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200 patients were studied using quantative metagenomics. Progression of cirrhosis, is associated with changes in gut-microbiome characterized by progressively reduced metagenomic species richness and increase in Peptostreptococous sp. Microbiome correlated with clinical outcomes, survival and functional changes.
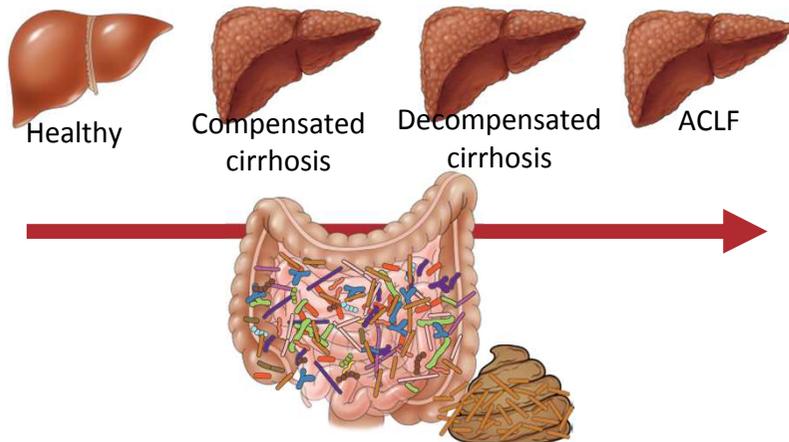
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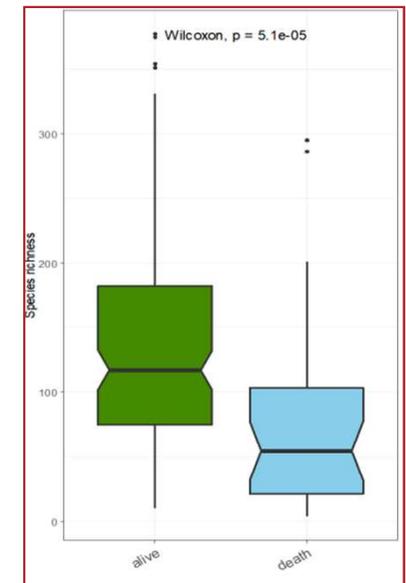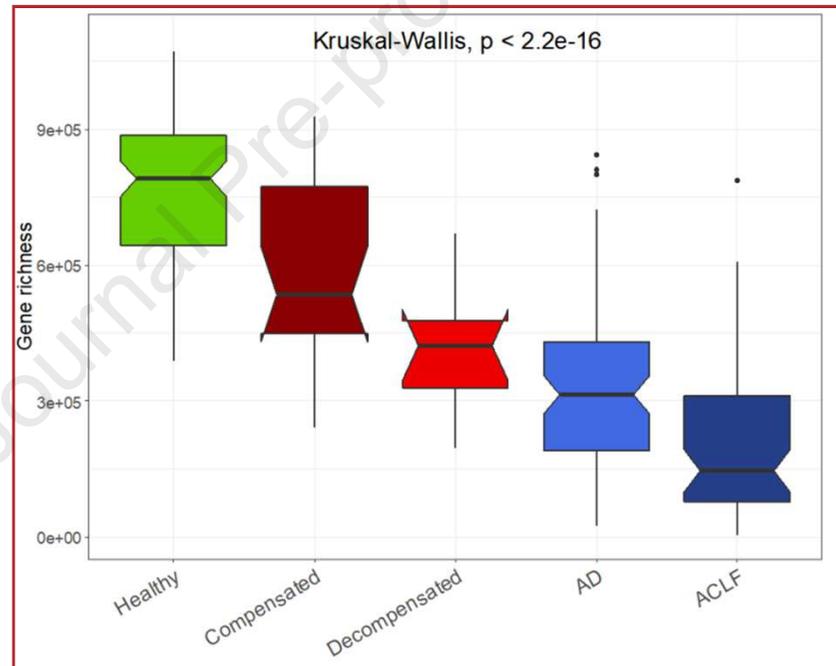
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Short title: Gut microbiome in cirrhosis and ACLF

Abbreviations: HE (hepatic encephaplopathy), ACLF (acute-on-chronic liver failure), MELD (model end-stage liver disease), CAG (co-abundance gene groups), MGS (metagenomic species), KEGG (Kyoto Encyclopedia Genes and Genomes), KO (KEEG Orthorlogy).

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of the manuscript for important intellectual content, obtained funding and study supervision

ABSTRACT

Background & Aims: Cirrhosis is associated with changes in gut microbiome composition. Although ACLF is the most severe clinical stage of cirrhosis, there is lack of information about gut microbiome alterations in ACLF using quantitative metagenomics. To investigate the gut microbiome in patients with cirrhosis encompassing the whole spectrum of disease: compensated, acutely decompensated without ACLF, and ACLF. A group of healthy subjects was used as controls.

Methods: Stool samples were collected prospectively in 182 patients with cirrhosis. DNA library construction and sequencing was performed using the Ion Proton sequencer. Microbial genes were grouped into clusters, denoted as metagenomic species(MGS).

Results: Cirrhosis was associated with a remarkable reduction in gene and MGS richness compared to healthy subjects. This loss of richness correlated with disease stages and was particularly marked in patients with ACLF and persisted after adjustment for antibiotic therapy. ACLF was associated with a significant increase of *Enterococcus* and *Peptostreptococcus sp*, and reduction of some autochthonous bacteria. Gut microbiome alterations correlated with MELD and Child-Pugh scores and organ failures and was associated with some complications, particularly hepatic encephalopathy and infections. Interestingly, gut microbiome predicted 3 month-survival with good stable predictors. Functional analysis showed that patients with cirrhosis had enriched pathways related to ethanol production, GABA metabolism, and endotoxin biosynthesis, among others.

Conclusions: Cirrhosis is characterized by marked alterations in gut microbiome that parallel disease stages with maximal changes in ACLF. Altered gut microbiome was associated with complications of cirrhosis and survival. Gut microbiome may contribute to disease progression and poor prognosis. These results should be confirmed in future studies.

Keywords: chronic liver diseases, gut-liver axis, infections, liver failure

INTRODUCTION

Cirrhosis of the liver is associated with marked alterations of the gut-liver axis that are believed to play a role in some complications of the disease (1). One of the most important consequences of the abnormal gut-liver axis is the development of pathological translocation of bacteria and/or bacterial products from the gut to lymph nodes (2). This increased translocation appears to be an important triggering factor of systemic inflammation characteristic of advanced cirrhosis and is key to the development of bacterial infections which are a major cause of morbidity and mortality (3).

It has been known for many years that in cirrhosis, particularly in advanced stages of the disease, there is an important gut dysbiosis (4,5). Earlier studies showed that this dysbiosis is characterized by an overgrowth of some potentially pathogenic bacteria together with reduced amounts of some beneficial autochthonous bacteria, which could contribute to bacterial translocation and increased risk of infections (6). Knowledge on alterations of gut microbiota in cirrhosis has improved in recent years with the use of techniques that allow identification and quantification of gut microbes. Several studies have shown a reduction in autochthonous taxa, including *Lachnospiraceae, Ruminococcaceae,* and Clostridiales XIV and an increase in pathogenic taxa such as *Enterococcaceae, Staphylococcaceae, and Enterobacteriaceae,* an alteration that appears to worsen as the disease progresses (6–8). Moreover, it has been shown that these abnormalities correlate with development of some complications of the disease, particularly hepatic encephalopathy (HE) (9). These studies used targeted sequencing of 16S ribosomal RNA, a technique that is limited to assessment of bacterial taxonomical composition, and does not provide a comprehensive study of bacterial genes. Many recent studies in a number of diseases such as obesity and diabetes

have used high-throughput methods of untargeted DNA sequencing in conjunction with human microbial gene catalogues, allowing microbial species-level and strain-level resolution and detailed function annotations of microbial communities (10,11). To our knowledge, there is only one study reporting the use of this technology in cirrhosis which showed a profound alteration of gut microbiome characterized by reduced gene and metagenomic richness and marked depletion of metagenomic species together with colonization of the gut by oral bacterial species (12). Nevertheless, in this study most patients had compensated hepatitis-B cirrhosis and the relationship between alterations in metagenomic species and disease stage or outcomes was not assessed. Moreover, the study did not evaluate gut microbiome in patients with acute-on-chronic liver failure (ACLF), a condition that represents the end of the clinical spectrum of cirrhosis characterized by one or more organ failures, frequent association with bacterial infections, and high mortality (13), in which the assessment of gut microbiome alterations is of marked relevance. On this background, we aimed at investigating gut microbiome using quantitative metagenomics in a large series of patients with cirrhosis encompassing the whole spectrum of the disease, from compensated to decompensated cirrhosis and ACLF. Our study demonstrates a profoundly abnormal gut microbiome in cirrhosis compared to healthy subjects that is exceptionally altered in patients with ACLF, and provides a characterization of abnormalities of metagenomic species throughout the progression of the disease, and their correlation with clinical features and mortality.

PATIENTS AND METHODS

Population and study design

This is a prospective study performed in 182 patients with cirrhosis seen at the Liver Unit of Hospital Clinic of Barcelona between March 2015 and February 2017. Eleven patients were studied on two time points when they were in different stages of cirrhosis, and only the first sample of those patients was taken into account for survival analysis. The study was aimed at assessing gut microbiome in cirrhosis and its relationship with clinical findings and disease stages. Inclusion criteria were: 1) age > 18 years; and 2) cirrhosis diagnosed by either liver biopsy or a combination of clinical, analytical, ultrasound, elastographic and/or endoscopic findings. Exclusion criteria were: 1) severe extrahepatic diseases; 2) hepatocellular carcinoma beyond Milan criteria; 3) previous organ transplantation; 4) HIV infection; and 5) lack of informed consent. Patients with cirrhosis were categorized in 4 groups: 1) patients with compensated cirrhosis (i.e without current complications of the disease); 2) ambulatory patients with stable decompensated cirrhosis; 3) patients hospitalized because of acute decompensation of cirrhosis without ACLF; and 4) patients with ACLF. In addition, a group of healthy subjects from the Spanish MetaHit project cohort were also included as controls (14).

At inclusion in the study, demographic, clinical, and laboratory data were recorded and a fecal sample was collected. For outpatients, subjects were asked to provide a stool within the following 7 days of the screening visit. For hospitalized patients, data were collected at admission and the fecal sample was collected when patient provided the stool (median of 2 days - IQR 1-4 days - after admission). Data collected was related to cirrhosis (etiology, alcohol consumption, laboratory variables, and complications prior to inclusion in the study as well as during and after hospitalization), current and past medications, and non-hepatic diseases. Special care was taken in the assessment of presence of infection as well as current or prior use of antibiotics. All patients were followed-up for at least 3 months since inclusion in the study.

Decompensated cirrhosis was defined when patients had one of the following complications: ascites, gastrointestinal bleeding, hepatic encephalopathy grade 2 or greater, and/or bacterial infections, without meeting the diagnostic criteria of ACLF. ACLF was defined according to the type and number of organ failures, as per the Canonic study and EASL guidelines (13,15).

