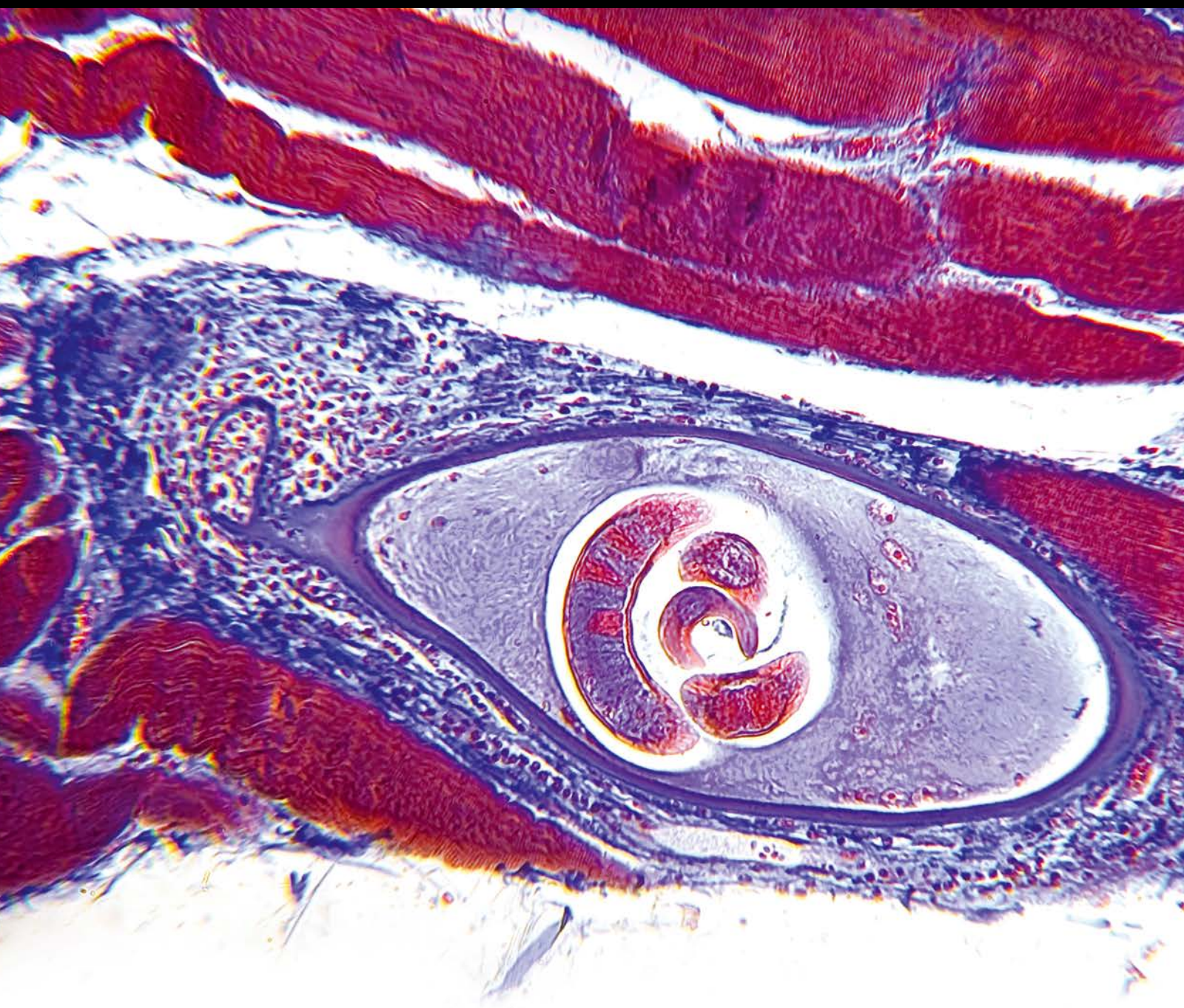
Gastroenterology Research and Practice

Gut Microbiota and Metagenomic Advancement in Digestive Disease

Guest Editors: Jinsheng Yu, Sharon Marsh, Junbo Hu, Wenke Feng, and Chaodong Wu



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Editorial

Gut Microbiota and Metagenomic Advancement in Digestive Disease

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Recent studies have made significant advances in understanding the mechanisms of gut microbiota involved in human health and disease [1, 2]. Now the gut microbiota has been recognized as a key player in a broad spectrum of human diseases from obesity associated liver and cardiovascular diseases to mental development and psychiatric diseases [3, 4]. Accordingly, modulations of gut microbial diversity and composition are expected to improve human health and to provide novel therapeutic modalities for human disease. The gut microbial modulators can be simply specific diets and drinks, natural tea and Chinese herbs, or specialized prebiotics and probiotics.

In this special issue, authors presented a number of very interesting studies on changes of gut microbiota in digestive diseases. These review and original articles of research and clinical studies cover a range of topics, including the pathogenesis of alcoholic and nonalcoholic fatty liver diseases (NAFLD), the outcome of intestinal bacterial translocation in advanced cirrhosis, the gut microbiota changes in an animal colitis model after treatment with a monoclonal antibody, and fecal microbiota transplantation (FMT) in elderly patients with refractory *Clostridium difficile* infection. In these articles, authors have described mechanisms of disease development as well as therapeutic effects of specific antibodies, probiotics, and FMT.

While we know the simple cause of alcoholic fatty liver disease (AFLD), its pathogenesis is complicated and it involves a wide range of changes in host metabolism and gut microbiota. Certainly, removal of the cause (alcohol abuse) is the first treatment for AFLD, and the use of probiotics can ameliorate its development as shown in recent clinical and animal studies [5–7]. Although sharing many pathological and clinical features with AFLD, NAFLD does not have a simple, single cause. Instead, NAFLD is the result of interactions between gut microbiota, host genetics, and diet. There have been a number of studies evaluating the effects and mechanisms of probiotics [8–10], natural tea [11, 12], and Chinese herbs/recipes [13, 14] on AFLD and NAFLD. These studies reported beneficial effects with promising perspectives for future development of novel therapeutic strategies.

However, in light of the nature of gut microbiota being highly diverse and constitutional, built during the early life of individuals, cautious optimism is necessary for any future therapeutic developments aiming at modulations of gut microbiota, as we do not yet know long-term effects from those initial efforts or indeed which of those microbiota and metabolomics changes are contributory, causative, or simply a cofounder in AFLD and NAFLD, or indeed any other condition. Thus far, it is not clear whether the modulations by specific diets and drinks including natural tea, even by probiotics, are able to change the constitutional nature of gut microbiota, although the relative abundance of microbial species is altered upon administrating modulators [12, 13, 15].

Moreover, current analytic strategies on metagenomics data are focused on major changes in gut microbial compositions and have not paid sufficient attention to or have even ignored the changes in minor species with less than 1% abundance. Furthermore, sampling of microbiota in intestinal lumen may not necessarily represent the mucosal portion or the whole population of gut flora [16]. In fact, the true signal and messengers governing the alterations of gut microbiota and host metabolism are largely elusive. Enteric short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate, are fermentation metabolites by intestinal bacteria. Recent studies have suggested a messenger role of SCFAs in several human diseases including metabolic syndrome [17] and autism [4]. In short, careful designs of future studies should take these factors and host genetic background under consideration to discover the real effectors and to discern the true effects of gut microbial modulators in alcoholic and nonalcoholic fatty liver disease and other human diseases.

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Review Article

The Pathogenesis of Nonalcoholic Fatty Liver Disease: Interplay between Diet, Gut Microbiota, and Genetic Background

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Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in the world, and it comprises a spectrum of hepatic abnormalities from simple hepatic steatosis to steatohepatitis, fibrosis, cirrhosis, and liver cancer. While the pathogenesis of NAFLD remains incompletely understood, a multihit model has been proposed that accommodates causal factors from a variety of sources, including intestinal and adipose proinflammatory stimuli acting on the liver simultaneously. Prior cellular and molecular studies of patient and animal models have characterized several common pathogenic mechanisms of NAFLD, including proinflammation cytokines, lipotoxicity, oxidative stress, and endoplasmic reticulum stress. In recent years, gut microbiota has gained much attention, and dysbiosis is recognized as a crucial factor in NAFLD. Moreover, several genetic variants have been identified through genome-wide association studies, particularly rs738409 (Ile748Met) in *PNPLA3* and rs58542926 (Glu167Lys) in *TM6SF2*, which are critical risk alleles of the disease. Although a high-fat diet and inactive lifestyles are typical risk factors for NAFLD, the interplay between diet, gut microbiota, and genetic background is believed to be more important in the development and progression of NAFLD. This review summarizes the common pathogenic mechanisms, the gut microbiota relevant mechanisms, and the major genetic variants leading to NAFLD and its progression.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in the world. It is present in 30% of the general adult population and found predominantly in obese people with high-fat diets and inactive lifestyles. In reality, NAFLD comprises a spectrum of hepatic abnormalities that are observable in liver histological slides, from a simple intrahepatic accumulation of fat (steatosis or nonalcoholic fatty liver, NAFL) to various degrees of necrotic inflammation (nonalcoholic steatohepatitis, NASH) [1–3]. Simple steatosis (i.e., NAFL) rarely progresses to advanced disease whereas, in approximately 20% of patients with NASH, it progresses to fibrosis and cirrhosis and potentially to hepatocellular carcinoma over a 15-year time period [4, 5]. The majority of patients with NAFLD are obese or even morbidly obese and have accompanying insulin resistance that plays a central role in the metabolic syndrome [6–9]. Thus, NAFLD is also deemed to be hepatic manifestation of metabolic syndrome which is a cluster of complex conditions including central obesity, hypertension, hyperglycaemia, hypertriglyceridemia, and low HDL (high density lipoprotein) that are predictive risk factors of cardiovascular disease, stroke, and diabetes [10, 11].

NAFLD has been considered a condition with a "two-hit" process of pathogenesis since 1998 when Day and James first proposed this hypothesis [12] with evidence from the Berson et al. study describing the role of lipid peroxidation in the liver injury [13]. Essentially, the first hit is the development of hepatic steatosis via accumulation of triglycerides in

hepatocytes, which increases the vulnerability of the liver to various possible "second hits" that in turn lead to the inflammation, fibrosis, and cellular death characteristics of NASH. The second hit can be a variety of factors, such as oxidative stress, endoplasmic reticulum stress, proinflammatory cytokines, and gut-derived bacterial endotoxin. As it has evidently emerged that (1) accumulation of triglycerides in hepatocytes may be a protective mechanism from liver damage and (2) hepatic inflammation can precede the simple hepatic steatosis and can also be a cause of steatosis, it has been believed that many "hit" factors may act simultaneously leading to the development of NAFLD, which supports the multihit model proposed in 2010 [14]. Indeed, among the proposed hit factors, many can interact with each other, forming a vicious circle. Recent advances in metagenomics complicate the understanding of the pathogenesis of NAFLD further in that dysbiosis and host-microbiota interactions are now also implicated. Moreover, genome-wide association studies have discovered several promising candidate genes, serving as the genetic background for the disease. These genetic players appear to distinguish subgroups of NFLD patients from obese and insulin resistance associated populations. Although a high-fat diet and inactive lifestyles are typical risk factors for NAFLD, the interplay between diet, gut microbiota, and genetic background can play a crucial role in the development and progression of NAFLD. This review summarizes the common pathogenic mechanisms, the gut microbiota relevant mechanisms, and the major genetic variants leading to NAFLD and its progression (Figure 1).

2. Common Pathogenic Mechanisms of NAFLD

Hepatic steatosis is a prerequisite to making a histological diagnosis of NAFLD [2]. Several mechanisms may lead to steatosis, including (1) increased fat supply such as high-fat diet and excess adipose lipolysis; (2) decreased fat export in the form of very low density lipoprotein-triglyceride; (3) decreased free fatty β-oxidation; and (4) increased *de novo* lipogenesis (DNL) [2]. Molecular mechanisms responsible for the accumulation of fat in the liver are not fully understood; however, certain cytokines derived from inflammation sites, particularly from extrahepatic adipose tissues, can trigger this process. In addition, the enhancement of hepatic DNL is deemed to be a unique feature in steatosis. More importantly, insulin resistance appears to be at center stage for the massive metabolic dysregulations of NAFLD that initiate and aggravate hepatic steatosis. At a certain point, the simple steatosis transforms to steatohepatitis in about 20–30% of NAFLD patients. This breakthrough-like process is mediated by the interplay of multiple hit factors. Pathological features of NASH include simple hepatic steatosis and, more characteristically, liver cell damage and accompanying inflammation and/or fibrosis. Currently, a number of common pathogenic mechanisms have been proposed and characterized for the transition from simple steatosis to NASH, such as lipotoxicity, oxidative stress, mitochondrial dysfunction, and endoplasmic reticulum stress.

2.1. Adipose Tissue Inflammation. What exactly initiates adipose tissue inflammation in obesity is uncertain; but hypoxia and death of rapidly expanding adipocytes are believed to play a role [15]. Adipocytes under inflammation secrete cytokines and chemokines, particularly tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and CC-chemokine ligand-2 (CCL2) [15, 16]. TNF-α was the first proinflammatory cytokine detected in adipose tissue and is involved in the regulation of insulin resistance. Studies indicated that neutralization of TNF-α activity by an anti-TNF-α monoclonal antibody improves insulin resistance and fatty liver disease in animals [17]. IL-6 is derived from many cells throughout the body including adipocytes. Serum levels of these cytokines correlate remarkably well with the presence of insulin resistance, and adipose tissue-derived TNF-α and IL-6 have been shown to regulate hepatic insulin resistance via upregulation of SOCS3, a suppressor of cytokine signaling [17]. CCL2 recruits macrophages to the adipose tissue, resulting in even more local cytokine production and perpetuating the inflammatory cycle; TNF-α and IL-6 induce a state of insulin resistance in adipocytes, which stimulates triglyceride lipolysis and fatty acid release into the circulation. At the same time, extrahepatic adipocytes are compromised in their natural ability to secrete adiponectin, an anti-inflammatory adipokine that facilitates the normal partitioning of lipid to adipocytes for storage [18]. Circulating adiponectin regulates hepatic fatty β-oxidation through AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC) signaling [19]. Together, these abnormalities accentuate fat loss from adipocytes and promote ectopic fat accumulation.

2.2. De Novo Lipogenesis (DNL). Presumably lipogenesis in liver could be increased due to the steatotic nature of NAFLD. A number of prior studies have shown that diets enriched in both saturated fat and simple sugar carry a high risk of hepatic steatosis, at least in part, through enhanced DNL [20–23]. The role of DNL in the development of hepatic steatosis is further supported by a recent study in subjects with metabolic syndrome and a high content of liver fat [24]. A 3-fold higher rate of *de novo* fatty acid synthesis is seen in these subjects. In addition, specific dietary compositions may have different effects. Basically since carbohydrates are substrates for DNL, the amount of carbohydrate in the diet will positively influence the amount of DNL in the liver. Simple sugars are converted to fatty acids more readily than complex carbohydrates [25, 26], and fructose is a more potent inducer of DNL than glucose [27, 28]. This is also supported by epidemiologic evidence linking dietary fructose to hepatic steatosis and NASH [20, 29]. It is worth noting that dietary fat, particularly saturated fat, stimulates DNL by upregulating SREBP-1 (sterol responsive element binding protein), a key regulator of the lipogenic genes in the liver [30]. However, not all individuals with hepatic steatosis had increased DNL nor upregulated SREBP-1 expression, as observed in the Mancina et al. study showing a paradoxical dissociation between hepatic DNL and hepatic fat content due to the PNPLA3 148M allele [31].

2.3. Insulin Resistance. Studies have highlighted the fact that insulin resistance is a characteristic feature of NAFLD [7–9]

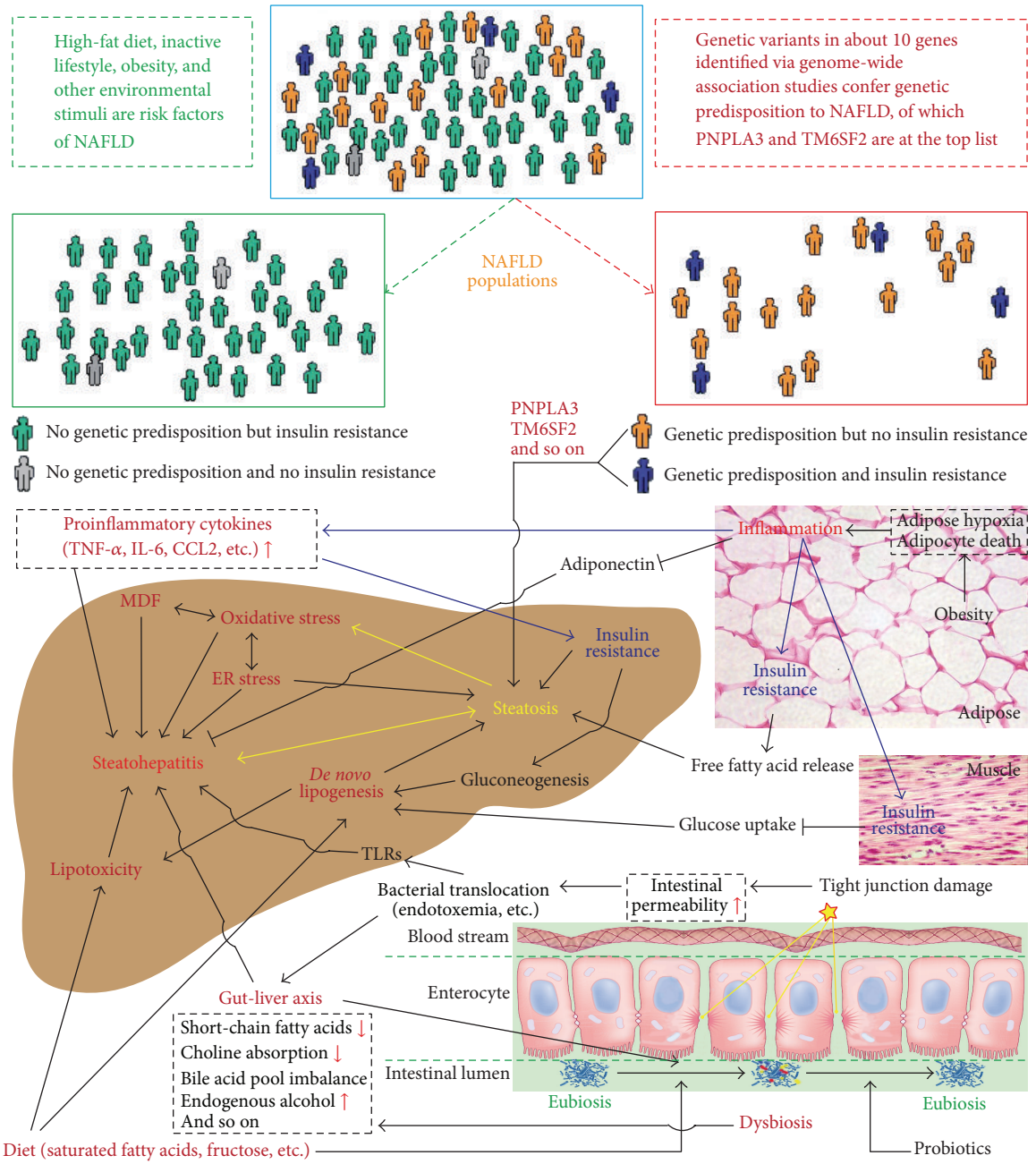

Figure 1: Overview at the pathogenesis of nonalcoholic fatty liver disease (NAFLD). The interplay between diet, microbiota, and host genetic variants plays a crucial role in the complex pathogenesis of NAFLD through a variety of mechanisms. The NAFLD patients can now be categorized into different populations based on their insulin sensitivity and genetic predisposition. Insulin resistance is at the center of the NAFLD pathogenic process, and a number of key factors are involved in the development of NAFLD, such as diet, dysbiosis, gut-liver axis, genetic predisposition genes (PNPLA3 and TM6SF2), oxidative stress, MDF (mitochondrial dysfunction), endoplasmic reticulum (ER) stress, *de novo* lipogenesis, lipotoxicity, and proinflammatory cytokines.

and is caused by a variety of factors, including soluble mediators derived from immune cells and/or adipose tissue, such as TNF-α and IL-6 [32]. Serine phosphorylation of insulin receptor substrates by inflammatory signal transducers such as c-jun N-terminal protein kinase 1 (JNK1) or inhibitor of nuclear factor-κB kinase-β (IKK-β) is considered one of the key aspects that disrupts insulin signaling [14]. On the other hand, insulin resistant subjects with NAFLD show reduced insulin sensitivity, not only at the level of the muscle, but also at the level of the liver and adipose tissue [7–9, 33], which can lead to a far more complex metabolic disturbance of lipid and glucose. However, not all people with NAFLD have increased insulin resistance, and NAFLD, *per se*, cannot be considered a cause for insulin resistance but rather a consequence

as shown by studies in subjects genetically predisposed to NAFLD. Mutations in *PNPLA3* (patatin-like phospholipase domain containing 3) [34, 35], *TM6SF2* (transmembrane 6 superfamily member 2) [6, 36], *DGAT1* (diacylglycerol O-acyltransferase 1) [37], or hypobetalipoproteinemia [38, 39] genes are not related to increased insulin resistance except for severely obese individuals in which it is associated [40]. It is worth noting that insulin resistance is characterized not only by increased circulating insulin levels but also by increased hepatic gluconeogenesis, impaired glucose uptake by muscle, and increased release of free fatty acids and inflammatory cytokines from peripheral adipose tissues [41], which are the key factors promoting accumulation of liver fat and progression of hepatic steatosis (Figure 1).

