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CLINICAL LIVER

Alterations in Gut Microbiome in Cirrhosis as Assessed by Quantitative Metagenomics: Relationship With Acute-on-Chronic Liver Failure and Prognosis

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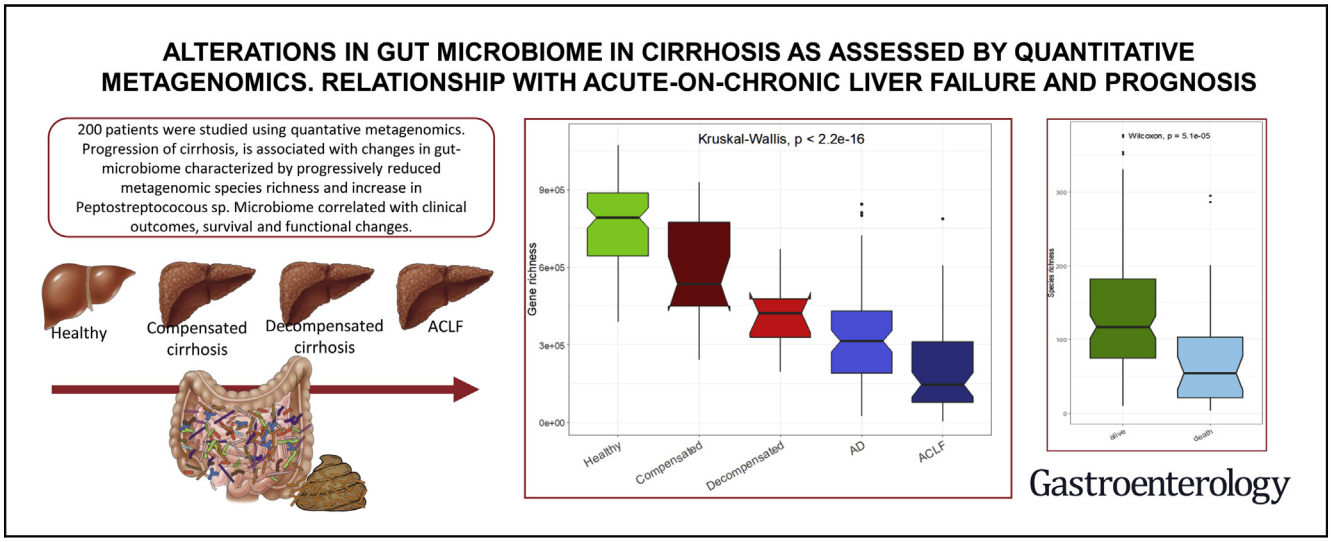

BACKGROUND AND AIMS: Cirrhosis is associated with changes in gut microbiome composition. Although acute-on-chronic liver failure (ACLF) is the most severe clinical stage of cirrhosis, there is lack of information about gut microbiome alterations in ACLF using quantitative metagenomics. We investigated 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 control subjects. **METHODS:** Stool samples were collected prospectively in 182 patients with cirrhosis. DNA library construction and sequencing were performed using the Ion Proton Sequencer (ThermoFisher Scientific, Waltham, MA). Microbial genes were grouped into clusters, denoted as metagenomic species. **RESULTS:** Cirrhosis was associated with a remarkable reduction in gene and metagenomic species richness compared with 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 a reduction of some autochthonous bacteria. Gut microbiome alterations correlated with model for end-stage liver disease and Child-Pugh scores and organ failure 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, γ-aminobutyric acid 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.

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 an abnormal gut-liver axis is the development of pathologic translocation of bacteria and/or bacterial products from the gut to the lymph nodes.[2] This increased translocation appears to be an important triggering factor of systemic inflammation that is 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 taxonomic 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, only 1 study has reported the use of this technology in cirrhosis that showed a profound alteration of gut microbiome characterized by reduced gene and metagenomic richness and marked depletion of metagenomic species (MGS) 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 MGS 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 1 or more organs in failure, 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 to investigate 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 with healthy subjects that is exceptionally altered in patients with ACLF and provides a characterization of abnormalities of MGS throughout the progression of the disease and their correlation with clinical features and mortality.

WHAT YOU NEED TO KNOW

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.

Patients and Methods

Population and Study Design

This prospective study was 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 at 2 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 age

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Abbreviations used in this paper: **ACLF, acute-on-chronic liver failure; FDR, false discovery rate; HE, hepatic encephalopathy; IHMS, International Human Microbiome Standards; KEGG, Kyoto Encyclopedia Genes and Genomes; MELD, model for end-stage liver disease; MGS, metagenomic species.**

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> 18 years and cirrhosis diagnosed by either liver biopsy or a combination of clinical, analytic, ultrasound, elastographic, and/or endoscopic findings. Exclusion criteria were severe extrahepatic diseases, hepatocellular carcinoma beyond the Milan criteria, previous organ transplantation, HIV infection, and lack of informed consent. Patients with cirrhosis were categorized in 4 groups: patients with compensated cirrhosis (ie, without current complications of the disease), ambulatory patients with stable decompensated cirrhosis, patients hospitalized because of acute decompensation of cirrhosis without ACLF, and patients with ACLF. In addition, a group of healthy subjects from the Spanish MetaHit project cohort were also included as control subjects.[14]

When patients were included in the study, demographic, clinical, and laboratory data were recorded and a fecal sample 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 the patient provided the stool (median, 2 days after admission [interquartile range, 1–4]). Data collected was related to cirrhosis (etiology, alcohol consumption, laboratory variables, and complications before inclusion in the study and during and after hospitalization), current and past medications, and nonhepatic diseases. Special care was taken in the assessment of the presence of infection and current or past use of antibiotics. All patients were followed for at least 3 months from study inclusion.

Decompensated cirrhosis was defined when patients had 1 of the following complications: ascites, gastrointestinal bleeding, HE grade $\geq$ 2, 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). All patients (and/or relatives) signed a written informed consent before entering in the study.

Procedures

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 24 hours. At the laboratory, 1 g of feces was mixed with 4 mL of stabilizing solution (RNAlater Stabilization Solution; ThermoFisher Scientific, Waltham, MA). Feces sampling from outpatients was done following the protocol IHMS SOP005, and self-collection samples were preserved in stabilizing solution at room temperature and handed to the biologic laboratory within 24 hours to 7 days after collection. All samples were homogenized and aliquoted to 200 mg subsamples that were kept at –80°C until DNA extraction.

DNA extraction. DNA was extracted from 1 subsample following IHMS SOP007 V2. DNA was quantitated using Qubit Fluorometric Quantitation (ThermoFisher Scientific) and qualified using DNA size profiling on a Fragment Analyzer (Agilent Technologies, Santa Clara, CA).

DNA library construction and sequencing. Three micrograms 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, MA), and DNA fragment library construction was performed using the Ion Plus Fragment Library and Ion Xpress Barcode Adaptaters Kits (ThermoFisher Scientific). Purified and amplified DNA fragment libraries were sequenced using the Ion Proton Sequencer (ThermoFisher Scientific), 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 to remove resilient sequencing adapters and to trim low-quality nucleotides at the 3′ side using a quality and length cutoff 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 2-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 (https://cran.r-project.org/web/packages/momr/index.html).[19]

Read downsizing. To decrease technical bias due to different sequencing depth and to 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 to 928,686 genes for the 182 samples, with an average of 325,147.5 genes. The resulting rarefied gene abundance table was normalized according to the FPKM strategy (Fragments Per Kilobase per Million mapped reads) (normalization by gene size and number of total mapped reads reported in frequency) to give the gene abundance profile table.

MGS construction. The gene catalogue was clustered by co-abundance as previously described,[20] which defined 6300 co-abundance gene groups with high correlations (Pearson correlation coefficient > 0.9). The 1529 largest of these, with more than 500 genes, were considered as MGS and are referred to as species throughout the article.

The abundance profiles of the co-abundance gene groups and MGS throughout the samples were determined as the mean abundance of 50 marker genes. Furthermore, the co-abundance gene groups and MGS were taxonomically annotated by summing up the taxonomic annotation of their genes as described by Nielsen et al.[20]

Microbial gene richness (gene count) was calculated by counting the number of genes 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 the Student *t* test or analysis of variance for normally distributed continuous variables, the Mann-Whitney U test or Kruskal-Wallis test for non-normally distributed continuous variables, and the χ^2 test or Fisher exact test for categorical variables. All statistical tests were 2-tailed, and $P < .05$ were considered significant. Statistical analysis was performed using SPSS Statistics Version 22.0 (IBM Corp, Armonk, NY).

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 $P < .05$. Comparison with the MetaHit cohort[14] was performed using a Wilcoxon rank-sum test.

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

Barcode visualization. MGS occurrence and abundance within samples are visualized using "barcodes," a heat map 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, light blue < 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 post hoc Dunn test were performed. A Benjamini-Hochberg correction[21] was applied to the results of the Kruskal-Wallis test. We identified a MGS marker for a Kruskal-Wallis–corrected $P < .05$ and Dunn $P < .05$.

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 1 significant correlation at the threshold of $P < .01$ were represented. Correlations with $P \leq .05$ were printed.

