in germ-free alcohol-treated mice that received FMT from SAH patients in comparison with those received FMT from non-SAH patients. Interestingly, second FMT from non-SAH patients can improve liver injury in mice who had previously received FMT from SAH patients

(123). Additionally, FMT from the alcohol-resistant mice to the alcohol-sensitive mice can protect the mice from alcohol injury (124).

In the past few years, numerous human studies have demonstrated that FMT is a safe and effective treatment for ALD (125). Phase 1 trial on 20 patients with AUD-related cirrhosis indicated that FMT is safe and exerts favorable microbial changes compared to placebo group. FMT enema from a donor enriched in *Lachnospiraceae* and *Ruminococcaceae* increases microbial diversity and SCFA-producing bacteria. Moreover, FMT also reduces AUD-related events over 6 months (126). Evaluating the efficacy of FMT in the treatment of severe alcohol-associated hepatitis (SAH) patients revealed that FMT is safe and improves short-term and medium-term survival in these patients (127). Numerous ongoing clinical trials are evaluating the efficacy of FMT for the treatment of patients with severe alcohol-associated hepatitis. A single center, randomized, and double-blind clinical trial (NCT05006430) is established in 2021 in Baylor College of Medicine, Houston, Texas, United States to assess the safety of lyophilized capsules containing microbiota suspension from health donors and evaluate survival in patients with severe alcohol-associated hepatitis receiving these capsules (n=25) comparing with standard care (n=25). Moreover, another clinical trial (NCT05285592) started in 2022 with the estimated enrollment of 84 participants assesses 3 month-mortality and liver transplant free survival in patients with alcohol-associated hepatitis receiving FMT in comparison to standard medical treatment group. A phase 3 single group clinical trial (NCT04758806) evaluates the efficacy of FMT in the treatment of severe alcohol-associated hepatitis (n=50) with the primary outcome of 28-day, 90-day, and 1-year overall mortality.

Bacteriophages

Conventional microbiota-based strategies cannot target a specific group of bacteria selectively. The selectivity of bacteriophages for specific bacteria without any tropism for human cells is an important advantage of bacteriophages. In the last few years, phage-mediated precise modulation of microbiota has evolved as an interesting new research field (128). The importance of phages in the treatment of ALD has been revealed recently.

As we previously mentioned, cytolysin-positive *E. faecalis* is closely associated with the prognosis of patients with alcohol-associated hepatitis. Liver injury and mortality rate increase following the transplantation of fecal microbiota containing cytolytic *E. faecalis* from alcohol-associated hepatitis patients to germ-free mice that were subjected to the chronic–binge feeding model. Cytolytic *E. faecalis*-specific bacteriophages are able to treat these transplanted mice which significantly reduce the hepatic levels of cytolysin and attenuates ALD (79). However, the application of phage therapy in clinical practice still requires more preclinical and human researches.

NLRP3 inflammasome-based treatment

Inhibiting the activity of the NLRP3 inflammasome may be a promising therapeutic strategy due to its crucial role in ALD and liver fibrosis. It has been identified that ursolic acid can reverse liver fibrosis by inhibiting NOX4/NLRP3 inflammasome pathway which is associated with intestinal bacterial dysbiosis (129). A study by Choudhury et al. showed that inhibition of HSP90 by HSP90 inhibitor, 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) inhibited the activity of NLRP3/CASP-1 pathway and reduces IL-1β and IL-18 leading to the improvement of ALD (130).

Conclusions

Based on recent exciting developments in the field of human gut microbiota, microbiota-based therapies will become an important component of future liver disease treatment. Experimental research convincingly established the close relationship between gut microbiota and the development of liver injury in alcohol consumers. Human gut microbiota is implicated in many underlying mechanisms of alcohol-associated liver injury. In addition to the bacterial microbiome, non-bacterial microbiota exerts its effects by interacting with the host and also with bacterial microbiota. Although human gut bacterial and non-bacterial microbiota signature has emerged as a diagnostic and prognostic biomarker for alcohol-associated liver injury, microbiota-related biomarker discovery is a

novel and promising field of interest to scientists. Given the central role of intestinal microbiota in the pathogenesis of alcohol-associated liver injury, many studies focused on the therapeutic efficacy of gut microbiota targeting for better management of these patients. However, further clinical trials are required to translate these findings into clinical practice.