The study was approved by the Ethics Committee of the Hospital Clínic of Barcelona (HCB/2014/0577), and all patients (and/or relatives) signed a written informed consent before entering in the study.

Methods

A quantitative metagenomic pipeline following the International Human Microbiome Standards (IHMS; http://www.microbiome-standards.org) was used to assess both composition and function of the gastrointestinal microbiome (16).

Fecal samples collection

Fecal samples from hospitalized patients were collected following the protocol IHMS SOP002 and 003, using when needed an anaerobic generator and processed within 24hours. At laboratory, 1gr of feces was mixed with 4mL of stabilizing solution (RNAlater Stabilization Solution, ThermoFisher Scientific, Waltham, US). Feces sampling from outpatients were done following the protocol IHMS SOP005, self-collection samples were preserved in stabilizing solution at room temperature and handled to the biological laboratory within 24h to 7 days after collection. All the samples were homogenized and aliquoted to 200 mg sub-samples which were kept at -80°C until DNA extraction.

DNA extraction

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DNA was extracted from one sub-sample following IHMS SOP007 V2. DNA was quantitated using Qubit Fluorometric Quantitation (ThermoFisher Scientific, Waltham, US) and qualified using DNA size profiling on a Fragment Analyzer (Agilent Technologies, Santa Clara, US).

DNA library construction and sequencing

3 µg of high molecular weight DNA (>10 kbp) was used to build the library. Shearing of DNA into fragments of approximately 150 bp was performed using an ultrasonicator (Covaris, Woburn, US) and DNA fragment library construction was performed using the Ion Plus Fragment Library and Ion Xpress Barcode Adaptaters Kits (ThermoFisher Scientific, Waltham, US). Purified and amplified DNA fragment libraries were sequenced using the Ion Proton Sequencer (ThermoFisher Scientific, Waltham, US), resulting in 21.9 ± 3 million (mean ± SD) single-end short reads of 150-baselong single-end reads on average.

Quality control reads

Reads were cleaned using Alien Trimmer24 in order (i) to remove resilient sequencing adapters and (ii) to trim low quality nucleotides at the 3' side using a quality and length cut-off of 20 and 45 bp, respectively. Cleaned reads were subsequently filtered from human and other possible food contaminant DNA (using Human genome RCh37-p10, Bos taurus and Arabidopsis thaliana with an identity score threshold of 97%).

Gene abundance profiling

The gene abundance profiling was performed using the 10.4 million gene integrated reference catalog of the human microbiome (12). Filtered high-quality reads were mapped with an identity threshold of 95% to the 10.4 million-gene catalogue using Bowtie (17) included in METEOR software (18). The gene abundance profiling table was generated by means of a two-step procedure using METEOR. First, the unique

mapped reads (reads mapped to a unique gene in the catalogue) were attributed to their corresponding genes. Second, the shared reads (reads that mapped with the same alignment score to multiple genes in the catalogue) were attributed according to the ratio of their unique mapping counts. The gene abundance table was processed for rarefaction and normalization and further analysis using the MetaOMineR (momr) R package (19).

Read downsizing

To decrease technical bias due to different sequencing depth and avoid any artifacts of sample size on low abundance genes, read counts were rarefied. The gene abundance table was downsized to 12 million mapped reads for each sample. After that, we found 4762 – 928686 genes for the 182 samples, with an average of 325147.5 genes. The resulting rarefied gene abundance table was normalized according to the FPKM strategy (normalization by the gene size and the number of total mapped reads reported in frequency) to give the gene abundance profile table.

Metagenomics species construction

The gene catalogue was clustered by co-abundance as it was previously described (20), which defined 6,300 co-abundance gene groups (CAGs) with high correlations (Pearson correlation coefficient > 0.9). The 1,529 largest of these, with more than 500 genes, were considered as metagenomics species (MGS) and referred to as species throughout the article.

The abundance profiles of the CAGs and MGS throughout the samples were determined as the mean abundance of 50 marker genes. Furthermore, the CAGs and MGSs were taxonomically annotated, by summing up the taxonomical annotation of their genes as described by Nielsen et al (20).

Microbial gene richness (gene count) was calculated by counting the number of genes that were detected at least once in a given sample, using the average number of genes counted in 10 independent rarefaction experiments. MGS richness (MGS count) was calculated directly from the MGS abundance matrix.

Statistical analysis

Clinical data. Comparisons between groups were performed using Student *t* test or ANOVA for normally distributed continuous variables, Mann-Whitney U test or Kruskal-Wallis test for non-normally distributed continuous variables, and chi-square test or Fisher exact test for categorical variables. All statistical tests were 2-tailed and p values <0.05 were considered significant. Statistical analysis was performed using SPSS statistical package (IBM SPSS Statistics 22.0).

Richness analysis. Global comparisons between groups were performed using a Kruskal-Wallis test and pairwise comparisons were performed with a post hoc Dunn test; we identified a significant difference if p-value was <0.05.

Comparison with the MetaHit cohort (14) was performed using a Wilcoxon rank-sum test.

Similarity between samples. Spearman correlation between the samples was performed using the abundance of the species detected in the samples. Hierarchical clustering of the samples was performed using the Ward's method.

Taxonomy distribution. The abundance of each genus was computed throughout samples as the sum of the abundance of all species belonging to the considered genus. Mean abundance of each genus in each group was next computed. Only genus representing more than 1% of the total composition were represented.

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Barcode visualization. MGS occurrence and abundance within samples is visualized using "barcodes", a heatmap of the frequency abundance table of 50 marker genes with samples in columns and genes in rows. A heat color code is used (white for 0, lightblue < blue < green < yellow < orange < red for increasing abundance, each color change corresponding to a 4-fold abundance change). In these barcodes, MGS appear as vertical lines (co-abundant marker-genes in the sample) colored according to gene abundance.

Gut metagenome analysis. To identify associations between metagenomics profiles and populations, a Kruskal-Wallis and a post hoc Dunn test were performed. A Benjamini-hochberg correction (21) was applied on the results of the Kruskal-Wallis test. We identified a MGS marker if Kruskal-Wallis corrected p-value <0.05 and Dunn p-value <0.05.

Coefficients of correlation between metagenomics profiles and clinical data, and their significance, were computed using a Spearman correlation. A Benjamini-Hochberg correction was applied and MGS with at least one significant correlation at the threshold of p-value<0.01 were represented. Correlations with a p-value≤0.05 were printed.

Functional analysis. The annotation of the genes based on the KEGG database (v82) was used. Through the functional annotation of the reference gene catalog to KEGG orthology (KO) groups, abundances of KO were computed for each sample. Gut metabolic modules (GMM) information (22) has also been used: gene counts for each KO have been summed and GMM module abundance were computed using an internal pipeline taking into account complex and alternative paths. An abundance matrix for the functional modules was obtained.

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Differentially abundant GMM were computed with a Wilcoxon rank-sum test between decompensated cirrhosis and ACLF samples and between healthy subjects and cirrhosis samples in additional results.

Model construction and validation. The predictive power of the gut microbiota for the prediction of mortality was assessed using a Penalized Logisitic Regression (PLR) model. Logistic regression is a supervised method for a two or multi-class classification problem (23). Model construction, parameter fine-tuning and validation was performed using the Caret package (Classification And REgression Training) (24).

We used PLR to build a predictive model of the mortality at three months based on the gut microbiota profiles using in input the species-level abundance data.

Input data were restricted to the samples with a status alive, A, (n_A=137) or deceased, D, (n_D=37, total n_T=174) at 3 months, and to the species with an occurrence >10% in the cohort. Training and discovery cohorts were respectively 65% (n_A=80, n_D=23, n_T=103) and 35% (n_A=43, n_D=11, n_T=54) of the global cohort, randomly drawn. Three pre-processing steps were applied on the features in the training cohort: near-zero variance and high-correlated variables were filtered, and linear combinations were removed, using caret built-in functions (25).

For each model, the performance was evaluated on the discovery cohort with the AUC, the sensitivity and specificity. The coefficients of the regression were used as a way to measure the importance of the features in the prediction of each status. A positive coefficient indicated alive status whereas a negative one deceased status.

To evaluate the global robustness and performance of the models, 300 repetitions were performed, each time with a new random draw of the samples in the training and discovery cohort. The mean value of AUC, sensitivity, specificity and number of predictors were computed. For each predictors, the mean value and the standard

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deviation of all the coefficients were computed. A list of stable predictors was obtained, composed of the features selected in more than 10% of all the models and for which abs(mean(coef)) > abs(sd(coef)), in order to keep only predictors which were always indicating the same status.

We also used random forest models with the Caret package based on the random forest R function. In out validation scheme, 80% of the cohort was used for model training and 20% for model testing, repeated 100 times. The number of trees was set to 500 by default, and the mtry parameter was adjusted during the training process. The accuracy for model on training data is based on the out-of-bag error. The unbalanced dataset was compensated by an oversampling strategy in order to give equal importance to the 'alive' and 'death' classes.

RESULTS

Characteristics of study population

Demographic, clinical, and laboratory data of patients included in the study are shown in table 1. Most patients were male (71%) and their mean age was 60±11 years. The most common causes of cirrhosis were excessive alcohol consumption and hepatitis C infection. As expected, patients with ACLF had more advanced liver disease compared to those of the other groups. In fact, they had higher frequency of ascites and hepatic encephalopathy, and more marked impairment of liver and renal function tests, and higher Child-Pugh and MELD scores compared to patients with decompensated cirrhosis without ACLF. By contrast, the frequency of bacterial infections in patients with decompensated cirrhosis without ACLF and in those with ACLF was similar. The type and characteristics of bacterial infections in both groups are shown in supplementary table 1. Finally, the number of patients receiving prophylactic antibiotics, either rifaximin or norfloxacin, was also similar between these two groups.

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Comparison of gut microbiome between healthy subjects and patients with cirrhosis

A hierarchical clustering analysis including all samples was performed and showed that patients with cirrhosis were clearly separated from healthy subjects. Moreover, gene and MGS richness were strikingly decreased in patients with cirrhosis compared to healthy subjects (supplementary figure 1). Overall, 613 MGS had significant differential abundance between healthy subjects and the whole series of patients with cirrhosis, 566 MGS were enriched in healthy subjects and 47 in patients with cirrhosis. Remarkably, *Enterococcus sp* and oral species such as *Streptococcus oralis* and *Streptococcus parasanguinis* were significantly enriched in patients with cirrhosis (supplementary figure 2).

To assess the existence of possible differences between healthy subjects and patients with early stages of cirrhosis, we compared the group of patients with compensated cirrhosis with that of healthy subjects. Thirty-six MGS had significant differential abundance between the two groups; 23 MGS were enriched in healthy subjects and 13 in compensated cirrhosis (Supplementary Figure 3). Patients with compensated cirrhosis had higher levers of *Clostridium sp*, *Erysipelatoclostridium ramosum* and *Streptococcus parasanguinis* as compared to healthy subjects. We performed prediction models to evaluate microbial profiles that could discriminate healthy subjects from patients with compensated cirrhosis. A model was obtained with 51 stables predictors (20 associated with healthy subjects and 30 associated with compensated cirrhosis) (AUROC of 0.81).