Functional analysis. The annotation of the genes based on the Kyoto Encyclopedia Genes and Genomes (KEGG) database (version 82; https://www.genome.jp/kegg/) was used. Through the functional annotation of the reference gene catalogue to KEGG orthology groups, abundances of KEGG orthology were computed for each sample. Gut metabolic modules information[22] was also used: Gene counts for each KEGG orthology have been summed and gut metabolic modules abundance were computed using an internal pipeline taking into account complex and alternative paths. An abundance matrix for the functional modules was obtained. Differentially abundant gut metabolic modules 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 gut microbiota for the prediction of mortality was assessed using a penalized logistic regression model. Logistic regression is a supervised method for a 2-class or multiclass classification problem.[23] Model construction, parameter fine-tuning, and validation were performed using the caret package (Classification And REgression Training).[24] We used penalized logistic regression to build a predictive model of mortality at 3 months based on the gut microbiota profiles using for 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 preprocessing steps were applied to 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 area under the curve, 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 coefficient indicated 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 the area under the curve, sensitivity, specificity, and number of predictors was computed. For each predictor, the mean value and SD of all coefficients were computed. A list of stable predictors was obtained, composed of the features selected in more than 10% of all models and for which abs(mean(coef)) > abs(sd(coef)), to keep only predictors that were always indicating the same status.

We also used random forest models with the caret package based on the random forest R function. In our 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 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 men (71%) with a mean age of 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 with other groups. In fact, they had higher frequencies of ascites and HE, more marked impairment of liver and renal

Table 1. Demographic and Clinical Data and Liver and Kidney Function Tests in All Patients

	Compensated (n = 24)	Decompensated Outpatients (n = 9)	Decompensated Inpatients Without ACLF (n = 84)	ACLF (n = 65)
Age, y	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[a]: alcohol and/or hepatitis C	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)
International normalized ratio	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 ($\times 10^9$/L)	104 (68-136)	88 (81-117)	93 (60-124)	71 (54-111)
Blood leukocytes ($\times 10^9$/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 (*mm Hg*)	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)
Child-Pugh score	5 (5-6)	8 (7-9)	8 (6-9)	11 (9-12)
Body mass index (kg/m^2)	28 (25-31)	28 (24-29)	27 (23-30)	27 (23-30)
Beta-blocker treatment[b]	11 (46)	1 (11)	31 (37)	28 (44)
Norfloxacin treatment[b]	1 (4)	1 (11)	17 (20)	11 (17)
Rifaximin treatment[b]	1 (4)	2 (22)	10 (12)	11 (17)
Lactulose/lactitol treatment[b]	1 (4)	4 (44)	29 (35)	23 (36)
Proton pump inhibitor treatment[b]	4 (17)	6 (67)	43 (51)	27 (42)
Metformin treatment[b]	4 (16)	1 (11)	7 (8)	8 (12)
Antibiotic treatment at fecal sample collection	0	0	52 (62)	44 (68)

NOTE. Values are median and (interquartile range) for quantitative variables and n (%) for qualitative variables.
[a]Other etiologies of cirrhosis were alcohol and hepatitis C (n = 1); NAFLD (6); primary biliary cholangitis (PBC) (1) in compensated cirrhosis, alcohol, and hepatitis C (1) in decompensated outpatients; alcohol and hepatitis C (7), hepatitis B (1), NAFLD (5), cryptogenetic (4), and PBC (2) in decompensated inpatients without ACLF; and alcohol and hepatitis C (10), hepatitis B (1), NAFLD (4), cryptogenetic (3), PBC (1), and hemochromatosis (1) in patients with ACLF.
[b]In hospitalized patients, refers to treatments received before hospital admission. Fecal samples for microbiome analysis were collected a median of 2 days after admission to the hospital.

function tests, and higher Child-Pugh and model for end-stage liver disease (MELD) scores compared with 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 2 groups.

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 with healthy subjects (Supplementary Figure 1). Overall, 613 MGS had significant differential abundance between healthy subjects and the whole series of patients with cirrhosis, and 566 MGS were

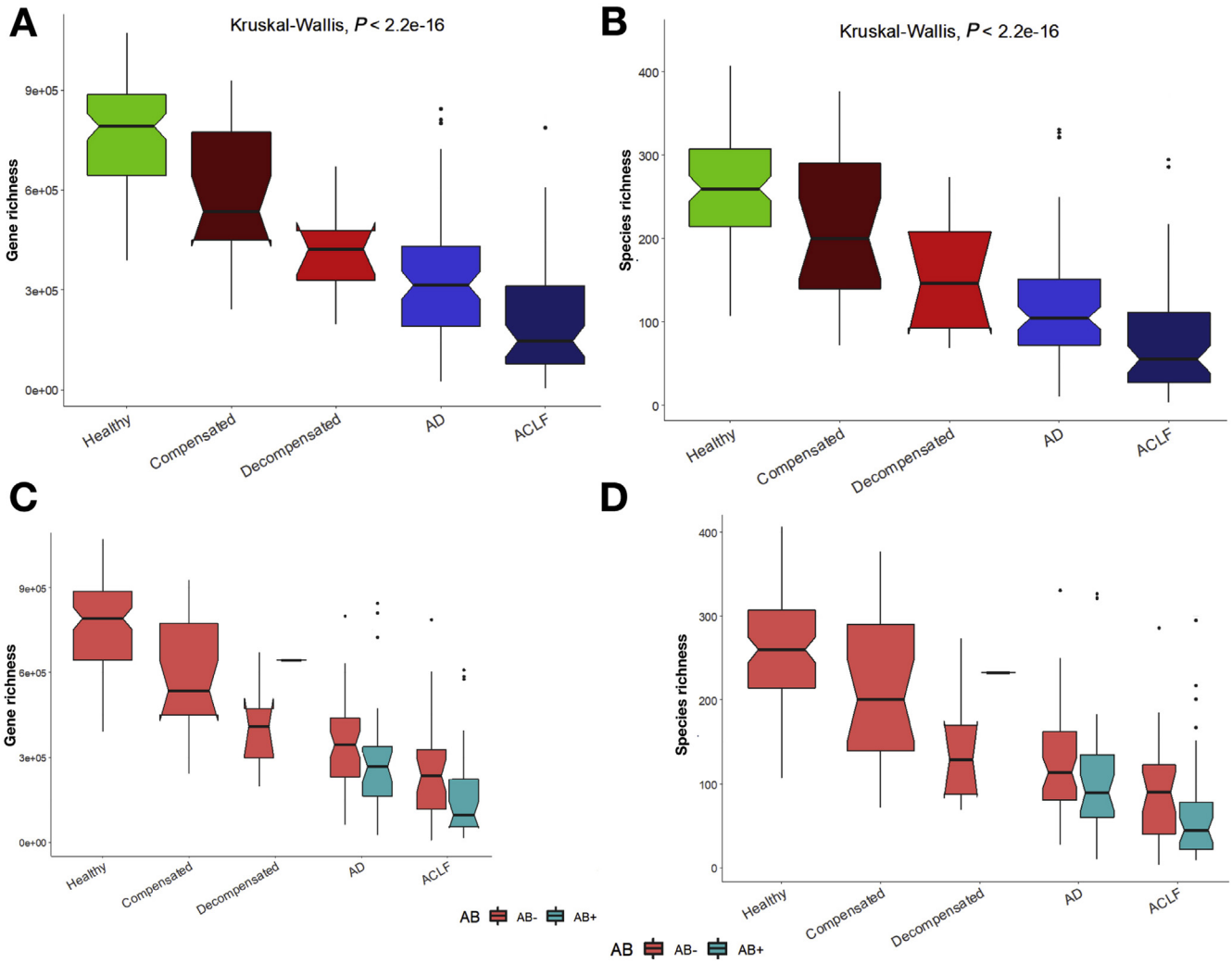

Figure 1. (*Top*) Comparison of gene (*left*) and MGS (*right*) richness in healthy subjects (n = 75, *green*) and in patients with cirrhosis divided according to disease stage: compensated cirrhosis (n = 24, *maroon*), decompensated outpatients (n = 9, *red*), decompensated inpatients without ACLF (n = 84, *blue*) and ACLF (n = 65, *dark blue*). (*Bottom*) 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*).

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 2 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 with 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 52 stable predictors (20 associated with healthy subjects and 32 associated with compensated cirrhosis) (area under the receiver operating characteristic curve, 0.81).

Characterization of Gut Microbiome Across Different Stages of Cirrhosis: Relationship With Complications and Disease Severity

Metagenomic sequencing revealed that gene richness and MGS richness significantly decreased with disease progression ($P < .001$) (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 ($P < .01$). By contrast, patients with compensated cirrhosis had the highest richness yet significantly lower than that of healthy subjects. Of interest, outpatients with stable decompensated

