Intestinal and hepatic microbiota changes associated with chronic ethanol administration in mice

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\textbf{ABSTRACT}
Alcohol-induced liver disease is closely related to translocation of bacterial products and bacteria from the intestine to the liver. However, it is not known whether bacterial translocation to the liver depends on certain intestinal microbiota changes that would predispose bacteria to translocate to the liver. In this study, we investigated the microbiota in the jejunum, ileum, cecum, feces and liver of mice subjected to chronic ethanol feeding using a Lieber DeCarli diet model of chronic ethanol feeding for 8 weeks. We demonstrate that chronic ethanol administration changes alpha diversity in the ileum and the liver and leads to compositional changes especially in the ileum. This is largely driven by an increase in gram-negative phyla – the source of endotoxins. Moreover, gram-negative \textit{Prevotella} not only increased in the mucus layer of the ileum but also in liver samples. These results suggest that bacterial translocation to the liver might be associated with microbiota changes in the distal gastrointestinal tract.

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\textbf{Introduction}
Alcohol consumption can lead to hepatic steatosis, which can progress to liver cirrhosis. Nearly 50% of cirrhosis-associated deaths are related to increased alcohol intake.\textsuperscript{1} Patients with alcohol abuse and alcohol-associated liver disease have a different intestinal microbial composition compared to healthy controls.\textsuperscript{2,3} This alcohol-associated dysbiosis is characterized not only by bacterial overgrowth in the small intestine, that is, an increase in numbers of bacteria,\textsuperscript{4,5} but also by compositional changes of the bacteria in the intestinal lumen.\textsuperscript{6} Microbial changes are thought to be crucial for the progression of alcohol-induced liver disease, as mice treated with nonabsorbable antibiotics had less features of liver disease.\textsuperscript{7–9}

Translocation of bacterial products such as lipopolysaccharide (LPS) in alcohol-induced liver disease is thought to be mediated by dysfunction of the intestinal barrier: antimicrobial peptides in the mucus layer are reduced,\textsuperscript{5,10} the epithelial cell function is disturbed,\textsuperscript{11} and intestinal inflammation is increased.\textsuperscript{12} The associated liver damage is mainly caused by toll-like receptor 4-induced activation of Kupffer cells (resident liver macrophages) with subsequent release of inflammatory cytokines in the liver.\textsuperscript{13,14}

We have recently shown that an ethanol-induced increase of bacteria in the intestinal mucus and epithelial cell layer in alcoholic liver disease is linked to increased bacterial translocation to mesenteric lymph nodes and the liver – as shown by bacterial cultures and increased 16S gene copies.\textsuperscript{15} If gastric acid production in mice is impaired, liver damage has even been associated with translocation of a certain bacterial species, that is, \textit{Enterococcus faecalis}.\textsuperscript{16} The microbial composition in the liver was investigated in patients with cholangiocarcinoma\textsuperscript{17} and hepatocellular carcinoma\textsuperscript{18} but not in patients with alcoholic liver disease.

The above demonstrates that alcoholic liver disease is closely related to translocation of bacteria and bacterial products to the liver, while nothing is known about the microbial composition in the liver, and whether bacterial translocation to the
liver depends on compositional microbiome changes at certain intestinal sites.

Here, we investigated changes in the microbial composition of ethanol-fed mice at different intestinal sites as well as in the liver to clarify which microbial compositions might facilitate bacterial translocation and which sites might be the source of translocating bacteria.

**Results**

*Bacterial diversity in control- versus ethanol-fed mice*

Wild-type C57BL/6 mice were fed a control- or ethanol-containing diet for 8 weeks, and microbiota was analyzed after 16S rRNA sequencing of feces and cecal contents, as well as lumen, mucus layer and epithelial cell layer of both jejunum and ileum, and from liver samples. Bacterial richness, that is, the numbers of different bacterial species within each sample, is represented by the Chao indices in Figure 1A. As expected, richness was highest in the feces and cecum and declined towards the oral gastrointestinal tract. Lowest values were found in the liver. A difference between ethanol feeding and controls was only present in the lumen of the jejunum, with ethanol leading to an increase in bacterial richness in the jejunal lumen. However, this difference was not present in the mucus/epithelial cell layer nor the liver.

Bacterial evenness, that is, how equal different bacterial species are distributed within each sample, is represented by the Shannon indices in Figure 1B. Low values for evenness correspond to bacterial communities that are dominated by only a few species. The distribution of bacterial species at most investigated sites was not significantly different between control and ethanol feeding. Evenness was increased only in the liver following chronic ethanol feeding, suggesting that chronic ethanol administration facilitates translocation of only certain bacterial species to the liver. In conjunction with the data from the Chao indices (Figure 1A), this indicates that ethanol leads to a more equal distribution of the few differing bacterial species that are present in the liver.

