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Alcoholic liver disease: The gut microbiome and liver crosstalk

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Abstract

Alcoholic liver disease is a leading cause of morbidity and mortality worldwide. Alcoholic fatty liver disease can progress to steatohepatitis, alcoholic hepatitis, fibrosis, and cirrhosis. Patients with alcohol abuse show quantitative and qualitative changes in the composition of the intestinal microbiome. Furthermore, patients with alcoholic liver disease have increased intestinal permeability and elevated systemic levels of gut-derived microbial products. Maintaining eubiosis, stabilizing the mucosal gut barrier or preventing cellular responses to microbial products protect from experimental alcoholic liver disease. Therefore, intestinal dysbiosis and pathological bacterial translocation appear fundamental for the pathogenesis of alcoholic liver disease. This review highlights causes for intestinal dysbiosis and pathological bacterial translocation, their relationship and consequences for alcoholic liver disease. We also discuss how the liver affects the intestinal microbiota.

Keywords

alcoholic liver disease; microbiome; bacterial translocation; intestinal bacterial dysbiosis; metabolome

Introduction

Alcohol-related liver cirrhosis was responsible for 0.9% of all global deaths and 47.9% of all liver cirrhosis-attributable deaths in 2010 (Hartmann et al., 2012, Rehm et al., 2013). Alcoholic liver disease (ALD) encompasses fatty liver, or hepatic steatosis, and the more serious entities alcoholic steatohepatitis, alcoholic hepatitis, fibrosis, cirrhosis, and liver cancer (Gao and Bataller, 2011). Already 50 years ago, Lieber et al. showed that alcohol-induced hepatic steatosis resolves within several weeks of abstinence (Lieber et al., 1965). In case of continued consumption of alcohol, fatty liver can progress to fibrosis and cirrhosis which can lead to portal hypertension or liver failure (Gao and Bataller, 2011, Liu, 2014). 10% of heavy drinkers will develop alcoholic liver cirrhosis (Levene and Goldin, 2012, Liu, 2014). Alcoholics and subjects with alcoholic liver cirrhosis display higher levels of bacterial products in their blood than healthy humans (Parlesak et al., 2000, Bajaj et al., 2014c). In addition, bacterial infections caused by pathological bacterial translocation increase the mortality in cirrhotic patients

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tremendously. 30% of these patients expire within one month, another 30% die by one year (Arvaniti et al., 2010).

This review provides an overview of causes for intestinal dysbiosis and describes changes in the intestinal microbiome during alcoholic liver disease. We will further discuss the relationship between dysbiosis and the onset of pathological bacterial translocation, as well as their contribution to ALD in animals and humans.

1. Intestinal dysbiosis

The intestine harbors a diverse community of bacteria. Microbial members of this community are beneficial for host metabolism and digestion, thereby creating a symbiotic relationship with the host. Intestinal dysbiosis is defined as an imbalance of the different microbial entities in the intestine with a disruption of symbiosis (McLoughlin and Mills, 2011). Intestinal dysbiosis can present as quantitative (intestinal bacterial overgrowth) and qualitative changes in the intestinal microbiota. It has been associated with ALD both in experimental animal models and patients (Kirpich et al., 2008, Mutlu et al., 2009, Chen et al., 2011, Yan et al., 2011, Mutlu et al., 2012, Hartmann et al., 2013, Leclercq et al., 2014, Chen et al., 2015).

1.1. Intestinal bacterial overgrowth in alcoholic liver disease

Chronic alcohol ingestion leads to small and large intestinal bacterial overgrowth and dysbiosis in animals and humans (Bode et al., 1984, Casafont Morencos et al., 1996, Yan et al., 2011, Hartmann et al., 2013). Intestinal bacterial overgrowth is defined as increased numbers of bacteria in the intestine, in animals most commonly evidenced by quantitative polymerase chain reaction (qPCR) using universal 16S ribosomal RNA bacterial primer sets in cecal samples; alternatively conventional culturing techniques of small and large intestinal contents or fecal samples can be used as well (Adachi et al., 1995, Yan et al., 2011, Hartmann et al., 2013). In humans, it is classically defined as at least 10^5 cultured colony forming units of bacteria per ml from jejunal aspirates (Kerlin and Wong, 1988, Bauer et al., 2000, Simren and Stotzer, 2006). The increase of both aerobic and anaerobic bacteria was most pronounced in the proximal small intestine following intragastric feeding of alcohol in mice (Yan et al., 2011). Large intestinal bacterial overgrowth develops as early as one week after intragastric alcohol feeding (Hartmann et al., 2013), and is also present in end-stage liver disease in rodents (Guarner et al., 1997, Sanchez et al., 2005). Similarly, subjects with moderate alcohol consumption as well as patients with alcoholic liver cirrhosis display small intestinal bacterial overgrowth (Casafont Morencos et al., 1996, Gabbard et al., 2014). Small intestinal bacterial overgrowth correlates well with the severity of the alcoholic cirrhosis (Casafont Morencos et al., 1996). Probiotics are live, non-pathogenic microorganisms promoting the growth of other beneficial microorganisms (Hartmann et al., 2012, Cicenia et al., 2014). Intriguingly, probiotics VSL#3 decrease small intestinal bacterial overgrowth in cirrhotic patients (Lunia et al., 2014). This finding is important since small intestinal bacterial overgrowth has been shown to be a risk factor for the occurrence of hepatic encephalopathy besides the Child-Pugh-Score itself (Lunia et al., 2014). Interestingly, selective intestinal decontamination with antibiotics as prevention and intervention can abrogate large intestinal bacterial overgrowth and alleviate subsequent liver damage in rodents (Adachi et al., 1995, Chen et al., 2014). However, a randomized, double-blind, placebo-controlled clinical trial in patients with alcoholic liver

disease (27 with cirrhosis, 23 without cirrhosis) using the non-absorbable, broad-spectrum antibiotic Paromomycin did not show an improvement in liver damage relative to placebo treated patients (Bode et al., 1997). Since endotoxin was not reduced after 4 weeks of treatment, the antimicrobial treatment might not have effectively reduced intestinal bacterial overgrowth, or the treatment length was not long enough. Another possible explanation could be that antibiotics induce dysbiosis (Cho et al., 2012) and possible pathogenic bacteria have been selected by the treatment with Paromomycin. There are indications that broad spectrum antibiotic treatment alone might decrease the expression of intestinal tight junction proteins (Cresci et al., 2013). Taken together, chronic alcohol abuse results in small and large intestinal bacterial overgrowth that (at least in rodents) represents an attractive target for therapy.