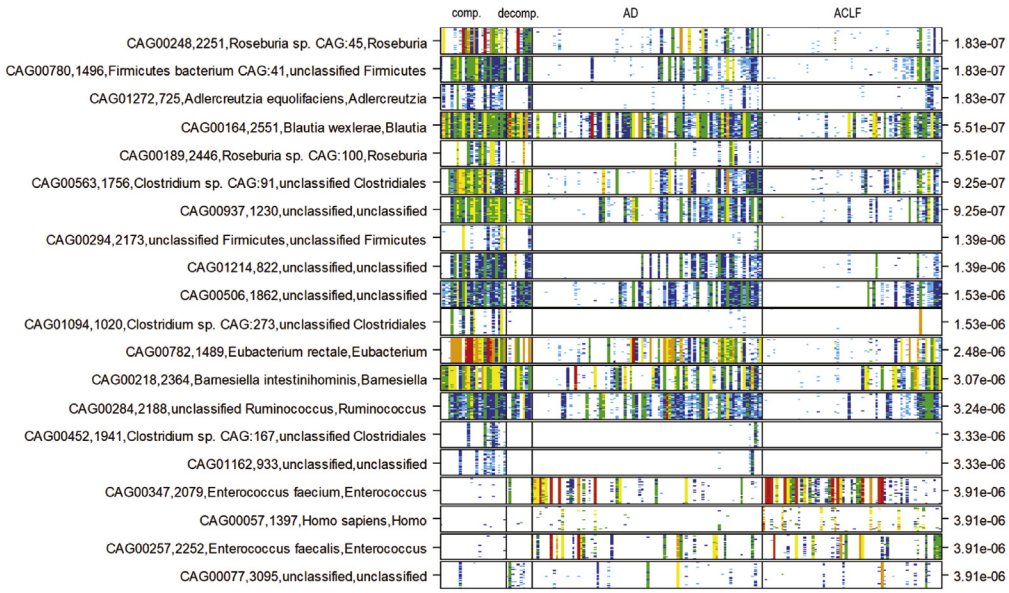

Figure 2. Differentially abundant MGS in patients with cirrhosis divided according to disease stages. MGS are in *rows*; MGS identification, genes number, and taxonomy (species name and genus) are indicated on the *left*. Abundance is indicated by color gradient from *white* (not detected) to *red* (most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

cirrhosis had higher gene and MGS richness compared with that of inpatients with decompensated cirrhosis. Interestingly, this loss of richness that paralleled disease progression persisted after adjustment for antibiotic therapy, suggesting 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 2 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 with their respective counterparts (Supplementary Figures 4–6), whereas differences according to alcoholic etiology and presence of infections were close to statistical significance. By contrast, chronic treatment with beta-blockers 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*, was 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 a community composed of MGS of the genus *Enterococcus* and oral bacteria like *Streptococcus* and *Veillonella* were positively correlated with 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

Table 2. Comparison of Metagenomics Richness According to Patient Characteristics

Category	Condition[a]	No. of Cases	Richness in MGS *P* value	Number of Significantly Contrasted MGS	
Etiology of cirrhosis (alcohol vs other)	Alcohol	123	.07	97 MGS	14 MGS
	Other	48			83 MGS
Alcohol consumption	Active alcohol	56	.01	172 MGS	10 MGS (including MGS of *S salivarius* and *E faeaclis*)
	Never	43			62 MGS
Hepatic encephalopathy	Yes	54	.04	83 MGS	17 MGS
	No	117			66 MGS
Infection	Yes	77	.06	98 MGS	7 (including MGS of *E faecium and E faecalis)*
	No	94			91 MGS
C-reactive protein				34 MGS[b]	2 MGS
					32 MGS
Leukocytes				27 MGS[b]	2 MGS
					25 MGS
Systemic inflammatory response syndrome	Yes	41	.05	47 MGS	3 MGS (including Homo sapiens, *E faecalis* and *E faecium*)
	No	130			44 MGS
Rifaximin treatment[c]	Yes	22	.01	78 MGS	8 MGS
	No	149			70 MGS
Norfloxacin treatment[c]	Yes	28	.05	55 MGS	5 MGS
	No	143			50 MGS
Proton pump inhibitor treatment[c]	Yes	75	.91	14 MGS	8 MGS
	No	96			6 MGS
Beta-blocker treatment[c]	Yes	65	.97	42 MGS	18 MGS
	No	105			24 MGS
Lactulose/lactitol treatment[c]	Yes	49	<.001	16 MGS	1 MGS
	No	121			15 MGS

NOTE. For patients evaluated on 2 different occasions (n = 11), only the first assessment is included in this table.
[a]In all cases, the presence of the condition is associated with lower richness compared with the absence of the condition. Alcohol etiology and alcohol consumption have lower richness compared with other etiologies and no alcohol consumption, respectively.
[b]MGS significantly correlated.
[c]In hospitalized patients, refers to treatments received before hospital admission. Fecal samples for microbiome analysis were collected a median of 2 days after admission to the hospital.

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 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 with those who survived (Figure 4*A*). At the MGS level, 17 were enriched in patients who died and 132 in patients who survived. Remarkably,

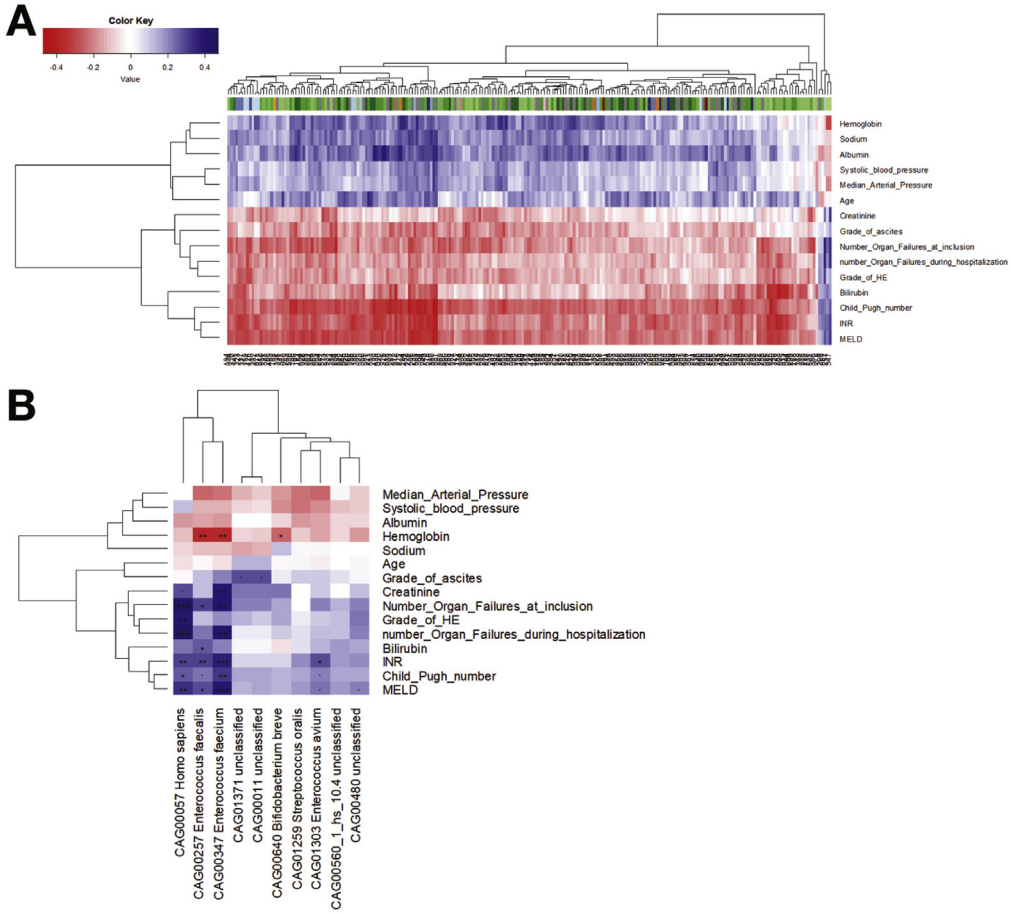

Figure 3. (*Top*) Heat map showing Spearman correlation between clinical variables and gut microbiome, global view. MGS were selected with at least 1 significant correlation ($q < 0.01$, FDR correction). Spearman correlation coefficient matrix with color-coded correlation (*blue* denotes positive correlation; *red* denotes negative correlation). (*Bottom*) Enlarged picture of the cluster in the *right* corner on MGS associated with disease severity. FDRs are denoted: $^{\cdot}q < 0.1$; $^{*}q < 0.05$; $^{**}q < 0.01$; $^{***}q < 0.001$. Spearman correlation coefficient matrix with color-coded correlation (*blue* denotes positive correlation; *red* denotes negative correlation).

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 area under the receiver operating characteristic curve 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 *E faecium, Streptococcus thermophilus, and Ruminococcus lactaris,* were predictors of poor short-term survival (Figure 4*B*). As an example, *R hominis* was found in 42% of patients who survived versus only 8% of those who died. By contrast, *E faecium* was found in 66% of patients who died versus 29% of patients who survived.

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 test sets; it was expectedly higher for both on the training than on the test sets, possibly because of 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 because of improving prediction of death rather than alive (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 were present in at least 1 sample. Eighty-two functional modules were significantly different between healthy subjects and patients with cirrhosis, 34 were more abundant in healthy subjects, and 48 were more abundant in patients with cirrhosis (Figure 5). Pathways enriched in cirrhosis were related to ethanol production, tryptophan degradation (amino acid degradation), lactose degradation (carbohydrate degradation), glycolysis, γ-aminobutyric acid 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.