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Table 1. Potential Prognostic Biomarkers of Alcohol-associated Liver Disease

Biomarker	Prognosis	Ref.
cytolysin-positive Enterococcus faecalis	Associated with increased mortality of alcohol-associated hepatitis patients	(79)
Haemophilus	Higher levels related to disease severity	(76)
Veillonella	Increased in more severe disease	(78)
Akkermansia	Decreased in more severe disease	(78)
Actinomycetaceae	Represent severity in alcohol-associated hepatitis patients	(80)
Coriobacteriaceae	Represent severity in alcohol-associated hepatitis patients	(80)
Atopobium	Represent severity in alcohol associated hepatitis patients	(80)
Fusobacteriaceae	Represent severity in alcohol-associated hepatitis patients	(80)
Saccharibacteria incertaesedis	Represent severity in alcohol-associated hepatitis patients	(80)
Veillonellaceae	Represent severity in alcohol-associated hepatitis patients	(80)
Closteridiales	Predict progression to alcohol-associated hepatitis in heavy drinkers	(80)
Lachnospiraceae	Predict progression to alcohol-associated hepatitis in heavy drinkers	(80)
Ruminococcaeae	Predict progression to alcohol-associated hepatitis in heavy drinkers	(80)
Streptococcus	Predicts severity and progression to cirrhosis in alcohol-associated liver disease patients	(83)
taurocholic acid	Predicts alcohol-associated liver disease progression	(38, 39)
taurochenodeoxycholic acid	Predicts alcohol-associated liver disease progression	(38, 39)
glycocholic acid	Predicts alcohol-associated liver disease progression	(38, 39)
glycochenodeoxycholic acid	Predicts alcohol-associated liver disease progression	(38, 39)
extracellular vesicles carrying sphingolipid cargo	predict survival in alcohol-associated hepatitis patients	(84)
Staphylococcus phages	associated with the severity of alcohol-associated hepatitis predict mortality	(21)
Herpesviridae	associated with the severity of alcohol-associated hepatitis predict mortality	(21)
Enterobacteria phages	predict progressive alcohol-associated liver disease	(22).

Lactococcus phages	predict progressive alcohol-associated liver disease	(22).
Candidalysin	predict the severity of alcohol-associated hepatitis, positively associated with the mortality	(27)
anti-Saccharomyces cerevisiae antibodies (ASCA)	Positively correlated with disease mortality	(25)

Table 2. Modulation of the Gut Microbiota using Probiotics for the Treatment of Alcohol-associated Liver Disease

Agent	Mechanism	Ref.
Roseburia spp	↑ gut barrier integrity through TLR5, ↑ tight junction protein Occludin, restoring the gut microbiota through ↑ IL-22 and REG3γ expression	(96)
Bifidobacterium animalis ssp. lactis Probio-M8 strain; M8	Restoring the gut microbiota diversity, richness and composition, ↓ liver inflammation and oxidative stress	(97)
Komagataeibacter hansenii	↑SCFAs, ↓ *Actinobacteria*, *Proteobacteria* and *Firmicutes*, ↑ *Bacteroidetes*	(98)
Lactococcus lactis	↑ diversification of the *Enterobacteriaceae*, modulating immunological changes after alcohol intake	(102)
Pediococcus pentosaceus	↑SCFAs, ↑ microbial diversity restoring *Lactobacillus*, *Pediococcus*, *Prevotella*, *Clostridium* and *Akkermansia*, ↑ tight junction protein ZO-1, mucin proteins (MUC-1, MUC-2 and MUC-4), ↑ Reg3β	(99)
Bacillus subtilis	Improving the intestinal barrier, restoring gut microbiota homeostasis, ↓ endotoxemia and hepatic inflammation via the TLR4 pathway	(103)
Akkermansia muciniphila	↓ gut leakiness, ↑ mucus thickness and tight-junction expression, ↓ hepatic injury and neutrophil infiltration.	(131)
Lactobacillus plantarum and Lactobacillus acidophilus	↑SCFA-producing bacteria, ↓ Gram-negative bacteria, improving intestinal permeability, ↓ serum LPS, ↓ liver lipid accumulation, oxidative stress, and inflammation by regulating TLR4/NF-κB pathway	(132)
Lactobacillus rhamnosus and acidophilus	↓ TLR4 expression	(91)
Lactobacillus rhamnosus	↓ endotoxemia, ↑ intestinal integrity by inhibition of miR122a, ↑ occludin, ↓ HIF-2α, ↓ TNF-α, ↓ free fatty acid production in liver, ↑ *Lactobacillus* and *Bifidobacterium*, ↓ *Clostridium perfringens* in ileum, ↓ Gram-negative bacteria *Proteobacteria*, *Campylobacterales*, and *Helicobacter* in cecum, ↓ hepatic apoptosis	(88-90, 133-135)
Lactobacillus plantarum	Improving the intestinal barrier, ↑ tight junction protein ZO-1, ↓ endotoxemia, ↓ oxidative stress, and inflammation by an EGF receptor-dependent mechanism	(94, 95)
Lactobacillus rhamnosus and inosine	Improving the gut ecosystem and intestinal barrier, immune homeostasis and liver injury, recovery of intestinal villi and tight junction proteins, ↓ hepatic inflammation, ↓ Tregs population, ↑ Th1 population	(92)
Lactobacillus rhamnosus 217-1 and Pueraria lobata, Lonicera japonica, and Crataegus pinnatifida	↓ endotoxemia, ↑ intestinal integrity, ↓ oxidative stress and inflammation	(93)
Faecalibacterium prausnitzii and potato starch	Modulating gut dysbiosis, improving hepatocyte and liver endothelial barrier integrity, ↓ steatosis and hepatocyte injury, influence luminal SCFA and SCFA transporters expression in the proximal colon and liver	(100, 101)
Akkermansia muciniphila	Restoring gut vascular barrier	(136)