1.2. Qualitative changes in the microbiome

The microbiome consists of several phyla which comprise classes that encompass orders. Orders consist of a number of families that finally comprise certain genera and species of bacteria (see Table 1, 2, and Supplementary Table 1). Gram staining can help to distinguish different phyla from each other: Firmicutes and Actinobacteria are generally Gram-positive, while Proteobacteria, Fusobacteria and Bacteroidetes are usually Gram-negative. However, members of the Firmicutes-class Negativicutes for example are, as the name implies, Gram-negative (Vesth et al., 2013). Therefore, more advanced technologies (e.g. deep pyrosequencing of bacterial 16S rRNA) are used to track microbial changes occurring in the intestine in alcoholic liver disease, as described below.

1.2.1. Qualitative changes in the microbiome in animals following chronic alcohol feeding—Although only a small number of enteric bacteria can be cultured using conventional culturing techniques (Gill et al., 2006, Yan et al., 2011, Hartmann et al., 2013), recent advances such as Length Heterogeneity PCR (LH-PCR) (Mutlu et al., 2009) and deep pyrosequencing of bacterial 16S rRNA (Yan et al., 2011, Chen et al., 2015) helped to explore the gut microbiome further. Alcohol administration for 10 weeks results in colonic dysbiosis in rats, which can be prevented by probiotic and prebiotic feeding (Mutlu et al., 2009). In mice ethanol feeding reduces the phylum Firmicutes (Yan et al., 2011, Bull-Otterson et al., 2013) and the genus *Lactobacillus* spp. within the phylum Firmicutes (Yan et al., 2011, Hartmann et al., 2013) (Table 1). *Enterococcus* spp., also belonging to Firmicutes, increases after alcohol administration (Yan et al., 2011, Campos Canesso et al., 2014). There is evidence that alcohol-treated mice show higher intestinal levels of Verrucomicrobia and one of their genera *Akkermansia muciniphila*, Actinobacteria with their genus *Corynebacterium* spp., and Proteobacteria and their genus *Alcaligenes* spp. (Yan et al., 2011, Bull-Otterson et al., 2013, Hartmann et al., 2013). Several studies in rodents (Yan et al., 2011, Bull-Otterson et al., 2013) and humans (Loguercio et al., 2005, Lata et al., 2007, Kirpich et al., 2008, Dhiman et al., 2014) demonstrate that supplementation with probiotic bacteria alleviates ALD and liver cirrhosis in general. Interestingly, administration of probiotic *Lactobacillus rhamnosus* GG during the last two weeks of a six week alcohol feeding experiment to mice reversed the aforementioned microbial findings so that Actinobacteria and *Corynebacterium* spp., and Proteobacteria and *Alcaligenes* spp., as well as Firmicutes and their genera *Lactobacillus* spp. and Ruminococcaceae *Incertae Sedis* increased significantly relative to mice fed alcohol alone (Bull-Otterson et al., 2013). Supplementation with saturated fatty acids prevents alcoholic liver

injury by restoring levels of Bacteroidetes, Firmicutes and *Lactobacillus rhamnosus* and spp. in the intestine of mice (Chen et al., 2015). Prebiotics, as complex carbohydrates that cannot be digested by the host but can specifically serve "good" bacteria as an energy source, reduce small intestinal bacterial overgrowth and ameliorate experimental alcoholic liver disease in an intragastric feeding model of ethanol (Yan et al., 2011).

1.2.2. Intestinal dysbiosis in alcoholics—Quantitative and qualitative changes in the intestinal microbiota occur in subjects with moderate alcohol consumption, alcoholics and alcoholic cirrhotics (Bajaj et al., 2014c, Gabbard et al., 2014, Leclercq et al., 2014). The term cirrhosis dysbiosis ratio, or CDR (Bajaj et al., 2014c), was proposed to reflect the changes of "good" vs. "bad" bacteria occurring in the intestine of cirrhotic patients. This ratio consists of the amount of the beneficial autochthonous bacteria Lachnospiraceae, Ruminococcaceae and the Clostridiales Family XIV *Incertae Sedis* divided by the amount of the potentially pathogenic taxa Enterobacteriaceae and Bacteroidaceae. It is postulated the lower the CDR, the more advanced is the cirrhosis (Bajaj et al., 2014c). The Lachnospiraceae (Chen et al., 2011, Bajaj et al., 2012b, Bajaj et al., 2014c), the Ruminococcaceae (Bajaj et al., 2012b, Kakiyama et al., 2013, Bajaj et al., 2014c), the Clostridiales Family XIV *Incertae Sedis* (Bajaj et al., 2012b, Bajaj et al., 2014c) are typically found at lower intestinal levels in subjects with at least partly alcohol-related liver cirrhosis, whereas Enterobacteriaceae (Chen et al., 2011, Bajaj et al., 2012b, Kakiyama et al., 2013, Bajaj et al., 2014c) including their prominent genus *Escherichia coli* (Liu et al., 2004) are found at higher levels (Table 2 and Supplementary Table 1). The Bacteroidaceae family showed a trend toward expansion in cirrhotic patients in some reports (Kakiyama et al., 2013, Bajaj et al., 2014c). Other studies showed a decreased abundance of Bacteroidaceae in patients with liver cirrhosis, in particular in alcoholic cirrhotics (Chen et al., 2011, Mutlu et al., 2012, Kakiyama et al., 2014). The CDR is lowest in alcoholic cirrhotic patients compared with cirrhotic subjects of another etiology; similarly, endotoxemia is higher and correlates with the expanding Gram-negative Enterobacteriaceae in these alcoholic patients (Bajaj et al., 2014c). Interestingly, administration of *Lactobacillus rhamnosus* GG to cirrhotic patients for four weeks resulted in an increase in Lachnospiraceae and the Clostridiales Family XIV *Incertae Sedis*, and a decrease in Enterobacteriaceae with an associated reduction of endotoxemia and serum TNF-alpha levels (Bajaj et al., 2014b). *Clostridium* spp. (Zhao et al., 2004, Bajaj et al., 2012a) as well as Enterococcaceae (Bajaj et al., 2014c) and their genus *Enterococcus* spp. (Zhao et al., 2004, Chen et al., 2011, Bajaj et al., 2012a) are found at greater quantities in the stools and colonic biopsy samples from cirrhotic patients. Fecal analysis in these patients also demonstrated a higher abundance of Fusobacteriaceae (Chen et al., 2011, Bajaj et al., 2012b), Staphylococcaceae (Bajaj et al., 2014c) and their genus *Staphylococcus* spp. (Liu et al., 2004). As mentioned above, alcoholics exhibit reduced numbers of the beneficial *Lactobacillus* spp. (Kirpich et al., 2008), and, similarly to cirrhotics, show lower fecal amounts of *Bifidobacterium* spp. (Zhao et al., 2004, Kirpich et al., 2008, Leclercq et al., 2014). Administration of probiotic *Lactobacillus* spp. and *Bifidobacterium* spp. to alcoholics increased levels of intestinal *Lactobacillus* spp. and *Bifidobacterium* spp., and improved liver enzymes (Kirpich et al., 2008). Likewise, mixtures of pre- and probiotics, or synbiotics (Cocktail 2000; Medipharm, Kagerod, Sweden; including *Lactobacillus* spp.), reduced amounts of *Staphylococcus* spp., *Fusobacterium* spp., *E. coli*, increased the abundance of *Lactobacillus* spp., and improved liver function in cirrhotic subjects

