Ascitic bacterial composition is associated with clinical outcomes in cirrhotic patients with culture-negative and nonneutrocytic ascites

Running title: Ascitic microbiota in liver cirrhosis

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Abstract

Background: Ascites bacterial burden is associated with poor clinical outcomes in patients with end-stage liver disease. However, the impact of ascitic microbial composition on clinical course was still not clear. In this study, the ascitic microbiota composition of 100 cirrhotic patients with culture-negative and nonneutrocytic ascites were researched. Results: By characterizing the ascitic microbial composition, two distinct microbial clusters were observed, Cluster 1 (86 patients) and Cluster 2 (14 patients). Cluster 1 showed lower microbial richness than Cluster 2. At the phylum level, Cluster 1 had greater abundance of Bacteroidetes and Firmicutes, but less abundance of Proteobacteria and Actinobacteria than Cluster 2. At the family level, family Bacteroidales S24-7 group, Prevotellaceae, Lachnospiraceae, Lactobacillaceae, Rikenellaceae, and Vibrionaceae were found over-represented in Cluster 1. And family Acetobacteraceae, Erysipelotrichaceae, Rickettsiaceae, and Streptococcaceae were found enriched in Cluster 2. The levels of plasma cytokine IL-17A, IL-7, and PDGF-BB were found significantly higher in Cluster 1 than in Cluster 2. There were four OTUs closely correlated with plasma cytokines, which were OTU 140 and OTU 271 (both from Bacteroidales S24-7 group), OTU 68 (Veillonellaceae), and OTU 53 (Helicobacteraceae). Patients from Cluster 1 showed significant higher short-term mortality than patients from Cluster 2. Conclusion: Our study demonstrated that the microbial composition of culture-negative and nonneutrocytic ascites in cirrhotic patients is associated with short-term clinical outcomes. The results here offer a rational for the identification of patients with high risk, and provide references for selective use of prophylactic methods.

Keywords: Ascitic fluids, Microbiome, End-stage liver disease, Bacterial translocation, Cytokines
Background

Bacterial translocation (BT) is increasingly recognized as a key driver in the development of complications in end-stage liver disease (1). BT can cause infections, in particular, spontaneous bacterial peritonitis (SBP). In the absence of overt infection, BT may further stimulate the immune system and contribute to haemodynamic alterations and complications. The accepted pathogenic theory of BT postulates that bacteria escape from the intestinal lumen and reach the mesenteric lymph nodes by crossing the intestinal, subsequently disseminating to the bloodstream and the ascitic fluid (AF). The presence of bacteria in AF has been investigated as a simple way for studying BT in cirrhosis.

Selective decontamination of the digestive tract (SDD) can prevent bacterial translocation and reduce severe infections and mortality in patients with end stage liver disease (2). SDD prevents secondary bacterial colonization through application of non-absorbable antimicrobial agents in the gastrointestinal tract (3). SDD is a widely evaluated but highly controversial intervention. One of the major concern about SDD is the development of bacterial resistance (4). Besides, SDD targets at both normal bacteria and potential pathogenic bacteria. The use of SDD might disturb the gut microbial balance and cause bad consequences. Hence, the identification of patients with high risk of short-term mortality and infections could provide a rational for selective use of SDD.

By culture, only a minority of ascitic bacteria can be isolated, even in the presence of overt infection. Using culture-independent techniques, Such et al. reported that bactDNA can be
commonly detected in culture-negative and nonneutrocytic AF (5). It was further confirmed by the same group that the presence of bactDNA in patients with cirrhosis during an ascitic episode is an indicator of poor prognosis, which may related to development of acute-on-chronic liver failure at short term (6). Fagan et al. found that ascites bacterial burden and immune cell profile are associated with poor clinical outcomes in the absence of overt infection (7).

With more sensitive molecular techniques such as 16S rRNA and shotgun metagenomic pyrosequencing, depth profile of microbial communities in AF have been characterized in small sample sizes recently (8, 9). Using 16S rRNA gene pyrosequencing, Rogers et al. found that differences in structure and membership of AF microbial communities correlated with severity of liver cirrhosis (8). In their research, propidium monoazide treatment were applied to ascitic samples to characterize only viable bacteria. However, the composition of translocated bacteria, both viable and non-viable, might worth studying. Circulation bacterial fragments are found with pathological significance in patients with inflammatory bowel disease (10), end stage disease (6), and chronic hemodialysis (11). Grade of soluble inflammatory response is mainly affected by plasmatic concentration of bactDNA. And, differences in inflammatory responses were observed between gram negative and gram positive bacterial fragment translocation (12).