2.4. Lipotoxicity. Studies have indicated that certain lipids can be harmful to hepatocytes in NAFLD. This is particularly true of the long-chain saturated fatty acids (SFAs) such as palmitate (C16:0) and stearate (C18:0), which are abundant in animal fat and dairy products and produced in the liver from dietary sugar. Under physiological conditions, SFAs are transported to mitochondria for β-oxidation or esterified for either excretion in the form of VLDL (very low density lipoproteins) or storage as lipid droplets. In the pathophysiology of NASH, multiple mechanisms are concurrently operative to produce liver injury in hepatocytes overwhelmed by SFA and by free cholesterol (FC) from *de novo* synthesis [42, 43]. FC accumulation leads to liver injury through the activation of intracellular signaling pathways in Kupffer cells (KCs), hepatic stellate cells (HSCs), and hepatocytes. The activation of KCs and HSCs promotes inflammation and fibrogenesis [44]. These lipids, including FC, SFA, and certain lipid intermediates from excessive SFA, can activate a variety of intracellular responses such as JNK1 and a mitochondrial death pathway, resulting in lipotoxic stress in the endoplasmic reticulum and mitochondria, respectively [42, 43, 45]. In addition, the toll-like receptor 4 (TLR4) is a pattern recognition receptor that activates a proinflammatory signaling pathway in response to excessive SFAs. This pathway is initiated by recruiting adaptor molecules such as toll/IL-1 receptor domain containing adaptor protein (TIRAP) and myeloid differentiation factor 88 (MyD88) that ultimately lead to activation of nuclear factor κB with production of TNF-α [46].

2.5. Mitochondrial Dysfunction. Mitochondria are the most important energy suppliers of the cell and play a pivotal role in fatty acid metabolism. Fatty acid oxidation is able to be upregulated to compensate for some degree of increased deposition of fat; however, multiple studies have shown that liver ATP levels are decreased in NAFLD [47–49]. This discrepancy implicates mitochondrial dysfunction in the state of liver fat overload that is characteristic of NAFLD. Although the mechanisms responsible for the mitochondrial dysfunction remain poorly understood in NAFLD, reduced enzymatic activities of mitochondrial electron transport chain (ETC) complexes may be attributed to increased generation of reactive oxygen species (ROS) as a result of ETC leakage during mitochondrial β-oxidation in energy production (in the form of ATP) [50]. Studies have found that ROS can damage the ETC [51] and even cause mutations in the mitochondria DNA [52].

2.6. Oxidative Stress. In the context of increased supply of fatty acids to hepatocytes, oxidative stress can occur and be attributable to raised levels of reactive oxygen/nitrogen species (ROS/RNS) and lipid peroxidation that are generated during free fatty acid metabolism in microsomes, peroxisomes, and mitochondria [53–55]. Peroxidation of plasma and intracellular membranes may cause direct cell necrosis/apoptosis and megamitochondria, while ROS-induced expression of Fas-ligand on hepatocytes may induce fratricidal cell death. Recent studies support the idea that oxidative stress may be a primary cause of liver fat accumulation and subsequent liver injury, and ROS may play a part even in fibrosis development [56, 57]. Importantly, these species can initiate lipid peroxidation by targeting polyunsaturated fatty acids (PUFAs), resulting in the formation of highly reactive aldehyde products, such as 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA). These reactive lipid derivatives have the potential to amplify intracellular damage by mediating the diffusion of ROS/RNS into the extracellular space, thus causing tissue damage.

2.7. Endoplasmic Reticulum (ER) Stress. The ER is a vast dynamic and tubular network responsible for the synthesis, folding/repair, and trafficking of a wide range of proteins [58]. Under pathological and/or stressful conditions such as NASH, it has been observed that ER efficiency in the protein-folding, repairing, and/or trafficking machinery is decreased while the demand of protein synthesis and folding/repair is increased [58, 59]. Such an imbalance between the load of needed protein-folding and the response-related capability of the ER is termed ER stress, which can lead to the accumulation of unfolded and/or misfolded proteins within the ER lumen. This type of cellular stress usually triggers an adaptive response, aimed at resolving ER stress, called unfolded protein response (UPR) [60–62]. The UPR is mediated by at least three different stress-sensing pathways: protein kinase RNA-like ER kinase (PERK), inositol-requiring protein 1α (IRE1α), and activating transcription factor 6 (ATF6) [62]. Coupled with inflammation, oxidative stress, insulin resistance, and apoptosis signaling, hepatic ER stress seems to play an important role in regulating the composition and size of lipid droplets as well as lipid synthesis, including cholesterol metabolism [58, 59, 63], through SREBP.

3. Microbiota Associated Mechanisms of NAFLD

Gut microbiota was first found to be altered in patients with chronic liver disease more than 80 years ago. Derangement of the gut flora, in particular small intestinal bacterial overgrowth (SIBO), occurs in a large percentage (20–75%) of patients with chronic liver disease. In recent years, the gut microbiome has gained much more attention due to the advancement of the high-throughput next-generation sequencing (NGS) technology. Prior studies of gut flora relied on culture dependent techniques, which were labor intensive

and limited only to a countable number of species, as over 80% of the gut microbes are not cultivatable [64]. In contrast, NGS-based taxonomic assignments of the uncultured, undefined microbes into operational taxonomic units (OTUs) represent an effective and revolutionary approach for studies on highly complex gut microbiota, which is based on clustering of the 16S rRNA sequences derived from the NGS platforms. This approach allows the characterization of both composition and diversity of the intestinal microbiota. According to evidence from relevant studies, the gut microbiota may contribute to the pathogenesis of NAFLD through several mechanisms, including (1) increased production and absorption of gut short-chain fatty acids; (2) altered dietary choline metabolism by the microbiota; (3) altered bile acid pools by the microbiota; (4) increased delivery of microbiota-derived ethanol to liver; (5) gut permeability alterations and release of endotoxin; and (6) interaction between specific diet and microbiota. Most recently, Musso et al. brought up a new mechanism by that chronic kidney disease may mutually aggravate NAFLD and associated metabolic disturbances through multiple paths including altered intestinal barrier function and microbiota composition [65]. In fact, diet can affect the composition and diversity of gut microbiota; thus any changes of gut microbiota that are observed in a diet-stratified study should be interpreted with caution because these changes could be either a direct effect of specific diets or an indirect effect of the gut-liver interactive axis which has been proposed and observed recently [66, 67].

3.1. Short-Chain Fatty Acids (SCFAs) Relevant Mechanisms. In the intestine, SCFAs are produced in the distal small intestine and colon where nondigestible carbohydrates like resistant starch, dietary fiber, and other low-digestible polysaccharides are fermented by saccharolytic bacteria which include the phyla Bacteroidetes, Firmicutes, and Actinobacteria. Acetate and propionate are the main products of Bacteroidetes phylum and butyrate is mainly produced by Firmicutes phylum. As an energy precursor, SCFAs are implicated in the pathogenesis of NAFLD because of their possible contribution to obesity. The first evidence regarding SCFAs was from Turnbaugh et al. study [68] showing that the cecum of ob/ob mice has an increased concentration of SCFAs and that transplantation of germ-free mice with the gut microbiome from ob/ob mice caused greater fat gain than transplants from lean animals. In humans, increased production of SCFAs by the gut microbiota was also observed in overweight and obese people, compared to lean subjects [69]. In metagenomics analysis, the majority of studies showed that ob/ob mice [70] and obese patients [71] exhibit reduced abundance of Bacteroidetes and proportionally increased abundance of Firmicutes. However, how these ratio changes affect energy imbalance leading to obesity and its complications including NAFLD needs further functional and species-level analyses. In fact, SCFAs have more beneficial effects than their obesity-causing effects in general [72]. Beneficial effects of SCFAs are through several ways, such as immunoregulation, enhanced intestinal barrier function, acting as a histone deacetylase 1 (HDAC) inhibitor to decrease expression of lipogenic genes and to increase carnitine palmitoyltransferase 1A expression [72], and a peroxisome proliferator-activated receptor-γ- (PPARγ-) dependent mechanism, shifting metabolism in adipose and liver tissue from lipogenesis to fatty acid oxidation [73].

3.2. Dietary Choline Mechanism. Dietary choline is required for very low density lipoprotein synthesis and hepatic lipid export; and dietary choline-deficiency has been linked with a variety of conditions including hepatic steatosis. Buchman et al. [74] found that, in patients with parenteral nutrition, diets deficient in choline can lead to increased hepatic steatosis, which can be reversed with choline supplementation. This study suggests a role of choline in fat export out of the hepatocytes. Recent studies indicate a role of the intestinal microbiota in the conversion of dietary choline to toxic methylamine, a substance that not only mimics a choline-deficient diet by decreasing effective choline levels but also exposes the host to an inflammatory toxic metabolite [75]. Very recently, a metagenomic analysis of the microbial communities living in the intestinal tracts of 15 women with a choline-depleted diet revealed that increased Gammaproteobacteria abundance and decreased Erysipelotrichi abundance were protective against developing steatosis [76].

3.3. Bile Acid Pool Related Mechanisms. Within hepatocytes, bile acids are synthesized from cholesterol through enzymatic pathways and then conjugated with either glycine or taurine before secretion into bile and released into the small intestine. In the small intestine, conjugated bile acids not only assist in lipid absorption and transport but have also been increasingly recognized to function as nuclear receptor binders and to have a putative role in altering the microbiome [77]. On the other hand, bacteria within the intestine can also chemically modify bile acids and thereby alter the composition of the bile acid pool [78, 79]. Besides the classic role as detergents to facilitate fat absorption, bile acids have also been recognized as important cell signaling molecules regulating lipid metabolism, carbohydrate metabolism, and inflammatory response [80]. These molecular functions are mediated through their binding and activation of the nuclear hormone receptor, farnesoid X receptor (FXR), and the G protein coupled cell surface receptor TGR5 [81]. Intestinal FXR activity upregulates endocrine FGF19 expression, which inhibits hepatic bile acid synthesis via CYP7A1 signaling [82]. McMahan et al. showed that activation of bile acid receptors with a receptor agonist was able to improve NAFLD histology in an obese mouse model [83]. Due to the nature of the complex interplay between the microbiome and the host bile acid pool, further studies are required in the context of risk for NAFLD and NASH.

3.4. Endogenous Alcohol Theory. The possible role for endogenous alcohol in NAFLD was first implicated in ob/ob mice. Cope et al. found that alcohol in the breath of obese animals is higher than that of lean animals [84], but they could not find any difference in the breath alcohol concentration between NASH patients and lean controls in a human study [85]. Recently, Zhu et al. found that NASH patients exhibited significantly elevated blood ethanol levels, while similar

blood ethanol concentrations were observed between healthy subjects and obese non-NASH patients [86]. Further, in this metagenomics study, the composition of NASH microbiomes was found to be distinct from those of healthy and obese microbiomes, and *Escherichia* stood out as the only abundant genus that differed between NASH and obese patients. Because *Escherichia* are ethanol producers, this finding is in agreement with their previous report that alcohol-metabolizing enzymes are upregulated in NASH livers [87]. However, Engstler et al. provided evidence against the alcohol theory [88]. In their study, ethanol levels were similar in portal vein and chyme obtained from different parts of the GI tract between groups, while ethanol levels in vena cava plasma were significantly higher in ob/ob mice, suggesting that more ethanol was not metabolized in the liver due to a significantly lower ADH activity observed in these ob/ob mice. They proposed that increased blood ethanol levels in patients with NAFLD may result from insulin-dependent impairments of ADH activity in liver tissue, rather than from an increased endogenous ethanol synthesis. Thus, the alcohol theory currently faces conflicting results from different investigators. To clarify these conflicting results, de Medeiros and de Lima have provided an interesting mechanistic framework explaining how NAFLD might be an endogenous alcohol fatty liver disease (EAFLD) [89]. However, this framework requires experimental evidence to be validated.

3.5. Intestinal Permeability and Endotoxemia. The gut microbiota plays a part in maintaining the integrity of the intestinal barrier [90]; and changes in the composition of microbiota can lead to increased intestinal permeability and subsequent overflow of harmful bacterial by-products to the liver that in turn triggers hepatic inflammation and metabolic disorders. Endotoxin, that is, lipopolysaccharide (LPS), is derived from Gram-negative bacteria, and it has long been implicated in chronic liver diseases. The first evidence in support of a role for LPS in the pathogenesis of NASH was the observation that endotoxemia readily induces steatohepatitis in obese rats and mice [91]. Further, murine NAFLD models of bacterial overgrowth develop compositional changes of the gut microbiota and present increased intestinal permeability, with a concurrent reduction in the expression of tight junction proteins [92]. In human studies, Miele et al. found evidence of a disruption in the intestinal barrier of biopsy-proven NAFLD patients, along with an increased rate of small bowel bacteria overgrowth, suggesting that alterations in the microbiome may have contributed to disruption of gut barrier integrity [93]. In addition, high-fat diets may facilitate LPS uptake through elevated chylomicron production in intestinal epithelial cells [94]. On the other hand, Yuan et al. did not find the correlation between Gram-negative bacteria abundance and the concentration of serum endotoxin and there was no endotoxemia in the majority of pediatric NASH patients [95], highlighting the multihit hypothesis for the pathogenesis of NASH. Nonetheless, LPS and other exogenous stimuli are responded to first by innate immunity through pattern recognition receptors such as toll-like receptors (TLRs) and NOD-like receptors (NLRs). Although TLRs might respond to nutritional lipids such as free fatty acids [96], studies have implicated the importance of LPS-TLR4/TLR9 signaling in the pathogenesis of NAFLD. Both TLR4- and TLR9-deficient mice are protected from high-fat diet-induced inflammation and insulin resistance [97, 98], while mice deficient in TLR5 develop all features of metabolic syndrome including hyperphagia, obesity, insulin resistance, pancreatic inflammation, and hepatic steatosis [99]. Metagenomic analysis indicated that TLR5 deficiency affected the composition of the gut microbiota and, remarkably, transfer of the microbiota from $TLR5^{-/-}$ mice to healthy mice resulted in transfer of disease [99]. Moreover, Wlodarska et al. found that NOD-like receptor family pyrin domain containing 6 (NLRP6) inflammasome deficiency leads to an altered transmissible, colitogenic gut microbiota [100]. When fed with a methionine and choline-deficient diet (MCDD), these inflammasome deficient mice developed NASH with significantly higher severity than wild-type animals [101].

3.6. Saturated Fatty Acids. It has been well known that animal meats are rich in saturated fatty acids (SFAs) which are highly correlated to an increased risk of obesity, diabetes, and cardiovascular diseases. Many studies have indicated that saturated fatty acids are more toxic than their unsaturated counterparts [102, 103]. It is worth noting that SFAs are protective in alcohol induced fatty liver disease [104–106]. However, in liver and hepatocytes not exposed to alcohol, SFAs appear to promote apoptosis and liver injury [107, 108]. It has been shown that SFAs increase the saturation of membrane phospholipids, thus initiating unfolded protein response (UPR) and leading to ER stress [108, 109]. SFAs also affect mitochondrial metabolism and promote ROS accumulation [23]. Furthermore, hepatocyte apoptosis has been shown to be dependent on the activation of JNK stress signaling pathways that respond to prolonged ER and oxidative stress [109]. In addition, SFAs can interact with gut microbiota to affect the progression of liver injury. For instance, by analyzing changes in the intestinal metagenome and metabolome of alcohol-fed mice, Chen et al. recently found that synthesis of saturated long-chain fatty acid is significantly reduced when compared with normal-chew mice and that supplementation of saturated long-chain fatty acids recovers intestinal eubiosis and reduces ethanol-induced liver injury in mice [110]. Moreover, de Wit et al. observed an overflow of SFAs to the distal intestine in mice on a high-SFA diet, which, rather than obesity itself, reduced microbial diversity and increased the Firmicutes-to-Bacteroidetes ratio in the intestine. Such a typical obesity microbiota profile stimulated by SFAs favors the development of obesity and hepatic steatosis [103].

3.7. Fructose. Fructose has been utilized as artificial sweetener in many commercial soft drinks that are consumed largely by adolescents and in a variety of social circumstances. A number of studies have found that excess fructose consumption is involved in the pathogenesis of NAFLD and that upregulated *de novo* lipogenesis and inhibited fatty acid β-oxidation are distinct metabolic processes for the development of hepatic steatosis in individuals with NAFLD [20, 24, 111–113]. Further, Abdelmalek et al. observed that

increased fructose consumption is associated with a higher fibrosis stage in patients with NAFLD, independent of age, sex, BMI, and total calorie intake [29]. Using a fructose-induced NAFLD mouse model, recent studies with metagenomics analysis found that fructose significantly decreased *Bifidobacterium* and *Lactobacillus* and tended to increase endotoxemia [114, 115]. Several probiotic bacterial strains of *Lactobacillus* protect mice from the development of high-fructose-induced NAFLD [116–118]. In addition, increased expression of TLRs has been implicated in the development of fructose-induced hepatic steatosis [119].

4. Genetic Background of NAFLD

Genomic variations that have a causative effect on the development of human diseases can be divided into two groups: ones in rare diseases and ones in common diseases. The former follow Mendelian inheritance patterns that are characterized by a single, highly penetrant but uncommon mutation in a specific gene being necessary and sufficient to cause the disease. The latter consist of causative mutations that are not subject to negative selection pressures, and disease susceptibility is due to the combined effects of multiple relatively common causative polymorphisms (minor allele frequency 1–5%) that are carried by affected individuals. Like most common diseases, NAFLD has been implicated in an inherited component to susceptibility, meaning that genetic variation does influence disease risk. As reviewed by Macaluso et al. in 2015 [120], dozens of genes with multiple polymorphisms have been discovered in genome-wide association studies (GWAS) that may be responsible for risk of NAFLD in certain populations. It is believed that as more large scale GWAS are complete, more genes could be identified. For instance, in early 2016 while we were preparing this review, a novel variant MBOAT7 rs641738 was reported to be associated with the development and severity of NAFLD in individuals of European descent [121]. Among all reported genes, only two of them (*PNPLA3* and *TM6SF2*) have been identified as potential genetic modifiers in more than one large scale study [120, 122], which are the focus of our review. According to genotypes in those key genes and sensitivity to insulin, NAFLD patients can be categorized into different subpopulations (Figure 1).

4.1. PNPLA3 (Patatin-Like Phospholipase Domain Containing 3). The *PNPLA3* gene (adiponutrin) encodes a transmembrane polypeptide chain exhibiting triglyceride hydrolase activity [123], which is highly expressed on the endoplasmic reticulum and lipid membranes of hepatocytes and adipose tissue [124]. It is also reported that *PNPLA3* is highly expressed in human stellate cells. The encoded protein has retinyl esterase activity and allows retinol secretion from hepatic stellate cells while the mutation causes intracellular retention of this compound [125–127]. As the first genome-wide association study with strong evidence for NAFLD, a report from Romeo et al. in 2008 showed that a genetic variant, an allele in *PNPLA3* (rs738409[G], encoding Ile148Met), confers susceptibility to the disease in individuals of several western populations [128]. This genetic variant was associated with increased liver fat and hepatic inflammation and fibrosis. This finding has subsequently been reproduced with solid evidence as shown in a meta-analysis comprising 16 studies [129]. Compared with noncarriers, homozygous carriers of the variant had a 73% higher liver fat content, a 3.2-fold greater risk of high necroinflammatory scores, and a 3.2-fold greater risk of developing fibrosis. The association between the *PNPLA3* variant and steatosis or severity of histological liver disease has been widely observed in the majority of subsequent genome-wide association studies [130] and several case-control studies, including those in Chinese, Korean, and Japanese populations [131–133]. It is worth noting that the link between *PNPLA3* I148M variant and NAFLD is independent of metabolic syndrome (MS) and its features; that is, most of patients carrying this variant are not associated with obesity, diabetes, and atherogenic dyslipidemia, as demonstrated in the recent meta-analysis [129]. Furthermore, the *PNPLA3* genotype seems to also influence steatosis development in patients with hepatitis B and hepatitis C and alcohol abuse, and it has been independently associated with the progression of hepatitis, including fibrosis, cirrhosis, and HCC occurrence [134–136]. The association between the *PNPLA3* variant I148M and the risk of HCC development has been robustly validated in patients with NAFLD [137, 138], and it has been estimated that the homozygous carriers of the p.148M mutation carry a 12-fold increased HCC risk as compared to p.I148 homozygotes [139]. Finally, as described earlier, subpopulations of NAFLD patients with PNLA3 mutation are not associated with insulin resistance, a hallmark of metabolic syndrome. Collectively, it seems that a distinct entity might exist in which the *PNPLA3* risk allele appears to be a major driver of disease progression in combination with viral infection, alcohol abuse, lifestyle (unhealthy diet and inactivity), and/or nonlifestyle (cryptogenic) causes, for example, *PNPLA3*-associated steatohepatitis ("PASH") [140].