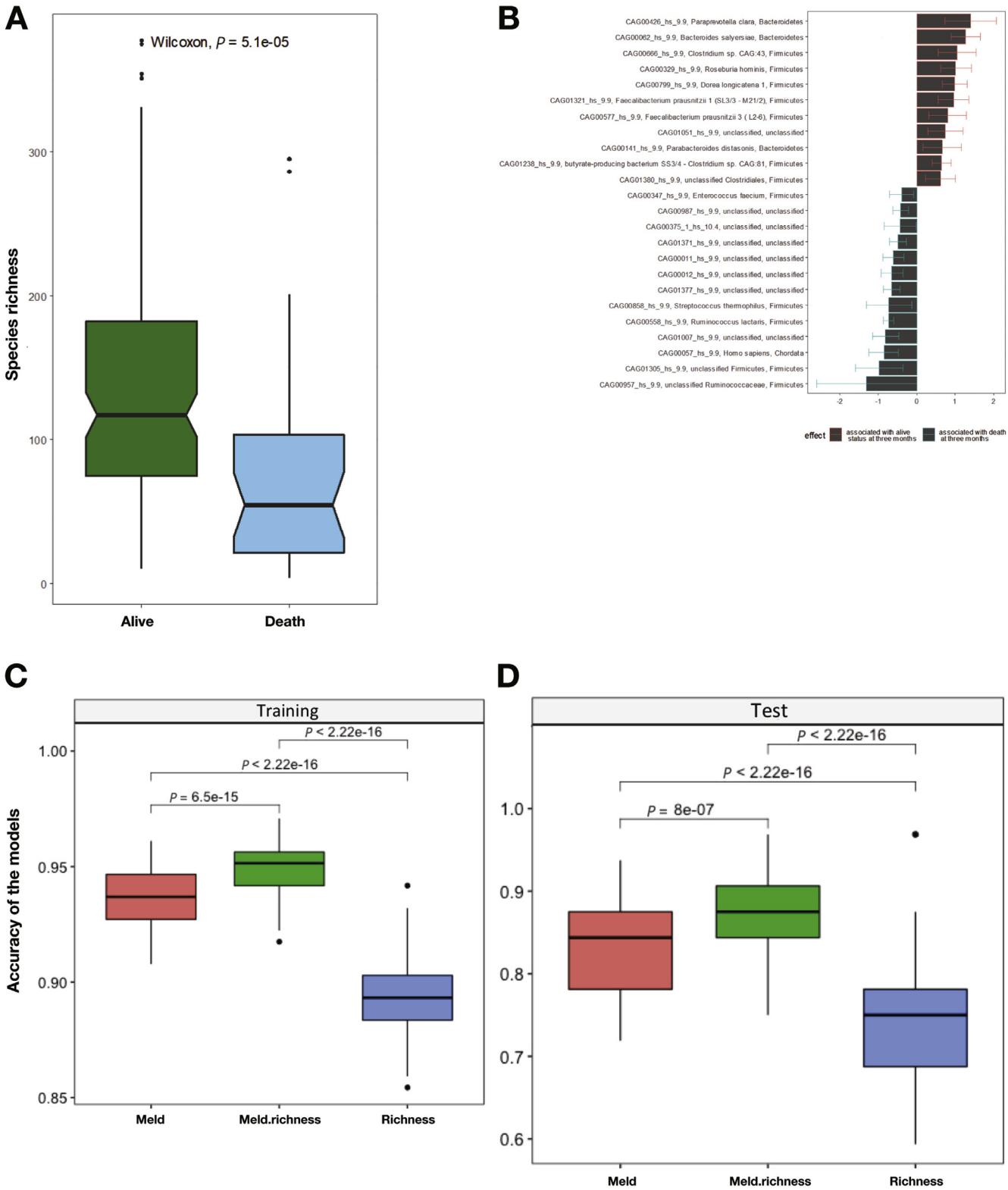

Figure 4. (*A*) MGS richness according to 3-month survival. (*B*) Stable set of predictors using gut microbiome for prediction of the 3-month mortality. *Red*, associated with alive status at 3 months. *Blue*, associated with death status at 3 months. MGS are in *rows*; MGS identification, species name,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

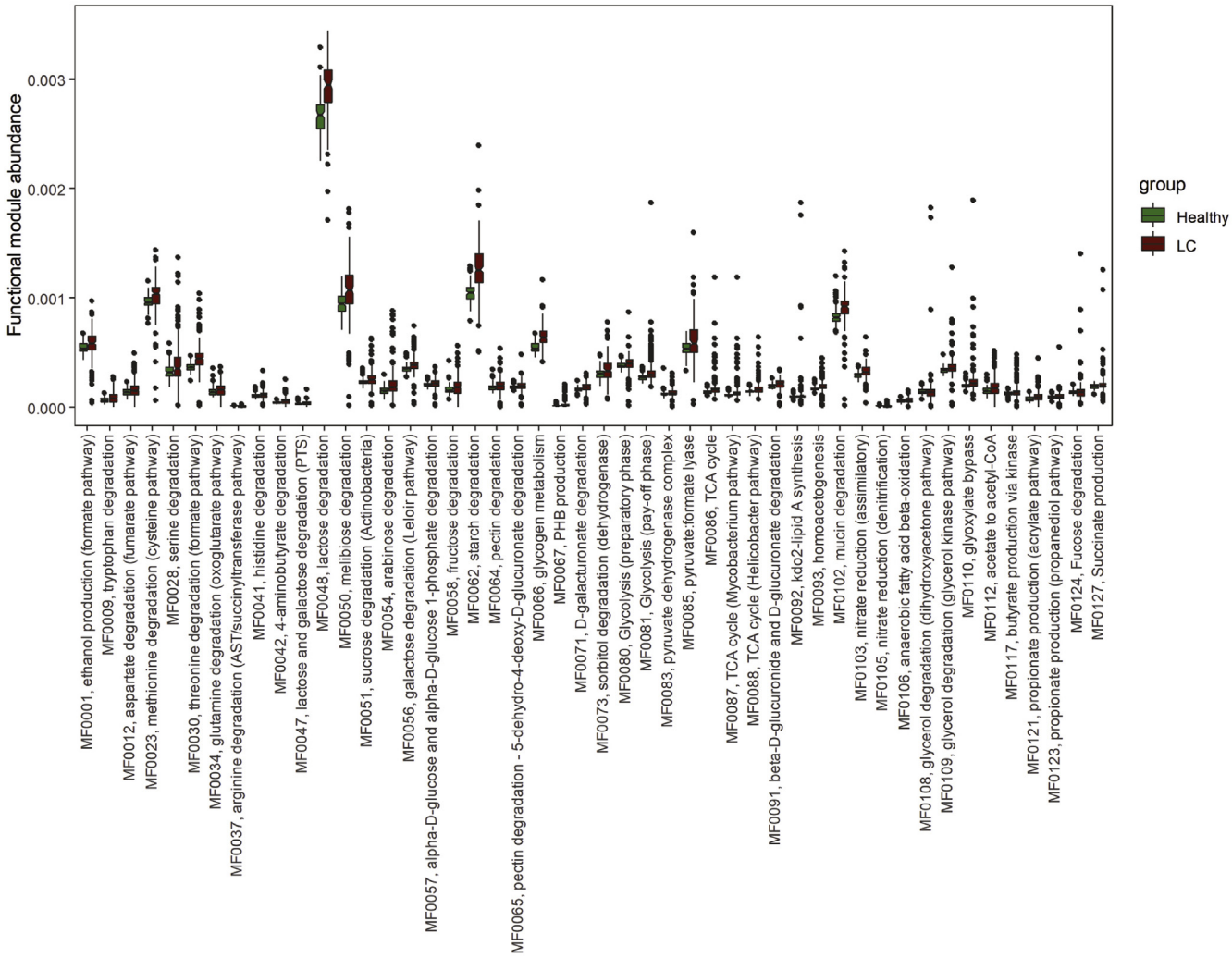

Figure 5. Contrasted functional modules between healthy subjects and patients with cirrhosis significantly enriched in patients with cirrhosis ($q < 0.05$, FDR correction).

Discussion

The current study demonstrates the existence of marked alterations in gut microbiome in cirrhosis that paralleled the disease stages, already obvious in compensated cirrhosis, progressing in decompensated cirrhosis, and 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 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 among the most remarkable seen in any disease condition studied so far using metagenomic sequencing.[10,14,19] 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 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.[26,27] The impairment in gut microbiome in decompensated versus 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,* and *Eubacterium,* among others, as compared with 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 versus those without; 17 MGS 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.[28] In fact, correction of gut dysbiosis by fecal microbiota transplantation has more recently been shown to prevent recurrent HE.[29,30] 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 with those not receiving rifaximin, with enrichment in 8 MGS with functional modules related with amino acid 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 spontaneous bacterial peritonitis 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 because of 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 2 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 *E faecium, S thermophilus,* and *R lactaris*, among others. By contrast, some species were associated with low risk of death. Of interest, microbiome richness improved the accuracy of the MELD score in outcome prediction.

Metagenomic technology allows the evaluation of functional modules that indicate pathways by which abnormalities in the 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; amino acid, carbohydrate, and lipid degradation; mucine degradation; nitrate metabolism; and γ-aminobutyric acid metabolism. Alteration in nitrate and γ-aminobutyric acid 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.

Some issues important to the interpretation of the current findings deserve discussion. First, this was 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 most patients hospitalized for management of decompensated cirrhosis, either with or without ACLF, receive antibiotics because of 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 played a pathogenic role in disease complications and mortality. Confirmation of this hypothesis requires prospective studies with a high number of patients focused on improving or modulating gut microbiome alterations, such as those already reported in patients with HE.[29,30]

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, with changes being maximal in patients with ACLF compared with 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.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org and at https://doi.org/10.1053/j.gastro.2020.08.054.

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Author contributions
Cristina Solé, Susie Guilly, Kevin Da Silva, Marta Llopis, Natalia Borruel, Magali Berland, Francesc Casellas, and Elsa Solà contributed to the conception and design of the study, acquisition of data, analysis and interpretation of data, and drafting the manuscript. Emmanuelle Le-Chatelier, Patricia Huelin, Marta Carol, Rebeca Moreira, Núria Fabrellas, Gloria De Prada, Laura Napoleone, Isabel Graupera, Elisa Pose, Adrià Juanola, David Toapanta, Francisco Guarner, and Jöel Doré participated in the analyses of the results, interpretation of data, and/or critical revision of the manuscript. Pere Ginès and Stanislav Dusko Ehrlich participated in the study concept, interpretation of the data, drafting the manuscript, critical revision of the manuscript for important intellectual content, obtained funding, and study supervision.