Characterization of gut microbiome across different stages of cirrhosis. Relationship with complications and disease severity

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Metagenomic sequencing revealed that gene richness and MGS richness significantly decreased with disease progression (p <0.001) (Figure 1). Among the different stages of cirrhosis, patients with ACLF had the lowest richness, which was significantly lower than that of patients with decompensated cirrhosis without ACLF (p<0.01). By contrast, patients with compensated cirrhosis had the highest richness, yet significantly lower than that of healthy subjects. Of interest, outpatients with stable decompensated cirrhosis had higher gene and MGS richness compared to that of inpatients with decompensated cirrhosis. Interestingly, this loss of richness that paralleled disease progression persisted after adjustment for antibiotic therapy, which suggests that findings observed could not be explained on the basis of a distinct or more broad-spectrum antibiotic therapy frequently given to patients with advanced stages of cirrhosis (Figure 1). To further explore the relationship between microbiome findings and disease stages, we then analyzed significantly different MGS in the different stages of cirrhosis. Overall, 354 MGS were significantly contrasted between at least two groups. The most contrasted MGS are shown in Figure 2. Of interest, 72 MGS contrasted between patients with decompensated cirrhosis without ACLF and patients with ACLF. Particularly, patients with ACLF were enriched in MGS of *Enterococcus* and *Peptostreptococcus* species. On the contrary, patients with ACLF had loss of some species *such as Roseburia, and Firmicutes*. To further assess the relationship between MGS and clinical features, we categorized patients according to relevant clinical findings, including etiology of cirrhosis, active alcohol consumption, treatment with beta-blockers, presence of complications, chronic antibiotic therapy, and laxative therapy (Table 2). Of note, active alcohol consumption, history of HE and chronic treatment with rifaximin, norfloxacin, or lactulose/lactitol were associated with significantly lower MGS richness compared to their respective counterparts (Supplementary figure 4-6), while differences according to alcoholic etiology and presence of infections were close to statistical significance. By contrast, chronic

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treatment with betablockers or proton pump inhibitors was not associated with significant differences in MGS richness.

Interestingly, in the overall group of patients with cirrhosis, a cluster of MGS positively correlated with the severity of cirrhosis, as estimated by MELD and Child-Pugh scores and the number of organ failures, indicating a strong relationship between disease severity and gut microbiome findings (Figure 3). Similar findings were observed when only patients not treated with antibiotics were analyzed separately (supplementary figure 7). The most relevant MGS that correlated significantly with MELD score are shown in supplementary figure 8. Thoroughly, 3 MGS correlated positively with MELD score, including *Enterococcus faecium, Enterococcus faecalis,* and the MGS Homo Sapiens. By contrast, 276 MGS correlated negatively with MELD score, indicating that the loss of some species, such as *Clostridiales, Faecalibacterium or Lachnoclostridium*, is associated with disease severity.

To reduce the complexity of the dataset, a network representation of MGS from patients with cirrhosis was performed (Supplementary Figure 9). Moreover, a Spearman correlation showed that community composed of MGS of the genus *Enterococcus* and oral bacteria like *Streptococcus* and *Veillonella* were positively correlated to the severity of the disease, as estimated by MELD score and negatively correlated with all other communities.

At the genus level, comparison between the overall group of patients with cirrhosis and healthy subjects showed an increase of genus *Bacteroides, Enterococcus*, and *Streptococcus* in patients with cirrhosis. On the contrary, in healthy subjects there was an increase of beneficial autochthonous bacteria, such as *Faecalibacterium, Eubacterium, and Ruminococcus.* Moreover, in parallel with cirrhosis progression, there was a significant increase of some pathogenic bacteria, particularly *Enterococcus* and *Peptostreptococcus,* and a significant decrease of some beneficial autochthonous

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bacteria, such as *Faecalibacterium Ruminococcus, Paraprevotella, Eubacterium, Phascolarctobacterium, Dorea, Oscillibacter, Lachnoclostridium Roseburia, and Blautia,* (supplementary figure 10).

Relationship between gut microbiome and prognosis

Of the 171 patients included in the analysis, 34 died during the 3-month follow-up period (7 from the decompensated cirrhosis group -8%- and 27 from the ACLF group -42%-). Patients who died had a significant loss of gene richness compared to those who survived (Figure 4 A). At MGS level, 17 were enriched in patients who died and 132 MGS were enriched in patients who survived. Remarkably, some *Enterococcus* species were more abundant in patients who died.

Prediction models were built to evaluate the capacity of gut microbiome to predict 3-month mortality. Overall, gut microbiome was a good predictor of mortality with an AUROC of 0.708. Some species were strongly associated with good prognosis, particularly *Paraprevotella clara, Bacteroides salyersiae, Clostridium sp, and Roseburia hominis.* On the contrary, other species such as *Enterococcus faecium, Streptococcus thermophilus, and Ruminococcus lactaris* were predictors of poor short-term survival (Figure 4 B). As an example, *Roseburia hominis* was found in 42% of patients who survived vs only 8% of those who died. By contrast, *Enterococcus faecium* was found in 66% of patients who died vs 29% of patients who survived.

In order to simplify and potentially improve the models predictive of 3-months mortality, we used random forest models based on microbiome richness and MELD taken separately or together. Accuracy was superior for MELD than for richness, both on the training and the test sets; it was expectedly higher for both on the training than on the test sets, possibly due to overfitting during training (Figure 4 C and D). However, accuracy of prediction by richness alone on the test sets was already high, with an

accuracy close to 0.75. Interestingly, accuracy was significantly improved by combining MELD and richness, above that obtained by each separately, approaching 0.9 on the test sets. This was mostly due by improving prediction of death rather than living (supplementary figure 11 A-D). These findings suggest that determining microbiome richness could have clinical relevance for prioritization for liver transplantation.

Functional analysis

Overall, 132 functional modules (FM) were present in at least one sample. Eighty-two FM were significantly different between healthy subjects and patients with cirrhosis, 34 FM were more abundant in healthy subjects, while 48 were more abundant in patients with cirrhosis (Figure 5). Pathways enriched in cirrhosis were related to: ethanol production, tryptophan degradation (aminoacid degradation), lactose degradation (carbohydrate degradation), glycolysis, GABA degradation/metabolism, endotoxin biosynthesis, gas metabolism, mucine degradation, nitrate metabolism, lipid degradation and organic acid metabolism. By contrast, pathways diminished in patients with cirrhosis were: protection against oxidative stress, carbohydrate, amino acid and lipid degradation, and gas metabolism such as butyrate production.

DISCUSSION

The current study demonstrates the existence of marked alterations in gut microbiome in cirrhosis that paralleled the disease stages, being already obvious in compensated cirrhosis, progressing in decompensated cirrhosis, and being striking in ACLF. The alterations of gut microbiome consisted of marked reduction in gene and metagenomic richness and progressive enrichment by unusual gut bacteria, particularly *Enterecoccus* species, some of them from the oral flora. The alteration of gut microbiome was associated with disease complications and impaired prognosis.

One of the main findings of the current study is that there was a clear progression in reduction of gene and metagenomic richness from compensated to decompensated cirrhosis and, finally, ACLF. Low richness of gut microbiota has been reported in patients with inflammatory bowel disorders, elderly patients, and obese individuals, and might be affected by treatments and genetic and individual factors (10). The alteration of gut microbiome found in the current study is amongst the most remarkable seen in any disease condition studied so far using metagenomic sequencing (10,14,26). One possible mechanism is that as liver disease progresses, the composition and richness of gut microbiome may be modified by altered composition of bile acids, and also influenced by agent(s) responsible for cirrhosis development, such as alcohol (5). In parallel, altered gut microbiome and low gene count may lead to altered functionality of microbiome, which may be a key factor for induction and maintenance of intestinal inflammation, disruption of intestinal barrier, and translocation of microbial material to lamina propia and adjacent organs, aggravating the systemic and liver inflammation and dysbiosis that exists in cirrhosis, which may contribute to progression of disease. Interestingly, the impairment in gut microbiome was not due to antibiotic therapy because differences persisted when patients with or without antibiotics were analyzed separately. This lack of relationship between impaired gut microbiome and antibiotic therapy is consistent with observations from previous studies (27,28). The impairment in gut microbiome in decompensated vs compensated cirrhosis has also been observed in prior studies using 16S methodology (7); however, the current study provides a more comprehensive analysis of changes at the metagenomic level. Moreover, it also provides a complete characterization of gut metagenomic changes in patients with ACLF. Patients with ACLF had significantly higher levels of *Enterococcus* and *Peptostreptococcus* species; by contrast patients with decompensated cirrhosis had higher levels of *Faecalibacterium, Ruminococcus, Eubacterium,* among others, as

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compared to ACLF patients. Indeed, a cluster of MGS was clearly associated with the presence and number of organ failures.

Abnormalities in gut microbiome were associated with some complications of cirrhosis, specifically HE and bacterial infections, the complications of cirrhosis most likely related pathogenically to alterations of gut liver axis (1). There were marked differences between patients with HE vs those without; 17 metagenomic species were enriched in patients with HE whereas 66 were enriched in patients without HE. These results extend the observations from previous studies using 16S technology in patients with recurrent HE and confirm the existence of profound abnormalities in gut microbiome characterized by higher abundance of *Streptococcus salivarius* that correlated with ammonia accumulation in patients with HE, indicating an important pathogenic role of gut microbiome in HE (29). In fact, correction of gut dysbiosis by fecal microbiota transplantation has recently been shown to prevent recurrent HE (30,31). An interesting observation of the current study was that patients under chronic treatment with rifaximin to prevent recurrence of HE had significant changes in gut microbiome composition compared to those not receiving rifaximin, with enrichment in 8 metagenomic species with functional modules related with aminoacid and carbohydrate degradation and gas metabolism. Although differences may in part be due to diverse populations, these results suggest that rifaximin affects the composition and functionality of gut microbiome. Differences in gut microbiome composition were also observed in patients under chronic norfloxacin treatment for prevention of SBP recurrence, and also in patients under laxative treatment. The effects of laxatives on gut microbiome are of interest and deserve further investigation. The effect of statins on gut microbiome, although of interest, could not be investigated in the current study due to the low number of patients treated (only 15 patients in the whole cohort).

Abnormalities in gut microbiome composition correlated with cirrhosis severity, as estimated by the two scores most commonly used in the assessment of prognosis in cirrhosis, Child-Pugh and MELD scores. A high risk of short-term mortality was associated with markedly reduced microbiome richness and enrichment with certain bacterial species, particularly *Enterococcus faecium, Streptococcus thermophilus, and Ruminococcus lactaris*, among others. By contrast, some species were associated with low risk of death. Of interest, microbiome richness improved the accuracy of MELD score in outcome prediction.