(partly due to alcohol) (Liu et al., 2004). Intriguingly, although some microbial changes were non-reversible such as a reduced amount of *Faecalibacterium prausnitzii*, alcohol abstinence alone resulted in a partial restoration of eubiosis. Suppressed levels of *Bifidobacterium* spp., *Lactobacillus* spp., and Ruminococcaceae recovered in alcohol dependent patients (Leclercq et al., 2014). These findings were associated with lower scores of depression, anxiety, and craving after 3 weeks of abstinence as well as a significantly improved intestinal permeability (Leclercq et al., 2014). The abundance of Veillonellaceae (Chen et al., 2011, Kakiyama et al., 2013, Kakiyama et al., 2014) and their genus *Megasphera* spp. (Leclercq et al., 2014) is greater in stools of alcoholics and cirrhotic patients compared with healthy subjects. On the other hand, treatment with Rifaximin causes a reduction of the Gram-negative Veillonellaceae and reduces endotoxemia in partially alcohol-induced cirrhotic subjects (Bajaj et al., 2013). Streptococcaceae seem to expand in patients with liver cirrhosis related to HBV and alcohol (Chen et al., 2011) which gets exacerbated after treatment with proton-pump inhibitors (PPI) (Bajaj et al., 2014a). The reduction of fecal levels of Lachnospiraceae and Ruminococcaceae worsens after PPI treatment, too (Bajaj et al., 2014a). Taken together, dysbiosis occurs after chronic alcohol administration and is commonly associated with a decrease in “good” bacteria. “Good” commensals such as *Lactobacillus* spp. diminish, and possible pathogenic, in this sense “bad” bacteria such as Enterobacteriaceae increase. Preventing dysbiosis or restoring eubiosis (e.g. by supplementing probiotics, prebiotics or synbiotics) appears a valid strategy for treatment of alcoholic liver disease. None of the intestinal bacteria that are induced after chronic alcohol administration was causatively linked to the onset or progression of alcoholic liver disease.

2. Factors contributing to intestinal dysbiosis after chronic alcohol consumption

How can we explain intestinal changes in the microbiota following chronic alcohol consumption? A number of factors might contribute to alcohol-associated dysbiotic changes (Figure 1).

Environmental factors

Environmental factors such as dietary habits, medications or xenobiotics are among the strongest determinants affecting the composition of the intestinal microbiome. For example, a western diet changes the gut microbiome dramatically (Ley et al., 2006). Alcohol and obesity synergistically worsen liver disease in experimental animal models and humans (Loomba et al., 2009, Xu et al., 2011). Whether microbiome changes contribute to this synergistic effect on steatohepatitis is not known. In addition, to what extent ethanol is used or produced by intestinal bacteria directly following chronic alcohol administration is also not known.

Genetics

Fatty liver disease develops in the majority of patients with chronic alcohol abuse, while fibrosis and cirrhosis occur in 40–60% of alcoholics (O’Shea et al., 2010). Genetic determinants are thought to contribute to the risk of developing progressive alcoholic liver disease. Women are more susceptible to alcohol-induced liver disease than men (Sato et al., 2001). Polymorphisms in cytochrome P4502E1 (CYP2E1) and alcohol-dehydrogenase-3 (ADH-3)

genes are risk factors for developing liver disease among alcoholics (Monzoni et al., 2001). Furthermore, variations in patatin-like phospholipase domain-containing protein 3 (PNPLA3) affect the risk of developing alcoholic liver cirrhosis as well (Tian et al., 2010). It is not clear whether genetic variants contribute to alcoholic liver disease by affecting the composition of the intestinal microbiota. Host genetics in general can influence the composition of the human gut microbiome, which subsequently can impact host metabolism (Goodrich et al., 2014). Further studies are needed to elucidate the impact of genetics on microbiota and ALD.

Intestinal dysmotility

Ethanol reduces the intestinal motility that might lead to a proliferation of luminal bacteria. Social drinkers demonstrate an increased orocecal transit time relative to teetotalers; alcoholics exhibit an even longer orocecal transit time than social drinkers (Addolorato et al., 1997). Similarly, cirrhotic patients exhibit small intestinal bacterial overgrowth with a prolonged transit time (Gupta et al., 2010). Cisapride as a prokinetic agent improves small intestinal motility, and, interestingly, inhibits bacterial proliferation in subjects with liver cirrhosis (Madrid et al., 2001). To which degree impaired intestinal motility contributes to alcoholic liver disease, is not known.

Increased gastric pH

Ethanol either has no impact on gastric acid release or even increases it in non-alcoholic subjects (Chari et al., 1993). Alcoholics, however, exhibit hypochlorhydria, or the state of reduced gastric acid production (Dinoso et al., 1972, Chari et al., 1993). This might be due to their significantly altered gastric histology with higher rates of superficial and atrophic gastritis (Dinoso et al., 1972, Chari et al., 1993). Hypochlorhydria is associated with small intestinal bacterial overgrowth in cirrhotic patients (Shindo et al., 1993). It is not known whether ethanol-induced hypochlorhydria alters the progression of alcoholic liver disease.

Altered bile flow

Chronic alcohol abuse leads to higher total bile acid levels in the stool (Kakiyama et al., 2014). However, once the alcoholic patient develops cirrhosis, the fecal amount of total bile acids decreases significantly (Kakiyama et al., 2014). This might be due to the diminished bile secretion into the intestine observed in cirrhotics (Raedsch et al., 1983). The major receptor for bile acids in intestinal cells, the nuclear receptor Farnesoid X Receptor (FXR), influences several antimicrobials, amongst them angiogenin 1 and RNAse family member 4. A reduction of these bactericidal proteins was linked to small intestinal bacterial overgrowth in mice (Inagaki et al., 2006). Remarkably, oral administration of bile acids to cirrhotic rats abolished the small intestinal bacterial overgrowth (Lorenzo-Zuniga et al., 2003). Therefore, a decreased bile flow in subjects with liver cirrhosis (Raedsch et al., 1983) could contribute to quantitative microbiome changes.

Altered immune response

Chronic alcohol consumption has profound effects on the host immune system. Host bactericidal molecules are central effectors of the intestinal innate immune system contributing to the composition of the intestinal microbiome. Antimicrobial molecules are secreted by

Paneth cells and intestinal epithelial cells. Two of these antimicrobials, Regenerating islet derived (Reg)-3b and Reg3g, were suppressed in murine and human small intestine after alcohol feeding and chronic ethanol abuse, respectively (Yan et al., 2011, Hartmann et al., 2013). The intestinal mucus layer serves as a means of protection in the gut, and its thickness increases in alcoholics (Hartmann et al., 2013). Whether alcohol-induced effects on the host immune system have either direct or indirect effects on the composition of the intestinal microbiome is an area that deserves to be studied in more detail.