Conflicts of interest
This author discloses the following: Pere Ginès has participated on Advisory Boards for Novartis, Grifols, Promethera, Sequana, Intercept, and Martin Pharmaceuticals and has received research support from Gilead Sciences and Grifols.

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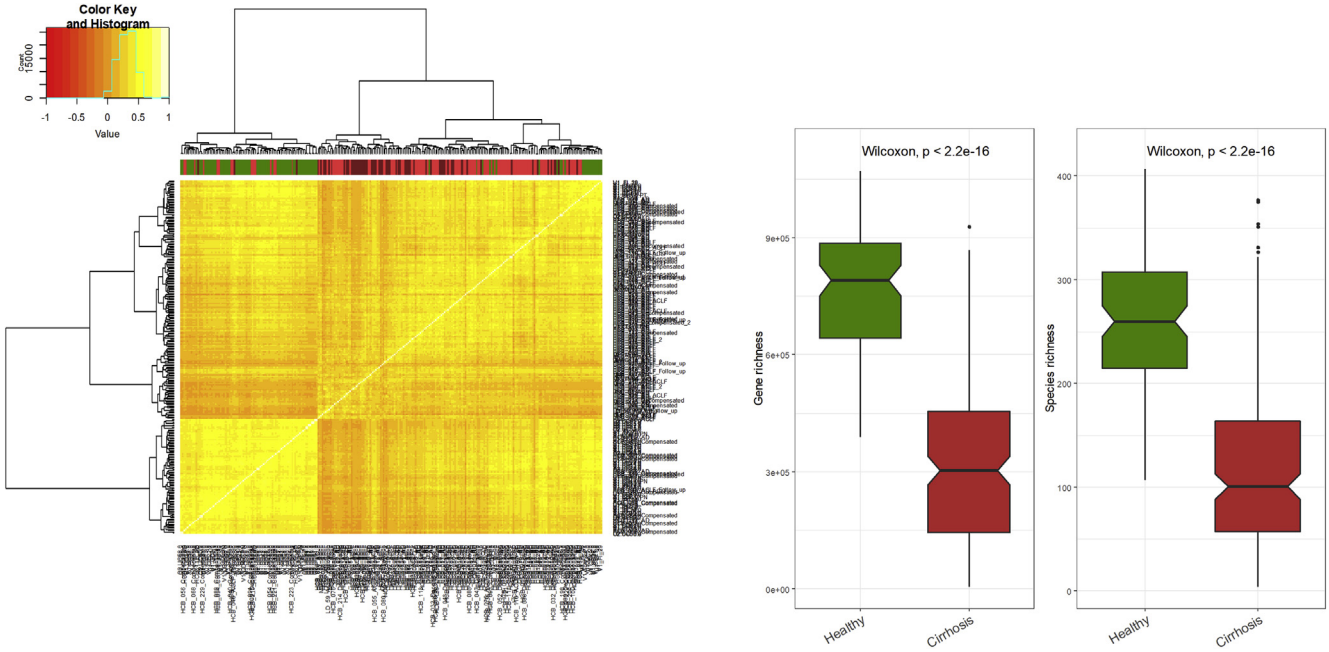
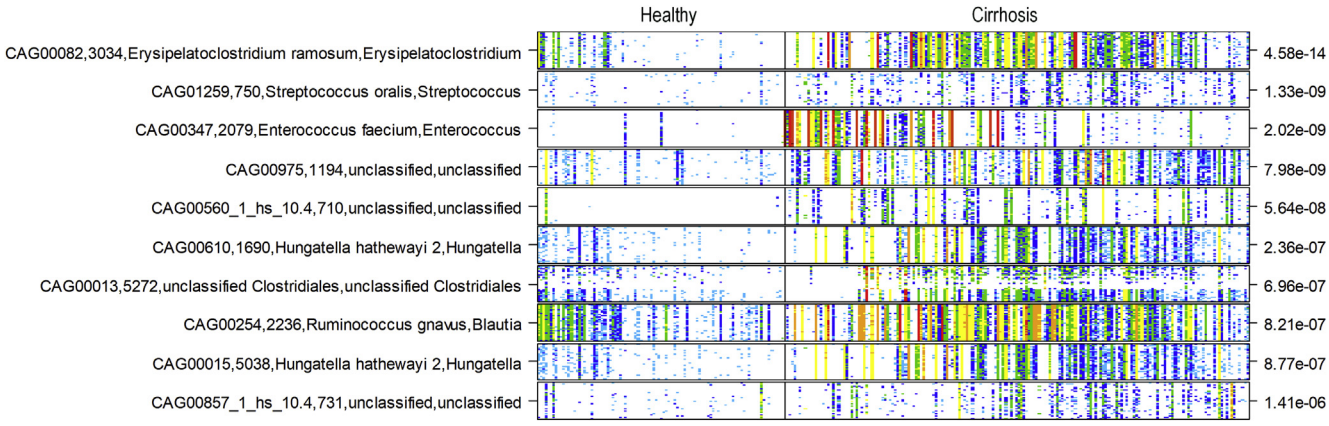
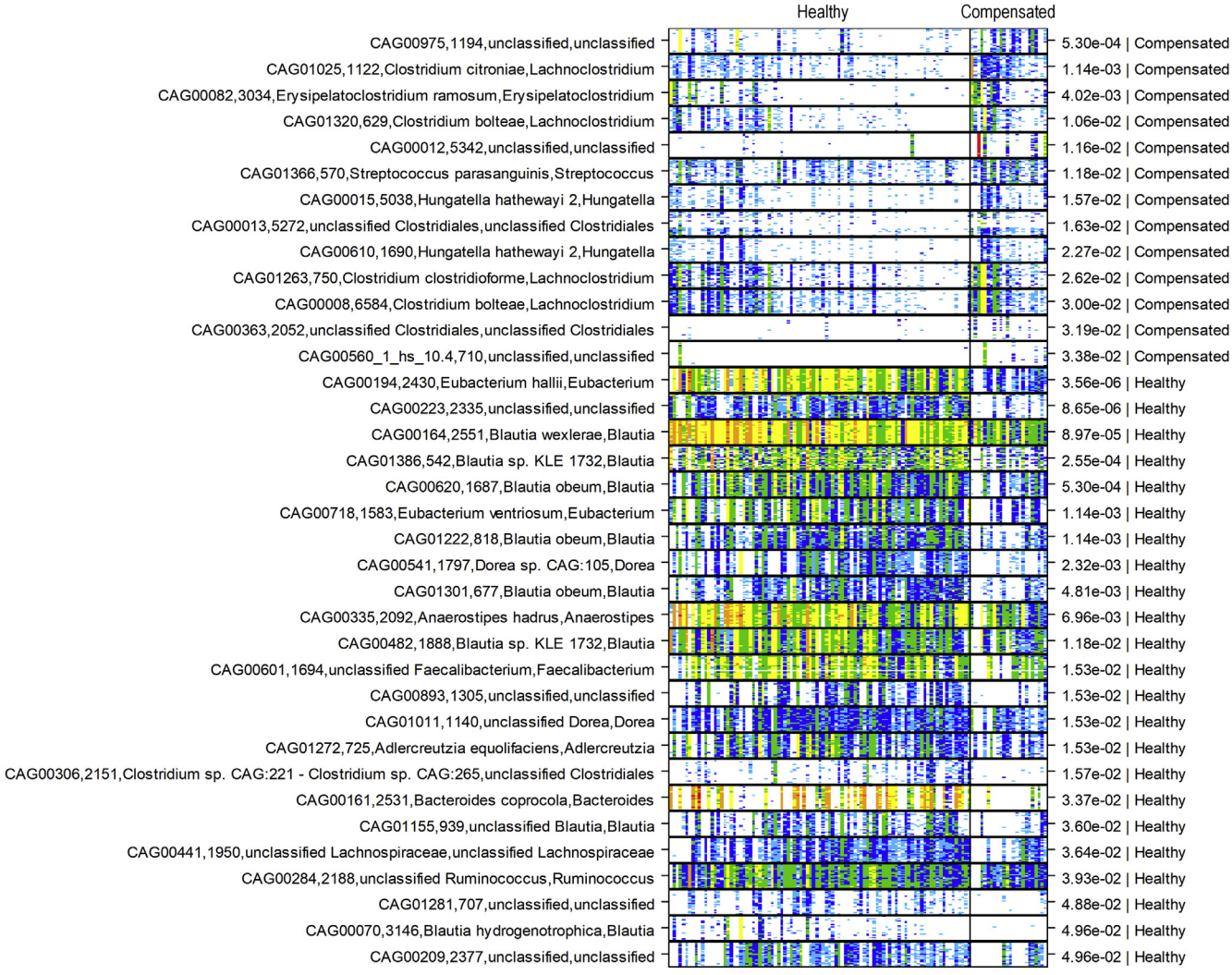