The aim of this prospective study has been to assess the associations between AF microbial composition and clinical outcomes in cirrhotic patients with culture-negative and nonneutrocytic ascites. We applied a combination of 16S rRNA pyrosequencing and enterotype-
like cluster analysis to identify AF microbial clusters. The resulting AF microbial clusters were
correlated with plasma cytokine profiles and clinical outcomes. This fact may become a relevant
clinical issue since it provides a reference for identification of high risk patients, who need
prophylactic SDD.

Methods

Patients. AF were obtained from 100 consecutive cirrhotic patients undergoing clinically
indicated therapeutic or diagnostic paracentesis for ascites at the First Affiliated Hospital of
Zhejiang University. Cirrhosis was diagnosed by histology or by clinical, laboratory, and/or
image findings. Inclusion criteria were the presence of cirrhosis and ascites fluids. Exclusion
criteria were infected AF with positive culture or > 250 polymorphonuclear, upper
gastrointestinal bleeding, intake of antibiotics in previous two weeks including norfloxacin as
prophylaxis of SBP, two or more of criteria of systemic inflammatory response syndrome
(temperature > 38°C or < 36°C, heart rate > 90 beats/minute, respiratory rate > 20
breaths/minute, blood white blood cells < 4000 or > 12000/mm³) (13).

AF samples were obtained when a large volume paracentesis were needed as a part of the
patient’s treatment. Paracentesis were performed under aseptic conditions following the usual
procedures. AF samples for routine biochemical study were obtained. Blood samples of the
same day was obtained for hematological, biochemical, coagulation and cytokine profile
analysis. Both blood and AF samples were kept under aseptic conditions. All the patients in
study were followed up for 90 successive days. The occurrence of death and complications during the 90 days were recorded.

**DNA extraction and 16S rRNA sequencing.** AF were stored at -80 °C immediately after collected. Total bacterial DNA was extracted from AF samples using QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to manufacture’s instruction. Bacterial 16S rRNA V3-V4 region was amplified using the 343F/798R primer set (343F 5′- TACGGRAGGCAGCAG -3′, 798R 5′- AGGGTATCTAATCCT -3’). PCR reaction was performed using phusion high-fidelity PCR Mastermix (Invitrogen, Carlsbad, CA, USA) with the following condition: 95 °C for 3 min (1 cycle), 95 °C for 30 s /55 °C for 30s /72 °C for 30s (35 cycles), 72 °C for 10 min. PCR product was purified using Agencourt AMPure XP beads (Beckman coulter, Brea, CA) according to manufacture’s protocol. Pyrosequencing was conducted on an Illumina Miseq 2*300 platform according to protocols.

**Pyrosequencing data bioinformatics analysis.** Raw reads were filtered according to length and quality criteria. Filter-pass reads were assembled. After assembly, chimeric sequences were removed using the Usearch software based on the Uchime algorithm (14). Operational Taxonomic Unit (OTU) was picked using de novo OTU picking protocol with a 97% similarity threshold. Taxonomy assignment of OTUs was performed by comparing sequences to Greengenes. Enterotype-like clustering was performed in R with package “BiotypeR” on Jensen-Shannon distance for the OTU-level relative abundance profile (15). The optimal number of
clusters was chosen based on Calinski-Harabasz (CH) values. To determine compositional features that were differentially abundant either between clusters, LEfSe was applied (16). The R package “phyloseq” was used for alpha diversity analysis (17).

Cytokine and chemokine measurements. The plasma levels of 27 cytokines and chemokines were measured using the Bio-Plex ProTM Human Cytokine Array 27-Plex Group I on a Luminex200TM (Luminex®Multiplexing Instrument, Merck Millipore) following the manufacturers’ instructions. Then, we analyzed the raw data using xPONENT 3.1 software (Merck Millipore). We assumed a value of 0.1 pg/mL for statistical purposes in cases in which the concentration was undetectable.