4.2. TM6SF2 (Transmembrane 6 Superfamily Member 2). Another widely validated and intriguing genetic player in NAFLD is the nonsynonymous variant rs58542926 (c.449 C>T) within the *TM6SF2* gene at the 19p13.11 locus, which encodes an E167K amino acid substitution. The role of variant E167K in *TM6SF2* was first described by Kozlitina et al. [36] in an exome-wide association study in a multiethnic, population-based cohort, highlighting the association of the *TM6SF2* variant with higher serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels—as surrogates for NASH—and with reduced plasma levels of triglycerides and low density lipoprotein- (LDL-) cholesterol. In addition, they performed a functional analysis for *TM6SF2* in mouse models by silencing the gene via adeno-associated viral vectors. Silencing of the gene showed a 3-fold increase in hepatic triglycerides levels and a decrease in plasma levels of triglycerides, LDL- and high density lipoprotein- (HDL-) cholesterols, and very low density lipoprotein (VLDL). Overall, their results demonstrated that the *TM6SF2* gene regulated hepatic triglyceride secretion and that the functional impairment of *TM6SF2* promoted NAFLD. An association between the *TM6SF2* rs58542926 SNP and the severity of liver disease has also been found in patients with biopsy-proven

NAFLD in a recent study reported by Liu et al. [141]. More intriguingly, the E167K variant in *TM6SF2* seems able to disconnect the risk of NAFLD/NASH progression from cardiovascular risk, which is supported mainly by the Dongiovanni et al. study [142] showing that 188 (13%) out of 1201 subjects who underwent liver biopsy for suspected NASH were carriers of the E167K variant and that these carriers had lower serum lipid levels than noncarriers, more severe steatosis, necroinflammation, ballooning, and fibrosis and were more likely to have NASH and advanced fibrosis after adjusting for metabolic factors and the *PNPLA3* I148M risk variant. In addition, E167K carriers had lower risk of developing carotid plaque; and in Swedish obese subjects assessed for cardiovascular outcomes, E167K carriers had higher ALT and lower lipid levels but also a lower incidence of cardiovascular events. Consequently, carriers of the *TM6SF2* E167K variant seem to be more at risk for progressive NASH, but at the same time they could be protected against cardiovascular diseases [143].

5. Interplay between Diet, Microbiota, and Host Genetics

One of the biggest lessons we learned from the metagenomic studies so far is that constitutive profiles of gut microbiota can determine liver pathology in response to a high-fat diet (HFD) in mice, reflecting a kind of interactive effect between diet and gut microbiota, that is, a net effect after the interplay. For instance, in a transplantation experiment [144], Le Roy et al. selected donor mice at first, based on their responses to a HFD. The "responders" developed hyperglycaemia and had a high plasma concentration of proinflammatory cytokines, and the "nonresponders" were normoglycaemic and had a lower level of systemic inflammation, although both developed comparable obesity on the HFD. Germ-free mice were then colonized with intestinal microbiota from either the responder or the nonresponder mice and then fed the same HFD. The responder-receiver (RR) group developed fasting hyperglycaemia and insulinaemia, whereas the nonresponder-receiver (NRR) group remained normoglycaemic. In contrast to NRR mice, RR mice developed hepatic macrovesicular steatosis, which was confirmed by a higher liver concentration of triglycerides and increased expression of genes involved in *de novo* lipogenesis. Pyrosequencing of the 16S ribosomal RNA genes revealed that RR and NRR mice had distinct gut microbiota including differences at the phylum, genera, and species levels. These results suggest that the gut microbiota can contribute to the development of NAFLD, independent of obesity but acting like a constitutional background of a host organ system. The interrelationship between diet, gut microbiota, and host genetics has been unraveled further in a recent study reported by Ussar and coworkers [145]. In this study, they utilized three commonly used inbred strains of mice: obesity/diabetes-prone C57Bl/6J, obesity/diabetes-resistant 129S1/SvImJ, and obesity-prone but diabetes-resistant 129S6/SvEvTac mice. Analysis of metabolic parameters and gut microbiota in all strains and their environmentally normalized derivatives revealed strong interactions between microbiota, diet, breeding site, and metabolic phenotype. More intriguingly, environmental reprogramming of microbiota resulted in obesity-prone 129S6/SvEvTac mice becoming obesity resistant. This study suggests that development of obesity/metabolic syndrome is the result of interactions between gut microbiota, host genetics, and diet.

6. Conclusions

NAFLD is best considered a multietiology disease trait, meaning that it is not caused by a single gene mutation genetically and is not associated with only a single factor environmentally; but it is the outcome of genetic variant-environmental factor interplay determining disease phenotype and progression. The genetic variants in *PNPLA3* and *TM6SF2* are only responsible for ~50% of NAFLD patients [120], and majority of *PNPLA3*-associated NAFLD patients are not obese and have no insulin resistance and its related diabetes and cardiovascular diseases [140]. In fact, like many common diseases, NAFLD is polygenic, where the heritable component to susceptibility variously accounts for up to 30–50% of relative risk [130]. Moreover, individual environmental factors, particularly the specific diets, interact with gut microbiota up front before a final beneficial or damaging signal is sent. Whether environmental factors, including lifestyle, are the cause of NAFLD will be steered by the interaction with the host genetics as well as the constitutional profile of gut microbiota. Thus, careful, multifaceted study designs are warranted in future analysis in order to "catch" the true causes to NAFLD.

Competing Interests

No competing interests are declared by the authors.

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Clinical Study

Fecal Microbiota Transplantation Using Upper Gastrointestinal Tract for the Treatment of Refractory or Severe Complicated *Clostridium difficile* Infection in Elderly Patients in Poor Medical Condition: The First Study in an Asian Country

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Background and Aims. Fecal microbiota transplantation (FMT) is a highly effective treatment option for refractory *Clostridium difficile* infection (CDI). FMT may be challenging in patients with a low performance status, because of their poor medical condition. The aims of this study were to describe our experience treating patients in poor medical condition with refractory or severe complicated CDI using FMT via the upper GI tract route. *Methods*. This study was a retrospective review of seven elderly patients with refractory or severe complicated CDI and a poor medical condition who were treated with FMT through the upper GI tract route from May 2012 through August 2013. The outcomes studied included the cure rate of CDI and adverse events. *Results*. Of these seven patients who received FMT via the upper GI tract route, all patients were cured. During the 11-month follow-up period, CDI recurrence was observed in two patients; rescue FMT was performed in these patients, which led to a full cure. Vomiting was observed in two patients. *Conclusions*. FMT via the upper gastrointestinal tract route may be effective for the treatment of refractory or severe complicated CDI in patients with a low performance status. Physicians should be aware of adverse events, especially vomiting.

1. Introduction

The incidence and severity of *Clostridium difficile* infection (CDI) have been increasing [1, 2]. Fecal microbiota transplantation (FMT) is an effective treatment modality for recurrent or refractory CDI. The therapeutic efficacy of FMT for the treatment of refractory CDI is >90% [3–5].

FMT can be performed via either the upper gastrointestinal (GI) tract route or the lower GI tract route. A systematic review reported that three out of four FMT procedures were performed via colonoscopy [6]. To date, no study has compared the therapeutic efficacy of CDI according to the infusion route. However, when FMT is performed using the upper GI tract route, the foul odor of the fecal suspension may cause discomfort, nausea, and vomiting in patients. This might contribute to the choice of the lower GI tract by physicians as an infusion route.

In FMT via the lower GI tract route, the fecal suspension is infused using colonoscopy or retention enema [6]. Although there is no guideline regarding the retention time of the fecal suspension when FMT is performed via this route, the fecal suspension should be retained in the colon as long as possible. One study reported that patients were asked to avoid defecation for 30–45 min [7].

Table 1: Karnofsky Performance Status score.

Score	Criteria
100	Normal; no complaints; no evidence of disease.
90	Able to carry on normal activity; minor signs or symptoms of disease.
80	Normal activity with effort; some signs or symptoms of disease.
70	Cares for self; unable to carry on normal activity or to do active work.
60	Requires occasional assistance but is able to care for most of their personal needs.
50	Requires considerable assistance and frequent medical care.
40	Disabled; requires special care and assistance.
30	Severely disabled; hospital admission is indicated although death is not imminent.
20	Very sick; hospital admission necessary; active supportive treatment necessary.
10	Moribund; fatal processes progressing rapidly.
0	Dead.

Old age and severe underlying comorbidities are risk factors for CDI and predictable risk factors for CDI recurrence [8, 9]. Because these patients cannot retain the fecal suspension sufficiently, FMT via the lower GI tract route may be challenging in this group of individuals. Therefore, in this subset of patients, FMT may be performed via the upper GI tract route. The aims of this study were to describe our experience treating 7 patients in poor medical condition with refractory or severe complicated CDI using FMT via the upper GI tract route.

2. Patients and Methods

2.1. Study Participants and Assessment. This study was a retrospective review of seven elderly patients with refractory or severe complicated CDI and a poor medical condition who were treated with FMT through the upper GI tract route at Seoul St. Mary's Hospital and Uijeongbu St. Mary's Hospital, Republic of Korea, from May 2012 through August 2013. The demographic characteristics, the characteristics of CDI, the clinical outcomes of the study participants and adverse events related to FMT were investigated. Patients' performance status was evaluated using the Karnofsky Performance Status (KPS) score which runs from 0 to 100 (Table 1) [10]. Patients' comorbidities were recorded using the Charlson Comorbidity Index score [11]. A total of 22 conditions were assigned with a score of 1, 2, 3, or 6. Points were assigned to each condition as follows: 1, myocardial infarct, congestive heart failure, peripheral vascular disease, dementia, cerebrovascular disease, chronic lung disease, connective tissue disease, ulcer, chronic liver disease, and diabetes; 2, hemiplegia, moderate or severe kidney disease, diabetes with end organ damage, tumor, leukemia, and lymphoma; 3, moderate or severe liver disease; 6, malignant tumor, metastasis, and acquired immune deficiency syndrome. The mental status and cognitive functions of patients were also assessed. WBC count and serum creatinine levels were recorded at the time of diagnosis of CDI, before FMT.

CDI was defined as a combination of a toxigenic stool culture and diarrhea ≥3/day [12]. The stool culture (chromID *C. difficile*; bioMérieux, Marcy l'Etoile, France) and a toxin assay using enzyme immunoassay (Wampole Tox A/B Quik Chek; Alere, Orlando, FL, USA) and polymerase chain reaction for the detection of toxin genes (*tcdA*, *tcdB*, *cdtA*, and *cdtB*) were performed. Refractory CDI was defined as an unresponsiveness to more than 14 days of a conventional therapy that included oral vancomycin. Severe, complicated CDI was defined as a combination of CDI and presence of abdominal distension, documented bowel dilatation on abdominal CT scan, and hemodynamic instability [12, 13]. Resolution of CDI was defined as the status in which all of the following criteria were met: (1) cessation of diarrhea 1-2 days after FMT and (2) negative conversion of a toxigenic stool culture. Recurrence was defined as the presence of the criteria used to define CDI at least 2 weeks after its resolution. Poor medical condition was defined as (1) KPS score ≤40, which warrants hospitalization and special treatment and/or (2) multiple comorbidities. This study's protocol was approved by the Institute Review Board of Seoul St. Mary's Hospital and of Uijeongbu St. Mary's Hospital.

2.2. Fecal Microbiota Transplantation. The donor-stool source was a family member or an unrelated healthy donor. Before FMT, we asked the patient's family to select the stool donor. Subsequently, the donor's medical history as well as stool and blood samples were screened. The hepatitis B surface antigen, the hepatitis C virus antibody, and the human immunodeficiency virus were checked, and a serological test for syphilis was performed. The test of the donor's stool included a white blood cell count, an ovum and parasite, *Salmonella* culture, and *C. difficile* toxin. The donor had not used antibiotics within the past year and had no history of chemotherapy. The donor's stool (>50 g) was collected within 24 h before FMT. Stool and normal saline (1 : 3) were placed in a blender (NJM-9060; NUC Electronics, Daegu, Korea) and ground for 3 min. The fecal suspension was passed through a stainless steel tea strainer, to remove large particles. Colonoscopy was performed in all patients before FMT, to detect pseudomembranous colitis (PMC). However, FMT was performed via the upper GI tract route, as the patients were not able to retain the fecal suspension because of their poor medical condition. The fecal suspension was infused using upper endoscopy or a percutaneous endoscopic gastrostomy (PEG) tube with a 50 mL syringe. A sedative was administered to patients whose vital signs were stable. The patients were kept in a 45° upright position for 4 h after FMT. Written informed consent was given by the patient or their family before FMT.

3. Results

3.1. Patient Characteristics. The medical records of seven patients were reviewed. There was no previous FMT history in six of the seven patients. One patient received two courses

of FMT via colonoscopy, which were not successful. The demographic and clinical data of the patients are shown in Table 2. All patients were immobilized and were treated as inpatients. The mean age was 75.6 years, and all patients had multiple comorbidities. The mean score on the KPS scale was 17.1 (range, 10–20). The median Charlson Comorbidity Index score was 3 (range, 1–14). One patient was intubated for the treatment of acute respiratory distress syndrome with sedation. Five of the seven patients had pneumonia as the index infection. The mean number of diarrhea events was 5.4/day, PMC was observed in five patients (71.4%), and the mean number of CDI episodes was 2.86. Five patients received FMT for refractory CDI, and two received FMT for severe, complicated CDI.

3.2. FMT and Post-FMT Data. Seven patients who received FMT via the upper GI tract route were initially cured that have met with the criteria of CDI resolution. One patient who had received two courses of FMT using colonoscopy before receiving FMT via the upper GI tract route exhibited symptoms that were compatible with severe, complicated CDI. The previous two courses of FMT, which were delivered via colonoscopy, were incomplete, as cecal intubation could not be performed because of severe abdominal distension and because the patient was not able to retain the fecal suspension sufficiently. CDI was cured in this patient after the FMT using upper endoscopy [14]. During the 11 months (mean) of the follow-up period (range, 5–17 months), recurrence was observed in two patients and occurred 90 and 130 days after FMT, respectively. *C. difficile*-provocative antibiotics were prescribed after FMT to each of these patients for 45 and 90 days, respectively. Rescue FMT was performed in patients with recurrence via upper endoscopy using the same donor. Recurrent CDI was cured, and recurrence was not observed 6 and 9 months after rescue FMT. Thus, the upper GI route of FMT was successful in all of the 7 patients, mostly in 1 session. The total number of episodes of CDI, including recurrence, was nine. Nine sessions of FMT were performed. The infusion route of the fecal suspension was as follows: upper endoscopy, eight sessions; PEG tube, one session. The mean amount of stool used was 91.2 g (range, 50–150 g). Five sessions of FMT (55.6%) were performed using a stool sample from a family donor. Sedatives were used in eight cases of FMT (88.9%).

3.3. Adverse Events. Among the seven patients included in the study, two vomited. One patient vomited 30 min after FMT and the other patient vomited 3 h after FMT. Although the amount of vomitus could not be measured, it was not significant. Other adverse events, including aspiration pneumonia, were not observed in these two patients.

4. Discussion

In this study, we treated refractory or severe complicated CDI in patients with a poor medical condition via FMT using the upper GI tract route; all patients were cured after FMT. Even though the indication of FMT has been increasing recently [15–17], there are few published articles on FMT in Asian countries [14, 18, 19]. This might be associated with the lower incidence of refractory CDI in these countries. A comprehensive single-center study of CDI performed in the Republic of Korea reported that the incidence of refractory CDI was 0.7% (2/320) in 2011 [20]. This lower incidence of refractory CDI is caused by the low prevalence of the hypervirulent strain B1/NAP1/027 in the Republic of Korea [21]. CDI is more prevalent in Western countries than it is in Asian countries. The incidence of hospital-acquired CDI in the Republic of Korea was reported as being up to 9.1 cases/10,000 patient hospital days [20, 21], which is lower than that observed in North America (28.1 cases/10,000 patient hospital days) [22]. Furthermore, the proportion of community-acquired CDI in North America was reported to be as high as 40% [23, 24], versus 3.4% in the Republic of Korea [18]. Well-known risk factors for CDI, including old age, hospitalization, and comorbidities, are absent in community-acquired CDI [25]. Antimicrobial exposure, use of proton-pump inhibitors, and *C. difficile* transmission in an outpatient setting are important risk factors for community-acquired CDI [25, 26]. B1/NAP1/027 was the most common (21.7%) strain isolated in community-acquired CDI [26]. This finding implies that CDI may be unresponsive to conventional treatment, even in a community setting, in North America. Most FMT procedures are performed in an outpatient manner in North America [4, 15, 27]. To our knowledge, there are no reports of refractory CDI in patients with community-acquired CDI in Asian countries. In these countries, refractory CDI that is unresponsive to conventional treatment might be complicated in patients with poor medical condition.

In our study, the medical condition of patients was poor because of old age, low performance status, or the presence of multiple comorbidities. FMT in patients with poor medical condition can be challenging. To minimize the risk of procedure-related complications, the procedure time should be shortened as much as possible. FMT via the upper GI tract route has some advantages compared with FMT performed via colonoscopy: (1) a shorter procedure time, (2) no need for bowel cleansing, and (3) a longer retention time in the large bowel, regardless of the patient's medical condition and consciousness. All FMT sessions were performed using the upper GI tract route. Only one patient received FMT via colonoscopy before the FMT using the upper GI tract. The two previous FMT sessions were partially effective, and abdominal distension and the number of diarrhea events were slightly decreased. However, the procedure was incomplete because cecal intubation was not performed and the patient was not able to retain the fecal suspension sufficiently. Diarrhea was improved, but not resolved. Drowsy patients cannot retain the fecal suspension sufficiently; therefore, we performed FMT using upper endoscopy. CDI was completely resolved after FMT via the upper GI tract route.

Recurrence of CDI was observed in two patients, 90 and 130 days after FMT, respectively. Continuous use of *C. difficile*-provocative antibiotics and comorbid conditions are well-known risk factors for CDI recurrence [28, 29]. *C. difficile*-provocative antibiotics were prescribed in patients with recurrence. Moreover, patients had multiple comorbidities.

Table 2: Pre-FMT and post-FMT data of the patients.

Patient number	Age	Sex	K-P scale	Mental status	Cognition	Index infection	CCIs	Number of diarrhea per day	WBC count (cell/mcL)	Cr (mg/dL)	PMC	Number of CDI before FMT	Days from first CDI diagnosis to FMT	Days of last course of antibiotic treatment for CDI	Indication of FMT	Adverse events
1	83	Male	20	Alert	Impaired	Pneumonia	2	5	4,200	0.42	Yes	1	15	15	Refractory CDI	None
2^	87	Male	20	Alert	Intact	Pneumonia	8	4	3,980	0.86	Yes	5	149	41	Refractory CDI	Vomiting
3*^	74	Male	10	Drowsy	Impaired	Pneumonia	14	10	5,200	2.57	Yes	2	29	10	Severe, complicated CDI	None
4	55	Male	20	Stupor	Impaired	Infectious colitis	3	5	6,240	0.68	No	5	486	37	Refractory CDI	None
5	75	Female	20	Alert	Intact	Urinary tract infection	3	5	18,830	2.3	No	4	459	7	Severe, complicated CDI	Vomiting
6	72	Male	20	Alert	Impaired	Pneumonia	6	4	12,350	0.97	Yes	2	237	27	Refractory CDI	None
7	83	Male	10	Sedated	Uncheckable	Pneumonia	1	5	8,440	0.64	Yes	1	37	37	Refractory CDI	None

FMT, fecal microbiota transplantation; K-P scale, Karnofsky Performance scale; CCIs, Charlson Comorbidity Index score; WBC, white blood cell; Cr, creatinine; PMC, pseudomembranous colitis; CDI, *C. difficile* infection.

^Patients who had recurrence after FMT.

*Patient who received 2 sessions of FMT using colonoscopy before FMT using upper endoscopy.

Because the time between FMT and recurrence was not short, FMT efficacy was not associated with recurrence; rather, recurrence seemed to be caused by the medical condition of the patients.

Patients with a poor medical condition have a high risk of adverse events after FMT [15]. In the current study, vomiting was observed in two of seven patients (28.6%), who vomited when they were sitting in an upright position after FMT. Vomiting and nausea are important adverse events after FMT performed via the upper GI tract route [5, 15]. Aspiration pneumonia after FMT may cause death [15]. Thus, FMT via the upper GI tract route should be performed with caution, and close monitoring of nausea and vomiting is necessary to prevent aspiration pneumonia in elderly patients with a poor medical condition. To prevent vomiting related to FMT delivered via the upper GI tract route, the fecal suspension may be infused into the proximal jejunum using push enteroscopy or balloon-assisted enteroscopy.

This work was the first study of FMT for the treatment of refractory or severe complicated CDI conducted in an Asian country. The limitations of the current study included its small sample size and retrospective design. Although this study was retrospective, the authors collected the data prospectively. We suggest that FMT via the upper GI tract route is an effective option for the treatment of refractory or severe complicated CDI in patients with old age and a poor medical condition who are not eligible for FMT through the lower GI tract route. Moreover, this method can be tried as a rescue or alternative treatment option when FMT using colonoscopy fails or is incomplete. Further protocol improvement is needed to reduce procedure-related adverse events.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

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Research Article

Systemic and Splanchnic Lipopolysaccharide and Endothelin-1 Plasma Levels in Liver Cirrhosis before and after Transjugular Intrahepatic Portosystemic Shunt

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Lipopolysaccharide (LPS) and endothelin- (ET-) 1 may aggravate portal hypertension by increasing intrahepatic resistance and splanchnic blood flow. In the portal vein, after TIPS shunting, LPS and ET-1 were significantly decreased. Our study suggests that TIPS can benefit cirrhotic patients not only in high hemodynamics related variceal bleeding but also in intestinal bacterial translocation associated complications such as endotoxemia.