Metagenomic technology allows the evaluation of functional modules that indicate pathways by which abnormalities in microbiome may theoretically influence the course of some disease states. Pathways enriched in the current series of patients with cirrhosis with respect to healthy subjects that may be of potential pathogenic significance are endotoxin biosynthesis, ethanol production, aminoacid, carbohydrate, and lipid degradation, mucine degradation, nitrate metabolism, and GABA metabolism. Alteration in nitrate and GABA modules was also found in a previous study (12). Changes in some functional pathways may represent a mechanism by which the marked abnormalities in gut microbiome can affect the progression of cirrhosis by causing profound alterations in body metabolism leading to some clinical consequences of cirrhosis. Confirmation of this hypothesis would require specific assessment of some key metabolic pathways and evaluation of changes after gut microbiota modulation.

There are some issues important to the interpretation of the current findings that deserve discussion. First, this is a single center study performed in a tertiary referral hospital; therefore, it is unknown whether our findings could be generalized to all settings. Second, many patients, particularly those with decompensated cirrhosis with and without ACLF were treated with antibiotics that could affect gut microbiome

composition; however, findings were quite similar in patients treated and not treated with antibiotics and differences among disease stages persisted after excluding patients receiving antibiotics; moreover, this is an intrinsic limitation of the study because the majority of patients hospitalized for management of decompensated cirrhosis, either with or without ACLF, receive antibiotics due to proven or suspected bacterial infections. Finally, although the alterations found in the gut microbiome are very remarkable and were associated with disease outcomes, it is unknown whether that play a pathogenic role in disease complications and mortality. Confirmation of this hypothesis would require prospective studies including a high number of patients focused on improving or modulating gut microbiome alterations, such as those already reported in patients with HE (30,31).

In conclusion, the results of the current study indicate that human cirrhosis is characterized by remarkable abnormalities in gut microbiome composition with profound reduction in gene and metagenomic richness and marked changes in microbiota composition, with enrichment by unusual gut species; changes being maximal in patients with ACLF compared to compensated cirrhosis and decompensated cirrhosis without ACLF. In addition, altered gut microbiome correlates with some complications, particularly HE and bacterial infections and short-term prognosis. Alterations in gut microbiome may contribute to disease progression and poor survival in cirrhosis.

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FIGURE LEGENDS

Figure 1. Upper panel: Comparison of gene (left) and metagenomic species (right) richness in healthy subjects (n=75, green) and patients with cirrhosis divided according to disease stage: compensated cirrhosis (n=24, maroon), decompensated-outpatients (n=9, red), decompensated-inpatients without ACLF (n=84, blue) and ACLF (n=65, dark blue). Lower panel: Comparison of gene (left) and MGS (right) richness for the same group of subjects shown above, categorized according to whether they were receiving antibiotics (turquoise) or not (pink).

Figure 2. Differentially abundant metagenomic species in patients with cirrhosis divided according to disease stages. Metagenomic species are in rows; MGS identification, genes number and taxonomy (species name and genus) are indicated in the left legend. Abundance is indicated by color gradient from white (not detected) to red (most abundant). Individuals ordered by increased richness for each disease stage (MGS mean) are in columns. Significance Kruskal-Wallis test (q value, FDR adjusted) is given in the right legend. Disease stages are: Comp., compensated; Decomp., decompensated-outpatients; AD, decompensated-inpatients without ACLF; ACLF, acute-on-chronic liver failure.

Figure 3. Upper panel: Heatmap showing spearman correlation between clinical variables and gut microbiome, global view. Metagenomic species were selected with at least one significant correlation (q<0.01, FDR correction). Spearman correlation coefficient matrix with color-coded correlation (blue color denotes positive correlation while red denotes negative correlation). Lower panel: Zoom on the cluster in the right hand corner on metagenomic species associated with disease severity. FDRs are denoted: ·, q<0.1; *, q<0.05; **, q<0.01; ***, q<0.001. Spearman correlation coefficient matrix with color-coded correlation (blue color denotes positive correlation while red denotes negative correlation).

Figure 4. Upper panel (*A*) Metagenomic species richness according to 3-month survival. (*B)* Stable set of predictors using gut microbiome for prediction of the 3-month mortality. Red, associated with alive status at 3 months. Blue, associated with death status at 3 months. Metagenomic species are in rows; MGS identification, species name and taxonomy (genus) are indicated in the left legend. Lower panel: Accuracy for predicting 3-month survival based on 100 random forest models. MELD is represented in red, MELD and richness is represented with green, and richness is represented in

blue. Results are divided according to model training with 80% of the data (*C*) and model testing with 20% of the data (*D*).

<u>Figure 5</u>. Contrasted Functional Modules between healthy subjects and patients with cirrhosis significantly enriched in patients with cirrhosis (q<0.05, FDR correction).

Table 1. Demographic and clinical data and liver and kidney function tests in all patients included.

	Compensated n= 24	*Decompensated outpatients n=9*	*Decompensated inpatients without ACLF n = 84*	*ACLF n= 65*
Age, yr	63 (57-70)	54 (51-59)	60 (53-67)	60 (50-65)
Male gender	17 (71)	9 (100)	57 (68)	47 (72)
Diabetes mellitus	6 (25)	2 (22)	31 (37)	21 (32)
Etiology*: Alcohol/ HCV	13(54)/3(13)	8(89)/0	51(61)/14(17)	40(62)/5(8)
Presence of ascites	0	7 (78)	45 (54)	55 (85)
Presence of encephalopathy	0	1 (11)	12 (14)	37 (57)
Presence of bacterial infection	0	0	47 (56)	38 (59)
Serum Creatinine (mg/dL)	0.8 (0.7-0.9)	0.7 (0.6-1)	0.84 (0.5-1.2)	2 (1.1-2.4)
Serum bilirubin (mg/dL)	1 (0.9-1.8)	1.9 (1-4)	1.9 (1-4)	5 (1.3-16)
INR	1.2 (1.1-1.3)	1.5 (1.3-1.6)	1.4 (1.2-1.7)	1.9 (1.4-2.2)
Serum sodium (mEq/L)	142 (141-143)	139 (134-142)	136 (134-139)	136 (131-138)
Serum albumin (g/L)	41 (37-45)	35 (30-39)	29 (25-34)	29 (26-34)
Platelets (x10^9/L)	104 (68-136)	88 (81-117)	93 (60-124)	71 (54-111)
Blood leukocytes (x10^9/L)	6 (4-7)	4 (3-7)	5 (4-7)	7 (5-12)
C-reactive protein (mg/dL)	-	-	2.2 (0.7-5)	2.4 (1-4)
Mean arterial pressure (mmHg)	98 (88-105)	90 (82-93)	81 (73-91)	80 (68-90)
MELD score	9 (8-12)	14 (11-17)	14 (10-19)	26 (18-31)

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Child-Pugh score	5 (5-6)	8 (7-9)	8 (6-9)	11 (9-12)
BMI (Kg/m^2)	28 (25-31)	28 (24-29)	27 (23-30)	27 (23-30)
Betablocker treatment**	11 (46)	1 (11)	31 (37)	28 (44)
Norfloxacin treatment**	1 (4)	1 (11)	17 (20)	11 (17)
Rifaximin treatment**	1 (4)	2 (22)	10 (12)	11 (17)
Lactulose/lactitol treatment**	1 (4)	4 (44)	29 (35)	23 (36)
PPI treatment**	4 (17)	6 (67)	43 (51)	27 (42)
Metformin treatment**	4 (16)	1 (11)	7 (8)	8 (12)
Antibiotic treatment at fecal sample collection	0	0	52 (62)	44 (68)

HCV; hepatitis C virus, BMI; Body Mass Index. PPI; Proton Pump inhibitor; INR; International Normalized Ratio; ACLF, Acute-on-chronic Liver Failure.

Data are median and (IQR) for quantitative variables and number and percentages (in brackets) for qualitative variables.

* Other etiologies of cirrhosis were alcohol and HCV (1), NAFLD (6), PBC (1) in compensated cirrhosis, alcohol and HCV (1) in decompensated outpatients, alcohol and HCV (7), HBV (1), NAFLD (5), cryptogenetic (4), and PBC (2) in decompensated inpatients without ACLF, and alcohol and HCV (10), HBV (1), NAFLD (4), cryptogenetic (3), PBC (1), hemochromatosis (1) in patients with ACLF.

** In hospitalized patients, refers to treatments received prior to hospital admission. Fecal samples for microbiome analysis were collected a median of 2 days after admission to hospital.

Table 2. Comparison of metagenomics richness according to patients' characteristics

Category	Condition*	n	Richness in MGS P value	Number of significantly contrasted MGS	
Etiology of cirrhosis (alcohol vs other)	Alcohol	123	0.07	97 MGS	14 MGS
	Other	48			83 MGS
Alcohol consumption	Active alcohol	56	0.01	172 MGS	10 MGS (including MGS of S.*Salivarius* and *E.faeaclis*)
	Never	43			62 MGS
Hepatic encephalopathy	Yes	54	0.04	83 MGS	17 MGS
	No	117			66 MGS
Infection	Yes	77	0.06	98 MGS	7 (including MGS of *E. Faecium* and *E. Faecalis*)
	No	94			91 MGS
CRP				34 MGS**	2 MGS
					32 MGS
Leukocytes				27 MGS**	2 MGS
					25 MGS
SIRS	Yes	41	0.05	47 MGS	3 MGS (including Homo sapiens, *E. Faecalis* and *E.Faecium*)
	No	130			44 MGS
Rifaximin treatment*	Yes	22	0.01	78 MGS	8 MGS
	No	149			70 MGS
Norfloxacin treatment*	Yes	28	0.05	55 MGS	5 MGS
	No	143			50 MGS
PPI treatment*	Yes	75	0.91	14 MGS	8 MGS
	No	96			6 MGS

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Betablocker treatment*	Yes	65	0.97	42 MGS	18 MGS
	No	105			24 MGS
Lactulose/lactitol treatment*	Yes	49	<0.001	16 MGS	1 MGS
	No	121			15 MGS

MGS, Metagenomic Species; E., Enterococcus. ; CRP, C-reactive protein; SIRS, systemic inflammatory response syndrome, PPI, proton pump inhibitor.

* In all cases, the presence of the condition is associated with lower richness compared to the absence of the condition. Alcohol etiology and alcohol consumption have lower richness compared to other etiologies and no alcohol consumption, respectively.

** MGS significantly correlated.

*** In hospitalized patients, refers to treatments received prior to hospital admission. Fecal samples for microbiome analysis were collected a median of 2 days after admission to hospital.

For patients evaluated on two different occasions (n=11), only the first assessment is included in this table

Supplementary table 1. Characteristics of bacterial infections and microbiological data in patients with decompensated cirrhosis without ACLF and in patients with ACLF.