Statistical analysis. Two-sided student’s t-test and Mann-Whitney U-tests was used to determine whether the differences in the alpha-diversity, cytokine and chemokine levels between groups were statistically significant. We used Spearman’s rank correlation coefficient analysis to analyze the linear correlation. The Benjamini & Hochberg method was used to control the false discovery rate for multiple testing corrections. The statistical tests and plotting were done in R with package “plyr”, “ggplot2”.

Results

Clinical characteristics and pyrosequencing data summary. A total of 100 cirrhotic patients were included in this AF microbial profiling study. Of the patients, 56 patients were Hepatitis-B-
virus related, 15 patients were alcoholic cirrhosis, 3 patients were primary biliary cirrhosis, and
other 26 patients were cryptogenic cirrhosis. Of all the patients, a total of 29 patients died in 90
days, with a short term mortality of 29%. A total of 1,595,225 high-quality sequences were
produced, accounting for 98.1% of valid sequences (average sequence length 419 bp).

Two clusters identified for ascitic microbiome. The partitioning around medoids method
using Jensen-Shannon distance for the OTUs-level relative abundance profile was used to
investigate whether AF microbiota can be classified into clusters. The Silhouette index for two
clusters was 1.86, which indicates strong support of two clusters. The two clusters were
visualized by between class analyze, and showed clear separations (Figure 1a). Two clusters
were confirmed with the highest CH value, as the optimal number of clusters (Figure 1b).

When comparing the diversity index, Cluster 1 were found to have significantly lower
richness than Cluster 2. After the sequencing depth normalized, the observed OTUs in Cluster 1
was significantly lower than in Cluster 2 (133±53 vs. 190±49, p=0.001) (Figure 1c), as well as the
index of Chao 1 (156±69 vs. 206±53, p=0.004) (Figure 1d).

Compositional analysis of AF microbial clusters. To identify signature taxa, we tested for
significant differences among taxa displaying >1% relative abundance in the whole dataset
(Figure 2). Major differences were observed between Cluster 1 and Cluster 2. At the phylum
level (Figure 2a), Bacteroidetes and Firmicutes were found over-represented in Cluster 1, while
Proteobacteria and Actinobacteria were found over-represented in Cluster 2.
At the class level (Figure 2b), *Bacteroidia*, *Bacilli*, *Clostridia*, and *Gammaproteobacteria* were found with significant higher relative abundance in Cluster 1 than in Cluster 2. And, *Alphaproteobacteria*, *Erysipelotrichia*, *Actinobacteria*, and *Coriobacteria* were enriched in Cluster 2.

At the family level (Figure 2c), *Bacteroidia* was linked to increased *S24-7, Prevotellaceae* and *Rikenellaceae*, while *Clostridia* expansion was linked to increased *Lachnospiraceae* in Cluster 1. Besides, *Lactobacillaceae* and *Vibrionaceae* were also found enriched in Cluster 1. The expansion of *Alphaproteobacteria* in Cluster 2 was linked to increased *Acetobacteraceae, LD12-freshwater group, Rickettsiaceae*, and *A0839*, while *Gammaproteobacteria* abundance was linked to increased *Moraxellaceae*.

At the genus level (Figure 2d), bacterial genus found over-represented in Cluster 1 included *Lactobacillus, Lachnospiraceae NK4A136 group, Alloprevotella, Vibrio, and Prevotella*. Bacterial genus *Saccharibacter, Commensalibacter, Erysipelotrichia, Allobaculum, Rickettsia*, and *Cupriavidus* were found enriched in Cluster 2.

**Plasma cytokine and chemokine levels correlated with the AF microbial compositions.** We performed multiples analyses of 27 cytokine and chemokine mediators using the plasma samples collected from the cirrhotic patients. The plasma cytokine and chemokine profile was measured in 78 patients (68 patients of Cluster 1 and 10 patients in Cluster 2). The level of plasma cytokine PDGF-BB was found significantly higher in patients of Cluster 1 than in patients...
of Cluster 2 (12.5±18 vs. 5.2±4.2, p=0.006). Plasma cytokine IL-7 (6.39±6.31 vs. 3.96±1.87, p=0.015) and IL-17A (9.03±29.72 vs. 0.35±0.77, p=0.019) were also found significantly higher in patients of Cluster 1 than in patients of Cluster 2 (Figure 3).