1. Introduction

Portal hypertension is a complication of liver cirrhosis. Cirrhotic nodules lead to altered intrahepatic architecture and are the initiating, irreversible pathophysiological feature of cirrhosis. The major pathophysiological mechanisms of portal hypertension are increased intrahepatic resistance and increased splanchnic blood flow, so intrahepatic vascular contraction and increased splanchnic blood flow may be key therapeutic targets in portal hypertension [1].

Bacterial translocation is a common problem and plays an important role in the pathogenesis and complications in patients with decompensated cirrhosis [2]. Bacterial endotoxin, such as lipopolysaccharide (LPS), is a prototypic microbe-derived inflammatory signal that regulates endothelin (ET) and NO synthesis [3]. The regulation is complex in advanced cirrhosis. Circulating LPS may have an important role in inducing intrahepatic sinusoidal and splanchnic vascular endothelial dysfunction. ET-1 is one of the most potent vasoconstrictors and binds to ET-A and ET-B receptors. ET-A receptors are typically located on vascular smooth muscle cells and mediate vasoconstriction, whereas ET-B receptors on endothelial cells stimulate endothelial NO synthase (eNOS) activity and NO release. ET-1 may play an important role in liver disease, especially in circulatory disorders such as portal hypertension and ischemia [4].

Transjugular intrahepatic portosystemic shunt (TIPS) is an important interventional procedure for treatment of the complications of advanced cirrhosis that have failed with medical management [5]. TIPS reduces the portal venous pressure gradient (PVPG) and gives the opportunity to obtain portal and hepatic venous blood directly, to determine concentrations of LPS and ET-1 and evaluate their contribution to intrahepatic and systemic hemodynamics. Thus, the aim of this study was to determine portal, hepatic, and systemic LPS and plasma ET-1 levels before and after TIPS in cirrhotic patients to better understand the portal hypertension and its complications.

2. Patients and Methods

2.1. Patients. We studied 30 consecutive patients with portal hypertension at high risk of acute variceal bleeding who underwent TIPS at Jinling Hospital, Nanjing, China,

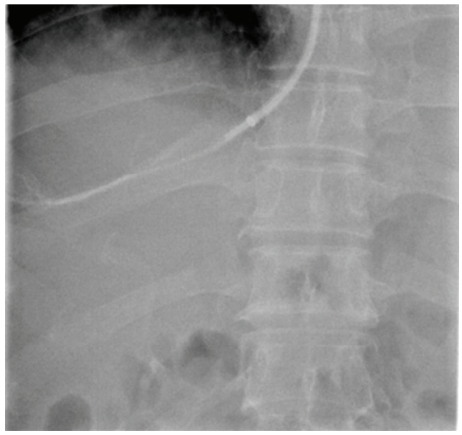

Figure 1: Hepatic venous blood samples were taken under guidance of X-rays.

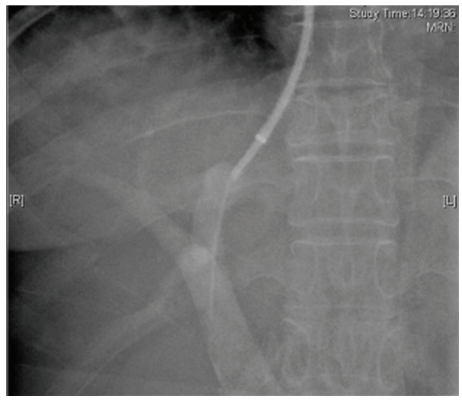

Figure 2: Portal venous blood samples were taken under guidance of X-rays.

between October 2013 and December 2014. Patients had severe esophageal varices upon endoscopy, had more than one episode of variceal bleeding, and had failed drug or endoscopic treatment. Diagnosis of cirrhosis was established by a combination of biochemical, clinical, ultrasonographic, and liver histological findings. The etiology of cirrhosis was alcohol in three patients, chronic Budd-Chiari syndrome in three patients, and viral hepatitis B in 24 patients. No alcohol abuse was detected 2 months before the procedure. The clinical indications for TIPS include repeated variceal bleeding despite appropriate secondary prophylaxis ($n = 25$) and ascites refractory to conventional treatment ($n = 5$).

2.2. Study Design. Blood samples were taken from the right atrium, hepatic vein, and portal vein before insertion of the TIPS stent and 7 days after the TIPS procedure (Figures 1 and 2). Plasma samples were centrifuged at 1800 g for 15 min at 4°C and immediately stored at −80°C until they were analyzed. Serum LPS and ET-1 were measured by enzyme linked immunosorbent assay (ELISA) as previously described [6]. Control samples and serum standards were jointly analyzed in each run. The interassay coefficient of variation in the current study (six runs) was ~10%. Serum NO was measured from the nitrate/nitrite content using a fluorometric assay (KGE 001; R&D Systems China, Shanghai, China). All other analyses were performed using standard laboratory methods.

Table 1: Basic characteristics of patients (plus/minus are means ± SD).

Gender (male/female)	24/6
Age (years)	52.5 ± 11.8
INR	1.3 (1.1–1.48)
Serum ALAT (U/L)	26.8 ± 13.5
Plasma albumin (g/L)	36 ± 4.29
Plasma creatinine (s)	87 (50–108)
Child-Pugh class (A/B/C)	5/18/7
Cirrhosis aetiology (n)	
HBV	24
Chronic Budd-Chiari	3
Alcohol	3
Indication	
Refractory ascites	25
Recurrent variceal bleeding	19

AST: aspartate aminotransferase; INR: international normalized ratio.

2.3. TIPS. TIPS was performed as described previously [7]. Stents were grafts covered with extended polytetrafluoroethylene (Fluency; BARD Peripheral Vascular, Tempe, AZ, USA) and inserted according to general guidelines. The covered stents were 8 or 10 mm in diameter. PVPG was measured during the procedure and 7 days after TIPS. The measurement of PVPG and the acquisition of different blood samples were conducted under the guidance of X-rays (Figures 1 and 2). Intravenous administration of a prophylactic broad-spectrum antibiotic was used after taking blood samples.

2.4. Statistical Analysis. Statistical analysis was performed with SPSS for Windows version 17. Quantitative variables are displayed as medians if not otherwise indicated. We used Student's t-test for comparing differences among continuous normally distributed data and a χ^2 test for categorical data. For analysis of correlation, we calculated the Spearman coefficient of correlation. Differences with $P < 0.05$ were considered significant.

2.5. Ethical Considerations. The Ethics Committee of Jinling Hospital approved this study. Written informed consent was obtained from each patient prior to the study.

3. Results

3.1. Patient Characteristics. The demographic and biochemical characteristics of the patients are listed in Table 1. TIPS was successfully placed in all of the patients. PVPG was significantly lowered from a median of 18 (range 12–32) to 10 (8–16) mmHg ($P < 0.05$). Three patients had transient,

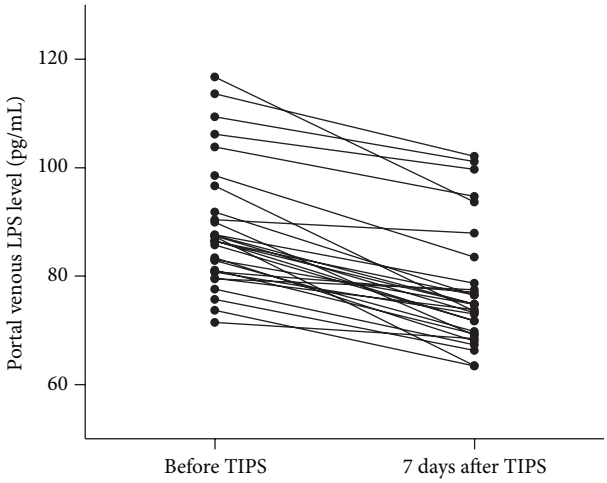

Figure 3: Portal venous plasma LPS concentration before and 5–7 days after TIPS procedure depicted as dot plots, illustrating median, range, and 50% interval with 25th and 75th percentile. The level of LPS in portal vein was decreased from 88 ± 8.63 to 77 ± 7.32 pg/mL ($P < 0.05$).

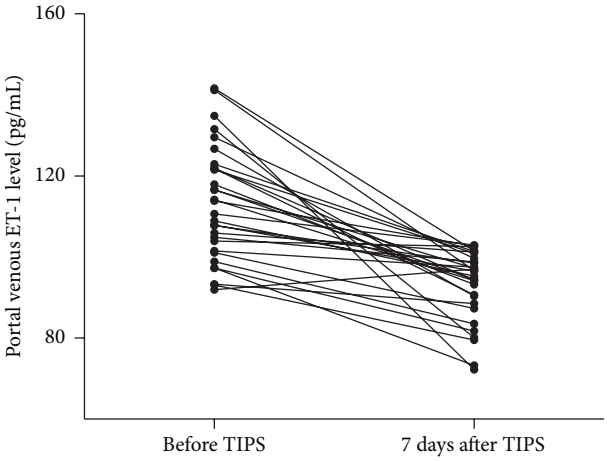

Figure 4: Portal venous plasma ET-1 concentration before and 5–7 days after TIPS procedure depicted as dot plots, illustrating median, range, and 50% interval with 25th and 75th percentile. The level of ET-1 in portal vein was decreased from 113 ± 3.51 to 93 ± 9.31 pg/mL ($P < 0.05$).

low-grade hepatic encephalopathy that was manageable by diet and laxatives without shunt reduction. No patient experienced upper gastrointestinal bleeding after TIPS during 6–13 (median 8) months of follow-up.

3.2. LPS and ET-1 in Portal and Hepatic Veins. Before TIPS, LPS level did not differ significantly between portal vein and hepatic vein plasma: 88 (56–105) versus 92 (54–110) pg/mL. In the portal vein, LPS level decreased significantly from 88 ± 8.63 to 77 ± 7.32 pg/mL ($P < 0.05$) (Figure 3) after TIPS placement. The level of ET-1 also decreased significantly from 113 ± 3.51 to 93 ± 9.31 pg/mL ($P < 0.05$) (Figure 4). There was no difference in the concentration of NO in the portal vein after TIPS placement (from 32.5 to 34.3 pg/mL; $P = 0.076$). Subgroup analysis demonstrated that median portal venous plasma LPS and ET-1 levels before TIPS were significantly higher in five patients with refractory ascites [113 (98–132) pg/mL] compared with 25 patients with repetitive variceal bleeding [86 (56–98) pg/mL] ($P < 0.05$).

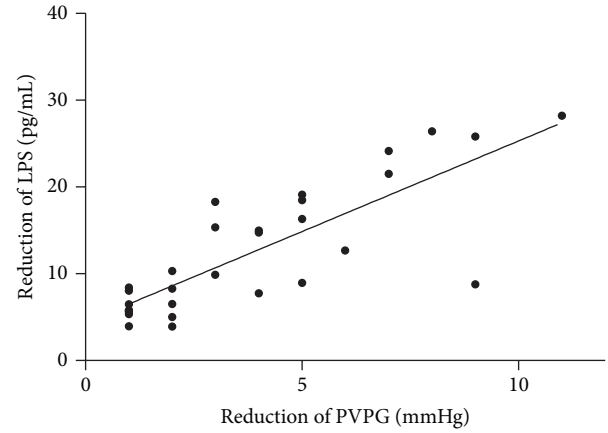

Figure 5: The reduction level of LPS was correlated with the reduction of the PVPG 7 days after the TIPS insertion (Spearman's $r = 0.67$; $P < 0.05$).

3.3. LPS and PVPG after TIPS. Regression analysis showed no significant correlation between right atrial and portal venous levels of LPS and ET-1 and PVPG before and after TIPS insertion. However, when compared with the PVPG at the time during TIPS insertion and 7 days after TIPS, PVPG decreased significantly from 10 (8–16) to 8 (6–14) mmHg ($P < 0.05$). From the time before TIPS and after TIPS insertion, there was a significant correlation between the reduction in portal venous LPS and the reduction in PVPG (Spearman's $r = 0.67$; $P < 0.05$) (Figure 5).

4. Discussion

The main findings of the present study were as follows. We found no intrahepatic gradient of LPS before TIPS placement. LPS and ET-1 levels were decreased in the portal vein after TIPS insertion. During the time before TIPS and after TIPS, there was a correlation between the reduction in portal venous LPS and the reduction in PVPG.

Bacterial translocation is defined as the passage of both viable and nonviable bacteria and bacterial products, such as endotoxin. It is common in decompensated cirrhosis and may be an important pathogenic event in several complications of cirrhosis [1]. LPS is a surrogate marker of bacterial translocation and is increased in systemic and portal circulation [8]. In this study, we did not find a significant difference in LPS levels between the portal and hepatic veins, which is in consistence with Trebicka et al. study [9]. The lack of hepatic endotoxin gradient in our patients may have resulted from the presence of extrahepatic collateral vessels and impaired liver function. We did not find a significant difference in

right atrial blood LPS levels before and after stent insertion. However, a recent study showed that TIPS increased LPS levels in peripheral blood 1 h after stent placement in patients with acute, uncontrolled bleeding [10]. This phenomenon might result from short-term hemodynamic changes caused by procedural trauma or acute bleeding [11].

We found reduced LPS levels in the portal vein; thus, TIPS may reduce LPS levels in the portal vein after stent insertion, possibly as a result of reducing the PVPG. Portal hypertension may be an important factor in the development of small bowel mucosal changes [12]. Abraldes et al. demonstrated that portal pressure is sensed in different vascular beds depending on the severity of portal hypertension, and small increases in portal pressure are first sensed by the intestinal microcirculation [13]. In patients with cirrhosis and portal hypertension, small bowel mucosal edema, red spots, and small bowel varices are attenuated after TIPS [14].

We found that ET-1 level was decreased in the portal vein after TIPS insertion. ET-1 may play an important role in liver disease, especially in circulatory disorders such as portal hypertension and ischemia. ET-1 acts as a paracrine hormone and its plasma levels could represent an overflow of locally produced peptides [15]. Fluid shear stress is a strong liberator of ET-1 from splanchnic vascular endothelial cells, and TIPS could markedly reduce the PVPG and fluid shear stress [16]. Kawanaka et al. found that splenectomy reduced portal venous pressure and normalized hepatic concentrations of ET-1 in patients with liver cirrhosis and portal hypertension. Splenectomy may decrease systemic and splanchnic circulation by eliminating spleen-derived ET-1. Vascular endothelial cells in enlarged spleen may be an important source of ET-1, and TIPS could reduce portal pressure and improve the enlarged spleen [17].

The reduction in portal venous LPS was well correlated with the reduction in PVPG after the TIPS procedure. Binding of ET-1 to ET-B receptors results in activation of eNOS and production of NO, which lead to vasodilation at the sinusoidal level [1]. During endotoxemia, the liver microcirculation becomes hypersensitive to ET-1-induced vasoconstriction. LPS inhibits ET-1-induced eNOS activation in hepatic sinusoidal cells. Therefore, the decrease in ET-1 and LPS levels in the portal vein may reduce intrahepatic vascular resistance owing to NO production in endothelial cells mediated by ET-B receptors [18].

It should be noted that the concentrations in our study have a preliminary character because of the limited numbers and the heterogeneous nature of the patients (e.g., different Child-Pugh classes, ascites, and acute bleeding). Additionally, the blood samples were measured before and 7 days after TIPS insertion separately, so the concentration may be attributed to the different times when the blood samples were obtained [9, 10].

In conclusion, we observed that, after the TIPS procedure, LPS and ET-1 levels in the portal vein both were decreased and the reduction in portal venous LPS was well correlated with the reduction in PVPG. Our study suggests that TIPS can benefit cirrhotic patients not only in high hemodynamics related variceal bleeding but also in intestinal bacterial translocation associated complications such as endotoxemia.

List of Abbreviations

LPS: Lipopolysaccharide
ET-1: Endothelin-1
TIPS: Transjugular intrahepatic portosystemic shunt
ELISA: Enzyme linked immunosorbent assay
NO: Nitric oxide
eNOS: Endothelial nitric oxide synthase
PVPG: Portal venous pressure gradient.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Jiaxiang Meng and Qing Wang contributed equally to this work.

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Research Article

Treatment with a Monoclonal Anti-IL-12p40 Antibody Induces Substantial Gut Microbiota Changes in an Experimental Colitis Model

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Background and Aim. Crohn's disease is associated with gut microbiota (GM) dysbiosis. Treatment with the anti-IL-12p40 monoclonal antibody (12p40-mAb) has therapeutic effect in Crohn's disease patients. This study addresses whether a 12p40-mAb treatment influences gut microbiota (GM) composition in mice with adoptive transfer colitis (AdTr-colitis). *Methods*. AdTr-colitis mice were treated with 12p40-mAb or rat-IgG2a or NaCl from days 21 to 47. Disease was monitored by changes in body weight, stool, endoscopic and histopathology scores, immunohistochemistry, and colonic cytokine/chemokine profiles. GM was characterized through DGGE and 16S rRNA gene-amplicon high-throughput sequencing. *Results*. Following 12p40-mAb treatment, most clinical and pathological parameters associated with colitis were either reduced or absent. GM was shifted towards a higher Firmicutes-to-Bacteroidetes ratio compared to rat-IgG2a treated mice. Significant correlations between 17 bacterial genera and biological markers were found. The relative abundances of the RF32 order (Alphaproteobacteria) and *Akkermansia muciniphila* were positively correlated with damaged histopathology and colonic inflammation. *Conclusions*. Shifts in GM distribution were observed with clinical response to 12p40-mAb treatment, whereas specific GM members correlated with colitis symptoms. Our study implicates that specific changes in GM may be connected with positive clinical outcomes and suggests preventing or correcting GM dysbiosis as a treatment goal in inflammatory bowel disease.

1. Introduction

Inflammatory bowel disease (IBD) arises from a loss of tolerance and excessive immune response to commensal bacteria in a genetically susceptible host, although environmental factors may influence as well. Ulcerative colitis (UC) and Crohn's disease (CD) are the major forms of IBD affecting the gastrointestinal tract [1]. Key clinical features of these diseases include abdominal pain, weight loss, and diarrhea, which can be hemorrhagic [1–3]. As many as 1.4 million in the United States and 2.2 million persons in Europe suffer from IBD and the numbers are increasing [4].

Animal models are widely used to study and explain the pathological mechanisms of gut inflammation. In order to study IBD and evaluate anti-inflammatory strategies, a variety of animal models have been developed and are traditionally divided into those with spontaneous development of colitis due to genetic manipulation (e.g., targeted deletion of the anti-inflammatory cytokine IL-10), chemically induced colitis (e.g., dextran sulfate sodium (DSS)), hapten-induced

colitis (e.g., 2,4,6-trinitrobenzene sulfonic acid (TNBS)), and adoptive transfer (AdTr) models (transfer of T cells from a donor mouse to a T cell deficient mouse) [5]. Compared to chemically inducible models of colitis such as those based upon DSS and TNBS, AdTr of $CD4^+CD25^-$ T cells into immune deficient mice more closely reflects the altered gene expression in human IBD [6]. Mouse models of colitis induced by AdTr of $CD25^-$ depleted $CD4^+$ T cells are highly suitable for pharmacological testing of new IBD drug candidates because they are easy and fast to perform (compared to other chronic models, e.g., SAMP-1/Yit) [7, 8], there is no generation of anti-drug antibodies, and it results in uniform and highly reproducible clinical and pathological signs of colitis [9]. Three weeks after adoptive cell transfer most recipient mice develop clear signs of colitis, characterized by weight loss, loose stools, increased white blood cell (WBC) count, and thickened and shortened colon [5].

Anti-IL-12/23p40 monoclonal antibody (mAb) targets the p40 subunit common to IL-12. IL-12 is formed by two chains, IL-12p35 and IL-12p40, which together form the active heteromer IL-12p70 [10], whereas the active heterodimer IL-23 is formed by IL-12p40 and IL-12p19. Both IL-12 and IL-23 are produced by monocytes, macrophages, and dendritic cells in response to microbial stimulation [10]. IL-12 induces the generation of T helper cells type 1 (T_H1) [11], and it enhances the cytotoxic activities of natural killer (NK) cells [12] leading to secretion of several cytokines, especially interferon-gamma (IFN-γ) [11]. IL-23 stimulates $CD4^+$ T cells to differentiate into a novel subset of T helper cells called T_H17 cells. T_H17 cells produce proinflammatory cytokines that enhance T cell priming and stimulate the production of other proinflammatory molecules such as IL-1, IL-6, and TNF-α, as well as chemokines resulting in inflammation [13–15]. CD has been associated with excess cytokine activity mediated by T_H1 and T_H17 cells. IL-12 and IL-23 are increased in patients with CD, but their production is downregulated after administration of IL-12/23p40 monoclonal antibody [7, 13, 16, 17]. Clinical trials with anti-IL12/23p40 therapy have shown encouraging results in CD patients not responding to the first-line biologic treatment, which is anti-TNF-α mAb. The reason why anti-IL12/23p40 works particularly well in this patient segment is currently unknown, but it could be due to specific genetic alterations, as well as microbiota/gut interactions common in the IL-12/23p40 pathway driving the pathogenesis in these patients. Moreover, alterations in gut microbiota (GM) have been shown as a predictor of relapse in CD patients [18].