	Decompensated inpatients without ACLF n = 47	*ACLF n = 38*	*P value*
Site of infection			
UTI	13 (28)	13 (34)	
SBP	5 (11)	6 (16)	
Pneumonia	6 (13)	9 (24)	0.461
Skin and soft tissue	7 (15)	4 (11)	
Spontaneous bacteriemia	3 (6)	1 (3)	
Other*	13 (28)	5 (13)	
SIRS	12 (25)	26 (70)	<0.001
Septic shock	0 (0)	17 (45)	<0.001
Positive cultures	25 (53)	24 (51)	0.273
Type of strain isolated			
Gram positive	14 (56)	10 (42)	
Gram negative	10 (40)	11 (46)	0.278
Fungi	1 (4)	3 (8)	
Blood leukocytes (x10^9/L)	5 (4-8)	8 (5-15)	0.001
C-reactive protein (mg/dL)	4 (1-6)	3 (1-6)	0.834
Antibiotic type**			
Cephalosporin	26	6	
Carbapenem	15	23	0.02
Antibiotics against Gram+	15	23	
Other	12	5	
Infection resolution	42 (91)	21 (57)	0.001

Data are number and percentages (in brackets) or mean and (IQR) for quantitative variables.
UTI; urinary tract infection, SBP; spontaneous bacterial peritonitis, CRP, C-reactive protein; SIRS, systemic inflammatory response syndrome.

*Other: Secondary bacterial peritonitis 1, spontaneous bacterial empyema 1, respiratory infection without pneumonia 7, biliary infection 1, endocarditis 1, signs of bacterial infection with negative cultures 11.
** Antibiotic treatment was categorized in four groups: 1) only cephalosporins, 2) carbapenem, 3) antibiotics against gram positive bacteria (including Vancomycin,

Teicoplanin, Tigeciclin, Ampicilin), and 4) others (Levofloxacin, Cirpofloxacin, Amikacin, Piperacilin-Tazobacatm, Linezolid). Patients could receive more than one antibiotic.

	comp.	decomp.	AD	ACLF	
CAG00248,2251,Roseburia sp. CAG:45,Roseburia					1.83e-07
CAG00780,1496,Firmicutes bacterium CAG:41,unclassified Firmicutes					1.83e-07
CAG01272,725,Adlercreutzia equolifaciens,Adlercreutzia					1.83e-07
CAG00164,2551,Blautia wexlerae,Blautia					5.51e-07
CAG00189,2446,Roseburia sp. CAG:100,Roseburia					5.51e-07
CAG00563,1756,Clostridium sp. CAG:91,unclassified Clostridiales					9.25e-07
CAG00937,1230,unclassified,unclassified					9.25e-07
CAG00294,2173,unclassified Firmicutes,unclassified Firmicutes					1.39e-06
CAG01214,822,unclassified,unclassified					1.39e-06
CAG00506,1862,unclassified,unclassified					1.53e-06
CAG01094,1020,Clostridium sp. CAG:273,unclassified Clostridiales					1.53e-06
CAG00782,1489,Eubacterium rectale,Eubacterium					2.48e-06
CAG00218,2364,Barnesiella intestinihominis,Barnesiella					3.07e-06
CAG00284,2188,unclassified Ruminococcus,Ruminococcus					3.24e-06
CAG00452,1941,Clostridium sp. CAG:167,unclassified Clostridiales					3.33e-06
CAG01162,933,unclassified,unclassified					3.33e-06
CAG00347,2079,Enterococcus faecium,Enterococcus					3.91e-06
CAG00057,1397,Homo sapiens,Homo					3.91e-06
CAG00257,2252,Enterococcus faecalis,Enterococcus					3.91e-06
CAG00077,3095,unclassified,unclassified					3.91e-06

	Healthy	Cirrhosis	
CAG00082,3034,Erysipelatoclostridium ramosum,Erysipelatoclostridium			4.58e-14
CAG01259,750,Streptococcus oralis,Streptococcus			1.33e-09
CAG00347,2079,Enterococcus faecium,Enterococcus			2.02e-09
CAG00975,1194,unclassified,unclassified			7.98e-09
CAG00560_1_hs_10.4,710,unclassified,unclassified			5.64e-08
CAG00610,1690,Hungatella hathewayi 2,Hungatella			2.36e-07
CAG00013,5272,unclassified Clostridiales,unclassified Clostridiales			6.96e-07
CAG00254,2236,Ruminococcus gnavus,Blautia			8.21e-07
CAG00015,5038,Hungatella hathewayi 2,Hungatella			8.77e-07
CAG00857_1_hs_10.4,731,unclassified,unclassified			1.41e-06

	Healthy	Compensated	
CAG00975,1194,unclassified,unclassified			5.30e-04 | Compensated
CAG01025,1122,Clostridium citroniae,Lachnoclostridium			1.14e-03 | Compensated
CAG00082,3034,Erysipelatoclostridium ramosum,Erysipelatoclostridium			4.02e-03 | Compensated
CAG01320,629,Clostridium bolteae,Lachnoclostridium			1.06e-02 | Compensated
CAG00012,5342,unclassified,unclassified			1.16e-02 | Compensated
CAG01366,570,Streptococcus parasanguinis,Streptococcus			1.18e-02 | Compensated
CAG00015,5038,Hungatella hathewayi 2,Hungatella			1.57e-02 | Compensated
CAG00013,5272,unclassified Clostridiales,unclassified Clostridiales			1.63e-02 | Compensated
CAG00610,1690,Hungatella hathewayi 2,Hungatella			2.27e-02 | Compensated
CAG01263,750,Clostridium clostridioforme,Lachnoclostridium			2.62e-02 | Compensated
CAG00008,6584,Clostridium bolteae,Lachnoclostridium			3.00e-02 | Compensated
CAG00363,2052,unclassified Clostridiales,unclassified Clostridiales			3.19e-02 | Compensated
CAG00560_1_hs_10.4,710,unclassified,unclassified			3.38e-02 | Compensated
CAG00194,2430,Eubacterium hallii,Eubacterium			3.56e-06 | Healthy
CAG00223,2335,unclassified,unclassified			8.65e-06 | Healthy
CAG00164,2551,Blautia wexlerae,Blautia			8.97e-05 | Healthy
CAG01386,542,Blautia sp. KLE 1732,Blautia			2.55e-04 | Healthy
CAG00620,1687,Blautia obeum,Blautia			5.30e-04 | Healthy
CAG00718,1583,Eubacterium ventriosum,Eubacterium			1.14e-03 | Healthy
CAG01222,818,Blautia obeum,Blautia			1.14e-03 | Healthy
CAG00541,1797,Dorea sp. CAG:105,Dorea			2.32e-03 | Healthy
CAG01301,677,Blautia obeum,Blautia			4.81e-03 | Healthy
CAG00335,2092,Anaerostipes hadrus,Anaerostipes			6.96e-03 | Healthy
CAG00482,1888,Blautia sp. KLE 1732,Blautia			1.18e-02 | Healthy
CAG00601,1694,unclassified Faecalibacterium,Faecalibacterium			1.53e-02 | Healthy
CAG00893,1305,unclassified,unclassified			1.53e-02 | Healthy
CAG01011,1140,unclassified Dorea,Dorea			1.53e-02 | Healthy
CAG01272,725,Adlercreutzia equolifaciens,Adlercreutzia			1.53e-02 | Healthy
CAG00306,2151,Clostridium sp. CAG:221 - Clostridium sp. CAG:265,unclassified Clostridiales			1.57e-02 | Healthy
CAG00161,2531,Bacteroides coprocola,Bacteroides			3.37e-02 | Healthy
CAG01155,939,unclassified Blautia,Blautia			3.60e-02 | Healthy
CAG00441,1950,unclassified Lachnospiraceae,unclassified Lachnospiraceae			3.64e-02 | Healthy
CAG00284,2188,unclassified Ruminococcus,Ruminococcus			3.93e-02 | Healthy
CAG01281,707,unclassified,unclassified			4.88e-02 | Healthy
CAG00070,3146,Blautia hydrogenotrophica,Blautia			4.96e-02 | Healthy
CAG00209,2377,unclassified,unclassified			4.96e-02 | Healthy