Table 3. Modulating Gut Microbiota by Prebiotics for Treatment of Alcohol-associated Liver Disease in Preclinical Step

Agent	Mechanism	Ref.
Inulin	↓ LPS-TLR4-Mψ axis, ↓ inflammation via SCFAs-inducing suppression of M1 and facilitation of M2 Mψ, ↑ *Allobaculum*, *Lactobacillus*, and *Lactococcus*, ↓ *Parasutterella*	(137, 138)
Butyrate	↓ gasdermin D-mediated pyroptosis, ↑ intestinal barrier function and ↓ gut leakage, ↓ endotoxemia	(28, 139)
Ursolic acid	↓ barrier dysfunction and gut leakiness, ↓ endotoxemia-mediated liver TLR-4 pathway induction, ↓ intestinal oxidative stress	(140)
Human beta defensin-2 (hBD-2)	↓ *Barnesiella*, *Parabacteroides*, *Akkermansia*, and *Alistipes*, immunomodulation	(108)
Antrodin A	↑ *Lactobacillus* and *Dubosiella*, ↓ *Clostridium*, *Lachnospiraceae*, *Prevotellaceae*, and *Prevotellaceae*, regulating glutathione, ascorbate, aldarate, taurine and hypotaurine metabolism, ↓ TNF-α and TLR-4,	(141, 142)
Lactoferrin	↑ *Akkermansia* and *Lactobacillus*, ↓ inflammation	(143)
Phosphoesterase complex	Modulating microflora and gut barrier, ↑ mucus layer thickness, ↓ inflammation	(144)
Iodomethylcholine (IMC) and fluoromethylcholine (FMC)	Inhibition of bacterial choline TMA lyase (CutC/D), modulating gut microbiota	(48)
Nicotinamide riboside	regulating lipid metabolism and the gut microflora-bile acid axis	(41)
Pectin	Modifying the overall BA composition, ↓ FXR signaling in the ileum, ↑ BA synthesis, ↓ BA serum levels by ↑ BA intestinal excretion	(109)
Astaxanthin	↓ *Bacteroidetes* and *Proteobacteria* and the genera *Butyricimonas*, *Bilophila*, and *Parabacteroides*, ↑ *Verrucomicrobia* and *Akkermansia*	(145)
Ganoderic acids	↑ *Ruminiclostridium*, *Prevotellaceae*, *Oscillibacter*, *Bilophila*, *Ruminococcaceae*, *Desulfovibrionaceae* and *Hydrogenoanaerobacterium*, ↓ *Clostridium*, modulating bile acid, riboflavin, tryptophan, and unsaturated fatty acids metabolism	(146)
Stearic acid	regulating the gut microbiota, improving the intestinal barrier, ↑ *Akkermansia muciniphila* and *Lactobacillus*, ↓ oxidative stress damage	(147)
Ellagic Acid	↓ oxidative stress, inflammatory response, steatosis, modulating the gut microbiota dysbiosis, ↓ *Actinobacteria* and *Verrucomicrobia*, ↓ gut barrier dysfunction, ↓ endotoxemia, ↓ tight junction, ↑ gut leakiness	(110, 111)
Rifaximin	↓ *Erysipelotrichales*, ↑ *Bacteroidales*, ↓ portal LPS, ↓ hepatic TLR4	(118)
Rhubarb	↑ *Akkermansia muciniphila* and *Parabacteroides goldsteinii*, ↑crypt depth, tissue weight, and the expression of antimicrobial peptides	(148)
Fucoidan	↑ ileac FXR, ↑ FGF15, ↓ CYP7A1 expression and total bile acid levels in the liver, ↑ *Prevotella*, ↓ *Paraprevotella* and *Romboutsia*	(149)
Allicin	↓ LPS-CD14-TLR4-induced hepatic inflammation pathway by ↓ LPS, CD14, TLR4, TNF-α, IL-1β, and IL-6.	(150)
Seladelpar (MBX-8025)	PPARδ agonist, ↓ serum total and secondary bile acids, ↓ total bile acid pool, ↓ *Coriobacteriaceae* and *Enterococcaceae*, ↑ *Rikenellaceae*	(112)
Kaempferol	↑ ZO-1 and occludin, butyrate receptors, and butyrate transporters in the ileum and proximal colon	(151)
Linderae radix	↑ *Firmicutes*, ↓*Bacteroidetes*, ↓TLR4, ↑ occludin and claudin-1,	(152)