Although all of these factors are affected by chronic alcohol consumption, more mechanistic studies are required to identify, whether these determinants and associated changes in the gut microbiome indeed impact alcoholic liver disease. Similarly, it is also not clear to what extent changes in liver function contribute to dysbiosis. Carefully designed future studies are required to determine whether pre-cirrhotic alcoholic liver disease affects the composition of the intestinal microbiome.

3. Consequences of intestinal dysbiosis in alcoholic liver disease

3.1. Pathological bacterial translocation

Pathological bacterial translocation is defined as the passage of viable bacteria or microbial products from the gastrointestinal tract to mesenteric lymph nodes or other extraintestinal organs (Berg and Garlington, 1979). The contribution of bacteria to liver disease is emphasized by an experiment where small intestinal bacterial overgrowth was experimentally induced which alone resulted in bacterial translocation and subsequent liver injury (Lichtman et al., 1990). Inversely, selective intestinal decontamination with antibiotics can reduce pathological bacterial translocation and endotoxemia, and ameliorate hepatic damage in rodents (Adachi et al., 1995, Chen et al., 2014).

Bacterial translocation is initiated when the intestinal epithelium is damaged and the intestine becomes more permeable (Parlesak et al., 2000, Purohit et al., 2008). What mechanisms are involved in the pathogenesis of that increased intestinal permeability? The ethanol metabolite acetaldehyde but not ethanol itself increases the permeability of Caco-2 cell monolayers (Rao, 1998). Furthermore, alcohol feeding to rats leads to acute injury of the colonic epithelial barrier via acetaldehyde, the metabolite of ethanol generated by gut bacteria, and an associated activation of mast cells (Ferrier et al., 2006). In addition, intestinal Cyp2E1 appears to play a role in alcohol-induced intestinal oxidative stress and intestinal permeability (Abdelmegeed et al., 2013). Taken together, possibly alcohol and its metabolite acetaldehyde directly cause tight junction disruption.

Intestinal inflammation is another mediator of intestinal barrier dysfunction. Pro-inflammatory mediators such as IL-1-beta or TNF-alpha are increased in the small intestine of mice after ethanol feeding (Fleming et al., 2001). Lamina propria monocytes and macrophages appear to be the source for increased cytokine production. These cells increase TNF-alpha production in the small intestine of mice and in the duodenum of humans after chronic alcohol consumption (Chen et al., 2014). Intestinal inflammation precedes the onset of alcohol-induced increased intestinal permeability in mice (Chen et al., 2014). Most importantly, alcohol-associated dysbiosis triggers this local intestinal inflammatory response. This was demonstrated by using

non-absorbable antibiotics, which reduce large intestinal bacterial overgrowth, intestinal inflammation and intestinal permeability. This evidently links intestinal dysbiosis with gut barrier dysfunction, although the triggering microbial metabolite or product is currently not known. TNF-receptor 1 mutant mice are protected from intestinal barrier dysfunction and alcoholic liver disease. Reactivation of TNF-receptor 1 on intestinal epithelial cells resulted in increased intestinal permeability and liver disease that is similar to wild type mice after alcohol feeding, suggesting that enteric TNF-receptor 1 promotes intestinal barrier dysfunction and mediates ALD (Chen et al., 2014). Mice lacking myosin-light chain kinase (MLCK), a intracellular downstream target of TNF-receptor 1 in intestinal epithelial cells, show partial protection from intestinal barrier dysfunction and ALD (Chen et al., 2014), suggesting that other intracellular signaling molecules are involved to mediate tight junction disruption downstream of the TNF-receptor 1. Inducible nitric oxide synthases (iNOS) could be a candidate, because intestinal iNOS expression is dependent on TNF-receptor 1 on enterocytes after chronic alcohol feeding (Chen et al., 2014). iNOS expression correlates with barrier function disruption in differentiated Caco-2 cells (Banan et al., 1999). Furthermore, an iNOS inhibitor attenuated alcohol-induced gut permeability, endotoxemia, and liver injury (Tang et al., 2009). Whether iNOS (possibly as genetic variant) affects the composition of the gut microbiota, is currently not known, but deserves future investigation.

Interestingly, not all patients with alcohol dependence show increased intestinal permeability. The group of alcoholics with increased gut permeability also had an altered composition and activity of the gut microbiota such as lower amounts of *Bifidobacterium* spp., Clostridiales Family XIV *Incertae Sedis*, and Ruminococcaceae when compared with healthy controls. Levels of these bacteria were not changed in alcoholics with a low intestinal permeability (Leclercq et al., 2014). This raises the question whether other factors than dysbiosis induce gut permeability. There are indications that host genetics influence the composition of the intestinal microbiome and the host metabolism (Goodrich et al., 2014). This might be involved in inducing gut permeability associated with ALD as well.

As a consequence of increased intestinal permeability, pathological bacterial translocation can occur and plasma levels of gut-derived microbial products increase. Lipopolysaccharide (LPS), or endotoxin, is a critical component of the outer membrane of Gram-negative bacteria (Fadl et al., 2005). Many studies have shown that alcohol administration correlates with plasma endotoxin levels in animal models (Nanji et al., 1993, Adachi et al., 1995, Tamai et al., 2000). Elevations in plasma LPS can be observed during early stages of ALD (Parlesak et al., 2000) as well as during advanced stages of cirrhosis (Bajaj et al., 2014c). The degree of liver injury correlates with endotoxemia in patients with liver cirrhosis (Lin et al., 1995), and is higher in alcoholic cirrhosis compared with other etiologies (Bajaj et al., 2014c). Additionally, peptidoglycan, the major cell wall component in Gram-positive bacteria, is elevated in rat plasma after acute alcohol administration (Tabata et al., 2002).

We recently used a genetic mouse model with an enhanced intestinal innate immune response and with resistance to alcohol-induced large intestinal bacterial overgrowth (Hartmann et al., 2013). Despite more permeable intestines due to the mechanical absence of mucin-2 (Muc2), Muc2-deficient mice had decreased plasma LPS levels and were consequently protected from

alcoholic liver disease (Hartmann et al., 2013). This suggests that failure of a physical barrier can be compensated by controlling the luminal bacterial burden.