At the OTU level, there were 14 OTUs correlated with plasma cytokines (Figure 4). The 14 OTUs were from six bacterial families, including \textit{S24-7 group} (6 OTUs), \textit{Lachnospiraceae} (2 OTUs), \textit{Rikenellaceae} (2 OTUs), \textit{Veillonellaceae} (2 OTUs), \textit{Helicobacteraceae} (1 OTU), and \textit{Prevotellaceae} (1 OTU). There were four OTUs closely correlated with multiple cytokines, which were OTU 140 and OTU 271 (\textit{S24-7 group}), OTU 68 (\textit{Veillonellaceae}), and OTU 53 (\textit{Helicobacteraceae}).

\textbf{Cluster 1 showed higher mortality rate but lower incidence of SBP.} Among the 100 patients in the study, a total of 86 patients (86%) were classified as AF Cluster 1, while other 14 patients (14%) fell into AF Cluster 2. All the patients in study were followed up for 90 successive days for occurrence of death and complications. For patients of AF Cluster 1, 29 patients died in 90 days, with a short term mortality of 33.7%. And in patients of AF Cluster 2, no patients died in 90 days. The short term mortality was significantly higher in Cluster 1 than in Cluster 2 (p=0.01) (Table 1). However, the incidence of SBP was found slightly higher in Cluster 2 than in Cluster 1 (p=0.06). Among the 86 patients in Cluster 1, only 27 patients had SBP in 90 days, with a prevalence of 31.4%. And in Cluster 2, 8 patients were diagnosed SBP in 90 days, with a prevalence of 57.1%. The severity of disease, as estimated by MELD score or Child Pugh score, was comparable between Cluster 1 and Cluster 2.
Discussion

Bacterial translocation is thought as a major mechanism of complications and mortality in end-stage liver disease. Previous studies have confirmed presence of bacterial DNA in ascites fluid, even in culture-negative and non-neutrocytic ascites. Also, positive associations between ascites bacterial burden and poor clinical outcomes was observed (7). This study demonstrated that not only the quantity of bacteria in AF but also the composition could impact the clinical outcomes of end-stage liver disease. The possible mechanism might be associated with systemic immune responses and cytokine expressions.

Based on the microbial composition of AF, the patients could be classified into two clusters. Cluster 1 has high mortality, and Cluster 2 has high prevalence of SBP. The group with higher mortality had more Bacteroidetes and Firmicutes than another group. Firmicutes and Bacteroidetes are the two most dominant bacterial phyla in human intestines (18). The translocation of these commensal bacteria into the abdominal cavity might indicate severe damage of intestinal mucosa barrier, which usually lead to poor outcomes. Structural and functional alterations in the intestinal mucosa that increase intestinal permeability to bacteria and its products have been described in cirrhosis (19). Altered gut microbiota profile is associated with high intestinal permeability, including less abundant of Ruminococcaceae and more abundant of Lachnospiraceae in subjects with high intestinal permeability compared with subjects with low intestinal permeability (20). Accordingly, it was observed here that ascitic microbiota of Cluster 1 have more abundance of Lachnospiraceae than that of Cluster 2. The
findings add to the evidence that bacterial translocation from the leaky gut participate in the progress of liver cirrhosis.

Our results showed over-represented of Alphaproteobacteria and Actinobacteria in AF of Cluster 2. Both Alphaproteobacteria and Actinobacteria are common members of marine and freshwater bacterioplankton assemblages. Genomic analysis demonstrated the genome characteristics of the bacterioplankton that render them to well adapted to such nutrient and energy-limited conditions (21). One possible reason is that the intestinal permeability of patients in Cluster 2 is not severely damaged as patients in Cluster 1, and bacteria which are able to translocate to AF need greater ability to survive in nutrient-limited circumstances.

In our study, the enrichment of gut commensal bacteria in AF showed no associations with SBP. Conflict results have been demonstrated regarding the correlation between intestinal permeability and infectious complications of cirrhotics. Several previous studies found that there was no relationship of intestinal permeability with the incidence of complications (22). And, no significant difference of intestinal permeability was observed between cirrhotic patients with and without SBP (23).