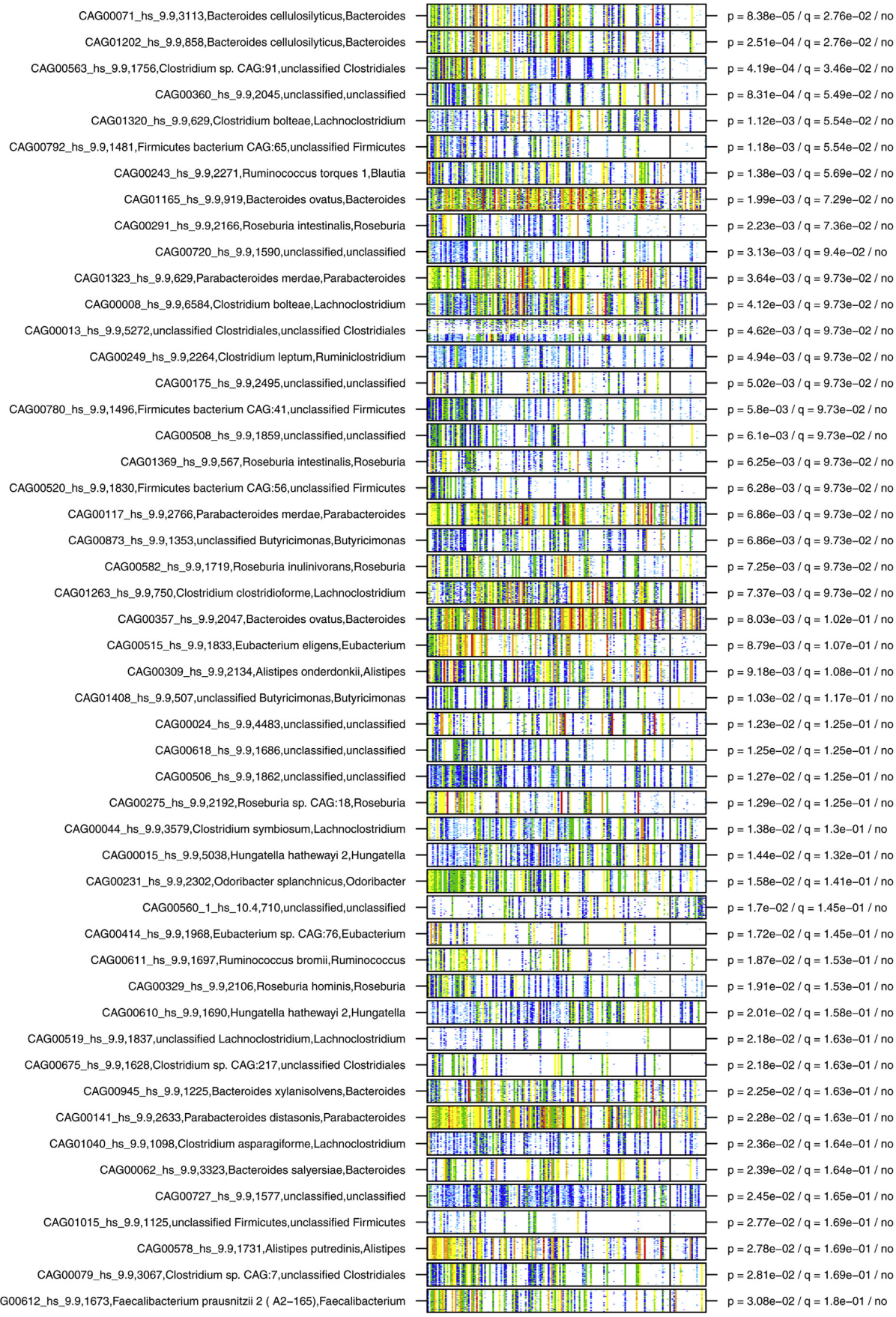
Supplementary Figure 1. (*Left*) Hierarchical clustering between healthy subjects and patients with cirrhosis. Healthy subjects are represented in *green*. Patients with cirrhosis are divided into those who received or did not receive antibiotics (*maroon* and *red*, respectively). (*Right*) Sequencing data and richness in genes and metagenomic species in healthy subjects (*green*) and patients with cirrhosis (*red*).



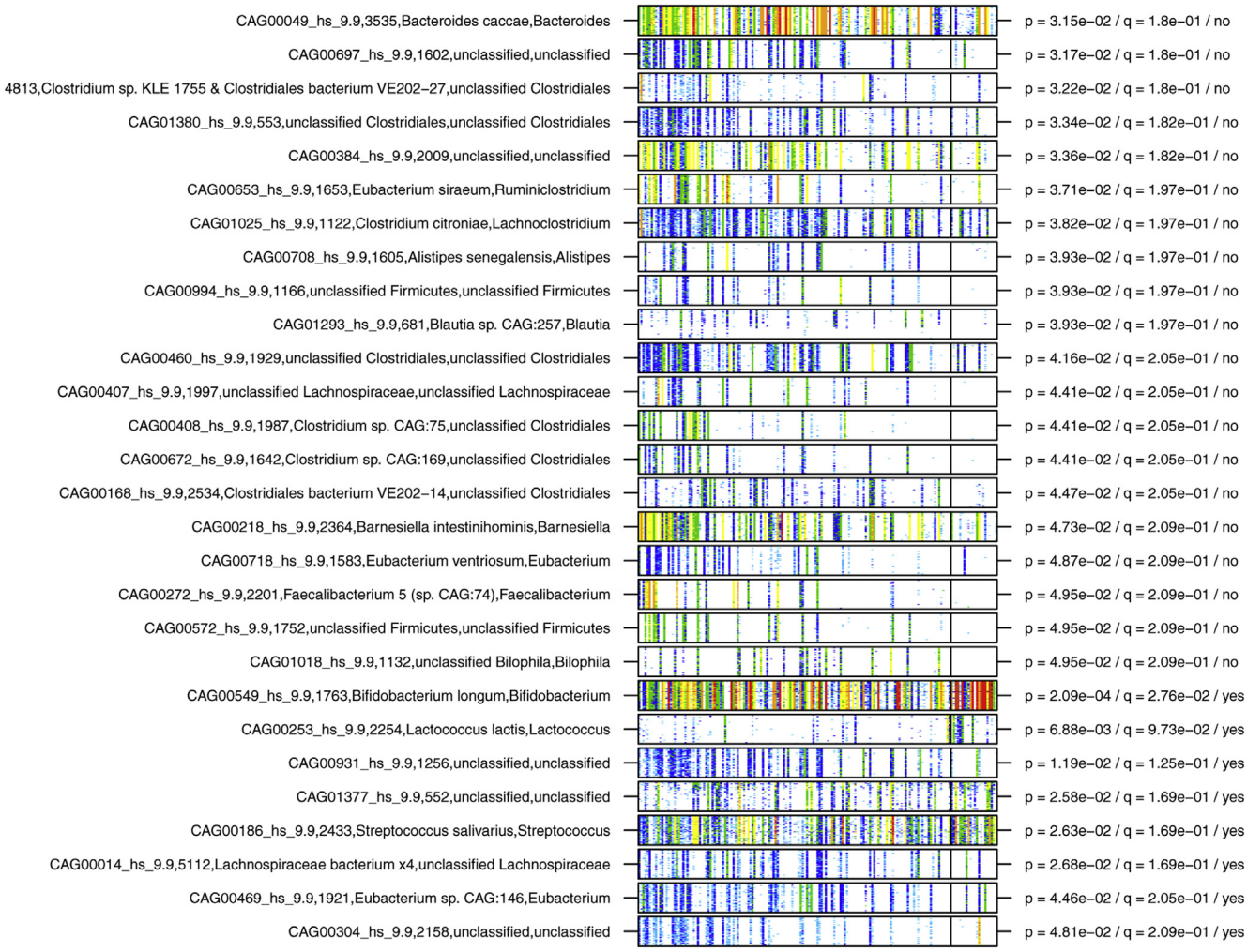
Supplementary Figure 2. The most contrasted metagenomic species significantly enriched in patients with cirrhosis compared with healthy subjects. Metagenomic species are in *rows*; MGS identification, genes number, and taxonomy (species name and genus) are indicated on the *left*. Abundance is indicated by color gradient from *white* (not detected) to *red* (most abundant). Individuals ordered by increased richness (MGS mean) are in *columns*. Significance Wilcoxon test (q value, FDR adjusted) is given on the *right*.



Supplementary Figure 3. Contrasted metagenomic species between healthy subjects and patients with compensated cirrhosis. Metagenomic species are in *rows*; MGS identification, genes number, and taxonomy (species name and genus) are indicated on the *left*. Abundance is indicated by color gradient from *white* (not detected) to *red* (most abundant). Individuals ordered by increased richness (MGS mean) are in *columns*. Significance of Wilcoxon test (q value, FDR correction) and prevalence group are given on the *right*.



Supplementary Figure 4. Contrasted metagenomic species significantly different between patients receiving or not receiving rifaximin. Metagenomic species are in *rows*; MGS identification, genes number, and taxonomy (species name and genus) are indicated on the *left*. Abundance is indicated by color gradient from *white*, not detected, to *red*, most abundant. Individuals ordered by increased richness (MGS mean) are in *columns*. Significance of the correlation (q value, FDR adjusted) and direction of the correlation are given on the *right*.