The innate immune system has conserved pattern recognition receptors, e.g. Toll-like receptors (TLRs), that recognize specific pathogen-associated molecular patterns (PAMPs) such as LPS, peptidoglycan, or bacterial DNA (Akira et al., 2006). The cellular receptor for LPS is TLR4 which plays an eminent role in the immune response to pathological bacterial translocation. TLR4 mutant C3H/Hej mice and TLR4-knockout mice – although exhibiting a similar gut permeability after alcohol feeding compared with wild-type mice – show less hepatic steatosis, inflammation and cell death following ethanol feeding relative to wild type mice (Uesugi et al., 2001, Hritz et al., 2008). Deficiency of its cellular co-receptor cluster of differentiation 14 (CD14) results in alleviated alcohol-induced liver injury (Yin et al., 2001). LPS binding to TLR4 initiates an intracellular downstream signaling cascade in immune cells and other liver cells. Kupffer cells, amongst other cells, are important in the pathogenesis of ALD. Inactivation of these cells via gadolinium chloride injections potently decreases ethanol-induced liver injury (Adachi et al., 1994). Furthermore, hepatic stellate cells (HSCs) play a central role in the pathogenesis of liver fibrosis (Karaa et al., 2008). Oxidative stress mediated by ethanol and its metabolite acetaldehyde sensitizes HSCs to activation by endotoxin, which results in liver fibrosis after chronic ethanol feeding combined with LPS (Karaa et al., 2008). TLR4 signaling in non-bone marrow-derived liver cells including HSCs is required for liver steatosis, inflammation, and a fibrogenic response after chronic alcohol treatment HSCs (Inokuchi et al., 2011). LPS induces apoptosis in hepatocytes, in particular in synergy with other hepatotoxic agents (Kudo et al., 2009). For subsequent cellular events in the liver following pathological bacterial translocation, we would like to refer to other in depth reviews (Seki and Schnabl, 2012, Szabo, 2015).

Interestingly, we recently showed that certain aspects of the commensal microbiota might protect against chronic liver disease. In the absence of the microbiota, liver injury and fibrosis induced by oral administration of thioacetamide or intraperitoneal injections of carbon tetrachloride is more pronounced in germ-free mice compared with conventional mice (Mazagova et al., 2014). Strikingly, hepatocytes were more susceptible to toxin-induced cell death in the absence of the microbiota or when lacking innate immune signaling (Mazagova et al., 2014). Future studies need to determine whether alcohol-induced hepatocyte death is similarly exacerbated in the absence of the microbiota. Furthermore, it will be crucial to identify microbial products or metabolites with cytoprotective properties.

3.2. Changes in intestinal metabolites

Metabolomic studies can determine intestinal metabolites that will reflect functional changes of the intestinal microbiota. Chronic ethanol administration to rats over 8 weeks results in a reduction of almost all amino acids including all three branched-chain amino acids (leucine, isoleucine, valine), perturbations of the steroid, lipid and carnitine metabolism (Xie et al., 2013b), as well as the bile acid metabolism (Xie et al., 2013a) in the intestine. Certain metabolites belonging to alcohols, alkanes, and benzenes (such as 1-nonanol, hexane, and styrene, respectively) could only be detected in the feces of alcohol dependent subjects but not in healthy controls. In contrast, other volatile organic compounds such as 2-methyl-1-butanol,

methyl-cyclopentane, and methanethiol were detectable in the feces of healthy humans but non-detectable in alcohol dependent patients (Leclercq et al., 2014).

Short-chain fatty acids (SCFAs) are bacterial fermentation products, and dysbiosis might cause differences in intestinal fermentation. And indeed, intestinal levels of SCFAs are lower after ethanol administration except for levels of acetic acid, which increases as metabolite of ethanol (Xie et al., 2013b). Supplementation of the SCFA butyrate improved the intestinal barrier function following acute, short-term or chronic alcohol exposure in mice, but liver injury was reduced only if ethanol was administered acute or short-term (Cresci et al., 2014).

Furthermore, ethanol directly decreases the biosynthesis of saturated LCFAs by gut microbiota as shown by metagenomics analysis. As a consequence, intestinal amounts of saturated long-chain fatty acids (LCFAs) diminish after alcohol feeding (Chen et al., 2015). Since *Lactobacillus* spp. are able to utilize saturated LCFAs *in vivo* and in culture for growth, lower levels of saturated LCFAs (Chen et al., 2015) could explain suppressed amounts of *Lactobacillus* spp. following chronic alcohol feeding (Kirpich et al., 2008, Yan et al., 2011, Hartmann et al., 2013, Leclercq et al., 2014). Administration of LCFAs to alcohol-fed mice increased levels of *Lactobacillus* spp., reduced intestinal inflammation, improved intestinal barrier function (Chen et al., 2015), and reduced alcoholic liver disease (Nanji et al., 1995, Nanji et al., 1997, Ronis et al., 2004, You et al., 2005, Kirpich et al., 2012, Zhong et al., 2013, Chen et al., 2015). Additionally, supernatant of *Lactobacillus* spp. alone has been shown to improve epithelial barrier function *in vitro* (Cicenia et al., 2014, Chen et al., 2015). Thus, microbial products or metabolites together with reduced amounts of *Lactobacillus* trigger intestinal inflammation, barrier dysfunction and liver disease following chronic alcohol feeding. This is a good example of how a connection between the microbial metabolome and host has been established. Although the taxonomic composition of the alcohol-associated gut microbiome has been characterized and has advanced our knowledge, we are just starting to understand the functional consequences of dysbiosis. Future studies are required to establish urgently needed links between microbial metabolites and the host that either confer protection against disease or mediate disease.

3.3. Bile acid metabolism

Bile acids are important communicators between the liver and the intestine. Conjugated bile acids are secreted from the hepatic biliary system into the duodenum, are modified in the intestine by bacteria and return to the liver via the enterohepatic circulation. As described earlier, patients with liver cirrhosis exhibit a reduced bile flow (Raedsch et al., 1983). Bile acids induce antimicrobial molecules by activating FXR in intestinal epithelial cells (Inagaki et al., 2006), therefore a reduced bile flow might contribute to intestinal bacterial overgrowth. Further evidence of the interplay between the intestinal microbiome and the bile acid metabolism is given by alcohol feeding experiments in rodents: Ethanol administration to rats results in decreased taurine-conjugated bile acids in liver and intestine, while levels of unconjugated and glycine-conjugated bile acids increase (Xie et al., 2013a). This could be partly explained by intestinal bacterial overgrowth because patients with gastrointestinal bacterial overgrowth exhibit an increased deconjugation of bile acids (Theisen et al., 2000). Patients with chronic alcohol abuse show higher total bile acids, higher lithocholic acid and

deoxycholic acid, higher secondary bile acids and a higher secondary-to-primary bile acid ratio in the stool (Kakiyama et al., 2014). However, once the patient develops cirrhosis (in particular if advanced), the fecal amount of total bile acids decreases significantly and serum levels of conjugated bile acids increase (Kakiyama et al., 2013, Kakiyama et al., 2014). Both phenomena might be due to the diminished bile acid secretion into the intestine observed in cirrhotics (Raedsch et al., 1983). Further studies are needed to clarify the interplay of gut microbiota and bile acids in ALD to better understand the pathogenesis and to develop novel pharmaceutical agents to ameliorate the treatment of patients with chronic alcohol abuse. This will better define how the liver communicates to the intestine, since this crosstalk is bidirectional.