	modulating LPS-TLR4-NF-κB pathway	
Dendropanax morbifera Leaf Extracts	↑ *Bacteroides* and *Allobaculum*, ↑ beneficial monounsaturated fatty acids such as oleate and palmitoleate, ↑ antioxidant enzymes activity	(116)
Schisandra chinensis Extract	↑ liver inflammation and oxidative/nitrosative stress, ↑ intestinal barrier function, ↑ SCFAs, ↑ *Lactobacillus* and *Bifidobacterium*.	(117)
Hippophae rhamnoides L.	↑*Firmicutes/Bacteroidetes* ratio, ↓ gram-negative *bacteroidetes*, ↓ *Akkermansia*, *Turicibacter*, *Alistipes* and *Ruminiclostridium*, ↑ *Lactobacillus*	(153)
Pleurotus geesteranus polysaccharides	↓ oxidative stress by ↑ Nrf2/HO-1 pathways, ↓ pro-inflammatory factors by ↓ TLR4/NF-κB pathways, improving the intestinal barrier, ↑ intestinal tight-junction protein and mucin expression ↑SCFAs producers	(154)
Korean Red Ginseng (Panax ginseng), urushiol (Rhus vernicifera Stokes)	↓TLR-4, Interleukin-1β, TNF-α level	(155)
fermented ginseng	↑ *Lactobacilli* and *Bifidobacteria*, ↓ *Bacteroidetes* phylum and the *Proteobacteria* genus of the *Sutterella* phylum, ↑ SCFA-producing bacteria such as *Akkermansia*, *Allobaculum*, *Ruminococcus*, *Adlercreutzia*	(156)
Vinegar extract	increasing the expression levels of ZO-1, occludin, claudin-1, Reg3b, and Reg3g, ↑ *Bacteroidetes*, *Verrucomicrobia*, *Akkermansia*, and *Lactobacillus*	(157, 158)
Auricularia auricula Melanin	↑ *Akkermansia*, *Bifidobacterium*, *Ronboutsia*, *Muribaculaceae*, *Lachnospiraceae*, regulatory effect on biosynthesis of unsaturated fatty acids	(159)
polysaccharides from Crassostrea gigas or polysaccharides from steamed oyster	↑ tight-junction proteins and ↓ inflammatory responses, ↑ *Lactobacillus reuteri* and *Roseburia spp.* and ↓ *Escherichia*, ↑ propionate and butyrate	(113)
polysaccharides (WIP) from Wolfporia cocos	↑ *Firmictues* to *Proteobacteria*, ↑ *Lachnospiraceae* including *Ruminoclostridium* and *clostridials*, ↑PPAR-γ signaling, ↓ inflammation in the colonic epithelia cell	(114)
fermented rice liquor	↑ SCFAs, restoring microbial composition, ↓ intestinal inflammation	(160)
Rice Bran Phenolic Extract	Improving intestinal microbiota dysbiosis and barrier dysfunction, ↓ LPS-TLR4-NF-κB pathway	(161)
okra seed oil	↓ *Proteobacteria*, ↑ *Bacteroidetes*, ↓ *Clostridium* and *Staphylococcus*, ↓ hepatic TNF-α, IL-1 and IL-6	(162)
Decaisnea insignis seed oil	↑ *Lactobacillus*, *Ruminoccoceae*, ↓ *Parabacteroides*, improving the intestinal permeability and tryptophan metabolism	(163)
Flaxseed oil	↓ *Proteobacteria* and *Porphyromonadaceae*, ↓ TNF-α	(164)