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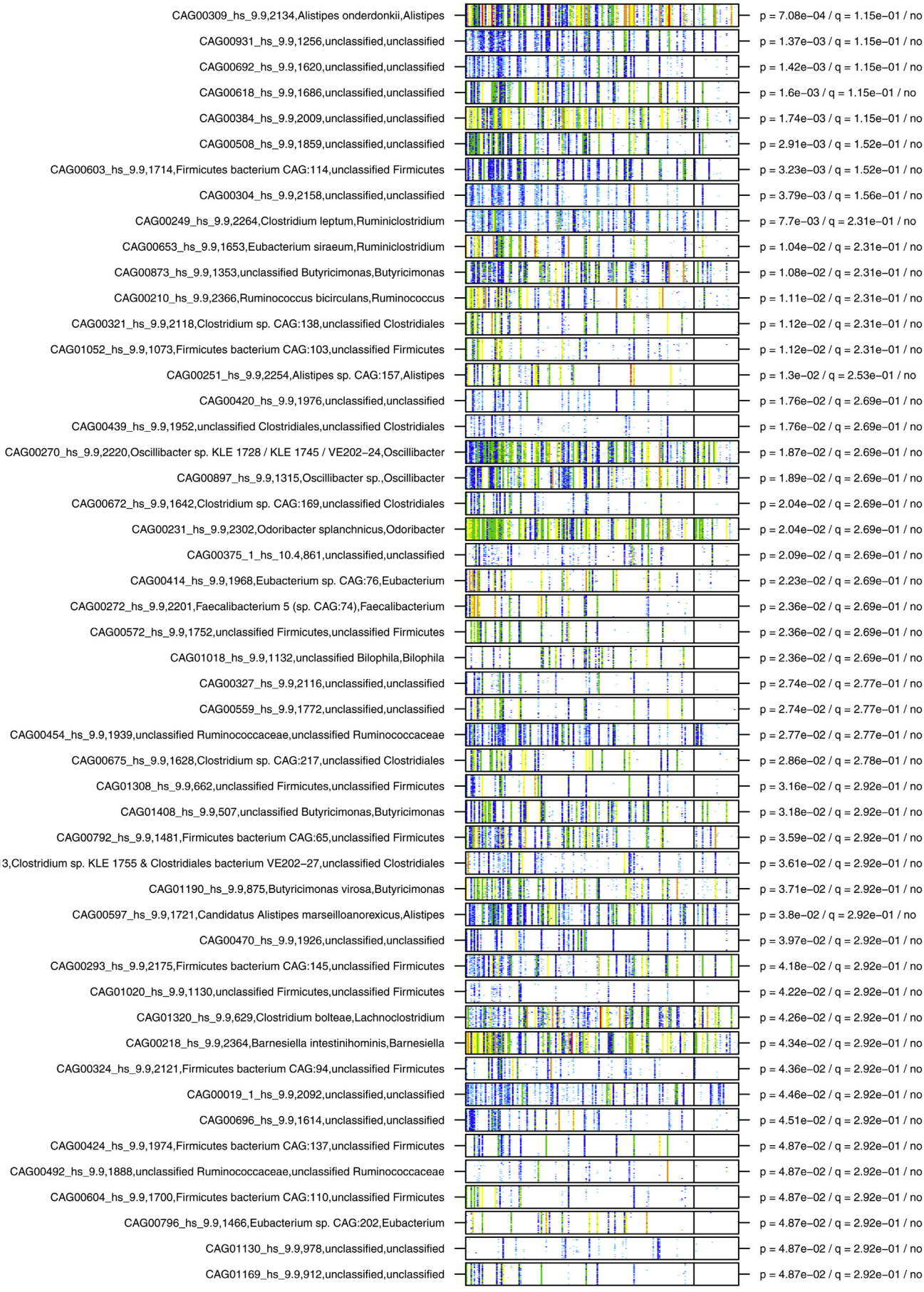

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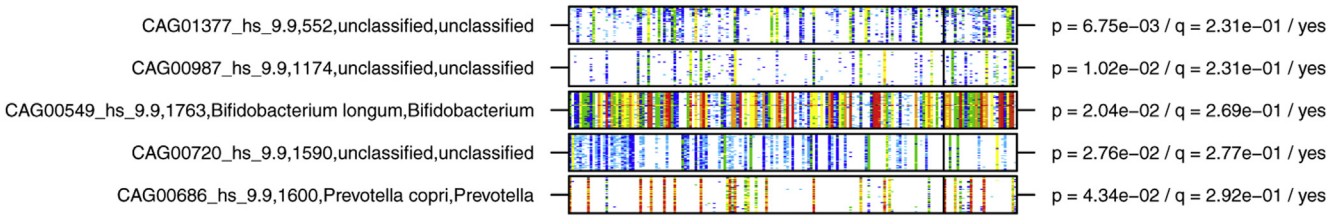