Consequences of intestinal dysbiosis that are relevant to the pathogenesis of alcoholic liver disease are illustrated and summarized in Figure 2.

Conclusion

Chronic alcohol consumption results in small and large intestinal bacterial overgrowth and changes in the taxonomic composition of the intestinal microbiome. Factors that shape the alcohol-associated microbiome are largely unknown. Ethanol and/or acetaldehyde could contribute to a dysfunction of the mucosal barrier by disrupting epithelial tight junctions. Recently, intestinal inflammation has been causatively linked to increased intestinal permeability. Intestinal inflammation precedes heightened gut permeability, and intestinal decontamination prevents intestinal inflammation and increased intestinal permeability. Which products or metabolites from the dysbiotic microbiota initiate intestinal inflammation requires further studies. Pathological bacterial translocation appears to be the only currently known pathogenic factor linking intestinal dysbiosis to progression of alcoholic liver disease. Given excellent examples of how metagenomic or metabolomic factors affect the progression of other liver diseases such as non-alcoholic fatty liver disease and steatohepatitis (NAFLD/NASH) (Schnabl and Brenner, 2014, Boursier and Diehl, 2015), it is unlikely that an increased gut permeability is the only critical intestinal component for progression of alcoholic liver disease. We have recently discovered metabolites, i.e. saturated long-chain fatty acids, whose reduced intestinal concentrations contribute to alcohol-associated dysbiosis and affect alcoholic liver disease (Chen et al., 2015). Identification of other pathways linking the microbiota to alcoholic liver disease is challenging, but could be a key for a better understanding of the gut-liver axis and for designing interventional trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ADH	alcohol dehydrogenase
ALD	alcoholic liver disease
CDR	cirrhosis dysbiosis ratio
CYP	cytochrome P450 enzyme
FXR	Farnesoid X Receptor
IL	interleukin
iNOS	inducible nitric oxide synthases
LCFAs	long-chain fatty acids
LPS	lipopolysaccharide
MLCK	myosin-light chain kinase
PAMPs	pathogen-associated molecular patterns
Reg3	regenerating islet-derived 3
SCFAs	short-chain fatty acids
TLR	Toll-like receptor
TNF	tumor necrosis factor

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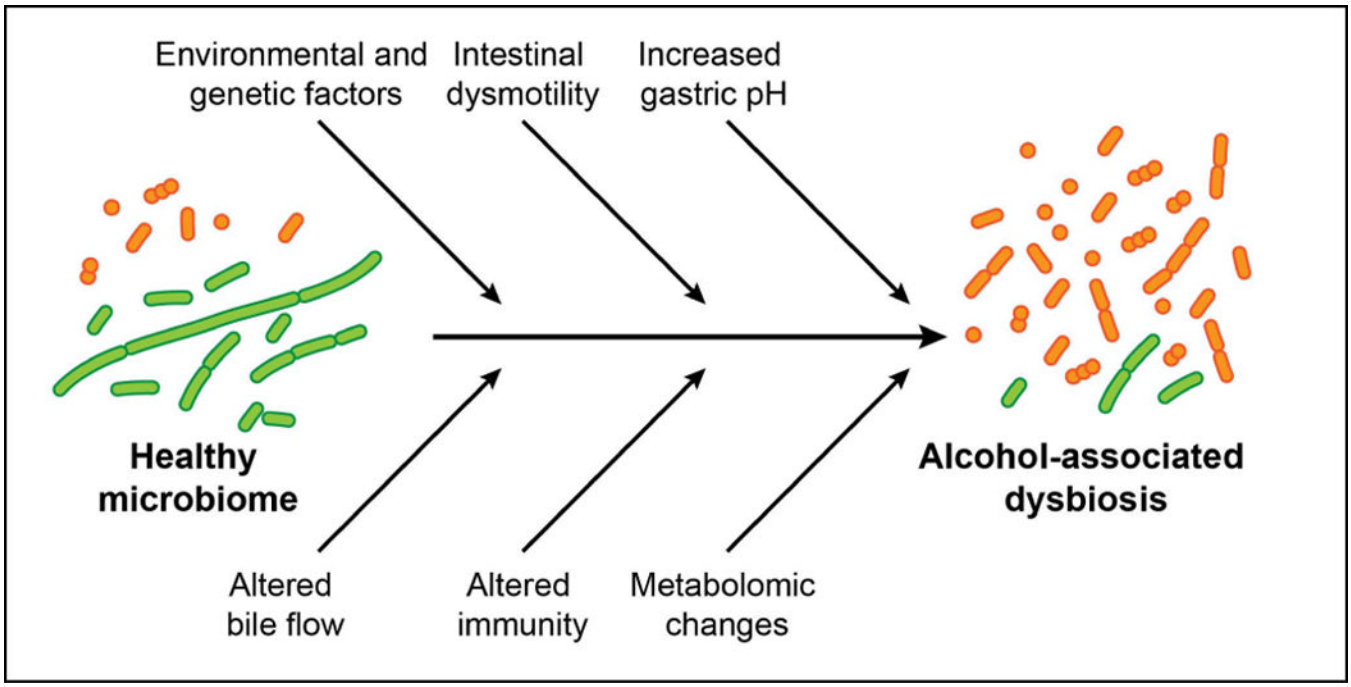

Figure 1. Contributing factors to intestinal dysbiosis after chronic alcohol consumption

Chronic alcohol administration results in a quantitative increase of intestinal bacteria and a qualitative change in the bacterial composition of the microbiota. Several factors might contribute to alcohol-associated dysbiotic changes in the intestine.