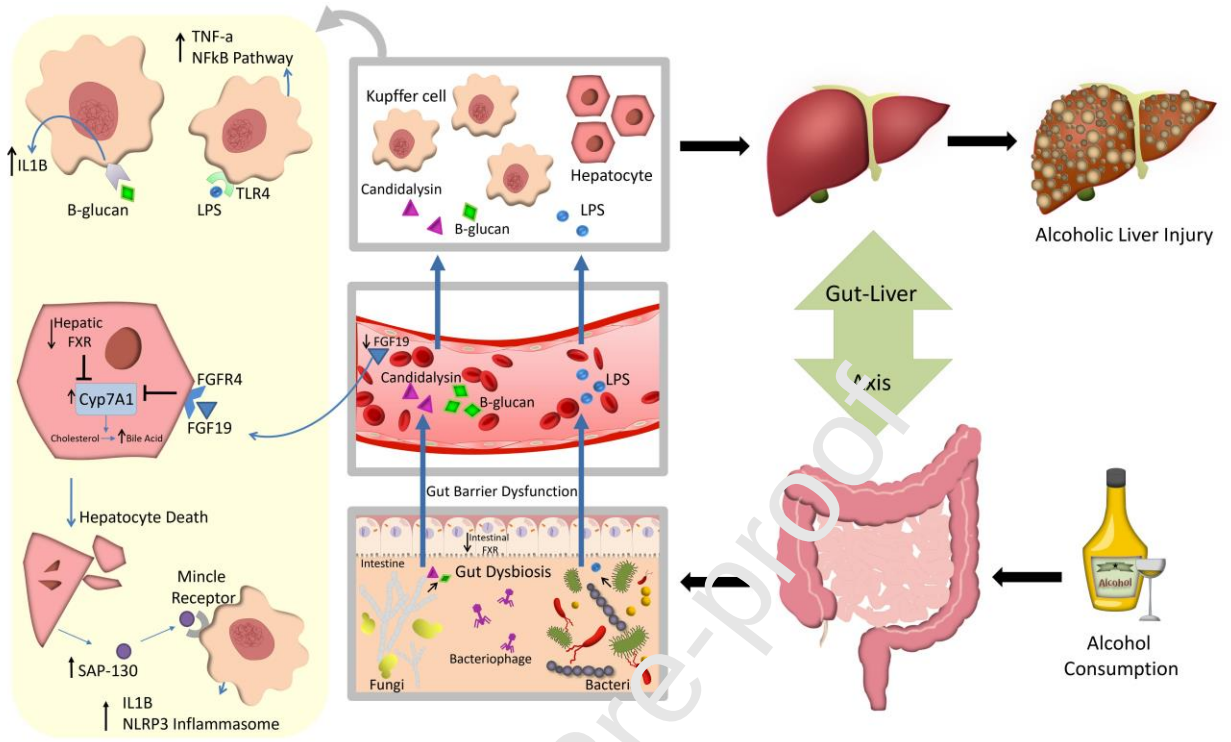

Figure 1: Potential pathogenic mechanisms behind the relationship between gut microbiota, alcohol, and liver injury. The gut-liver axis is implicated in the development and progression of ALD. Alcohol consumption alters gut microbial features and disrupts the intestinal epithelial integrity leading to elevated serum levels of LPS. Bacterial-derived LPS travels through the liver and interacts with Toll-like receptor 4 (TLR4) on the surface of Kupffer cells. Subsequent release of Tumor necrosis factor-alpha (TNF-α) by Kupffer cells causes inflammation and liver fibrosis. FXR reduces bile acid synthesis by modulating the activity of CYP7A1 which is the rate-limiting enzyme in bile acid synthesis. Alcohol consumption increases bile acid production in the liver by suppressing intestinal and hepatic FXR. increased translocation of bacteria and bacteria-derived particles such as LPS into the liver through the portal vein activates NLRP3 inflammasome which contributes to liver injury. Intestinal mycobiome plays their role in the pathogenesis of ALD by producing beta-glucan and candidalysin. Emerging evidence revealed that intestinal virome especially bacteriophages takes part in ALD pathogenesis.

Credit

Majid Ghayour-Mobarhan, Gordon A Ferns, Mohammad Ali Kiani and Amir Avan conceived of the presented idea. Ghazaleh Pourali, Majid Khazaei, Mohammadreza Nassiri and Seyed Mahdi Hassanian developed the theory and performed the data collection and analysis. Nima Zafari, Mahla Velayati, Mostafa Fahim and Mina Maftooh[1] provided the initial draft of the manuscript. All authors commented on previous versions of the manuscript, discussed the results and contributed to the final manuscript.

Graphical abstract