![Figure 1. Alpha diversity in gut and liver samples.](image)

**Bacterial community changes with chronic ethanol feeding**

Combined principal component analysis (PCA) plots of bacterial communities are displayed in Figure 2A. PCA plots separated by site are displayed in Figure 2B. To test for the ethanol-related variability in species composition, we applied

| Sample site          | Control | Ethanol |
|----------------------|---------|---------|
| Liver                | 7       | 11      |
| Jejunum, epithelial cells | 5       | 10      |
| Jejunum, mucus layer | 5       | 10      |
| Jejunum, lumen       | 5       | 10      |
| Ileum, epithelial cells | 5       | 10      |
| Ileum, mucus layer   | 5       | 10      |
| Ileum, lumen         | 5       | 10      |
| Cecum                | 7       | 11      |
| Feces                | 6       | 7       |
Figure 2. Bacterial communities in all samples. (A) PCA plots of relative OTU abundances from all investigated sites. Sites and diet of mice are indicated within the figure. (B) PCA plots of relative OTU abundance per site as indicated below each panel. EtOH: Lieber DeCarli ethanol diet; Ctrl: isocaloric control diet. Numbers of biological replicates are given in Table 1.
Bray–Curtis distance analyses (Figure 3).\textsuperscript{19,20} Ethanol-related differences in bacterial community composition were detected in feces, cecum (Figure 3A) and the epithelial cell layer of the ileum (Figure 3C). Using unweighted PCA with Bray–Curtis dissimilarity matrices compared by permutational multivariate analysis of variance (PERMANOVA) as a secondary approach, chronic ethanol feeding induced significant differences in the microbiota of both cecum and feces and, in addition, in microbiota from livers and luminal contents of the jejunum.

**Figure 3.** Beta diversity in gut and liver samples. Bray-Curtis dissimilarities in (A) cecum, feces and liver; (B) lumen, mucus layer and epithelial cell layer of the jejunum; and (C) lumen, mucus layer and epithelial cell layer of the ileum. EtOH: Lieber DeCarli ethanol diet; Ctrl: isocaloric control diet. Numbers of biological replicates are given in Table 1.

**Chronic ethanol feeding leads to changes in bacterial colonization patterns**

To elucidate, if above-mentioned bacterial community changes might be driven by distinct bacterial phyla, we further analyzed bacterial colonization pattern and community compositions. Figure 4 depicts the colonization pattern of the 20 most prevalent bacteria at the investigated sites. Ethanol-associated changes in bacterial colonization pattern were distinct in the intestine and liver at the phylum (Figure 4A) and the genus level (Figure 4B).
Gram-negative bacteria are the source of LPS, which is considered an endotoxin. LPS is an important mediator during experimental alcoholic liver disease, it is increased in binge-ethanol consumption and it correlates with mortality in cirrhosis. Grouping of bacteria into gram-negative and gram-positive phyla revealed that the proportion of gram-negative bacteria was reduced in feces, cecal contents, jejunal sites and the liver of ethanol-fed mice. In the ileum, ethanol rather seemed to increase the proportion of gram-negative bacteria, and this ethanol-associated change was more pronounced as the above-mentioned change was more pronounced as the above-mentioned changes at other sites (Figure 5A).

Looking further into the most abundant gram-negative (Bacteroidetes) and gram-positive (Firmicutes) phyla in the mucus layer of the ileum, we found changes in several genera following chronic ethanol feeding supporting the above findings (Figure 5B). Relative abundance of most...
genera belonging to Bacteroidetes increased, while there were several genera belonging to Firmicutes that decreased following chronic ethanol feeding. Significant changes with ethanol feeding were seen for *Bacteroides*, *Prevotella* and *Parabacteroides* (increase), as well as for *Blautia* and *Lactobacillus* (decrease). Next, we were interested if any of those genera can be found in liver samples as well and if ethanol-induced changes would be similar. And indeed, we saw an increase in *Prevotella* in the livers of ethanol-fed mice (Figure 5C), suggesting that the relative increase of *Prevotella* in the mucus layer of the ileum is associated with bacterial translocation to the liver.

**Discussion**

We have previously shown that ethanol impairs the expression of intestinal antimicrobial proteins, which induces a quantitative increase of bacteria in the mucus and epithelial cell layer. This was linked to increased bacterial translocation to mesenteric lymph nodes and the liver, and to increased ethanol-induced liver disease in mice. Conversely, transgenic overexpression of regenerating islet derived (REG) 3g in intestinal epithelial cells reduced ethanol-associated bacterial overgrowth in the mucus and epithelial cell layer, and decreased bacterial translocation and ethanol-induced liver disease. This study investigated ethanol-associated compositional microbiome changes along the intestinal tract and in the liver of mice. Ethanol increased bacterial richness in the jejunum and changed bacterial communities in the ileum by inducing a shift towards gram-negative bacteria. The relative abundance of gram-negative *Prevotella* was increased in the mucus layer of the ileum, and *Prevotella* was also detected in the liver.
suggesting that bacterial translocation possibly occurs from the distal gastrointestinal tract in ethanol-induced liver disease.