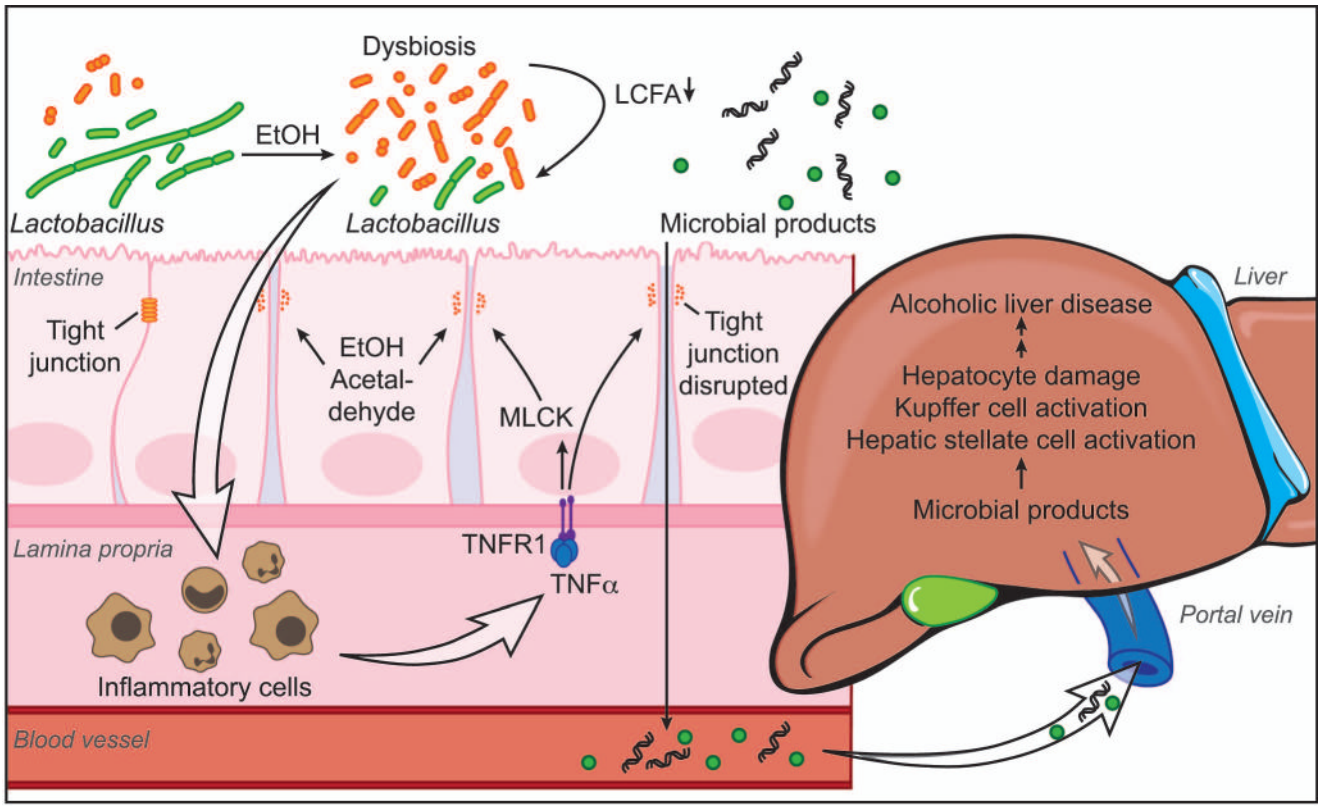

Figure 2. Consequences of intestinal dysbiosis relevant to the pathogenesis of alcoholic liver disease

Chronic alcohol consumption leads to intestinal bacterial overgrowth and dysbiosis. Metabolomic changes such as lower bacterial synthesis of long-chain fatty acids (LCFA) result in smaller amounts of 'good' bacteria, e.g. *Lactobacillus* spp. Yet unknown microbial metabolites or products cause intestinal inflammation. While anti-inflammatory properties of intestinal lactobacilli suppress intestinal inflammation during health, reduced amounts of lactobacilli associated with chronic alcohol administration are not any longer able to maintain intestinal homeostasis. Inflammatory cells of the intestinal lamina propria are activated and secrete tumor necrosis factor (TNF)-alpha. TNF-alpha then binds to its receptor TNFR1 on enterocytes, which results in a disruption of tight junctions, partly mediated via myosin-light chain kinase (MLCK). Ethanol and its metabolite acetaldehyde might contribute to a dysfunction of the gut barrier. Microbial products can therefore translocate from the intestinal lumen to the portal venous blood. Translocated microbial products activate hepatic stellate cells and Kupffer cells, and damage hepatocytes. This synergizes with a direct hepatotoxic effect of alcohol and its metabolites to cause progression of alcoholic liver disease.

Table 1

Changes in intestinal microbiota associated with experimental alcoholic liver disease in animals

		Implicated microbiota						
Condition	**Comparison**	**Phylum**	**Class**	**Order**	**Family**	**Genus/Species**	**Methodology**	**Reference**
Intragastric Tsukamoto-French mouse model for 3 weeks	Isocaloric vs. ethanol fed (n=3 vs. 3)	Bacteroidetes ↑ Firmicutes ↓	Bacteroidia ↑ Bacilli	Bacteroidales Lactobacillales ↓	Bacteroidaceae Enterococcaceae Lactobacillaceae ↓	*Bacteroides* spp. ↑ *Enterococcus* spp. ↑ *Lactobacillus* spp. *Pediococcus* spp. *Leuconostoc* spp. *Lactococcus* spp. ↓	16S rRNA gene pyrosequencing, quantitative real-time PCR Cecum samples	Yan and colleagues (2011)
		Proteobacteria ↑ Verrucomicrobia ↑	Erysipelotrichia Verrucomicrobiae	Erysipelotrichales Verrucomicrobiales	Leuconostocaceae Streptococcaceae Erysipelotrichaceae ↑ Verrucomicrobiaceae	*Akkermansia muciniphila* ↑		
Intragastric Tsukamoto-French mouse model for 1 week	Isocaloric vs. ethanol fed (n=7 vs 9, and 3 vs. 5)	Firmicutes Verrucomicrobia	Bacilli Verrucomicrobiae	Lactobacillales Verrucomicrobiales	Lactobacillaceae Verrucomicrobiaceae	*Lactobacillus* spp. *Akkermansia muciniphila* ↑	Quantitative real-time PCR Cecum samples	Hartmann and colleagues (2013)
Lieber-DeCarli Liquid diet mice for 6 weeks	Isocaloric vs. ethanol fed (n=8 vs. 8)	Actinobacteria ↑ Bacteroidetes ↓ Firmicutes ↓ Proteobacteria ↑	Actinobacteria Bacteroidia Bacilli Clostridia Erysipeltrichia Betaproteobacteria	Actinomycetales Bacteroidales Bacillales Lactobacillales Clostridiales Erysipeltrichales Burkholderiales	Corynebacteriaceae Bacteroidaceae Porphyromonadaceae Prevotellaceae Listeriaceae Aerococcaceae Lactobacillaceae Ruminococcaceae Erysipeltrichaceae Alcaligenaceae	*Corynebacterium* spp. ↑ *Bacteroides* spp. *Parabacteroides* spp. *Tannerella* spp. *Hallella* sp. *Listeria* spp. ↑ *Aerococcus* spp. ↑ *Lactobacillus* spp. ↑ *Acetivibrio* spp. ↑ *Incertae Sedis* *Allobaculum* spp. ↑ *Alcaligenes* spp. ↑	16S rRNA gene pyrosequencing Stool sample	Bull-Otterson and colleagues (2013)
	Ethanol fed vs. ethanol fed+ *Lb. rhamnosus* (n=8 vs. 4)	Actinobacteria ↓ Firmicutes ↑ Proteobacteria ↓	Actinobacteria Bacilli Clostridia Betaproteobacteria	Actinomycetales Lactobacillales Clostridiales Burkholderiales	Corynebacteriaceae Lactobacillaceae Ruminococcaceae Alcaligenaceae	*Corynebacterium* spp. ↓ *Lactobacillus* spp. ↑ *Incertae Sedis* ↑ *Alcaligenes* spp. ↓		
Ethanol liquid diet for 7d + oral binge on day 7 in mice	Isocaloric vs. ethanol fed (n=5-7)	Proteobacteria Firmicutes	Gammabacteria Bacilli	Enterobacteriales Lactobacillales ↑	Enterobacteriaceae ↑ Enterococcaceae	*Enterococcus* spp. ↑	Quantitative culturing of stool samples	Campos Canesso and colleagues (2014)
Intragastric Tsukamoto-French mouse model including supple- ments	Isocaloric vs. ethanol fed with unsaturated FAs	Bacteroidetes ↑ Firmicutes ↓	Bacilli	Lactobacillales	Lactobacillaceae	*Lb. rhamnosus* *Lactobacillus* spp. ↓	16S rRNA gene pyrosequencing, quantitative real-time PCR Cecum samples	Chen and colleagues (2015)
	Ethanol fed vs. ethanol fed with unsaturated FAs	Bacteroidetes ↑ Firmicutes ↓	Bacilli	Lactobacillales	Lactobacillaceae	*Lb. rhamnosus* *Lactobacillus* spp. ↓	16S rRNA gene pyrosequencing, quantitative real-time PCR Cecum samples	Chen and colleagues (2015)