Feature	Statistics
CAG00309_hs_9.9,2134,Alistipes onderdonkii,Alistipes	p = 7.08e−04 / q = 1.15e−01 / no
CAG00031_hs_9.9,1256,unclassified,unclassified	p = 1.37e−03 / q = 1.15e−01 / no
CAG00692_hs_9.9,1620,unclassified,unclassified	p = 1.42e−03 / q = 1.15e−01 / no
CAG00618_hs_9.9,1686,unclassified,unclassified	p = 1.6e−03 / q = 1.15e−01 / no
CAG00384_hs_9.9,2009,unclassified,unclassified	p = 1.74e−03 / q = 1.15e−01 / no
CAG00508_hs_9.9,1859,unclassified,unclassified	p = 2.91e−03 / q = 1.52e−01 / no
CAG00603_hs_9.9,1714,Firmicutes bacterium CAG:114,unclassified Firmicutes	p = 3.23e−03 / q = 1.52e−01 / no
CAG00304_hs_9.9,2158,unclassified,unclassified	p = 3.79e−03 / q = 1.56e−01 / no
CAG00249_hs_9.9,2264,Clostridium leptum,Ruminiclostridium	p = 7.7e−03 / q = 2.31e−01 / no
CAG00653_hs_9.9,1653,Eubacterium siraeum,Ruminiclostridium	p = 1.04e−02 / q = 2.31e−01 / no
CAG00873_hs_9.9,1353,unclassified Butyricimonas,Butyricimonas	p = 1.08e−02 / q = 2.31e−01 / no
CAG00210_hs_9.9,2366,Ruminococcus bicirculans,Ruminococcus	p = 1.11e−02 / q = 2.31e−01 / no
CAG00321_hs_9.9,2118,Clostridium sp. CAG:138,unclassified Clostridiales	p = 1.12e−02 / q = 2.31e−01 / no
CAG01052_hs_9.9,1073,Firmicutes bacterium CAG:103,unclassified Firmicutes	p = 1.12e−02 / q = 2.31e−01 / no
CAG00251_hs_9.9,2254,Alistipes sp. CAG:157,Alistipes	p = 1.3e−02 / q = 2.53e−01 / no
CAG00420_hs_9.9,1976,unclassified,unclassified	p = 1.76e−02 / q = 2.69e−01 / no
CAG00439_hs_9.9,1952,unclassified Clostridiales,unclassified Clostridiales	p = 1.76e−02 / q = 2.69e−01 / no
CAG00270_hs_9.9,2220,Oscillibacter sp. KLE 1728 / KLE 1745 / VE202−24,Oscillibacter	p = 1.87e−02 / q = 2.69e−01 / no
CAG00897_hs_9.9,1315,Oscillibacter sp.,Oscillibacter	p = 1.89e−02 / q = 2.69e−01 / no
CAG00672_hs_9.9,1642,Clostridium sp. CAG:169,unclassified Clostridiales	p = 2.04e−02 / q = 2.69e−01 / no
CAG00231_hs_9.9,2302,Odoribacter splanchnicus,Odoribacter	p = 2.04e−02 / q = 2.69e−01 / no
CAG00375_1_hs_10.4,861,unclassified,unclassified	p = 2.09e−02 / q = 2.69e−01 / no
CAG00414_hs_9.9,1968,Eubacterium sp. CAG:76,Eubacterium	p = 2.23e−02 / q = 2.69e−01 / no
CAG00272_hs_9.9,2201,Faecalibacterium 5 (sp. CAG:74),Faecalibacterium	p = 2.36e−02 / q = 2.69e−01 / no
CAG00572_hs_9.9,1752,unclassified Firmicutes,unclassified Firmicutes	p = 2.36e−02 / q = 2.69e−01 / no
CAG01018_hs_9.9,1132,unclassified Bilophila,Bilophila	p = 2.36e−02 / q = 2.69e−01 / no
CAG00327_hs_9.9,2116,unclassified,unclassified	p = 2.74e−02 / q = 2.77e−01 / no
CAG00559_hs_9.9,1772,unclassified,unclassified	p = 2.74e−02 / q = 2.77e−01 / no
CAG00454_hs_9.9,1939,unclassified Ruminococcaceae,unclassified Ruminococcaceae	p = 2.77e−02 / q = 2.77e−01 / no
CAG00675_hs_9.9,1628,Clostridium sp. CAG:217,unclassified Clostridiales	p = 2.86e−02 / q = 2.78e−01 / no
CAG01308_hs_9.9,662,unclassified Firmicutes,unclassified Firmicutes	p = 3.16e−02 / q = 2.92e−01 / no
CAG01408_hs_9.9,507,unclassified Butyricimonas,Butyricimonas	p = 3.18e−02 / q = 2.92e−01 / no
CAG00792_hs_9.9,1481,Firmicutes bacterium CAG:65,unclassified Firmicutes	p = 3.59e−02 / q = 2.92e−01 / no
4813,Clostridium sp. KLE 1755 & Clostridiales bacterium VE202−27,unclassified Clostridiales	p = 3.61e−02 / q = 2.92e−01 / no
CAG01190_hs_9.9,875,Butyricimonas virosa,Butyricimonas	p = 3.71e−02 / q = 2.92e−01 / no
CAG00597_hs_9.9,1721,Candidatus Alistipes marseilloanorexicus,Alistipes	p = 3.8e−02 / q = 2.92e−01 / no
CAG00470_hs_9.9,1926,unclassified,unclassified	p = 3.97e−02 / q = 2.92e−01 / no
CAG00293_hs_9.9,2175,Firmicutes bacterium CAG:145,unclassified Firmicutes	p = 4.18e−02 / q = 2.92e−01 / no
CAG01020_hs_9.9,1130,unclassified Firmicutes,unclassified Firmicutes	p = 4.22e−02 / q = 2.92e−01 / no
CAG01320_hs_9.9,629,Clostridium bolteae,Lachnoclostridium	p = 4.26e−02 / q = 2.92e−01 / no
CAG00218_hs_9.9,2364,Barnesiella intestinihominis,Barnesiella	p = 4.34e−02 / q = 2.92e−01 / no
CAG00324_hs_9.9,2121,Firmicutes bacterium CAG:94,unclassified Firmicutes	p = 4.36e−02 / q = 2.92e−01 / no
CAG00019_1_hs_9.9,2092,unclassified,unclassified	p = 4.46e−02 / q = 2.92e−01 / no
CAG00696_hs_9.9,1614,unclassified,unclassified	p = 4.51e−02 / q = 2.92e−01 / no
CAG00424_hs_9.9,1974,Firmicutes bacterium CAG:137,unclassified Firmicutes	p = 4.87e−02 / q = 2.92e−01 / no
CAG00492_hs_9.9,1888,unclassified Ruminococcaceae,unclassified Ruminococcaceae	p = 4.87e−02 / q = 2.92e−01 / no
CAG00604_hs_9.9,1700,Firmicutes bacterium CAG:110,unclassified Firmicutes	p = 4.87e−02 / q = 2.92e−01 / no
CAG00796_hs_9.9,1466,Eubacterium sp. CAG:202,Eubacterium	p = 4.87e−02 / q = 2.92e−01 / no
CAG01130_hs_9.9,978,unclassified,unclassified	p = 4.87e−02 / q = 2.92e−01 / no
CAG01169_hs_9.9,912,unclassified,unclassified	p = 4.87e−02 / q = 2.92e−01 / no
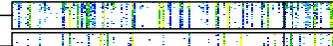
CAG01377_hs_9.9,552,unclassified,unclassified p = 6.75e−03 / q = 2.31e−01 / yes

CAG00087_hs_9.9,1174,unclassified,unclassified p = 1.02e−02 / q = 2.31e−01 / yes

CAG00549_hs_9.9,1763,Bifidobacterium longum,Bifidobacterium p = 2.04e−02 / q = 2.69e−01 / yes

CAG00720_hs_9.9,1590,unclassified,unclassified p = 2.76e−02 / q = 2.77e−01 / yes

CAG00686_hs_9.9,1600,Prevotella copri,Prevotella p = 4.34e−02 / q = 2.92e−01 / yes

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Label	Value
CAG00347,2079,Enterococcus faecium,Enterococcus	3.28e-04 | POS
CAG00057,1397,Homo sapiens,Homo	1.76e-03 | POS
CAG00257,2252,Enterococcus faecalis,Enterococcus	1.19e-02 | POS
CAG01380,553,unclassified Clostridiales,unclassified Clostridiales	7.49e-06 | NEG
CAG00506,1862,unclassified,unclassified	3.59e-05 | NEG
CAG00931,1256,unclassified,unclassified	3.59e-05 | NEG
CAG00231,2302,Odoribacter splanchnicus,Odoribacter	5.01e-05 | NEG
CAG00270,2220,Oscillibacter sp. KLE 1728 / KLE 1745 / VE202-24,Oscillibacter	5.01e-05 | NEG
CAG00304,2158,unclassified,unclassified	5.01e-05 | NEG
CAG00508,1859,unclassified,unclassified	5.20e-05 | NEG
CAG00218,2364,Barnesiella intestinihominis,Barnesiella	6.93e-05 | NEG
CAG00164,2551,Blautia wexlerae,Blautia	8.19e-05 | NEG
CAG00672,1642,Clostridium sp. CAG:169,unclassified Clostridiales	1.55e-04 | NEG
CAG00025,4407,Hungatella hathewayi 1,Hungatella	1.74e-04 | NEG
CAG00577,1718,Faecalibacterium prausnitzii 3 (L2-6),Faecalibacterium	1.74e-04 | NEG
CAG00612,1673,Faecalibacterium prausnitzii 2 (A2-165),Faecalibacterium	1.74e-04 | NEG
CAG01025,1122,Clostridium citroniae,Lachnoclostridium	1.74e-04 | NEG
CAG01263,750,Clostridium clostridioforme,Lachnoclostridium	1.91e-04 | NEG
CAG00249,2264,Clostridium leptum,Ruminiclostridium	2.20e-04 | NEG
CAG00937,1230,unclassified,unclassified	2.34e-04 | NEG
CAG01094,1020,Clostridium sp. CAG:273,unclassified Clostridiales	2.64e-04 | NEG
CAG00578,1731,Alistipes putredinis,Alistipes	2.95e-04 | NEG
CAG01028,1103,Ruminococcaceae bacterium LM158,unclassified Ruminococcaceae	2.95e-04 | NEG