Several studies have investigated ethanol-induced microbiome changes in rodent cecal, fecal, ileal and colon samples.\(^3,22\) A recent publication assessed ethanol-induced changes of the fecal microbiome and metabolome in mice.\(^24\) In line with our findings, alpha diversity measures were similar and community clustering differed in control- versus ethanol-fed mouse feces. In a study with rats, ethanol reduced abundance of *Lactobacillus* and increased *Bacteroides* and *Parabacteroides*, which was also observed in our study.\(^25\) In human stool samples, patients with high intestinal permeability and ethanol consumption had increased *Blautia* and *Dorea* and less *Ruminococcus* and *Clostridia*.\(^2\) In contrast, we saw less *Blautia* in the ileum, while the other genera were unchanged not only in the ileum but also in feces (data not shown). However, neither the animal nor the human studies have investigated the ethanol-associated microbiota changes in the lumen, mucus layer and epithelial cell layer at different intestinal sites.

As one major finding we saw an increase of *Prevotella* in the mucus layer of the ileum and in the liver. The available literature on *Prevotella* in liver disease shows inconsistent results: In duodenal aspirates of patients with ethanol-related cirrhosis, *Prevotella* was decreased compared to patients with hepatitis C virus-related cirrhosis.\(^26\) With respect to chronic ethanol consumption, *Prevotella* was also decreased in stool samples of cirrhotics but not in alcohol-dependent subjects without cirrhosis.\(^27\) In contrast, ethanol consumption increased *Prevotella* in the oral cavity.\(^28\) In addition, *Prevotella* was increased in patients with cirrhosis (for various etiologies) versus controls in saliva and stool samples.\(^29\) *Prevotella* was also increased in duodenal mucosa samples of patients with hepatitis B-cirrhosis.\(^30\) The varying results may be explained by high genetic diversity of *Prevotella* species.\(^31\)

*Prevotella* are gram-negative, obligate anaerobes.\(^32\) LPS released from *Prevotella* could enhance ethanol-induced liver disease as LPS is an important mediator of alcoholic liver disease.\(^33,33\) Increased levels of *Prevotella copri* (detected in human stool samples) correlated with endotoxin levels and insulin resistance in type 2 diabetes.\(^34,35\) Because *Prevotella* are abundant in the human gingival groove, their role in the development of periodontitis has been extensively studied, and some of the disease-promoting effects might also play a role for liver disease.\(^32\) *Prevotella intermedia* was found to be capable to invade oral epithelial cells, as it secretes exopolysaccharides to form biofilms and is a producer of several proteases.\(^36–39\) In addition, *P. intermedia* induced the secretion of tumor necrosis factor-alpha (TNF) from human monocyte-derived THP-1 macrophages.\(^40\) Other species, *P. gingivalis* and *P. nigrescens*, induced arthritic bone erosions by toll-like receptor-2- and interleukin (IL)-1-dependent Th17-cell activation in mice.\(^41\) IL-1\(\beta\) mediates ethanol-induced liver disease in mice.\(^42\)

In the gut, mucosal invasion of *Prevotella* was detected in Crohn’s disease.\(^43\) Moreover, a proinflammatory microbiome containing *Prevotella* induced spontaneous colitis in mice by intestinal induction of CC-chemokine ligand 5 (CCL5).\(^44\) CCL5 expression in the liver is increased in ethanol-induced liver disease.\(^16,45,46\) It induces T-cell mediated inflammation,\(^47\) and just recently inhibition of CCL5 signaling by a CCR2/5 inhibitor prevented and reversed ethanol-induced liver injury in mice.\(^45\)

The above shows that besides the release of LPS, which is known to enhance ethanol-induced liver disease, *Prevotella*-induced release of either TNF, IL-1\(\beta\) or CCL5 might trigger ethanol-induced liver injury. However, it is a limitation of our study that we can only speculate about a functional link between *Prevotella* and ethanol-induced liver disease, as well as bacterial translocation from the ileum to the liver. Further studies are required to determine whether *Prevotella* promotes experimental ethanol-induced liver disease, and if so, which virulence factors mediate a liver disease-promoting effect. In addition, functional studies with labeled bacteria to enable tracking of bacterial translocation might elucidate, if viable *Prevotella* actually translocate from the ileum to the liver.