		Implicated microbiota						
Condition	**Comparison**	**Phylum**	**Class**	**Order**	**Family**	**Genus/Species**	**Methodology**	**Reference**
of un-saturated or saturated fatty acids for 3 weeks								
	Ethanol fed with unsaturated FAs vs. ethanol fed with saturated FAs (n=2-7)	Bacteroidetes ↓ Firmicutes ↑	Bacilli	Lactobacillales	Lactobacillaceae	*Lb. rhamnosus* ↑ *Lactobacillus* spp. ↑		

A comparison of condition A vs condition B; ↑, increase in condition B relative to condition A; ↓, decrease in condition B relative to condition A.

Taxonomy was updated using the National Center for Biotechnology Information (NCBI) Taxonomy Browser.

Table 2

Changes in intestinal microbiota associated with alcoholic liver disease in humans – Selection, for full list see Supplementary Table 1

		Implicated microbiota						
Disease	**Comparison**	**Phylum**	**Class**	**Order**	**Family**	**Genus/Species**	**Methodology**	**Reference**
Healthy (n=24) Alcoholic patients (n=66)	Healthy vs. alcoholic patients	Actinobacteria Firmicutes	Actinobacteria Bacilli	Bifidobacteriales Lactobacillales	Bifidobacteriaceae Enterococcaceae Lactobacillaceae	*Bifidobacterium* spp. ↓ *Enterococcus* spp. *Lactobacillus* spp. ↓	Quantitative culturing of stool samples	Kirpich and colleagues (2008)
	Alcoholic patients without probiotics vs. alcoholic patients with probiotics	Actinobacteria Firmicutes	Actinobacteria Bacilli	Bifidobacteriales Lactobacillales	Bifidobacteriaceae Enterococcaceae Lactobacillaceae	*Bifidobacterium* spp. ↑ *Enterococcus* spp. ↑ *Lactobacillus* spp. ↑		
Healthy (n=18) Alcoholics with- out cirrhosis (n=29) Alcoholics with cirrhosis (=19)	Healthy vs. alcoholic cirrhotic patients	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae ↓		16S rRNA gene pyrosequencing Sigmoid mucosal biopsy	Mutlu and colleagues (2012)
	Healthy vs. alcoholics without cirrhosis	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae ↓			
	Nondysbiotic alcoholics vs. dysbiotic alcoholics	Bacteroidetes Firmicutes Proteobacteria Verrucomicrobia	Bacteroidia ↓ Sphingobacteria ↑ Bacilli ↑ Clostridia ↓ Gammaproteobacteria ↑ Verrucomicrobiae ↓					
Healthy (n=25) Cirrhotic patients (only alcoholic=43, not alcoholic=170)	Cirrhotic patients other than solely alcoholic vs. alcoholic cirrhotic patients	Firmicutes Proteobacteria	Clostridia Gammabacteria	Clostridiales Enterobacteriales Oceanospirillales	Family XIV *Incertae Sedis* ↓ Lachnospiraceae ↓ Ruminococcaceae ↓ Enterobacteriaceae ↑ Halomonadaceae ↑		16S rRNA gene pyrosequencing Stool sample only	Bajaj and colleagues (2014c)
Nonalcoholic cirrhotic patients (n=5) Alcoholic cirrhotic patients with active alcohol abuse (n=5)	Nonalcoholic cirrhotic patients vs. alcoholic cirrhotic patients	Bacteroidetes Firmicutes	Bacteroidia Negativicutes	Bacteroidales Selenomonadales	Bacteroidaceae ↓ Veillonellaceae ↑		16S rRNA gene pyrosequencing Stool sample	Kakiyama and colleagues (2014)
Healthy (n=15) Alcohol dependent patients with high or	Alcohol dependent patients with	Actinobacteria Firmicutes	Actinobacteria Clostridia Negativicutes	Bifidobacteriales Clostridiales Selenomonadales	Bifidobacteriaceae Clostridiaceae Family XIII *Incertae Sedis* ↓ Family XIV *Incertae Sedis* ↑	*Bifidobacterium* spp. ↓ *Clostridium* spp. ↓ *Blautia* spp. ↑	16S rRNA gene pyrosequencing, quantitative real-	Leclercq and

		Implicated microbiota						
Disease	**Comparison**	**Phylum**	**Class**	**Order**	**Family**	**Genus/Species**	**Methodology**	**Reference**
low intestinal permeability (n=26, or 34, out of whom 6 or 7 were used for microbiota studies, respectively)	low intestinal permeability vs. alcohol dependent patients with high intestinal permeability				*Lachnospiraceae* ↑ Oscillospiraceae Ruminococcaceae ↓ Veillonellaceae	*Dorea* spp. ↑ *Oscillibacter* spp. *F. prausnitzii* ↓ *Ruminococcus* spp. *Subdoligranulum* spp. *Megasphaera* spp. ↑	time PCR Stool sample	colleagues (2014)
	Alcohol dependent patients with high intestinal permeability pre- vs. post-alcohol abstinence	Actinobacteria Firmicutes	Actinobacteria Bacilli Clostridia Erysipeltrichia	Bifidobacteriales Lactobacillales Clostridiales Erysipeltrichales	Bifidobacteriaceae Lactobacillaceae Ruminococcaceae ↑ Erysipeltrichaceae ↓	*Bifidobacterium* spp. ↑ *Lactobacillus* spp. ↑ *Holdemania* spp. ↓		

A comparison of condition A vs condition B: ↑, increase in condition B relative to condition A; ↓, decrease in condition B relative to condition A.

Taxonomy was updated using the National Center for Biotechnology Information (NCBI) Taxonomy Browser.