Our research found that several cytokines were upregulated in patients of Cluster 1, including IL-17A, IL-7, and PDGF-BB. IL-17A is a pro-inflammatory cytokine, mainly produced by Th17 cells (24). IL-17A plays dual roles including protection host from bacterial and fungi infections, and participating in the autoimmunity diseases (25). Gut microbiota is supposed to induce Th17
differentiation and thus regulate IL-17A production and functions (26). Decreased *Firmicutes/Bacteroidetes* ratio, has been reported in systemic lupus erythematosus, that is associated with increased Th17 activation and differentiation (27). In consistent with the previous study, the AF microbiota of Cluster 1 showed decreased *Firmicutes/Bacteroidetes* ratio with increased plasma IL-17A level. The results confirmed associations between gut microbiota and Th17 differentiation. IL-17A plays important roles in fighting against pathogenic microbes using several mechanisms including induction of antimicrobial peptides, such as α-defensins, β-defensins, and lysozyme (28) (29) (30). This might explain the relative lower prevalence of SBP in patients of Cluster 1. However, Cluster 1 patients had significantly higher short-term mortality. IL-17A is able to up-regulate inducible nitric oxide synthase (iNOS), an enzyme that participates in the production of nitric oxide (31). The importance of iNOS-derived nitric oxide production in promoting BT has been evidenced experimentally in several studies. The mechanisms included that induces gastric mucosal damage, decreases the viability of rat colonic epithelial cells, directly dilates TJs in intestinal epithelial monolayers, inhibits ATP-formation and hence, increases intestinal permeability (1).

Our study demonstrated close correlations between *S24-7* family and plasma cytokines. *Bacteroidales* *S24-7* family is one of the substantial component of gut microbiota, which has not been successfully cultured. Multiple studies have since reported the altered abundance of *S24-7* family members in association with different physical conditions. *S24-7* is more abundant in diabetes-sensitive mice fed a high-fat diet, in particular when chow is supplemented with gluco-oligosaccharides (32). In mouse model of colitis, enrichment of *S24-7* was observed following
treatment-induced remission of colitis in mice (33). Some members of the S24-7 family are
targeted by innate immune system by inducing high-affinity antigen-specific IgA responses and
become highly coated with IgA (34). It was proposed that bacterial species highly coated with
IgA could stimulate intestinal immunity and drive intestinal disease.

Using comparative genomics analysis, a subset of S24-7 family was found to have urease
encoding genes (35). In the intestine, bacterial urease converts host-derived urea to ammonia
and carbon dioxide, contributing to hyper-ammonemia associated neurotoxicity and
encephalopathy in patients with liver disease (36). Urease is also a recognized virulence factor
in both bacterial and fungal infection (37). The enrichment of urease-positive species in Cluster
1, such as S 24-7 family and Lactobacillus, might suggest a possible mechanism of bacterial
produced ammonia in progression of end-stage liver disease.

Bacterial class Gammaproteobacteria was found with significantly higher relative abundance
in Cluster 2 than in Cluster 1. The Moraxellaceae and Acinetabacter were responsible for the
enrichment of Gammaproteobacteria at the family and genus level, respectively. Acinetobacter
species have become increasingly important nosocomial pathogens worldwide and can result in
a wide range of infections, including bacteremia, pneumonia, urinary tract infection, peritonitis,
among other (38). In end stage liver disease, Acinetobacter spp. is among the major pathogens
that responsible for SBP (39). Acinetobacter species infections in liver transplant patients have
been researched in several studies. Acinetobacter baumannii and Acinetobacter Iwoffii were
two major pathogenic species isolated (40). Acinetobacter infections after liver transplantation
were associated with a high mortality (41). Preoperative MELD scores were more likely to be higher among the non–*Acinetobacter baumannii* compared with the *Acinetobacter baumannii*–infected group (40).