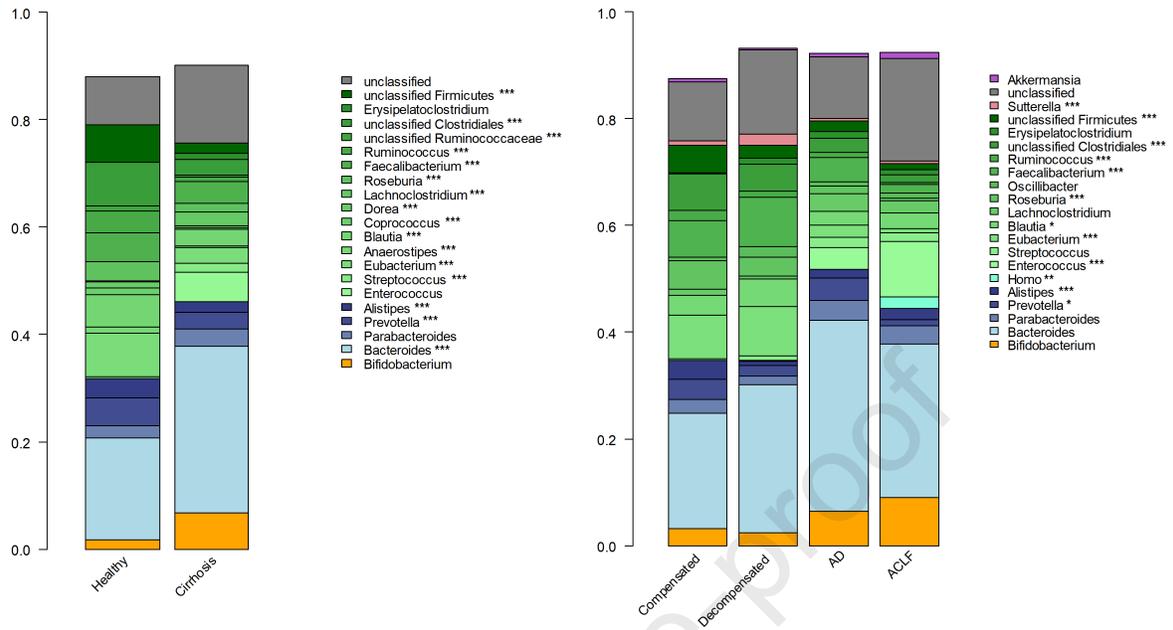| CAG label	Statistics
CAG00071_hs_9.9,3113,Bacteroides cellulosilyticus,Bacteroides	p = 8.38e−05 / q = 2.76e−02 / no
CAG01303_hs_9.9,858,Bacteroides cellulosilyticus,Bacteroides	p = 3.51e−04 / q = 2.76e−02 / no
CAG00563_hs_9.9,1756,Clostridium sp. CAG:91,unclassified Clostridiales	p = 4.19e−04 / q = 3.46e−02 / no
CAG00360_hs_9.9,2045,unclassified,unclassified	p = 8.31e−04 / q = 5.49e−02 / no
CAG01320_hs_9.9,629,Clostridium bolteae,Lachnoclostridium	p = 1.12e−03 / q = 5.54e−02 / no
CAG00792_hs_9.9,1481,Firmicutes bacterium CAG:65,unclassified Firmicutes	p = 1.18e−03 / q = 5.54e−02 / no
CAG00243_hs_9.9,2271,Ruminococcus torques 1,Blautia	p = 1.38e−03 / q = 5.69e−02 / no
CAG01165_hs_9.9,919,Bacteroides ovatus,Bacteroides	p = 1.99e−03 / q = 7.29e−02 / no
CAG00291_hs_9.9,2166,Roseburia intestinalis,Roseburia	p = 2.23e−03 / q = 7.36e−02 / no
CAG00720_hs_9.9,1590,unclassified,unclassified	p = 3.13e−03 / q = 9.4e−02 / no
CAG01323_hs_9.9,629,Parabacteroides merdae,Parabacteroides	p = 3.64e−03 / q = 9.73e−02 / no
CAG00008_hs_9.9,6584,Clostridium bolteae,Lachnoclostridium	p = 4.12e−03 / q = 9.73e−02 / no
CAG00013_hs_9.9,5272,unclassified Clostridiales,unclassified Clostridiales	p = 4.62e−03 / q = 9.73e−02 / no
CAG00249_hs_9.9,2264,Clostridium leptum,Ruminiclostridium	p = 4.94e−03 / q = 9.73e−02 / no
CAG00175_hs_9.9,2495,unclassified,unclassified	p = 5.02e−03 / q = 9.73e−02 / no
CAG00780_hs_9.9,1496,Firmicutes bacterium CAG:41,unclassified Firmicutes	p = 5.8e−03 / q = 9.73e−02 / no
CAG00508_hs_9.9,1859,unclassified,unclassified	p = 6.1e−03 / q = 9.73e−02 / no
CAG01369_hs_9.9,567,Roseburia intestinalis,Roseburia	p = 6.25e−03 / q = 9.73e−02 / no
CAG00520_hs_9.9,1830,Firmicutes bacterium CAG:56,unclassified Firmicutes	p = 6.28e−03 / q = 9.73e−02 / no
CAG00117_hs_9.9,2766,Parabacteroides merdae,Parabacteroides	p = 6.86e−03 / q = 9.73e−02 / no
CAG00873_hs_9.9,1353,unclassified Butyricimonas,Butyricimonas	p = 6.86e−03 / q = 9.73e−02 / no
CAG00582_hs_9.9,1719,Roseburia inulinivorans,Roseburia	p = 7.25e−03 / q = 9.73e−02 / no
CAG01263_hs_9.9,750,Clostridium clostridioforme,Lachnoclostridium	p = 7.37e−03 / q = 9.73e−02 / no
CAG00357_hs_9.9,2047,Bacteroides ovatus,Bacteroides	p = 8.03e−03 / q = 1.02e−01 / no
CAG00515_hs_9.9,1833,Eubacterium eligens,Eubacterium	p = 8.79e−03 / q = 1.07e−01 / no
CAG00309_hs_9.9,2134,Alistipes onderdonkii,Alistipes	p = 9.18e−03 / q = 1.08e−01 / no
CAG01408_hs_9.9,507,unclassified Butyricimonas,Butyricimonas	p = 1.03e−02 / q = 1.17e−01 / no
CAG00024_hs_9.9,4483,unclassified,unclassified	p = 1.23e−02 / q = 1.25e−01 / no
CAG00618_hs_9.9,1686,unclassified,unclassified	p = 1.25e−02 / q = 1.25e−01 / no
CAG00506_hs_9.9,1862,unclassified,unclassified	p = 1.27e−02 / q = 1.25e−01 / no
CAG00275_hs_9.9,2192,Roseburia sp. CAG:18,Roseburia	p = 1.29e−02 / q = 1.25e−01 / no
CAG00044_hs_9.9,3579,Clostridium symbiosum,Lachnoclostridium	p = 1.38e−02 / q = 1.3e−01 / no
CAG00015_hs_9.9,5038,Hungatella hathewayi 2,Hungatella	p = 1.44e−02 / q = 1.32e−01 / no
CAG00231_hs_9.9,2302,Odoribacter splanchnicus,Odoribacter	p = 1.58e−02 / q = 1.41e−01 / no
CAG00560_1_hs_10.4,710,unclassified,unclassified	p = 1.7e−02 / q = 1.45e−01 / no
CAG00414_hs_9.9,1968,Eubacterium sp. CAG:76,Eubacterium	p = 1.72e−02 / q = 1.45e−01 / no
CAG00611_hs_9.9,1697,Ruminococcus bromii,Ruminococcus	p = 1.87e−02 / q = 1.53e−01 / no
CAG00329_hs_9.9,2106,Roseburia hominis,Roseburia	p = 1.91e−02 / q = 1.53e−01 / no
CAG00610_hs_9.9,1690,Hungatella hathewayi 2,Hungatella	p = 2.01e−02 / q = 1.58e−01 / no
CAG00519_hs_9.9,1837,unclassified Lachnoclostridium,Lachnoclostridium	p = 2.18e−02 / q = 1.63e−01 / no
CAG00675_hs_9.9,1628,Clostridium sp. CAG:217,unclassified Clostridiales	p = 2.18e−02 / q = 1.63e−01 / no
CAG00945_hs_9.9,1225,Bacteroides xylanisolvens,Bacteroides	p = 2.25e−02 / q = 1.63e−01 / no
CAG00141_hs_9.9,2633,Parabacteroides distasonis,Parabacteroides	p = 2.28e−02 / q = 1.63e−01 / no
CAG01040_hs_9.9,1098,Clostridium asparagiforme,Lachnoclostridium	p = 2.36e−02 / q = 1.64e−01 / no
CAG00062_hs_9.9,3323,Bacteroides salyersiae,Bacteroides	p = 2.39e−02 / q = 1.64e−01 / no
CAG00727_hs_9.9,1577,unclassified,unclassified	p = 2.45e−02 / q = 1.65e−01 / no
CAG01015_hs_9.9,1125,unclassified Firmicutes,unclassified Firmicutes	p = 2.77e−02 / q = 1.69e−01 / no
CAG00578_hs_9.9,1731,Alistipes putredinis,Alistipes	p = 2.78e−02 / q = 1.69e−01 / no
CAG00079_hs_9.9,3067,Clostridium sp. CAG:7,unclassified Clostridiales	p = 2.81e−02 / q = 1.69e−01 / no
CAG00612_hs_9.9,1673,Faecalibacterium prausnitzii 2 (A2–165),Faecalibacterium	p = 3.08e−02 / q = 1.8e−01 / no

CAG00049_hs_9.9,3535,Bacteroides caccae,Bacteroides — p = 3.15e−02 / q = 1.8e−01 / no

CAG00697_hs_9.9,1693,unclassified,unclassified — p = 3.17e−02 / q = 1.8e−01 / no

...4813,Clostridium sp. KLE 1755 & Clostridiales bacterium VE202−27,unclassified Clostridiales — p = 3.22e−02 / q = 1.8e−01 / no

CAG01380_hs_9.9,553,unclassified Clostridiales,unclassified Clostridiales — p = 3.34e−02 / q = 1.82e−01 / no

CAG00384_hs_9.9,2009,unclassified,unclassified — p = 3.36e−02 / q = 1.82e−01 / no

CAG00653_hs_9.9,1653,Eubacterium siraeum,Ruminiclostridium — p = 3.71e−02 / q = 1.97e−01 / no

CAG01025_hs_9.9,1122,Clostridium citroniae,Lachnoclostridium — p = 3.82e−02 / q = 1.97e−01 / no

CAG00708_hs_9.9,1605,Alistipes senegalensis,Alistipes — p = 3.93e−02 / q = 1.97e−01 / no

CAG00994_hs_9.9,1166,unclassified Firmicutes,unclassified Firmicutes — p = 3.93e−02 / q = 1.97e−01 / no

CAG01293_hs_9.9,681,Blautia sp. CAG:257,Blautia — p = 3.93e−02 / q = 1.97e−01 / no

CAG00460_hs_9.9,1929,unclassified Clostridiales,unclassified Clostridiales — p = 4.16e−02 / q = 2.05e−01 / no

CAG00407_hs_9.9,1997,unclassified Lachnospiraceae,unclassified Lachnospiraceae — p = 4.41e−02 / q = 2.05e−01 / no

CAG00408_hs_9.9,1987,Clostridium sp. CAG:75,unclassified Clostridiales — p = 4.41e−02 / q = 2.05e−01 / no

CAG00672_hs_9.9,1642,Clostridium sp. CAG:169,unclassified Clostridiales — p = 4.41e−02 / q = 2.05e−01 / no

CAG00168_hs_9.9,2534,Clostridiales bacterium VE202−14,unclassified Clostridiales — p = 4.47e−02 / q = 2.05e−01 / no

CAG00218_hs_9.9,2364,Barnesiella intestinihominis,Barnesiella — p = 4.73e−02 / q = 2.09e−01 / no

CAG00718_hs_9.9,1583,Eubacterium ventriosum,Eubacterium — p = 4.87e−02 / q = 2.09e−01 / no

CAG00272_hs_9.9,2201,Faecalibacterium 5 (sp. CAG:74),Faecalibacterium — p = 4.95e−02 / q = 2.09e−01 / no

CAG00572_hs_9.9,1752,unclassified Firmicutes,unclassified Firmicutes — p = 4.95e−02 / q = 2.09e−01 / no

CAG01018_hs_9.9,1132,unclassified Bilophila,Bilophila — p = 4.95e−02 / q = 2.09e−01 / no

CAG00549_hs_9.9,1763,Bifidobacterium longum,Bifidobacterium — p = 2.09e−04 / q = 2.76e−02 / yes

CAG00253_hs_9.9,2254,Lactococcus lactis,Lactococcus — p = 6.88e−03 / q = 9.73e−02 / yes

CAG00931_hs_9.9,1256,unclassified,unclassified — p = 1.19e−02 / q = 1.25e−01 / yes

CAG01377_hs_9.9,552,unclassified,unclassified — p = 2.58e−02 / q = 1.69e−01 / yes

CAG00186_hs_9.9,2433,Streptococcus salivarius,Streptococcus — p = 2.63e−02 / q = 1.69e−01 / yes

CAG00014_hs_9.9,5112,Lachnospiraceae bacterium x4,unclassified Lachnospiraceae — p = 2.68e−02 / q = 1.69e−01 / yes

CAG00469_hs_9.9,1921,Eubacterium sp. CAG:146,Eubacterium — p = 4.46e−02 / q = 2.05e−01 / yes

CAG00304_hs_9.9,2158,unclassified,unclassified — p = 4.81e−02 / q = 2.09e−01 / yes

CAG00360_hs_9.9,2045,unclassified,unclassified — p = 5.49e−06 / q = 6.36e−03 / no

CAG00243_hs_9.9,2271,Ruminococcus torques 1,Blautia — p = 6.43e−05 / q = 2.48e−02 / no

CAG00520_hs_9.9,1830,Firmicutes bacterium CAG:56,unclassified Firmicutes — p = 9.87e−05 / q = 2.86e−02 / no

CAG00508_hs_9.9,1859,unclassified,unclassified — p = 1.62e−04 / q = 3.75e−02 / no

CAG00071_hs_9.9,3113,Bacteroides cellulosilyticus,Bacteroides — p = 2.26e−04 / q = 3.79e−02 / no

CAG00873_hs_9.9,1353,unclassified Butyricimonas,Butyricimonas — p = 2.31e−04 / q = 3.79e−02 / no

CAG01202_hs_9.9,858,Bacteroides cellulosilyticus,Bacteroides — p = 2.62e−04 / q = 3.79e−02 / no

CAG00025_hs_9.9,4407,Hungatella hathewayi 1,Hungatella — p = 6.47e−04 / q = 4.68e−02 / no

CAG00140_hs_9.9,2638,Subdoligranulum sp. 4_3_54A2FAA,Subdoligranulum — p = 5.03e−04 / q = 4.68e−02 / no