To the best of our knowledge, we present the first comprehensive microbiome analysis from samples of the entire intestinal tract and the liver.
of ethanol-fed mice. Microbial differences between the investigated intestinal sites are important to consider for future studies. In particular, human studies might have to include mucosal biopsies from the ileum.

**Materials and methods**

**Mice**

The analysis was done with tissues obtained during a recently published mouse study by Wang et al.\textsuperscript{15} Female wild-type littermate mice (C57BL/6 background) were used. Mice were housed in pairs and received a liquid ethanol-containing Lieber DeCarli diet to mimic chronic alcohol feeding for 8 weeks.\textsuperscript{15} Pair-fed control mice received a similar diet with ethanol being replaced by isocaloric amounts of dextrose. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of the University of California, San Diego.

**DNA extraction**

16S rRNA sequencing was done from DNA isolated from fecal pellets, cecal content, lumen (i.e., content), mucus layer and epithelial cell layer of the jejunum and ileum, as well as from the liver. Numbers of biological replicates are shown in Table 1. The different layers from jejunum and ileum were obtained as described.\textsuperscript{15} In brief, luminal contents from 2-cm pieces of intestine were obtained from flushing the intestinal pieces with 1 ml of phosphate-buffered saline (PBS); mucus layer was obtained by subsequent cutting of the intestine and vigorous washing with 1 ml of PBS; the epithelial cell layer was analyzed from the remaining tissue. DNA extraction was done as described previously:\textsuperscript{48} the materials were homogenized in 1 ml sterile PBS using lysing matrix C tubes (MP116912, MP Biomedicals, Santa Ana, CA) and a Mini-BeadBeter-96 (GlenMills, Clifton, NJ). This was followed by digestion with proteinase K (Am2546, Thermo Fisher Scientific, Waltham, MA), RNase A (19101, Qiagen, Valencia, CA) and 10% sodium dodecyl sulfate (SDS; L3771, Sigma-Aldrich, St. Louis, MO) at 55°C for 1 h. Suspensions were transferred to lysing matrix B tubes (MP116911, MP Biomedicals, Santa Ana, CA) and homogenized after addition of phenol (15513, Thermo Fisher Scientific, Waltham, MA). Using phenol/chloroform/isoamyl alcohol (15593, Thermo Fisher Scientific, Waltham, MA), the lysate was extracted three times followed by one extraction with chloroform and sodium acetate buffer solution (S7899, Sigma-Aldrich, St. Louis, MO). DNA was finally precipitated and washed using ethanol and resuspended in sterile water (BP561, Thermo Fisher Scientific, Waltham, MA).

**16S rRNA profiling**

The extracted DNA was amplified using primers that target the V4 region of the 16S rRNA gene.\textsuperscript{49} These primers included the i5 and i7 adaptor sequences for dual index Illumina MiSeq pyrosequencing as well as unique 8 bp indices incorporated onto both primers such that each sample receives its own unique barcode pair. Using approximately 100 ng of extracted DNA, the amplicons were generated with Platinum Taq polymerase (Life Technologies, Carlsbad, CA). Amplicons were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA), quantified using Tecan fluorometric methods (Tecan Group, Maennedorf, Switzerland), normalized and finally pooled in preparation for Illumina MiSeq sequencing using the dual index V2 chemistry 2 × 250 bp format (Illumina, San Diego, CA) following the manufacturer’s protocol.

**Statistical analyses**

Analyses and data plots from OTU counts were done with R, version 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism 7.04 (GraphPad Software, Inc., La Jolla, CA), respectively. Differences between groups were assessed by Mann-Whitney or Wilcoxon rank-sum test. The permutational multivariate analysis of variance (PERMANOVA) calculations were performed using the VEGAN package for R. Significant differences are marked with asterisk if \( p < 0.05 \).
Abbreviations

CCL5 CC-chemokine ligand 5
Ctrl Control
EtOH Ethanol feeding
IL Interleukin
LPS Lipopolysaccharide
PERMANOVA Permutational multivariate analysis of variance
TNF Tumor necrosis factor alpha

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Accession Numbers

Sequence data were registered at NCBI under BioProject PRJNA512118. Sequence reads are available at NCBI under the following consecutive BioSample IDs (SAMN10661899–SAMN10662037).

Disclosure of interest

The authors report no conflict of interest.

Author Contributions

S.B. wrote this manuscript, analyzed data and generated figures and tables. L.W. provided mice and specialized knowledge. M.T., C.K., K.M., H.S. and D.E.F. performed microbiota sequencing and analyses and generated figures. H.S. performed PERMANOVA analyses. D.E.F. also supervised the microbiome sequencing, performed microbiome data submission, wrote text and edited the manuscript. B. S. supervised the study and edited the manuscript. All authors approved the final version of this manuscript.

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