Genetically engineered animal models of IBD do not develop fulminant colitis under germ-free conditions, but gut inflammation evolves when they are colonized by bacteria, which points out the important role of the GM in the initiation and development of colitis [19, 20]. Moreover, antibiotics may ameliorate experimental colitis even in a therapeutic setting [7]. Several studies have shown that IBD is accompanied by a shift in GM towards higher abundance of proinflammatory bacteria, such as Enterobacteriaceae, while the abundance of, for example, lactobacilli and bifidobacteria is reduced [21–24]. Furthermore, a number of studies indicate that GM diversity is reduced in colitis [21, 25, 26]. However, detailed knowledge on how GM composition changes in relation to colitis is still limited and more studies are required to verify the alternations in the overall composition of GM during intestinal inflammation and to which extent such alternations are of importance in a preventive or therapeutic intervention. Identifying relevant links between GM composition and clinical parameters of colitis is also of great importance.

Consequently, the aim of the present study was to evaluate how microbiota composition in colonic content correlated with clinical signs of IBD in mice treated with monoclonal anti-IL12/23p40 antibody (12p40-mAb) in an adoptive T cell transfer model of colitis (AdTr-colitis).

2. Materials and Methods

2.1. Mice. All experiments were conducted in accordance with the European Communities Council Directive 86/609/ECC for the protection of animals used for experimental purposes, approved by the Danish Animal Experiments Inspectorate, Ministry of Food, Fisheries and Agriculture, Denmark, and the internal Ethical Review Council at Novo Nordisk A/S.

C.B-Igh-1b/IcrTac-Prkdcscid (C.B-17 SCID) and BALB/cAnNTac female mice (8–10 weeks) bred under barrier protected conditions (Taconic, Ll. Skensved, Denmark) were housed at Novo Nordisk A/S. Mice were identified using Plexx microchips (Plexx, Elst, Netherlands) and were randomized in the cages to reduce cage effects. Dirty cage bedding was transferred to the individual cages before the experiment was initiated as well as once weekly during the experiment. Health monitoring was performed according to FELASA guidelines [27].

2.2. Purification of Cells and Induction of Colitis. Colitis was induced by AdTr of $CD4^+CD25^-$ T cells (AdTr-colitis) from spleen of MHC-compatible BALB/c mice to C.B-17 SCID recipients as previously described [9]. Briefly, splenocytes of BALB/c donor mice were subjected for positive selection of $CD4^+$ T cells using Dynabeads and DETACHaBEAD (Life Technologies Europe, Ballerup, Denmark) and depletion of $CD25^+$ from the $CD4^+$ T cell suspensions using the CD25 MicroBead kit. Flow cytometry was used to analyze purity of the cells and showed that more than 98% of the $CD4^+$ cells were $CD25^-$ cells. Each recipient was reconstituted with 300,000 cells by intraperitoneal injection. Two or three weeks after transfer, peripheral blood from all mice was analyzed by flow cytometry for the presence of $CD4^+$ T cells. Only animals with successful transplantation of cells were included in the study.

2.3. Experimental Groups. The mice were divided into four groups. One group of nontreated C.B-17 SCID mice (SCID control) were not subjected to AdTr-colitis ($n = 4$), while the three groups of adoptively transferred C.B-17 SCID mice were treated with a neutralizing rat anti-mouse IL-12/23p40 monoclonal antibody (12p40-mAb) (clone C17.8) ($n = 15$) or rat-IgG2a monoclonal isotype antibody (clone 2A3) ($n = 13$) or NaCl ($n = 14$) from day 21 until termination at day 47.

The antibodies were purchased from Bio X Cell (New Hampshire, USA) and had been tested for endotoxins. The antibodies were injected intraperitoneally three times weekly and dosed according to body weight at 25 mg kg^{-1}.

2.4. Monitoring of Disease. Animals were weighed three times per week and mice that lost more than 20% of the initial weight were sacrificed. Fecal samples were evaluated and scored from 0 to 4 according to their consistency (normal stool = 0; slightly soft stool = 1; soft but formed stool = 2; not formed stool = 3; liquid stools or no feces in the colon at sacrifice = 4). Disease activity index score (DAI) was calculated based on data obtained from weight loss and feces type as previously described by Murthy et al. [28]. As fecal blood is rarely observed in AdTr-colitis it was not monitored. Mice were anaesthetized by isoflurane (Isoba Vet, MSD Animal Health, Ballerup, Denmark) and blood was obtained by retroorbital puncture. All blood samples were collected in EDTA K2 coated microtubes (Milian, Gahanna, Ohio, USA). EDTA-stabilized peripheral whole blood samples (20 μL) were used for monitoring the number of white blood cells (WBC) per liter with Medonic CA 620 (Boule Nordic, Denmark) blood analysis apparatus according to the manufacturer's protocol. Colonoscopy was performed on days 21 and 34. Mice were anaesthetized with isoflurane and placed in dorsal recumbency. A rigid telescope (HOPKINS Straight Forward, 0°) was connected to a light source/air pump (Xenon 175) and camera (Telecam SL) as described by Becker et al. [29] The endoscope (2 mm) was coated with a lubricant containing lidocaine hydrochloride (Farco-Pharma, Köln, Germany) and introduced via the anus into the distal 4 cm of the colon. The evaluation of the colonoscopic findings was done by two blinded observers using the murine endoscopic index of colitis severity (MEICS) score (Supplemental Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/4953120), as outlined by Becker et al. [30].

2.5. Postmortem Analysis. Mice were sacrificed by cervical dislocation on day 47. However, one mouse in the rat-IgG2a group was sacrificed at day 45 due to severe weight loss. After the animals were sacrificed the colon was excised and opened longitudinally. Fecal and colonic content was collected in a sterile Eppendorf tube and tissue was gently rinsed with saline and its weight (W, mg) and length (L, cm) were measured. The left halves of colon were used for cytokine measurements and the right halves were fixed on a plastic plate with pins and processed for histological analysis. Cecum was excised and its contents were collected in sterile Eppendorf tubes.

2.6. Histology. Tissue for histology was fixed in 4% paraformaldehyde (VWR-Bie & Berntsen, Herlev, Denmark) for approximately 24 hours at 4°C. Subsequently, the samples were transferred to 70% ethanol and stored at 4°C until processed for histopathology. The samples were processed in a Leica Asp300S histoprocessor (Leica Microsystems, Ballerup, Denmark) overnight, embedded in paraffin blocks using a Shandon Histocentre 3 (Thermo Electron Corporation, Marietta, Ohio), and sectioned at a thickness of 3 μm in a Leica Microtome RM 2165 (Leica Microsystems, Ballerup, Denmark). Subsequently, the slides were stained with hematoxylin (Ampliqon, Skovlunde, Denmark) and eosin (Sigma-Aldrich, Brøndby, Denmark) (H&E) for light-microscopic examination, Olympus Ax70 microscope. The severity of the histopathological lesions of colon segments was examined in a blinded manner, using the criteria described in Supplemental Table 2.

For immunohistochemical (IHC) detection of CD3 or S100A8 (calprotectin) paraformaldehyde fixed colon sections were mounted on adhesive slides (Superfrost Plus, Menzel-Gläser, Germany) dried at 60°C and kept at 4°C until processed. Tissue sections were processed through xylene and rehydrated. For CD3 IHC the tissue sections were boiled in Tris-EGTA buffer and quenched with H_2O_2 (0,5%). Tissue sections were blocked (with goat and mouse serum, BSA, and skim milk) and slides were incubated with polyclonal rabbit anti-human CD3 antibody (RM-9107-S; SP7, Thermo Scientific). After washing in TBS buffer secondary antibody-polymer complex (Envision, K4003) was applied. Then, slides were developed with diaminobenzidine (DAB) and counterstained in Meyer's hematoxylin and mounted with Pertex.

For calprotectin IHC staining the tissue sections were boiled in citrate buffer and quenched with H_2O_2 (0,5%). Endogenous biotin was blocked using an avidin-biotin blocking kit. Nonspecific binding was blocked by incubation with TBS containing skimmed milk, donkey serum, and mouse serum. The primary antibody (Rat-a-Mo S100A8 (MRP-8), MyBiosource) and secondary antibody (Biotin-SP Donkey Anti-Rat IgG, Jackson ImmunoResearch) were diluted in Tris-buffer containing skimmed milk and donkey and mouse sera and incubated for 60 min each at room temperature. Next, an amplification step was performed by incubation with Vectastain ABC Peroxidase Kit for 30 min followed by a chromogenic reaction with DAB. Nuclei were counterstained with Meyer's hematoxylin and the sections were rehydrated, cleared in xylene, and mounted with Pertex. Control immunostainings were run without the primary antibody and with a nonsense polyclonal antibody of the same concentration as the primary antibody for both CD3 and calprotectin.

Automated digital image analyses were performed on the IHC positive areas using the Visiopharm Integrator System (VIS, version 4.5.1.324, Visiopharm, Hørsholm, Denmark). On individual digital images of the proximal and distal colon sections, a region-of-interest (ROI) was automatically defined of both colon sections using K-means clustering classification. Subsequently, an analysis was run using threshold classification inside the ROI to detect the brown DAB staining of the specific calprotectin IHC immunostaining. The results are given as area stained with CD3 or calprotectin of the entire colon sections (%).

2.7. Cytokine Measurements. Tissue for cytokine analysis was weighed and transferred to individual 3.6 mL CryoTubes containing Tissue Homogenate Lysis Buffer (Ampliqon, Skovlunde, Denmark). The buffer was a solution of 200 mM NaCl, 5 mM EDTA, 10 mM Tris, 10% glycerin, 1 mM PMSF, 1 μg mL^{-1} leupeptin, and 28 μg mL^{-1} aprotinin (pH 7.4).

The buffer was kept cold (approx. 4°C) at all times. The tubes were immediately snap-frozen in liquid nitrogen and stored at −80°C until homogenized. Using an Ultra-Turrax T25 basic disperser (IKA-Werke, Staufen, Germany) the colon segments were homogenized and the homogenates were centrifuged three times for 15 min at 10,000 ×g and 4°C, twice in Eppendorf tubes and the last time in an Ultrafree MC-Centrifugal Filter device, 5 μm pore size (Millipore, Billerica, Massachusetts, USA). The supernatants were analyzed for levels of colonic cytokines and chemokine using Milliplex (Millipore, Billerica, Massachusetts, USA). The assays were run according to the manufacturer's guidelines with the exception that the standard and test samples were diluted in the Tissue Homogenate Lysis Buffer rather than the kit-supplied assay diluents.

2.8. Gut Microbiota Characterization. Cecal, colonic, and fecal contents collected at euthanasia were homogenized three times for 20 sec at speed 5.5 m/sec prior to extraction using a Bead Beater (FastPrep-24, MP Biomedicals, Santa Ana, USA). DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Concentration and quality of the extracted DNA were measured using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA). A preliminary screening of the GM diversity (based on profiles of 16S rRNA amplicons) of cecal, colonic, and fecal contents was performed using denaturing gradient gel electrophoresis (DGGE) as outlined in Pyndt Jørgensen et al. [31]. Only DNA extracted from colon contents was subjected to high-throughput sequencing (Illumina MiSeq). The V3-V4 region of the 16S rRNA gene (amplicon size ~460 bp) was amplified with primers including adapters for the Nextera Index Kit (Illumina, California, USA). The primers sequences, conditions for amplification and tagmentation (1st and 2nd round of PCR), purification, and sequencing were performed as previously described [31]. Pair-ended reads (with corresponding quality scores) were trimmed and merged using the CLC Genomic Workbench 7.0.4 (CLC bio, Aarhus, Denmark) [31]. The UPARSE algorithm [32] was used for OTU clustering (97%) and filtering of chimeric sequences, while the Green-Genes database 97% (version 12.10) was used for OTU picking [33]. The dataset was further analyzed using the Quantitative Insight Into Microbial Ecology (QIIME versions 1.7.0 and 1.8.0) [34].

Relative quantification of *Akkermansia muciniphila* was carried out as previously described [35]. Briefly, the reaction mixture (20 μL) contained 10 μL of 1x SYBR green PCR Master Mix (Applied Biosystems), 1 μL of either *A. muciniphila* specific-primers or 16S rRNA universal primers (each primer was used at a final concentration 0.5 μM), 4 μL of nuclease-free water, and 5 μL of DNA template (20 ng μL^{-1}). The temperature profile for qPCR was as follows: 95°C for 5 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. For generation of the standard curve, serial tenfold dilutions of *A. muciniphila* DSMZ 22959 genomic DNA were used.

2.9. Statistical Analyses. General statistical analyses were carried out using GraphPad Prism version 5.01 (GraphPad Software, La Jolla, California, USA). Unpaired Student's t-test was used when comparing the means of normally distributed parametric data from two groups and a Mann-Whitney U test was performed when comparing non-Gaussian distributed data from two groups. When three or more groups with parametric data were compared one-way ANOVA was used with Tukey's posttest. The Kruskal-Wallis with Dunn's posttest was used on multiple datasets that did not assume Gaussian distributions. For high-throughput sequencing, the number of sequences used for subsample (−e value, Alpha and Beta Diversity; −d value, multiple rarefactions) was set to 90% of the most indigent sample. The Jackknife Beta Diversity workflow was used to generate PCoA plots (based on 10 distance matrices that were determined using 10 subsampled OTU tables). Differences between categories in UniFrac distance matrices were assessed with analysis of similarities (ANOSIM). Alpha Diversity was measured and expressed as observed species (97% similarity OTUs) and computed with 10 rarefied OTU tables. Comparison of Alpha Diversities was made through nonparametric t-test method (Monte Carlo, 999 permutations). The occurrence and absence of OTUs associated with a given group of mice were assessed with the G-test of independence, whereas differences in the abundance of OTUs were tested with ANOVA. Finally, differences in the relative abundance of *A. muciniphila* determined by qPCR were evaluated using two-tailed Student's t-test.

3. Results

3.1. Monitoring of Disease (DAI Score, Weight Change, and Pathology). The $CD4^+CD25^-$ T cell transfer was well tolerated by the mice, and no adverse events directly related to 12p40-mAb, rat-IgG2a isotype control, or NaCl were detected. Mice were randomized according to body weight (BW) on day 19. No apparent signs of disease were observed within the first two weeks after AdTr-colitis (data not shown). The change in BW over the course of the experiment from days 21 to 47 is shown in Figure 1(a). Mice started to lose weight in the 3rd-4th week following transfer. The weight loss progressed until the termination of the study. Mice treated with 12p40-mAb did not show any apparent weight loss and were similar to SCID control mice, whereas mice treated with the isotype control and NaCl developed severe weight loss ($p < 0.0001$ and $p < 0.0007$). No significant differences were observed between NaCl and rat-IgG2a treated groups. The disease activity index (DAI) is a combined index from 0 to 8 containing weight loss (score 0–4) and stool score (score 0–4), which gives an overall evaluation of the disease intensity during the study. DAI was significantly lower in mice treated with 12p40-mAb compared to rat-IgG2a ($p < 0.001$) and NaCl ($p < 0.006$) (Figure 1(b)). In animal models of AdTr-colitis, disease can be associated with thickening and shortening of the colon wall as a host inflammatory response. As expected, the colon $W : L$ ratio was increased in mice receiving NaCl and rat-IgG2a. A significant treatment effect was observed with the 12p40-mAb compared to the isotype treated group ($p < 0.0002$) and NaCl ($p < 0.0009$) (Figure 1(c)), whereas no differences were observed between their controls groups.

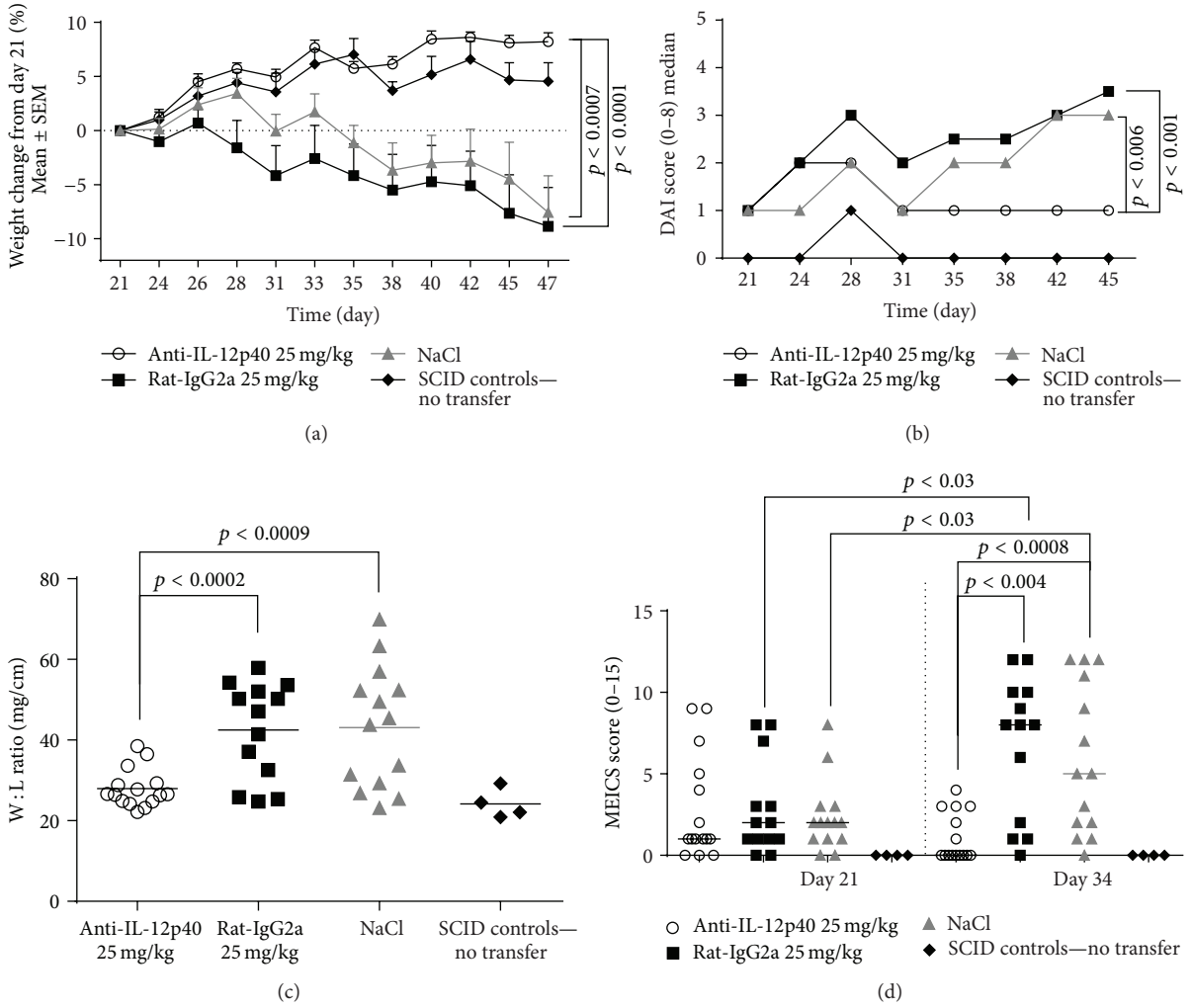


Figure 1: Induction of AdTr-colitis is associated with weight loss, loose stools, and colonic inflammation. (a) Delta weight loss from day 21 is shown as mean ± SEM for individual groups. Significant differences between the areas under the curve (AUC) for individual mice were determined by Student's t-test. (b) DAI was determined with a combined scale from 0 to 8 by combining weight loss (0–4) and stool score (0–4). Differences in DAI scores were calculated as AUC for individual mice from 21 to 45 days using Mann-Whitney U test. (c) The colon weight- (mg) to-length (cm) ratio was calculated for individual mice. Comparisons between groups were performed using Student's t-test. (d) Endoscopic analysis of colon at 21 and 34 days was graded with MEICS. All mice were scored semiquantitatively in a blinded fashion. Significant differences were determined with Mann-Whitney U test for unpaired analysis and Wilcoxon for paired analysis.

Endoscopic pictures were acquired for monitoring and grading inflammation using MEICS (encompassing the thickening of the colon, changes of vascular pattern, visible fibrin, granularity of mucosal surface, and stool consistency). After randomization but prior to treatment (day 21), the majority of mice had clear signs of colonic inflammation; nevertheless, no significant difference between the groups could be detected (Figure 1(d)). At day 34, however, mice treated with the 12p40-mAb had a significant reduction in the endoscopic score, compared to mice treated with rat-IgG2a ($p < 0.004$) and NaCl ($p < 0.0008$). No differences were observed between the 12p40-mAb treated and healthy SCID control mice. Similarly, no difference was observed between the rat-IgG2a and NaCl treated mice at day 34 (Figure 1(d)). When comparing disease progressions within the groups at day 21 versus day 34, mice treated with rat-IgG2a and NaCl increased their endoscopic score, from day 21 to day 34 (both $p < 0.03$). In comparison, mice treated with the IL12p40-mAb showed signs of disease amelioration, $p < 0.09$ (Figure 1(d)).