CAG00218_hs_9.9,2364,Barnesiella intestinihominis,Barnesiella — p = 5.78e−04 / q = 4.68e−02 / no

CAG00309_hs_9.9,2134,Alistipes onderdonkii,Alistipes — p = 4.22e−04 / q = 4.68e−02 / no

CAG00320_hs_9.9,2121,Phascolarctobacterium sp. CAG:207,Phascolarctobacterium — p = 6.12e−04 / q = 4.68e−02 / no

CAG00460_hs_9.9,1929,unclassified Clostridiales,unclassified Clostridiales — p = 5.44e−04 / q = 4.68e−02 / no

CAG00780_hs_9.9,1496,Firmicutes bacterium CAG:41,unclassified Firmicutes — p = 5.42e−04 / q = 4.68e−02 / no

CAG01380_hs_9.9,553,unclassified Clostridiales,unclassified Clostridiales — p = 5.19e−04 / q = 4.68e−02 / no

CAG00549_hs_9.9,1763,Bifidobacterium longum,Bifidobacterium — p = 1.99e−05 / q = 1.15e−02 / yes
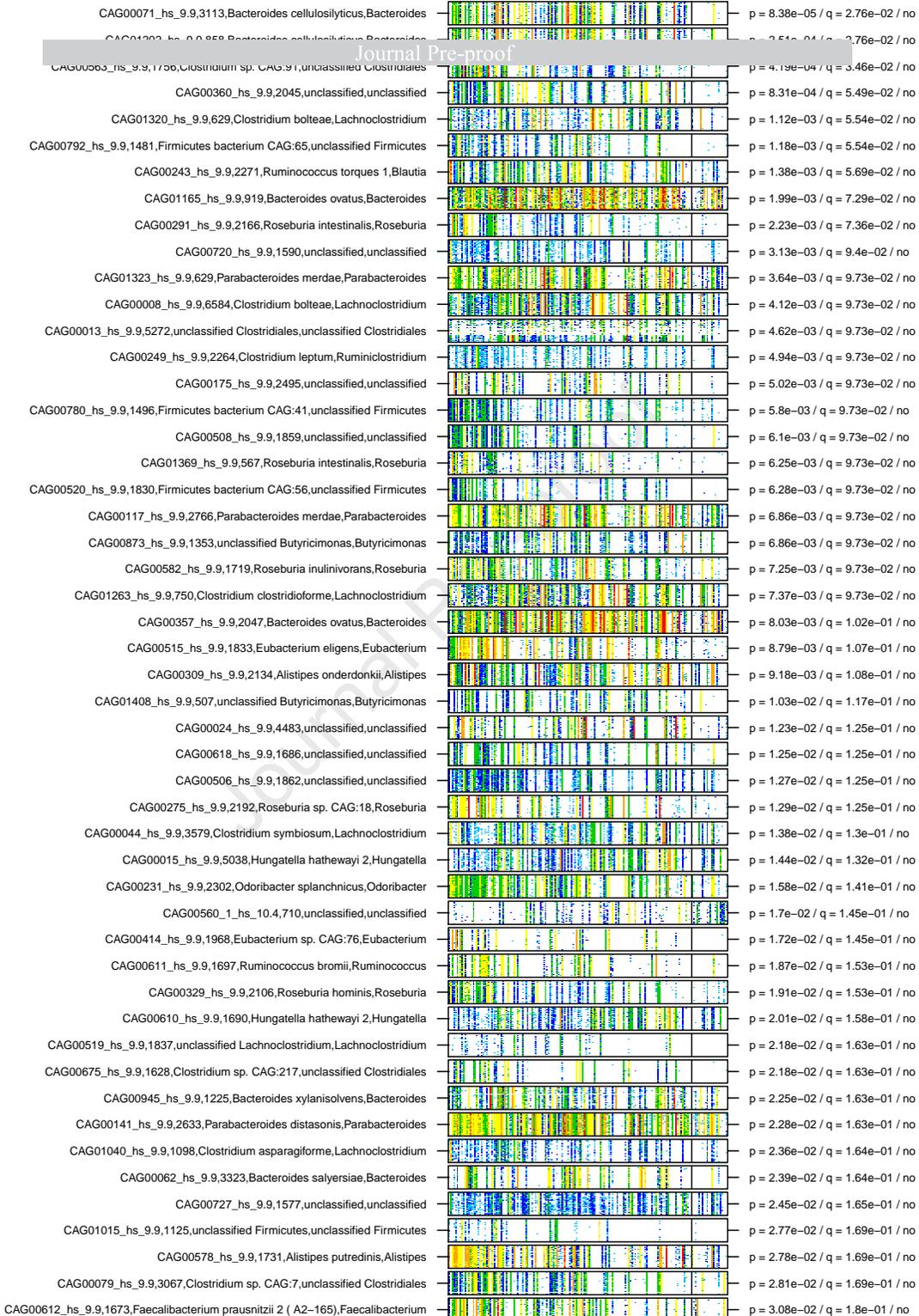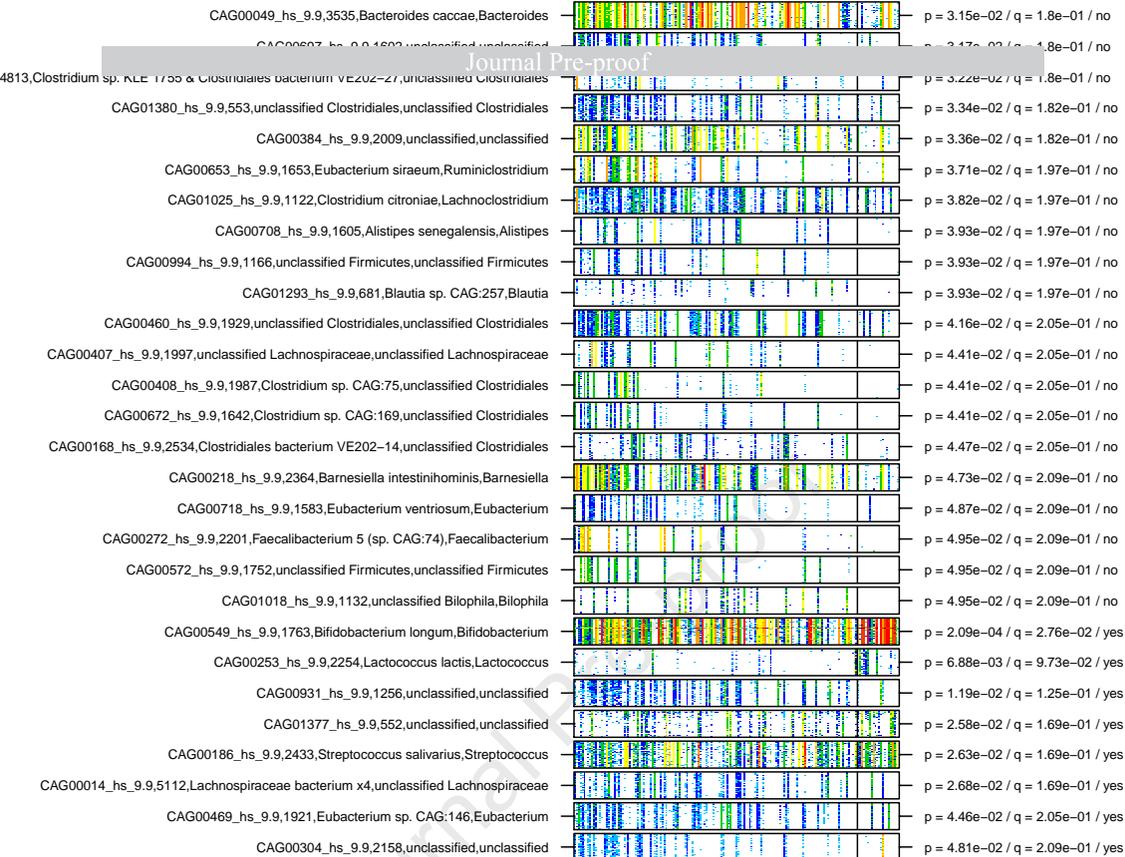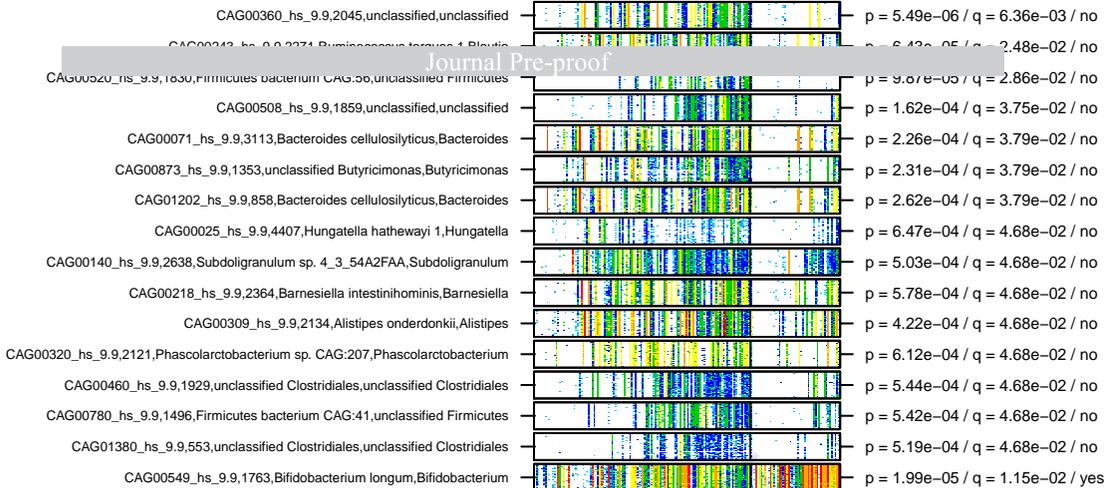
BACKGROUND AND CONTEXT

Cirrhosis is associated with alterations in gut microbiome. However, little information exists on gut microbiome using quantative metagenomics in cirrhosis. We investigated gut-microbiome using quantative metagenomics in the whole-spectrum of the disease, from compensated cirrhosis to ACLF.

NEW FINDINGS

Using high-throughput analysis, progression of cirrhosis was associated with profound reduction of gene and metagenomic species richness, that are particularly intense in ALCF. Gut microbiome predicted survival and was associated with functional changes.

LIMITATIONS

Patients with decompensated cirrhosis and ACLF frequently receive antibiotics for treatment of infections, which can affect gut microbiome. This a single center study with a relatively low number of patients.

IMPACT

This is the most in depth analysis of gut-microbiome in patients with ACLF. Strategies to modify gut microbiome composition and functionality in patients with decompensated cirrhosis and ACLF should be investigated.

LAY SUMMARY

Using metagenomics, we demonstrated that progression of cirrhosis, from compensated to decompensated cirrhosis and ACLF, is associated with parallel remarkable changes in gut-microbiome. Microbiome findings correlated with clinical outcomes, survival and functional changes.