Conclusions

Our study demonstrated that the microbial composition of AF in cirrhotic patients have correlations with short-term clinical outcomes. The possible mechanisms include intestinal permeability-dependent bacterial translocation and immune responses triggered by different translocated microbes. While the present study strengthens the association between AF microbiota and clinical outcomes, it has limitations. The intestinal microbiota and intestinal permeability of the participants was not analyzed simultaneously. We were unable to identify a direct pathway among gut dysbiosis, intestinal permeability, and immune response in end-stage liver disease. Future in vivo and in vitro studies are in need to investigate the direct correlations among intestinal permeability, translocated bacteria and immune responses.

List of abbreviations

BT, bacterial translocation; SBP, spontaneous bacterial peritonitis; AF, ascitic fluids; SDD, selective decontamination of the digestive tract; OTU, operational taxonomic unit; CH, Calinski-Harabasz index;

Declarations

*Ethics approval and consent to participate*. The ethics committee of the First Affiliated
Hospital, College of Medicine, Zhejiang University approved all the work on February 27th 2014, IRB ID#2014073. All patients gave written informed consent for inclusion in the study. The data were analyzed without personal identifiers.

**Consent for publication.** Not applicable

**Availability of data and material.** All data generated or analysed during this study are included in this published article and its supplementary information files.

**Competing interests.** The authors declare they have no competing interests.

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**Authors’ contributions.** YFC and JG participated in the design of the study, collected biopsy samples, performed the statistical analysis, and write the paper. DS and DQF carried out the DNA extraction, and performed 16S rRNA gene PCR amplification. CLC carried out the cytokine profile analysis. LJL conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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References


Table

Table 1 Clinical characteristics of patients with different ascitic microbiota clusters

<table>
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<th>Variable</th>
<th>Cluster 1 (n=86)</th>
<th>Cluster 2 (n=14)</th>
<th>p-value</th>
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<tr>
<td>Age (yr)</td>
<td>56±11</td>
<td>54±15</td>
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<td>Gender (M/F)</td>
<td>16/70</td>
<td>4/10</td>
<td>0.7</td>
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<td>Child-Pugh score</td>
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<td>9±2</td>
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<td>MELD score</td>
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<td>1.43±0.57</td>
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<td>90-days mortality</td>
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<td>0%</td>
<td>0.01</td>
</tr>
<tr>
<td>90-days SBP</td>
<td>31.4%</td>
<td>57.1%</td>
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</table>

Abbreviation: SBP, Spontaneous bacterial peritonitis

Figure legends

Figure 1. Two clusters were observed in ascitic fluids microbiota. a. The principal coordinate analysis of the Jensen-Shannon distance generated from the OTU-level relative abundance profiles. Samples are colored by clusters identified by the partitioning around medoids clustering algorithm. Dark blue, Cluster 1; red, Cluster 2. b. Two clusters were supported with the highest Calinski-Harabasz (CH) pseudo F-statistic value, as the optimal number of clusters. c. Boxplot comparison of the number of observed OTUs between Cluster 1 and Cluster 2. d.
Boxplot comparison of the Chao1 index between Cluster 1 and Cluster 2. **p < 0.01 based upon Mann-Whitney U-tests with Benjamini & Hochberg correction.

**Figure 2.** Compositional analysis of ascitic microbial clusters. **a.** Pie chart comparison of bacterial phyla represented in two clusters (upper: Cluster 1, lower: Cluster 2). **b.** Histograms showing differentially enriched bacterial classes between Cluster 1 and Cluster 2. Blue histograms, Cluster 1; red histograms, Cluster 2. **c.** LEfSe analysis revealed differentially enriched bacterial families associated either with Cluster 1 (blue) or Cluster 2 (red). **d.** LEfSe analysis revealed differentially enriched bacterial genus associated either with Cluster 1 (blue) or Cluster 2 (red).

**Figure 3.** Plasma cytokine levels with statistical difference between Cluster 1 and Cluster 2. Blue histograms, Cluster 1 (n = 68); red histograms, Cluster 2 (n = 10). Significance values are indicated: * p < 0.05 **p < 0.01 based upon Mann-Whitney U-tests with Benjamini & Hochberg correction.

**Figure 4.** Correlation analysis of ascitic bacterial OTUs and plasma cytokines. Spearman rank correlation was performed. Only correlations with a coefficient r > 0.40 are displayed. The colors of OTU nodes and lines indicate bacterial families as labeled on the lower right.