3.2. Histopathology and Immunohistochemical Staining. At day 47, a significant reduction in histopathology scores was observed in mice treated with 12p40-mAb compared to the corresponding isotype control ($p < 0.0001$) and NaCl ($p < 0.0008$) (Figures 2(a) and 2(d)), while no significant differences were observed between the control groups. Since pathogenic CD4 T cells drive disease progression in AdTr-colitis,

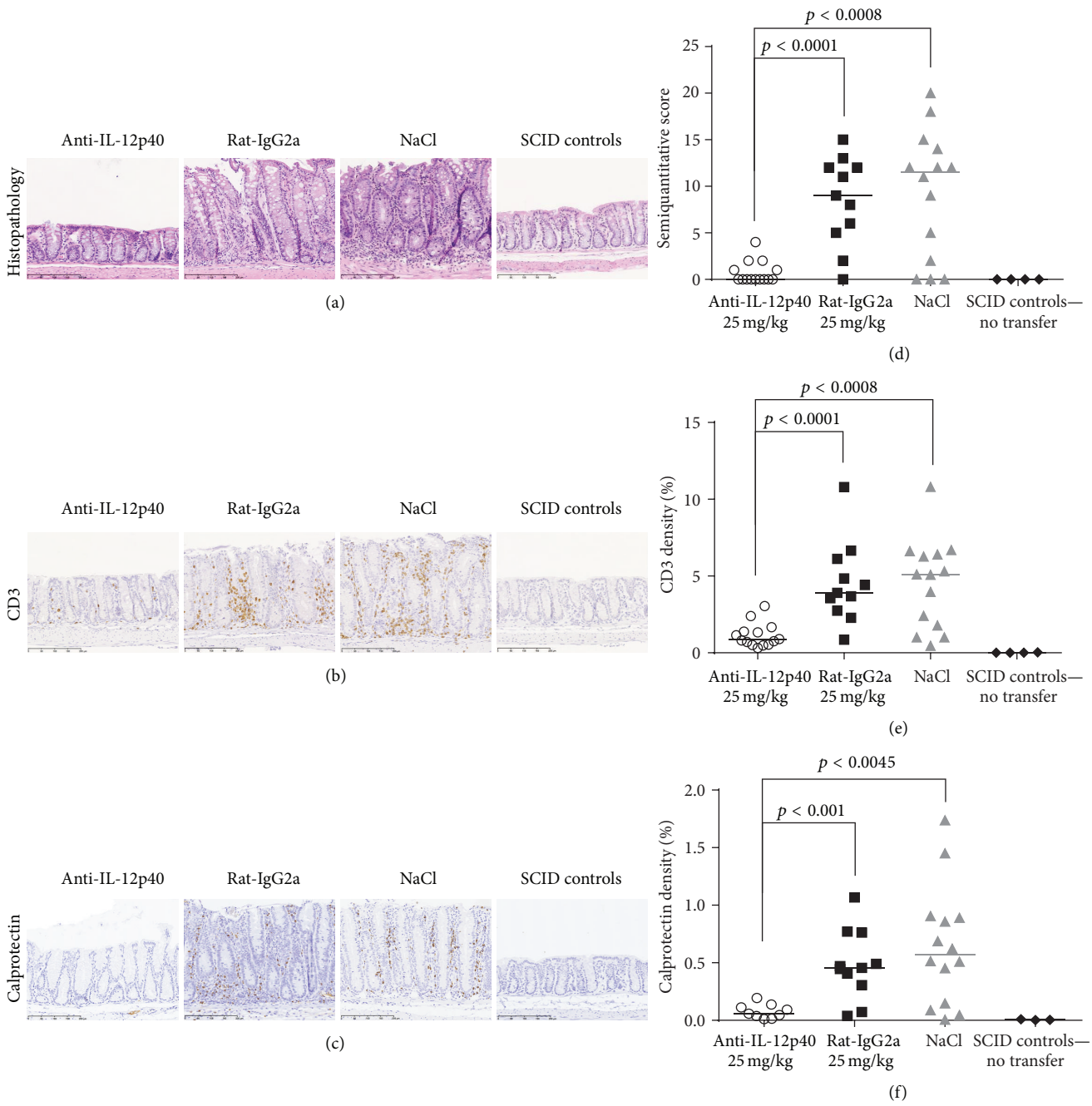


FIGURE 2: Histology and immunohistochemistry of representative areas of mouse colon. The left panel (a, b, c) shows the histopathological and IHC evaluation of the colon. After H&E staining (a), the 12p40-mAb treated mice had normal morphology as seen in the SCID control mice. Rat-IgG2a and NaCl treated mice had major hyperplasia of the mucosal layer and infiltration of immune cells. The CD3 (b) and calprotectin (c) IHC identify these immune cells as being CD3 or calprotectin positive cells as visualized with the brown DAB staining of the positive cells in the mucosa layer and submucosa. The scale bar indicates 200 μm and all images are captured at 20x magnification. The images are from the mouse with the results representing the group median for the specific staining of the four mice groups. The right panel (d, e, f) depicts the results from the histopathological (d) evaluation using a semiquantitative score (range 0–24). CD3 (e) and calprotectin (f) staining were quantitatively assessed by digital image analysis; data is represented as the density of the IHC reactivity (%).

colonic sections were stained for a T cell marker (CD3). The percentage of CD3 stained colon tissue was then used to monitor the effect of 12p40-mAb on colonic T cell inflammation. Mice treated with 12p40-mAb had significantly lower density of CD3 as compared to the isotype control and NaCl ($p < 0.0001$ and $p < 0.0008$, resp.) (Figures 2(b) and 2(e)). Calprotectin is primarily found in the cytosol of neutrophils and is dramatically upregulated in areas of inflammation,

Table 1: Cytokine and chemokine levels in colon.

pg/100 mg colon[A]	Mice group			Significance	Significance
	SCID healthy control	12p40-mAb	rat-IgG2a	12p40-mAb versus rat-IgG2a	12p40-mAb versus SCID
CCL5	192 ± 72	229 ± 35	493 ± 62	<0.001	0.600
IL-2	182 ± 24	156 ± 19	95 ± 11	0.010	0.430
IL-5	69 ± 15	118 ± 37	31 ± 3	0.020	0.400
IL-9	1,489 ± 180	1,379 ± 194	683 ± 97	0.004	0.730
IL-10	164 ± 30	156 ± 22	91 ± 10	0.010	0.840
IL-13	932 ± 145	763 ± 122	392 ± 52	0.009	0.430
IP-10	926 ± 567	965 ± 180	3,446 ± 606	<0.001	0.930
KC	224 ± 44	167.5 ± 31	440 ± 61	<0.001	0.310
TNF-α	28 ± 5	24 ± 5	60 ± 7	<0.001	0.630

[A]Cytokine and chemokine levels in colon were evaluated by Luminex assay and are depicted as pg per 100 mg colon tissue. Only three of the study groups were included in the analysis (12p40-mAb, rat-IgG2a, and SCID control mice).

whereas it is seldom observed in healthy tissue. Compared to the isotype control and NaCl treatments, mice treated with 12p40-mAb had lower density of calprotectin (or neutrophil inflammation) in the colon ($p < 0.001$ and $p < 0.0045$, resp.) (Figures 2(c) and 2(f)). No significant differences were determined between the control groups.

3.3. Cytokines. Total colonic cytokine/chemokine levels were determined in colon biopsies from mice treated with 12p40-mAb or rat-IgG2a or healthy controls. Several proinflammatory cytokines and chemokines (TNF-α, CCL5, IP-10, and KC) were significantly elevated in mice treated with rat-IgG2a compared to 12p40-mAb treated mice (Table 1). The cytokine/chemokine levels in 12p40-mAb treated mice were not different from the healthy control mice (Table 1). Moreover, mice treated with 12p40-mAb had significantly higher levels of T_H2 (IL-5, IL-9, and IL-13) and the immunosuppressive cytokine IL-10 compared to rat-IgG2a treated mice (Table 1). Thus, different T cell environments may be induced by the two treatment settings.

3.4. Gut Microbiota Composition. Significant changes of 16S rRNA amplicons profiles, based on denaturing gradient gel electrophoresis (DGGE), were observed within gut contents of the NaCl and 12p40-mAb treated mice (Supplemental Figure 1). Since significant differences between the colon contents of 12p40-mAb and rat-IgG2a were also observed, the DNA from colonic contents was subjected to high-throughput sequencing (Illumina MiSeq). The number of reads obtained by sequencing of 16S rRNA gene (V3-V4 region) amplicons from 32 colonic fecal samples was 1,697,168 (mean sequence length of 341 bp). After preprocessing (trimming, quality control, sorting, and chimera filtering), the number of high quality reads obtained was 990,505, whereas the number of sequences per sample varied from 14,534 to 61,423 (average of 30,953 ± 9,561). The estimated average number of observed OTU-species in the 12p40-mAb treated group ($n = 9$), SCID control ($n = 3$), rat-IgG2a treated group ($n = 9$), and NaCl group ($n = 11$) was not significantly different [a reduced number of samples were subjected to sequencing due to limited sequencing capacity; samples were randomly selected and all treatments were represented] (t-test, $p > 0.05$; Supplemental Table 3). Similarly, no differences in Beta Diversity were observed between the 12p40-mAb and SCID control (nonactive disease) or between the isotype and NaCl treated mice (active colitis) (ANOSIM, $p > 0.05$). Principal coordinate analysis of weighted UniFrac distance matrices, however, showed significant separation of the nonactive disease and active colitis mice (ANOSIM, $p < 0.01$, weighted $r = 0.18$; Figure 3). Furthermore, their GM showed changes in the relative abundance of 7 genera (Table 2) [not statistically different after correcting for multiple comparisons] and at the phylum level a significantly lower (t-test, $p > 0.05$) Firmicutes : Bacteroidetes ratio was seen in the colitis active mice (0.30) as compared to the nonactive disease mice (0.83) (however, when only 12p40-mAb and rat-IgG2a were compared no significant differences were observed, putatively due to low number of observations and high intersample variation). Such lower relative abundance of operational taxonomic units (OTUs) assigned to phylum Firmicutes was partially caused by a drop in the relative abundance of Clostridiales from 34.5% to 15.9% (constituted by members of *Ruminococcus*, *Oscillospira*, and an unclassified genus).

3.5. Correlation of Gut Microbiota Composition to Disease Parameters. Seventeen genus-level OTUs correlated either positively or negatively with changes in host parameters (Figure 4 and Supplemental Table 4). Within highly abundant members, *Bacteroides* (with a range in relative distribution of 1.5–89%) showed strong positive correlations with most host parameters (with exception of weight AUC) as well as inverse correlations against IL-2, IL-9, and IL-13. Two unclassified genera of Clostridiales (1.2–63%, unclassified Clostridiales and unclassified Lachnospiraceae), *Oscillospira* (0.1–8.5%), and S24-7 family (3–60%) correlated only negatively with the host parameters, while the latter group correlated positively with IL-2, IL-9, IL-10, and IL-13 (Figure 4). The rest of the OTUs that showed significant correlations did not exceed 15% of relative distribution.

Upon categorization of host parameters (intervals scores) the prevalence of two OTUs at the genus-level, assigned

Table 2: Changes in taxa abundance between active colitis (rat-IgG2a and NaCl) and nonactive disease (12p40-mAb and healthy) determined by ANOVA.

Phylum	Class	Order	Family	Genus	Relative distribution		Significance	
					Active colitis	Nonactive disease	p value	q-value[A]
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	37.19%	11.32%	0.002	0.060
Bacteroidetes	Bacteroidia	Bacteroidales	[Paraprevotellaceae]	Unclassified	0.14%	0.05%	0.004	0.072
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Ruminococcus*	0.47%	3.89%	0.004	0.054
Firmicutes	Clostridia	Clostridiales	Unclassified	Unclassified	13.22%	26.45%	0.010	0.100
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Oscillospira*	2.20%	4.21%	0.029	0.225
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	*Parabacteroides*	0.68%	0.15%	0.044	0.288
TM7	TM7-3	CW040	F16	Unclassified	0.01%	0.02%	0.045	0.249

[A]q-values were determined with the False Discovery Rate correction method.

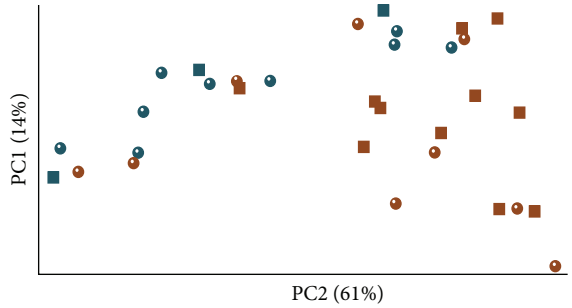
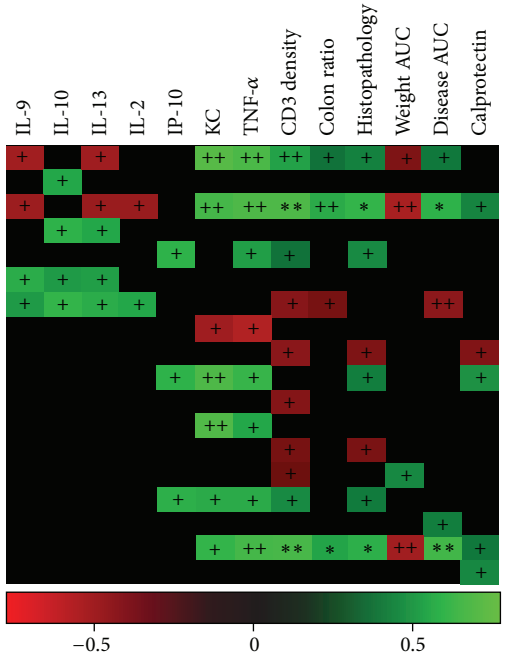


Figure 3: Beta Diversity analysis separates nonactive disease [light-blue] (12p40-mAb [circles] and healthy [squares]) from active colitis mice [orange] (rat-IgG2a [circles] and NaCl [squares]). PCoA plot (unscaled) was determined through weighted UniFrac metrics (ANOSIM, $p < 0.01$, $r = 0.18$) of the colonic microbiota from 32 mice. The ellipsoids highlight the degree of variation around each sample.

to *Akkermansia* (relative abundance of 0–7.8% and ≤0.001–11.6% as determined by Illumina sequencing and species-specific qPCR, resp.) and an unclassified member of the RF32 order (0.1 to 9.2%, Alphaproteobacteria related to *Rhodospirillum rubrum*), was associated (G-test, $p < 0.05$) with high histopathology scores (Figure 5). Furthermore, species-specific qPCR confirmed that an increase in relative abundance of *Akkermansia muciniphila* was associated with high scores of the same host parameter (Figure 5). Likewise, the prevalence of both bacteria was significantly associated also with high scores of CD3 density (scores > 2.7), disease AUC (scores > 39.5), and calprotectin (scores > 0.15). Additionally, prevalence of RF32 order was also observed in high scores of colon ratio (>33.7).

4. Discussion

Several studies support a causal role of a dysfunctional mucosal barrier in the manifestation of IBD. Recent findings have shed light on the mechanisms by which intestinal epithelial cells, microbiota, and immune cells interact and react in such an environment and how loss of normal regulatory processes may lead to IBD [17–20]. We have focused on the regulation of GM following treatment with a monoclonal antibody against IL-12p40 in an AdTr-colitis mouse model, in which IBD development was clearly prevented by the treatment. Most clinical and pathological signs in treated mice were either clearly reduced or not apparent at all. In other words, mice subjected to both AdTr-colitis and 12p40-mAb treatments appeared similar to SCID control mice, whereas mice treated with the isotype antibody developed severe signs of IBD. Comparable results have been achieved by others in the DSS model [36]. Changes in GM seem to be a shared primary phenomenon in both IBD patients and AdTr-colitis mice, and our results show that treatment with 12p40-mAb restores the GM composition, that is, similar to SCID mice. In addition, the colonic cytokine response with increased levels of IL-5, IL-9, IL-10, and IL-13 clearly shows that it induces a T_H2 response, which is expectable when IL-12/IL-23 is prevented from inducing a T_H1/T_H17 profile. In contrast to what has been observed in CD patients [37] and the mice treated with the isotype antibody, rodents subjected to the 12p40-mAb treatment had a higher ratio of Firmicutes : Bacteroidetes. Furthermore, in CD patients a reduction in bacterial diversity as well as lower relative distribution of bacteria with anti-inflammatory properties has been reported [18, 38]. In our study, however, the overall diversity of the GM was not changed by the treatment. Whether dysbiosis or even specific bacterial strains can be future surrogate markers for inflammation, incomplete disease control, relapse, and response remains uncertain. However, caution should be taken when extrapolating from mouse to human. On the other hand, the increased Firmicutes : Bacteroidetes ratio in mice treated with 12p40-mAb was mainly due to the fact that the treated mice had higher proportion of Clostridiales members, many of which correlated inversely to clinical symptoms and TNF-α levels. *Clostridium* spp. are strong inducers of Foxp3-positive regulatory T cells in the colonic mucosa of mice [39], and transfer of a mixture of 17 human *Clostridium* spp. or related bacteria to mice also increased their level of regulatory T cells [40]. This fits nicely with our observation that 12p40-mAb significantly upregulated IL-10 in colon. In our study,



Phylum	Class	Order	Family	Genus
Bacteroidetes	Bacteroidia	Bacteroidales	Paraprevotellaceae	Unclassified
Bacteroidetes	Bacteroidia	Bacteroidales	[Paraprevotellaceae]	[*Prevotella*]
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*
Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	*Prevotella*
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	*Parabacteroides*
Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Unclassified
Bacteroidetes	Bacteroidia	Bacteroidales	S24-7	Unclassified
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	*Lactococcus*
Firmicutes	Clostridia	Clostridiales	Unclassified	Unclassified
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Unclassified
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Unclassified
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	*Coprococcus*
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Oscillospira*
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Ruminococcus*
Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	Unclassified
Proteobacteria	Alphaproteobacteria	RF32	Unclassified	Unclassified
Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	*Sutterella*
Unassigned				

Figure 4: Pearson's correlation of immunological and host parameters with changes in the relative distribution of microbiota members of the colonic content. On top of the heat map the immunological and host parameters are depicted. The lineages are marked on the right and are represented by five major taxonomic ranks. Symbols describing significant correlations were defined as follows: for p value, + ≤ 0.05 and ++ ≤ 0.01. For q-value (False Discovery Rate correction), ∗ ≤ 0.05 and ∗∗ ≤ 0.01. A matrix containing the r-values for every significant correlation is shown in Supplemental Table 4.

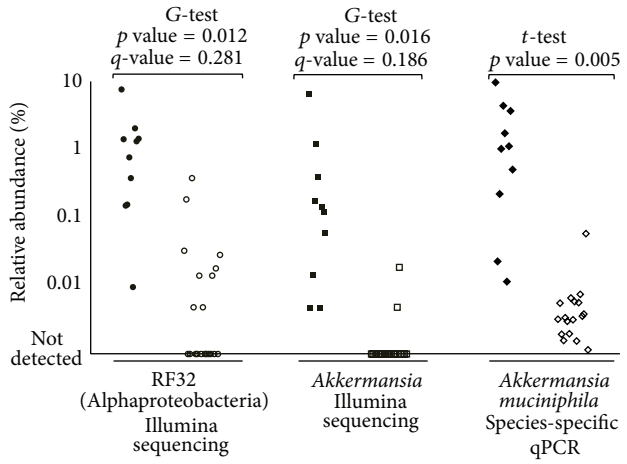


Figure 5: Bacterial prevalence in mice with high and low histopathology scores. Filled marks illustrate scores between 6 and 24, while empty marks illustrate those between 0 and 2. Samples in which RF32 (Alphaproteobacteria), *Akkermansia* (Illumina sequencing), and *A. muciniphila* (species-specific qPCR) were detected are represented by their relative distribution. Samples that were negative (no bacteria detected) are depicted at the no detection line. q-values were determined with the False Discovery Rate correction method.

an unclassified genus of Clostridiaceae family correlated positively to clinical symptoms, presence of effector cells and proinflammatory cytokines, but this is not necessarily in conflict with the other observations, as there are also a number of Clostridiales families in mice, which are known to induce inflammation [41].

Mice treated with 12p40-mAb had lower relative abundance of *Bacteroides*, and we observed a positive correlation between the abundance of *Bacteroides* with clinical symptoms and TNF-α levels. This is in accordance with the fact that both *B. vulgatus* [42] and *B. fragilis* [43] are known to induce intestinal inflammation in rodent models. It is of interest to note that the abundance of *Prevotella* correlates positively with IL-10 levels. In the DSS model they are known to increase severity of IBD [44, 45], but it is difficult to compare the DSS model with the AdTr model in this aspect as only few of the IL-10 producing cells are present in the recipient SCID background, and thus, their IL-10 levels will be different at baseline. It is well known that shifts in GM may lead to a change in the $T_H1/T_H2/T_H17$ balance, which again may prevent symptoms of T_H1/T_H17 induced diseases such as CD. It, however, seems to be novel that an anti-T_H1/T_H17 treatment, such as 12p40-mAb, also leads GM towards an anti-inflammatory direction. In our controlled setup, correlations between mice GM members and biological markers were significantly associated, but this is seldom the case in humans [18]. On the other hand some mechanisms seem to be shared despite this lack of heterogeneity.

Surprisingly *A. muciniphila* correlated with high expression of clinical IBD symptoms as determined by histopathology. *A. muciniphila* is a mucin-degrader with high mucinase

activity [46], which accounts for more than 1% of the bacterial cells in human feces [47]. Extracellular vesicles of *A. muciniphila* protect against DSS induced IBD in mice [48], and its prevalence is decreased manyfold in patients with either UC or CD [49]. Nevertheless, one possible explanation of our results is that intestinal inflammation may induce higher production of mucin that would primarily be degraded by *A. muciniphila* [50]. In the azoxymethane/DSS model of colon cancer *A. muciniphila* also seems to correlate to an increased number of colon tumors [51]. The prevalence of Alphaproteobacteria was found to be associated with high histopathological scores, they have previously been reported to be highly prevalent in fecal microbiota of UC patients [52], and their role in IBD pathogenesis seems to be associated with proinflammatory changes and GM dysbiosis [53].

5. Conclusions

Here we report that 12p40-mAb treatment in an AdTr-colitis mouse model of colitis leads to GM changes and that specific GM composition and members correlate with histopathological changes and cytokine responses. In addition, increasing the proportion of a number of Clostridiales members seems to be associated with prevention and attenuation of colitis symptoms. Thus, our results encourage the search for biomarkers based on GM and prevention or correction of dysbiosis as a potential treatment in IBD.

Abbreviations

IBD:	Inflammatory bowel disease
UC:	Ulcerative colitis
CD:	Crohn's disease
12p40-mAb:	Rat anti-mouse IL-12/23p40 monoclonal antibody
IHC:	Immunohistochemical
DAI:	Disease activity index
MEICS:	Murine endoscopy index of colitis severity
GM:	Gut microbiota
OTU:	Operational taxonomic unit
SCID:	Severe combined immunodeficiency
AdTr-colitis:	Adoptive transfer colitis.

Conflict of Interests

Peter Helding Kvist, Bodil C. Søndergaard, and Thomas Lindebo Holm work for Novo Nordisk A/S.

Authors' Contribution

Josué Castro-Mejía and Maja Jakesevic contributed equally to the study. Josué Castro-Mejía and Maja Jakesevic drafted and wrote part of the paper. Josué Castro-Mejía carried out preparation of sequencing-libraries, qPCR experiments and analyzed the high-throughput sequencing dataset. Bodil C. Søndergaard and Peter H. Kvist performed histology and immunohistochemistry experiments. Maja Jakesevic performed DGGE and collected the microbiome samples. Łukasz Krych participated in molecular experiments and preprocessing of the high-throughput sequencing data. Lars H. Hansen carried out DNA sequencing and helped to draft the paper. Thomas L. Holm performed the animal study and supported clinical assays including cytokine/chemokine analysis. Dennis S. Nielsen, Axel K. Hansen, and Thomas L. Holm conceived the study and experimental design and drafted and wrote parts of the paper. All authors read the paper, provided critical inputs, and approved the final version of the paper.

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Review Article

Probiotics and Alcoholic Liver Disease: Treatment and Potential Mechanisms

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Despite extensive research, alcohol remains one of the most common causes of liver disease in the United States. Alcoholic liver disease (ALD) encompasses a broad spectrum of disorders, including steatosis, steatohepatitis, and cirrhosis. Although many agents and approaches have been tested in patients with ALD and in animals with experimental ALD in the past, there is still no FDA (Food and Drug Administration) approved therapy for any stage of ALD. With the increasing recognition of the importance of gut microbiota in the onset and development of a variety of diseases, the potential use of probiotics in ALD is receiving increasing investigative and clinical attention. In this review, we summarize recent studies on probiotic intervention in the prevention and treatment of ALD in experimental animal models and patients. Potential mechanisms underlying the probiotic function are also discussed.

1. Introduction

Chronic alcohol consumption is a major cause of liver injury. Alcoholic liver disease (ALD) encompasses a broad spectrum of stages including fatty liver, inflammation, fibrosis, cirrhosis, and even hepatocellular carcinoma [1]. Although almost all heavy drinkers develop hepatic steatosis, only a small portion progress to advanced liver diseases. Despite many years of extensive research, the cellular and molecular mechanisms underlying the progression of ALD are not fully understood.

Abstinence is likely the best choice for management of ALD in subjects with early disease stages [2]. Classic treatment of ALD includes nutritional support, corticosteroids, and a phosphodiesterase and TNF-α (tumor necrosis factor-alpha) inhibitor (pentoxifylline), based on disease severity and other complications [3–5]. Recently, targeting the inflammatory response has received substantial investigative attention. The immunosuppressive drug, prednisolone, and interleukin-22 have been tested in animals and patients with ALD [6, 7]. However, despite intensive studies in the last two decades, there are still no FDA-approved therapies for the treatment of ALD.

The liver acts as the major organ in alcohol metabolism. The oxidative pathway of alcohol metabolism mediated by alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH) generates large amounts of acetaldehyde, which is considered to be the key toxin in alcohol-mediated liver injury [8, 9]. The oxidation of alcohol can also occur via cytochrome P450 2E1 (CYP2E1), which causes tissue injury by the production of reactive oxygen species (ROS) [10, 11].

Although alcohol is mainly metabolized in the liver, it is well known that alcohol consumption causes gut lumen bacterial overgrowth and dysbiosis, intestinal mucosal damage, and increased intestinal permeability, leading to increased translocation of bacteria and their products, endotoxin (mainly lipopolysaccharide, LPS), into the portal circulation. Bacteria and their products stimulate the production of ROS and proinflammatory cytokines and chemokines, resulting in damage to liver cells and the development of liver injury [12, 13]. Gut bacteria-derived endotoxin acts through pattern recognition receptors such as toll-like receptors (TLRs) which

are expressed in liver resident macrophages, Kupffer cells, as well as other cell types in the liver. The major endotoxin, LPS, is derived from the cell walls of Gram-negative bacteria in the gut lumen and recognizes TLR4 and its coreceptors, CD14 and MD2, in the liver when penetrating the intestinal barrier and entering into blood stream. Deficiency in the TLR4 complex, such as mutation of TLR4 and lack of CD14 and/or MD2, protects mice from alcohol-induced liver injury. It has been widely demonstrated that alcohol consumption induces endotoxemia [14, 15]. These observations suggest that gut bacteria homeostasis, intestinal barrier integrity, and hepatic TLRs are important in the pathogenesis of ALD.

Therefore, approaches targeting this gut-liver axis may be useful for treating/preventing ALD. In this review, we briefly summarize the recent studies using probiotic intervention for ALD in patients and animal models.

2. ALD: Intestinal Dysbiosis and Leaky Gut

Intestinal dysbiosis is defined as an imbalance of the various microbial entities in the intestine with a disruption of symbiosis [16]. Both chronic and acute alcohol consumption lead to bacterial overgrowth and dysbiosis in both the small and large intestine in experimental animals [10, 16–18]. In a rat model of ALD using intragastric gavage feeding, Mutlu and colleagues showed that alcohol ingestion did not change microbiota composition in 4–6-week feeding but significantly altered the mucosa-associated microbiota in the colon after 10-week feeding [14]. A recent study by Yan et al. further demonstrated that 3-week alcohol ingestion in mice led to bacterial overgrowth in the proximal small intestine and dysbiosis, which was associated with the suppression of antimicrobial peptides, Reg3b and Reg3g. The authors also observed an increase in Bacteroidetes and Verrucomicrobia abundance and a decrease in Firmicutes level in alcohol-fed mice. Interestingly, an overgrowth of *Akkermansia muciniphila* was observed in alcohol-fed mice, and this is believed to be responsible for mucin degradation. Moreover, the population of *Lactobacilli* was depleted in alcohol-fed mice [19]. More recently, using metagenomics-based techniques, we observed a decline in the abundance of both Bacteroidetes and Firmicutes phyla, with a proportional increase in the Gram-negative Proteobacteria and Gram-positive Actinobacteria phyla. Genera analysis showed the greatest expansion in Gram-negative alkaline tolerant *Alcaligenes* and Gram-positive *Corynebacterium*. These alterations were accompanied by the changes in colonic pH and liver steatosis [17]. Canesso and colleagues studied the intestinal bacteria composition in germ-free mice and conventional mice after acute alcohol ingestion [18]. This 7-day treatment of alcohol in the drinking water caused a bacterial overgrowth and dysbiosis in conventional mice. Germ-free mice had less fat in the liver after alcohol feeding compared to conventional mice. Moreover, transplantation of intestinal contents from conventional mice to germ-free mice induced inflammation in both intestine and liver.

Intestinal dysbiosis and bacterial overgrowth have also been studied in human alcoholic subjects [20–25]. In 1984, Bode and coworkers found that the bacterial population was increased in the jejunum of alcoholics compared with hospitalized control patients [20]. These same investigators further showed a higher prevalence of small intestine bacterial overgrowth in chronic alcoholics compared to controls using a breath test [21]. These observations were later confirmed by other groups [22, 23]. Recently, Bajaj et al. studied intestinal bacterial composition in 244 alcoholic cirrhotic patients and 25 age-matched controls. Using an index, cirrhosis dysbiosis ratio (CDR, a low number indicating dysbiosis), the authors found that intestinal dysbiosis was more severe in decompensated cirrhotics compared to compensated cirrhotics [24]. Using the lactulose breath test, Gabbard and coworkers observed that moderate drinking was a strong risk factor for small intestine bacterial overgrowth [25].

Recently, Mutlu and colleagues investigated the mucosa-associated colonic microbiome in alcoholics with and without cirrhosis and in controls. Pyrosequencing analysis of colon biopsy samples revealed that mucosa-associated bacteria were persistently altered in a subset of alcoholics, and this was correlated with endotoxemia [26]. This clinical study confirmed the authors' preclinical observation in mice [14].

Taken together, as yet, there is no specific intestinal bacterial pattern identified that has an ethologic role in the development of ALD. However, that fact that alcohol consumption causes bacterial overgrowth and dysbiosis provides an opportunity for the treatment and/or prevention of ALD by targeting intestinal microbiota to prevent dysbiosis and bacterial overgrowth.

3. Probiotics and Prebiotics

Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host", according to the FAO/WHO definition [27]. The beneficial effects of probiotics have been widely investigated in multiple animal models and clinical studies of a variety of disease conditions in the gastrointestinal system such as inflammatory bowel disease, nonalcoholic steatohepatitis (NASH), cirrhosis, and ALD [28, 29]. Ideal probiotic strains for this kind of application should be resistant to bile, hydrochloric acid, and pancreatic juice; be able to tolerate stomach and duodenum conditions and gastric transport; and have the ability to stimulate the immune system, thereby improving intestinal function via adhering to and colonizing the intestinal epithelium. In addition, probiotic strains must be able to survive during manufacture and storage in order to exert considerable healthful outcomes [30]. Currently, the most often used probiotics are *Bifidobacteria,* lactic acid bacteria (LAB), *Propionibacteria*, yeasts (*Saccharomyces boulardii*), and the Gram-negative *Escherichia coli* strain Nissle 1917. *Lactobacilli,* major contributors to the LAB group, are frequently used probiotics. Various species and strains of *Lactobacilli* have been used in the practice in animals and humans, including *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, and *Lactobacillus helveticus*. Most of these species belong to the phylum Firmicutes. *Bifidobacterium*, which produces lactic acid, is another commonly used probiotic genus and belongs to the Actinobacteria phylum. To date, a large number of probiotics

have been reported to be suitable for the treatment of a variety of diseases, and this number is still growing.

Unlike probiotics, prebiotics, which have also been frequently used for disease treatment, are not live bacteria but rather nondigestible carbohydrates. Prebiotics serve as an energy source for "good" bacteria and stimulate the growth and activities of specific bacteria in the gut [19]. The major fermentation products of prebiotics metabolism are short-chain fatty acids (SCFAs), including acetate, propionate, and butyrate. In particular, butyrate has been recognized as a beneficial metabolite associated with many biological functions in the gut. One of the important functions of butyrate is its ability to regulate gene expression through epigenetic mechanisms [31]. Butyrate enhances cell proliferation and inhibits cell apoptosis in normal cells, but not in the transformed cells [32]. A combination of probiotics and prebiotics (synbiotics) is also used in clinical practice and animal models of diseases.

4. Probiotics Treatment/Prevention of Experimental ALD

Probiotics are used in experimental animals and, to some extent, in humans, to modulate gut microbial homeostasis and to manage liver diseases including cirrhosis with hepatic encephalopathy, nonalcoholic fatty liver disease (NAFLD), and ALD. A PubMed search using key words "probiotics and alcoholic liver disease" generated 20 publications that described studies using probiotics for management of ALD. Of these, 14 publications were experimental animal studies using several models of ALD (Table 1), including chronic alcohol exposure, single dose acute alcohol exposure, multiple dose alcohol exposure, and alcohol exposure plus LPS challenging. A variety of probiotic strains have also been used, such as *Lactobacillus rhamnosus* GG, *Lactobacillus acidophilus*, *Lactobacillus helveticus*, *Bifidobacterium*, *VSL#3*, heat-killed *Lactobacillus brevis* SBC8803, and *Lactobacillus rhamnosus* GG supernatant.

Among those, *Lactobacillus rhamnosus* GG (LGG) is the most frequently used strain. LGG is a Gram-positive bacterial strain of the *Lactobacillus rhamnosus* species that was isolated in 1983 by Barry R. Goldin and Sherwood L. Gorbach [33]. In several models of ALD in rats and mice, LGG administration showed significant protective effects. LGG reduced plasma endotoxin level, improved liver enzymes alanine transaminase (ALT) and aspartate transaminase (AST), and reduced hepatic steatosis and injury.

Nanji and coworkers were one of the earliest groups demonstrating the effectiveness of LGG in experimental ALD [34]. LGG was administrated to Wistar rats at 10^{10} CFU and reduced alcohol-induced endotoxemia and liver injury. In another study, a combination treatment using *Lactobacillus acidophilus*, *Lactobacillus helveticus*, and *Bifidobacterium* in rats with alcohol pancreatitis-related liver damage effectively protected against endotoxin/bacterial translocation, as well as liver damage in the course of acute pancreatitis and concomitant heavy alcohol consumption [35]. Additional studies using LGG in rats demonstrated reduced alcohol-induced gut leakiness, oxidative stress, and inflammation in both intestine and liver [36] and improved intestinal dysbiosis [14]. Another frequently used probiotic mixture, VSL#3, was shown to be effective in modulating gut microbiota and protecting against alcohol-induced intestinal barrier dysfunction [37].

Recently, our group fed mice with the Lieber-DeCarli liquid diet containing 5% alcohol for 8 weeks to produce hepatic fatty liver and injury. These mice were treated with LGG culture broth at 10^9 CFU (Colony Forming Unit) for the final 2 weeks along with continued chronic alcohol administration. LGG supplementation reversed established alcoholic hepatic steatosis and injury [38]. This beneficial effect was associated with a reduction in circulating LPS and improved intestinal barrier function mediated, at least in part, by intestinal hypoxia-inducible factor- (HIF-) modulated mucus layer regulation.

5. Probiotics Treatment in Patients with ALD

While many reports have studied the effects of probiotics in experimental ALD, clinical trials are limited (Table 2). Stadlbauer and coworkers evaluated the effectiveness of the probiotic *Lactobacillus casei* Shirota on alcoholic cirrhosis (AC) patients ($n = 12$) and healthy controls ($n = 13$) in a small open-labeled study [39]. Compared to control group, cirrhotic patients who received the probiotics for 4 weeks had a significantly lower TLR4 expression and Il-10, sTNFR1 (soluble TNF receptor), and sTNFR2 levels, along with a restored neutrophil phagocytic activity, suggesting that the probiotic is safe and may be effective in the treatment of patients with defective immunity. In a brief report, Loguercio et al. [40] showed that treatment with a synbiotic mixture of different bacteria strains and a prebiotic in 10 AC patients, who were all persistent alcohol users with a median daily intake of pure ethanol of 150 g, significantly improved liver damage and function compared to basal values. Patients were treated with the synbiotic for 2 months, followed by 1 month of a washout period. The ALT and γGT (Gamma Glutamyl Transferase) levels were slightly, but not significantly, increased after the washout period. These results indicate that the effects of synbiotic treatment partially persisted beyond the end of treatment. The same group [41] also reported that a commonly used probiotics mixture, VSL#3, was beneficial in liver disease. This open study involved 22 NAFLD and 20 alcoholic cirrhosis (AC) patients and 36 hepatitis C virus-(HCV-) positive patients with and without liver cirrhosis for comparison. VSL#3 treatment significantly improved plasma levels of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) in NAFLD and AC patients, but cytokines (TNF-α, IL-6, and IL-10) improved only in AC patients. More recently, Dhiman et al. [42] reported that probiotic VSL#3 treatment reduced liver disease severity and hospitalization in a double-blind trial in patients with cirrhosis including AC ($n = 89$, 46 probiotics, 43 placebos; patients who had alcohol using history in the previous 6 weeks were excluded). Lata and colleagues [43] showed in a double-blind, randomized study that treatment with the probiotic *Escherichia coli* Nissle for 42 days in 34 cirrhosis patients (19 on probiotics; 15 on placebo) who had an alcoholic etiology of their cirrhosis improved

Table 1: Probiotics application in experimental ALD.

Animal	Alcohol feeding model	Probiotics/(prebiotics) treatment	Effect	Mechanism based on the study	Reference
Rat	Liquid diet containing ethanol and corn oil for 1 month	A daily bolus of *Lactobacillus rhamnosus* GG at 10^{10} CFU/mL for 1 month	Improved liver pathology score and lowered plasma endotoxin level	Prevention of endotoxemia, improved barrier and immune function	[34]
Rat	15 g/kg/day ethanol consumption for 2 weeks	Liquid diet through an intragastric tube containing *Lactobacillus acidophilus*, *Lactobacillus helveticus*, and *Bifidobacterium* pretreatment for 1 week	Normalized AST/ALT levels, improved liver histological score, and lowered plasma endotoxin level	Prevention of endotoxemia, improved barrier and immune function	[35]
Mouse	Lieber-DeCarli diet (5% EtOH, w/v) for 4/5 weeks	Heat-killed *Lactobacillus brevis* SBC8803 orally administered at 100/500 mg/5 mL/kg/day for 5 weeks	Reduced serum ALT and AST, TG, and liver total cholesterol	Reduction of gut-derived endotoxin through induction of heat shock protein	[52]
Rat	Gavage with gradually increased ethanol concentration to 8 g/kg/day in 10 weeks	Gavage with *Lactobacillus rhamnosus* GG at 2.5×10^7 CFU/mL/day or oats 10 g/kg/day for 10 weeks	Normalized colonic microbiota composition, reduced hepatic steatosis, and improved alcoholic steatohepatitis	Prevention of colonic mucosa-associated dysbiosis, reduction of oxidative stress in intestine and liver	[14, 36]
Mouse	Lieber-DeCarli liquid diet (5% EtOH, w/v) for 8 weeks	Culture broth of *Lactobacillus rhamnosus* GG added to the diet at 10^9 CFU/mouse/day for the last 2 weeks	Reduced plasma ALT, endotoxin level, liver steatosis, and inflammation	Increasing HIF-mediated mucosal protecting factors and tight junction proteins, positive modification of gut microflora, reduction of endotoxemia, and desensitization of macrophage to endotoxin	[17, 38, 71]
Mouse	Acute binge, one dose of 6 g/kg ethanol	Gavage with *Lactobacillus rhamnosus* GG supernatant at a dose of equivalent to 10^9 CFU/mouse/day pretreatment for 5 days	Reduced liver enzymes, hepatic steatosis, hepatic ROS, and serum endotoxin level	Increasing HIF-mediated mucosal protecting factors and intestinal tight junction proteins	[53]
Rat	3 doses of 5 g/kg ethanol administration every 12 hours	Intragastric feeding of VSL#3/heat-killed VSL#3 at 0.6 g/kg body wt. pretreatment for 30 min	Lowered plasma endotoxin level	Regulation of the ecological balance of gut microbiota, prevention of TNF-α, decreasing epithelial permeability, and increasing tight junction proteins—ZO-1, occludin	[37]
Mouse	Multiple doses of 5 g/kg/day ethanol, plus multiple LPS for a total of 11 weeks	Intragastric feeding of *Lactobacillus rhamnosus* R0011 and *Lactobacillus acidophilus* R0052 (1 mg/mL/day) for the last 2 weeks	Improved hepatitis activity, increased body weight	Modulation of the gut-liver axis: reduction of ALT, TLR4, TNF-α, and IL-1β expression; increasing IL-10 expression	[67]
Mouse	Lieber-DeCarli liquid diet (5% EtOH, w/v) for 4 weeks	Liquid diet containing *Lactobacillus rhamnosus* GG supernatant at a dose of equivalent to 10^9 CFU/mouse/day pretreatment for 4 weeks	Reduced hepatic steatosis and inflammation, reduced endotoxemia; normalized fatty acid levels in mouse liver and feces	Restoration of occludin in ileum mediated by the inhibition of miR122a expression, increasing hepatic AMPK activation, and inhibition of hepatic apoptosis; increasing intestinal and decreasing hepatic fatty acid and increasing amino acid concentration	[54–56]

Table 2: Probiotics in ALD—clinical evidence.

Disease	Treatment and duration	Observations	Reference
Alcoholic cirrhosis patients, $n = 10$	VSL#3 treatment for 3 months	Reduced plasma ALT, AST, and GGT levels; normalized plasma TNF-α, IL-6, and IL-10 levels; and decreased MDA, 4-HNE, and S-NO levels	[40]
Alcoholic cirrhosis patients, $n = 20$	*Lactobacillus casei* Shirota for 4 weeks of treatment	Normalized phagocytic capacity, decreased TLR4, sTNFR1, sTNFR2, and IL10 levels	[41]
Alcoholic cirrhosis patients, $n = 34$	*Escherichia coli* Nissle for 42 days of treatment	Improvement in intestinal colonization, restored microflora in feces, and reduced endotoxin levels in blood	[43]
Alcoholic cirrhosis patients, $n = 12$	A mixture of different lactic acid bacteria strains treated for 2 months	Positive effects on ecological balance of enteric commensals, reduced ALT, γ-GT, and TNF-α levels	[39]
Patients with alcoholic psychosis and liver disease, $n = 66$	*Bifidobacterium bifidum* and *Lactobacillus plantarum* 8PA3 for 5 days of treatment	Increased numbers of both *Bifidobacteria* and *Lactobacilli*; reduction in ALT, AST, GGT, LDH, and total bilirubin	[23]
Alcoholic and nonalcoholic cirrhosis and hepatic encephalopathy patients $n = 89$	VSL#3 treatment for 6 months	Reduced risk of hospitalization for HE (hepatic encephalopathy), improved CTP (Child-Turcotte-Pugh) and MELD (model for end-stage liver disease) scores	[42]

colonic colonization and liver function. In an open-labeled, randomized study which involved 66 patients who were diagnosed with alcoholic psychosis and liver disease as well as 24 matched healthy controls, Kirpich et al. [23] demonstrated that, after 5 days of treatment with *Bifidobacterium bifidum* and *Lactobacillus plantarum* 8PA3, mild alcoholic hepatitis patients had a significant end-of-treatment reduction of ALT and AST, lactate dehydrogenase, and total bilirubin. Compared to standard therapy, probiotic treatment significantly reduced serum ALT. This liver function improvement was associated with changes in the fecal commensal bacteria *Bifidobacteria* and *Lactobacilli*.

Taken together, clinical studies suggest that targeting the gut-liver axis through the use of probiotics may have a therapeutic role in the treatment of patients ranging from those with mild alcoholic hepatitis to those with severe alcoholic cirrhosis. As noted, further studies with larger sample sizes for testing the effects of probiotics on ALD are needed. Developing novel probiotic strains and related products, including isolating new probiotic bacteria with improved potency for inhibiting pathogenic bacterial growth, strengthening intestinal barrier function, and improving immunoregulation, and engineered probiotic bacteria producing specific metabolites, will provide more selectivity for treating ALD patients at different disease stages.

Accumulating evidence demonstrates the protective effect of probiotics on multiple pathological disorders. However, these treatments are not always effective because, in many cases, live bacteria must colonize the gut to confer their beneficial effects. The spectrum of pathogenic bacteria varies from patient to patient. Drugs, in particular, antibiotics, used by patients may be harmful to live probiotics. Therefore, an unstable and variable effect of live probiotics may occur. Moreover, the clinically recommended dose of probiotics usually consists of billions of live bacteria. Generally, probiotics are considered safe, but several reports have raised safety concerns about ingesting such large amounts of bacteria, especially when the intestinal function and the patient's immune response are compromised [44–47]. In fact, soluble factors secreted from probiotics and dead probiotics have been used in the treatment of several diseases conditions such as inflammatory bowel disease, colitis, and arthritis [48–50]. Yan et al. demonstrated that soluble proteins produced by probiotic bacteria regulate intestinal epithelial cell survival and growth [51]. Interestingly, the beneficial effects of probiotics on ALD appear to not be restricted to viable probiotic bacteria. Segawa and colleagues demonstrated that oral administration of heat-killed *Lactobacillus brevis* SBC8803 induced the expression of cytoprotective heat shock proteins and improvement of intestinal barrier function leading to amelioration of experimental ALD [52]. Recently, we evaluated the effectiveness of LGG culture supernatant in the prevention of acute and chronic alcohol-induced hepatic steatosis and liver injury [53–55]. Pretreatment with LGG supernatant (LGG-s) reduced hepatic fat accumulation in mice subsequently exposed to acute-binge alcohol [53]. Furthermore, coadministration of LGG supernatant with alcohol in the Lieber-DeCarli liquid diet for 4 weeks significantly prevented alcohol-induced intestinal barrier dysfunction, endotoxemia, fatty liver, and inflammation in mice [54, 55]. The use of probiotic culture supernatant opens a new avenue for the probiotic application. Further characterization of the LGG-s active components will enhance our understanding of the protective effect of probiotics in ALD and advance the development of new therapeutic strategies for ALD.

6. Potential Mechanisms of Probiotics in ALD

Despite many proof-of-effectiveness studies of probiotics on the treatment of both experimental and human ALD,

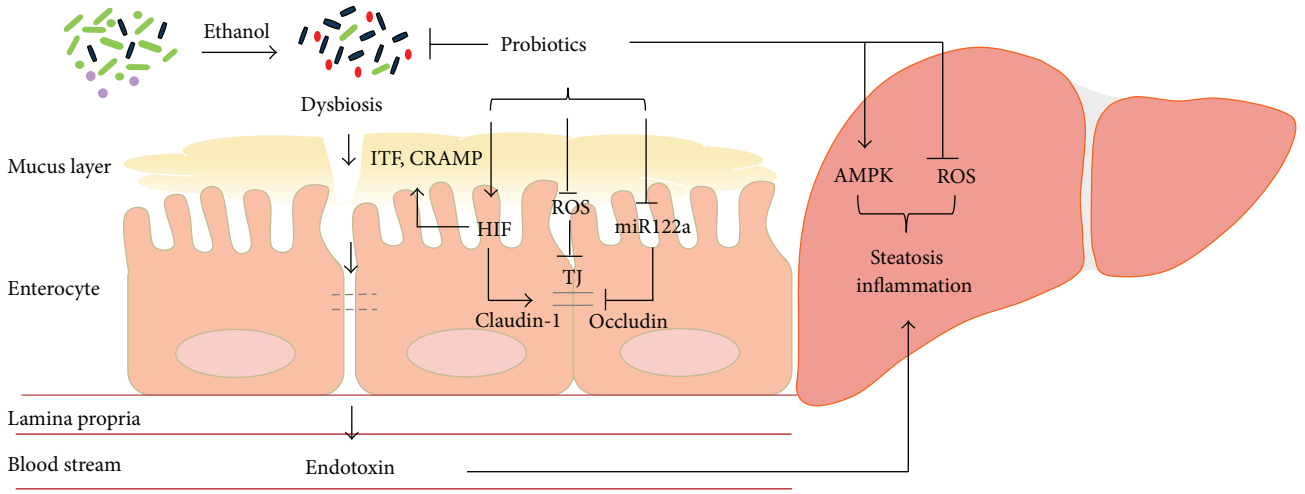

Figure 1: Proposed mechanisms of probiotic function in ALD. Ethanol consumption causes a gut bacterial overgrowth and a dysbiosis leading to impaired mucus layer and dysfunctional tight junctions. The damaged epithelial barrier function results in endotoxemia. Elevated endotoxin activates Kupffer cells in the liver and induces hepatic steatosis and inflammation. Probiotics and related products prevent ethanol-induced effects in the intestine and the liver through multiple mechanisms: (1) positive modification of gut microbiota; (2) reduction of ROS production in intestine and liver; (3) enhancement of mucus layer component, ITF, and antimicrobial peptide, CRAMP, and tight junction protein claudin-1 expression through increased HIF signaling; (4) inhibition of miR122a expression leading to occludin upregulation; and (5) activation of hepatic AMPK.

the mechanisms by which probiotics function are still poorly understood. To date, several important mechanisms including the modification of gut microbiota, improvement of the intestinal epithelial barrier function, regulation of the immune system and inflammation, and alteration of hepatic lipid homeostasis have been proposed. These mechanisms involve gene expression regulation in both intestinal and hepatic tissues. Figure 1 summarizes many of the proposed mechanisms of probiotic function in ALD.

Alterations of gut microbiota have been recognized widely as one of the major mechanisms underlying probiotic function. One of the first studies in rats with ALD showed a dysbiosis in colon lumen contents, which was prevented by probiotic and prebiotic treatment [14]. Several other studies also demonstrated that supplementation with probiotics restored gut microbiota homeostasis and alleviated alcohol-induced liver injury [17, 19, 23, 40, 42, 43]. We have shown that, in mice fed with a 6-week course of alcohol plus 2-week treatment with LGG with continued alcohol intake, the LGG positively modified the alcohol-induced dysbiosis [17]. Chronic ethanol feeding caused a decline in the abundance of both Bacteroidetes and Firmicutes phyla, with a proportional increase in Proteobacteria and Actinobacteria phyla. Gram-negative alkaline tolerant *Alcaligenes* and Gram-positive *Corynebacterium* were the bacterial genera that showed the greatest expansion. In parallel with the qualitative and quantitative alterations in the microbiome, ethanol caused an increase in plasma endotoxin, fecal pH, hepatic inflammation, and injury. Notably, the ethanol-induced pathogenic changes in the microbiome and the liver were prevented by LGG supplementation [17] (Figure 2). Clearly, due to the critical role of microbiota in gut-liver axis, restoration of gut microbiota contributes to the beneficial effects of probiotics in ALD.

One of the major functions of gut bacteria is to metabolize food to produce metabolites that are beneficial (or harmful in the case of harmful bacteria) to the host. In our recent study [56] using a metabolomics approach, we demonstrated that heptadecanoic acid (C17:0), a long chain fatty acid produced only by bacteria, was reduced by alcohol ingestion and increased by probiotic treatment. Interestingly, supplementation of heptadecanoic acid attenuated ALD in mice [57]. Moreover, short-chain fatty acids, which have multiple roles in the intestine including serving as energy source and immunoregulation, were reduced by alcohol and increased by probiotics [58–60]. We also showed that probiotic supplementation normalized the abundance of several amino acids in the liver and in the gut [56]. These results demonstrate that LGG-s attenuates ALD by mechanisms involving increasing intestinal fatty acids and amino acid metabolism.

Gut barrier function and endotoxemia are at the center of gut-liver axis in multiple disease conditions. Probiotic administration has been shown to reinforce the intestinal barrier and reduce endotoxin levels in both NAFLD and ALD. The intestinal epithelial barrier is a complex system composed of cellular, physical, and chemical components [61]. The epithelial cells form a lining with the paracellular space sealed by tight junctions (TJ) and adherens junctions [62], and this is covered by a protective mucin layer that physically blocks most particles from direct contact with the epithelial cells [63]. Alcohol consumption, both acute-binge and chronic, directly affects the gut intestinal barrier at multiple levels including tight junctions, production of mucin, and recruitment and activation of inflammatory cells

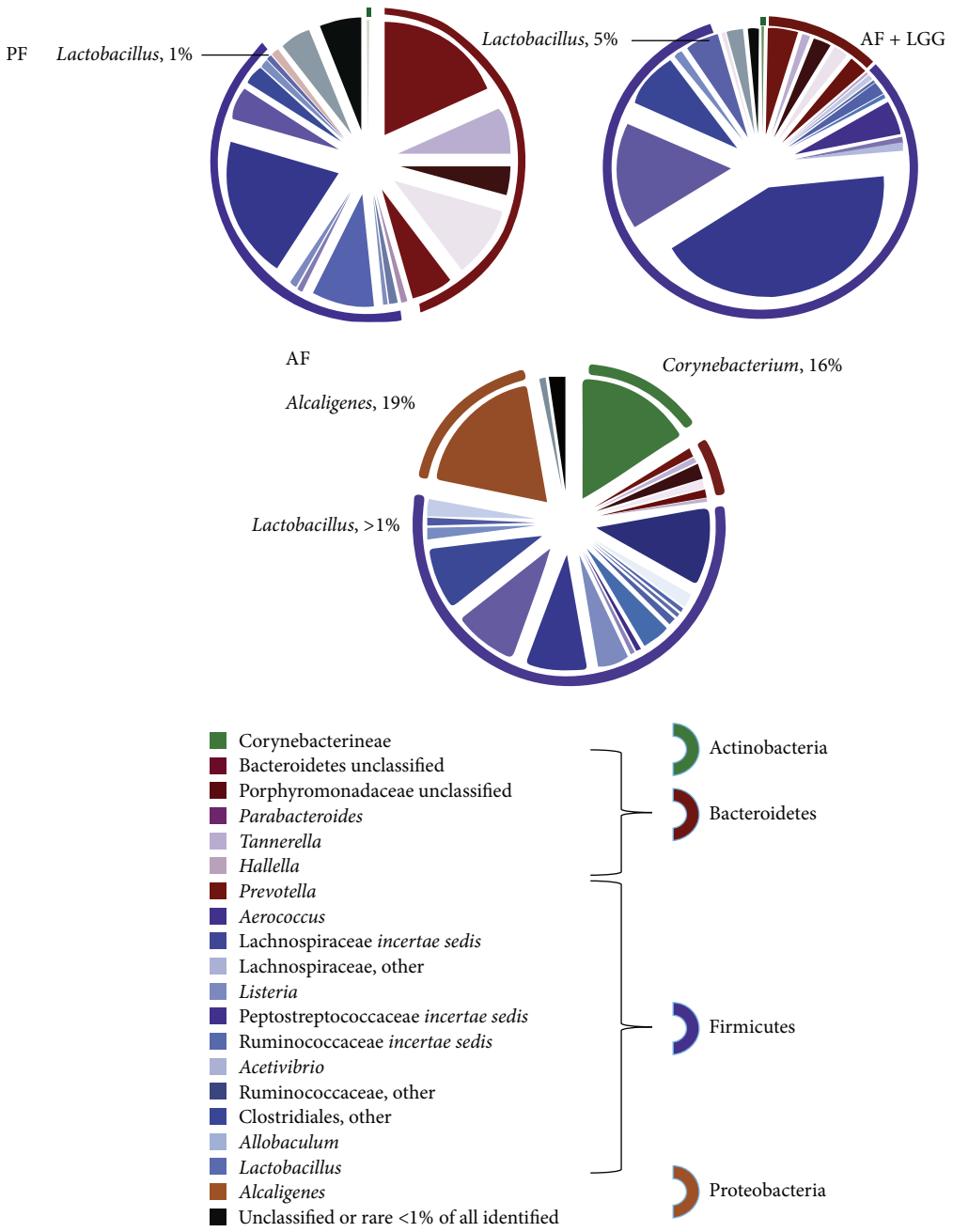

Figure 2: The relative distribution of the bacterial phyla and genera in response to ethanol feeding and LGG supplementation. Mice were fed with Lieber-DeCarli diet containing 5% EtOH or pair-fed with maltose dextrin for 6 weeks. *Lactobacillus rhamnosus* GG was supplemented at a dose of 10^9 CFU/day for the last 2 weeks with continued alcohol feeding. The fecal samples were analyzed by a metagenomic approach. The microbiome of the PF, AF + LGG, and AF mice is shown in the pie charts and color coordinated by genus and phylum. The different shades of color represent the different genera and the common color spectrum (reds, purples, green, and orange) represents the phyla. The outer ring around the pie charts also depicts the different phyla. The microbiome of AF mice is characterized by greater abundance of *Alcaligenes* and *Corynebacterium* and loss of *Tannerella*. The AF + LGG group shows a much greater abundance of *Lactobacillus* and nonspecific Ruminococcaceae *incertae sedis* compared to the other exposure groups (PF: pair-feeding; AF: alcohol feeding; and AF + LGG: alcohol feeding plus *Lactobacillus rhamnosus* GG, adapted from [17]).

to the intestinal wall [64]. Our studies evaluated the effects of probiotics LGG and LGG-s on epithelial cell permeability and severity of hepatic steatosis using *in vivo* (mouse) and *in vitro* (epithelial cell culture) models [38, 53, 55]. Probiotics administration increased the expression of tight junction proteins claudin-1, ZO-1, and occludin at both protein and mRNA levels and normalized barrier function by decreasing intestinal permeability using *ex vivo* measurement in the ileum or transepithelial electrical resistance (TEER) in Caco-2 monolayers. In addition, we also showed that

LGG and LGG-s restored the expression of mucus-related genes including intestinal trefoil factor (ITF), as well as P-glycoprotein (P-gp), and cathelin-related antimicrobial peptide (CRAMP), which were decreased by alcohol ingestion in mice.

How do probiotic bacteria affect gut barrier function? Gut bacteria metabolize ethanol to acetaldehyde by cytochrome P450 2E1 (CYP2E1) that produces a large number of reactive oxygen species (ROS), which could damage intestinal barrier components including mucus layer and tight junctions. A recent study also demonstrated that bacterial metabolism produces endogenous ethanol, which might also have deleterious effects on the gut barrier [65]. Probiotics, therefore, could contribute to intestinal barrier function by modulating certain gut bacteria leading to reduced metabolism of alcohol and ROS production in the intestine. Intestinal inflammatory cells such as mast cells also affect alcohol-induced epithelial barrier dysfunction [66]. Alcohol-induced barrier dysfunction is associated with local and systemic production of proinflammatory cytokines such as TNF-α and IL-1β. Several studies showed that probiotic administration decreased alcohol-induced systemic and intestinal TNF-α and IL-1β levels [37, 52, 67], which might contribute to the beneficial effects of probiotics on gut barrier integrity in ALD.

The intestinal mucosa experiences profound fluctuations in blood flow and metabolism. Alcohol metabolism in the intestine could cause tissue hypoxia that triggers induction of a master transcription factor, hypoxia-inducible factor (HIF). HIF is important for maintaining barrier function by increasing global mucosal protective mechanisms including mucin production and stabilization via regulation of ITF, xenobiotic clearance by P-gp, and various other nucleotide signaling pathways [68]. However, alcohol-induced ROS could damage this compensatory role of HIF leading to barrier dysfunction [38]. LGG administration restored intestinal HIF expression and function in ALD in mice. In addition, the intestinal level of another important HIF target, CRAMP, was decreased by alcohol exposure and increased by LGG-s treatment in mice, implying a potential role of probiotics in the regulation of gut microbiota in ALD [53]. Additional studies reported that antimicrobial proteins Reg3g and Reg3b were downregulated by chronic alcohol exposure, which may contribute to the quantitative and qualitative changes in the gut flora, and prebiotics treatment can partially restore Reg3g levels, leading to decreased intestinal bacterial overgrowth, and ameliorates alcoholic steatohepatitis [19]. A recent study identified one of the major tight junction molecules, claudin-1, as being a HIF transcriptional target suggesting that probiotics may protect the gut barrier directly through the HIF-tight junction axis [69].

Tight junction proteins are regulated by multiple mechanisms. Ye et al. demonstrated that intestinal occludin is a target of microRNA 122a [70]. TNF-α induced an increase in miR122a leading to a reduction of intestinal occludin protein expression. Similarly, alcohol ingestion increased miR122a levels in the intestine. Probiotic LGG-s administration decreased miR122a levels and therefore increased occludin expression [54].

In addition to intestinal mechanisms in ALD, probiotic bacteria also act on the immune system through TLRs. We have shown that two weeks of LGG supplementation reduced hepatic inflammation and markedly reduced TNF-α expression in a murine model of ALD. We also demonstrated that, in an *in vitro* system using human peripheral blood monocytes-derived macrophages, incubation with ethanol primes, both lipopolysaccharide- and flagellin-induced TNF-α production, and LGG-s reduced this induction in a dose dependent manner [71].

In a recent study [55], we further demonstrated that probiotics may function as a direct mediator in regulating hepatic lipid metabolism and apoptotic cell death. LGG-s administration prevented alcohol-increased expression of genes involved in lipogenesis and alcohol-decreased genes involved in fatty acid β-oxidation. Importantly, these lipid regulatory effects were mediated through probiotic action on adenosine-monophosphate-activated protein kinase (AMPK) phosphorylation. LGG-s also decreased Bax expression and increased Bcl-2 expression, which attenuated alcohol-induced hepatic apoptosis. Thus, probiotics likely exert their beneficial effects, at least in part, through modulation of hepatic AMPK activation and Bax/Bcl-2-mediated apoptosis in the liver.

Myosin light-chain kinase (MLCK) is a downstream target of TNF-α. MLCK can be phosphorylated in intestinal epithelial cells after alcohol consumption, thus playing a vital role in regulation of the epithelial barrier integrity. Ma et al. [72] found that ethanol can stimulate MLCK activation and monolayer permeability in Caco-2 cells, which can be effectively inhibited by the MLCK inhibitor, ML-7. A similar finding was demonstrated by Su and coworkers [73] using MLCK intestinal epithelial specifically transgenic (Tg) mice in a colitis model. Tg mice demonstrated significant barrier loss and a more severe form of colitis than controls. Recently, Chen et al. [74] further demonstrated the partial contribution of MLCK to intestinal barrier dysfunction and liver disease after chronic alcohol feeding using MLCK-deficient mice. Whether probiotics exert their beneficial effects through inhibition of MLCK in ALD has not been demonstrated yet, but a newly published study by Sun and coworkers [75] indicated that *Lactobacillus acidophilus* treatment of traumatic brain injury (TBI) mice can efficiently prevent the damage of interstitial cells and improve the terminal ileum villus morphology via decreased MLCK concentration.

7. Conclusion

In conclusion, with the growing body of studies demonstrating that ALD is closely associated with gut microbial alterations and that gut bacteria/bacterial products play an important role in ALD progression, using probiotics for the prevention and/or treatment of ALD continues to attract more investigative and clinical attention. Although increasing numbers of probiotic strains and related products have been identified as being useful in ALD, the precise mechanisms underlying the role of probiotics in regulating gut microbiota, intestinal barrier function, and eventfully alcoholic liver disease need further investigation. It is likely that probiotics work through multiple mechanisms. Specific actions may

be particularly important in specific disease processes and individual people; thus, this may be a unique form of personalized medicine.

Conflict of Interests

No conflict of interests is declared by the authors.

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