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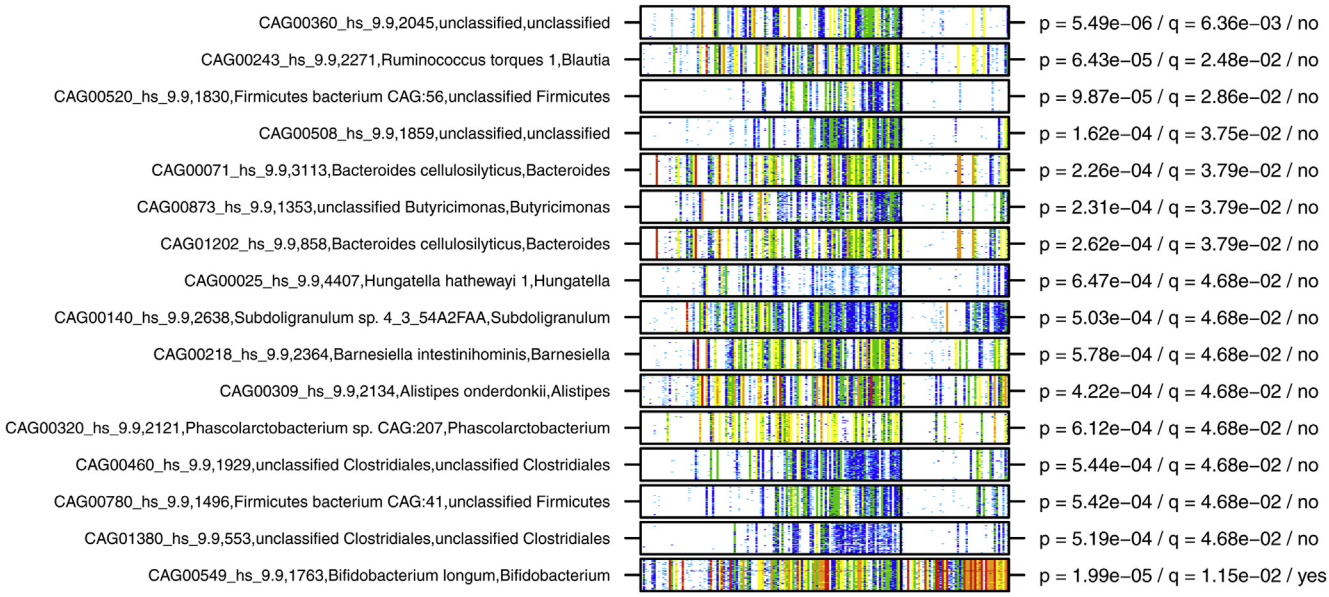
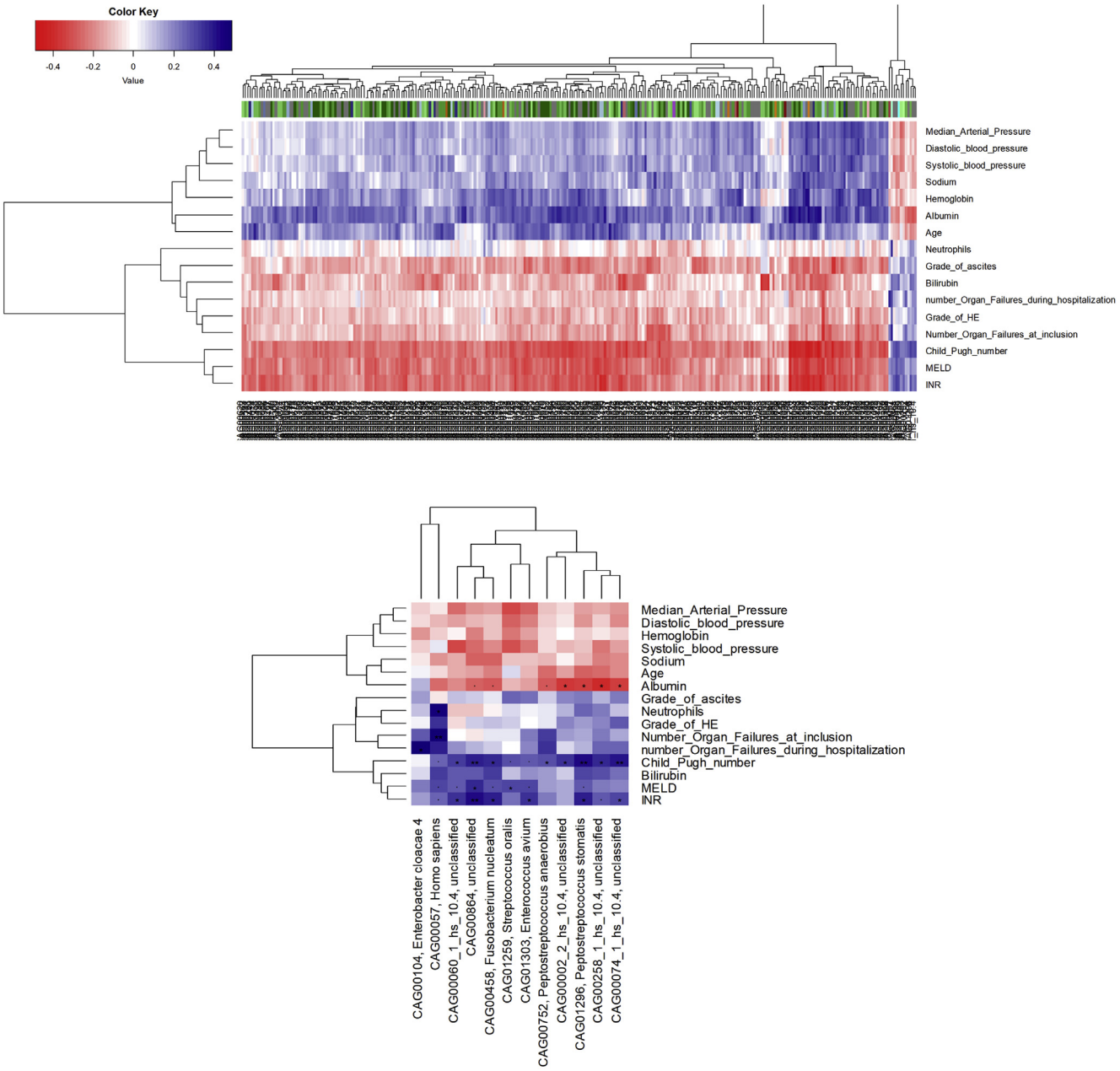
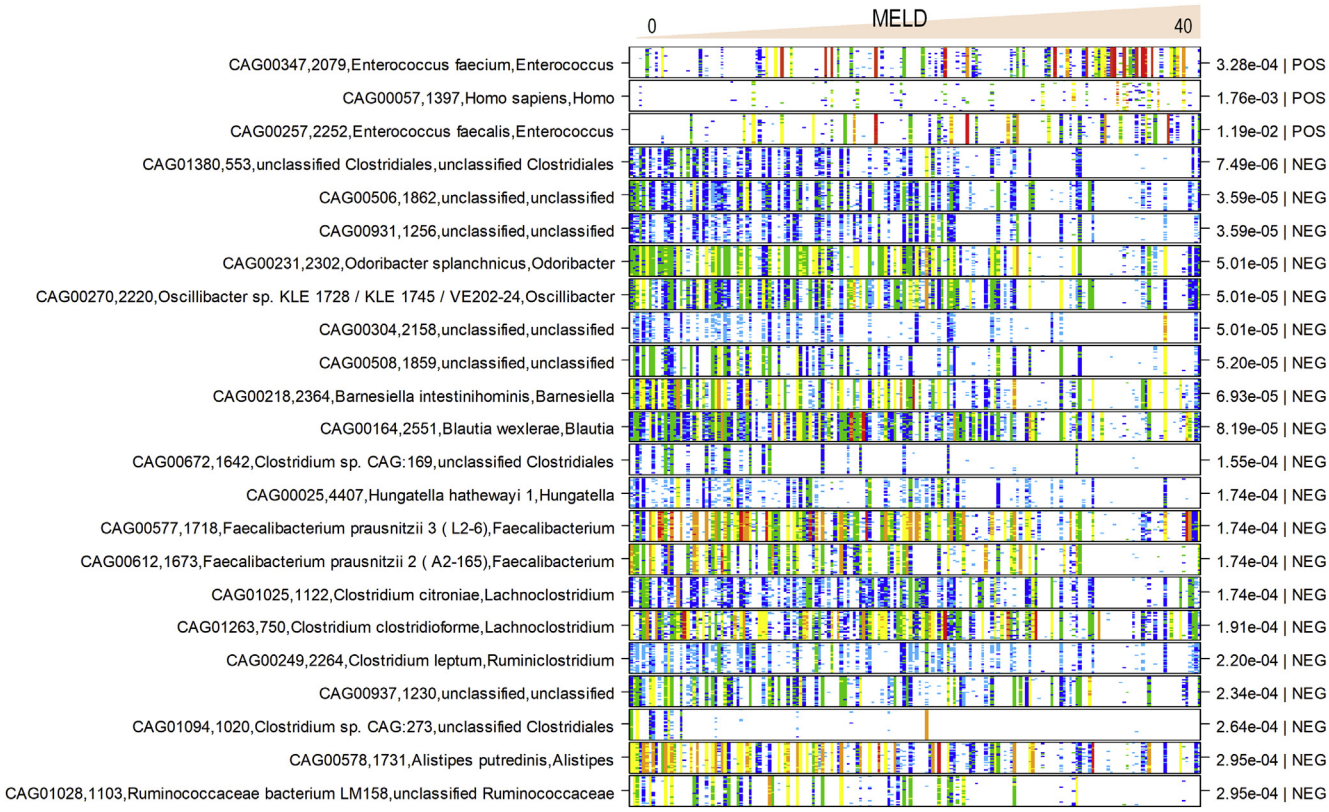
Supplementary Figure 5. Contrasted metagenomic species significantly different between patients receiving or not receiving norfloxacin. Metagenomic species are in *rows*; MGS identification, genes number, and taxonomy (species name and genus) are indicated on the *left*. Abundance is indicated by color gradient from *white*, not detected, to *red*, most abundant. Individuals ordered by increased richness (MGS mean) are in *columns*. Significance of the correlation (q value, FDR adjusted) and direction of the correlation are given on the *right*.



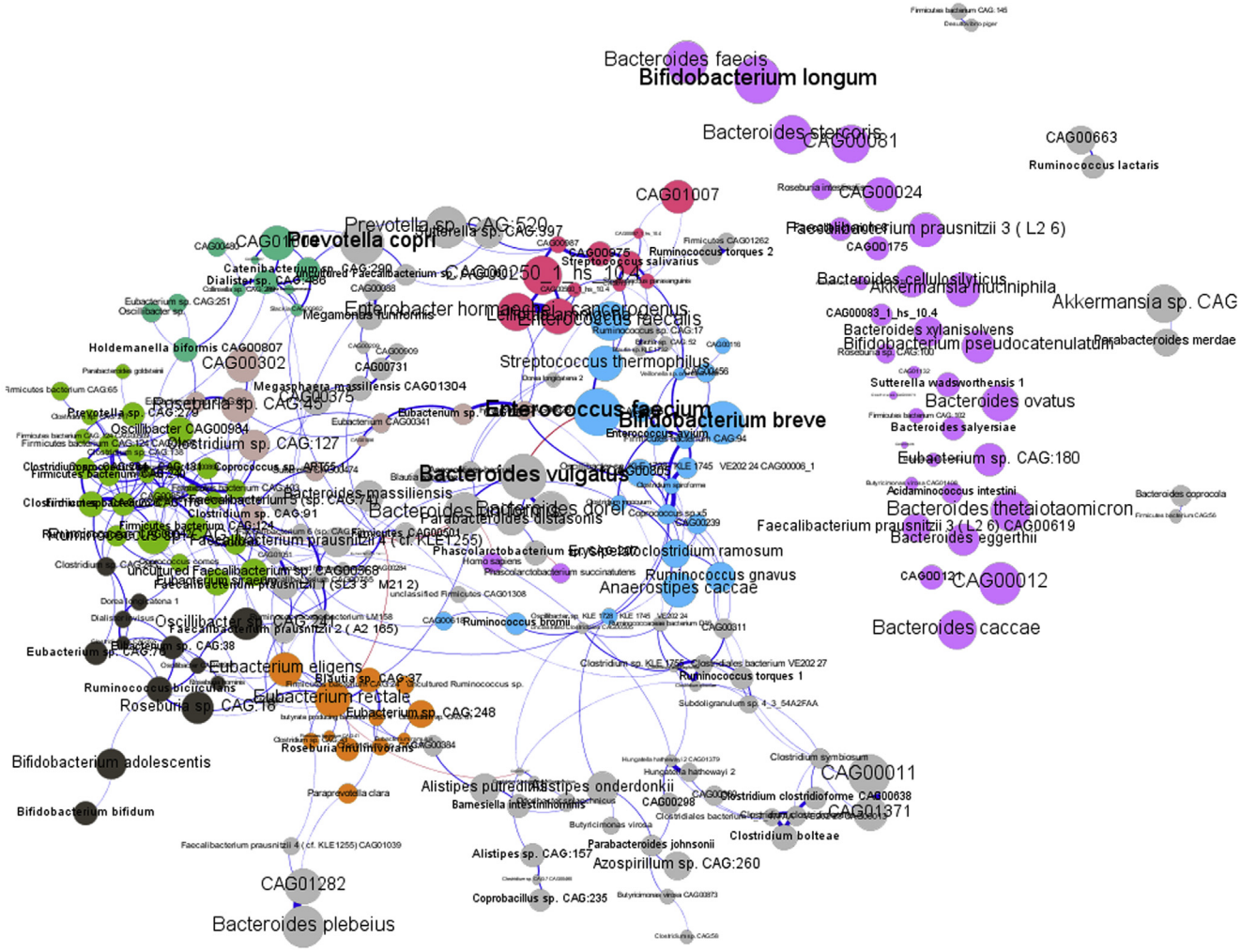
Supplementary Figure 6. Contrasted metagenomic species significantly different between patients receiving or not receiving laxative treatment. Metagenomic species are in *rows*; MGS identification, genes number, and taxonomy (species name and genus) are indicated on the *left*. Abundance is indicated by color gradient from *white*, not detected, to *red*, most abundant. Individuals ordered by increased richness (MGS mean) are in *columns*. Significance of the correlation (q value, FDR adjusted) and direction of the correlation are given on the *right*.



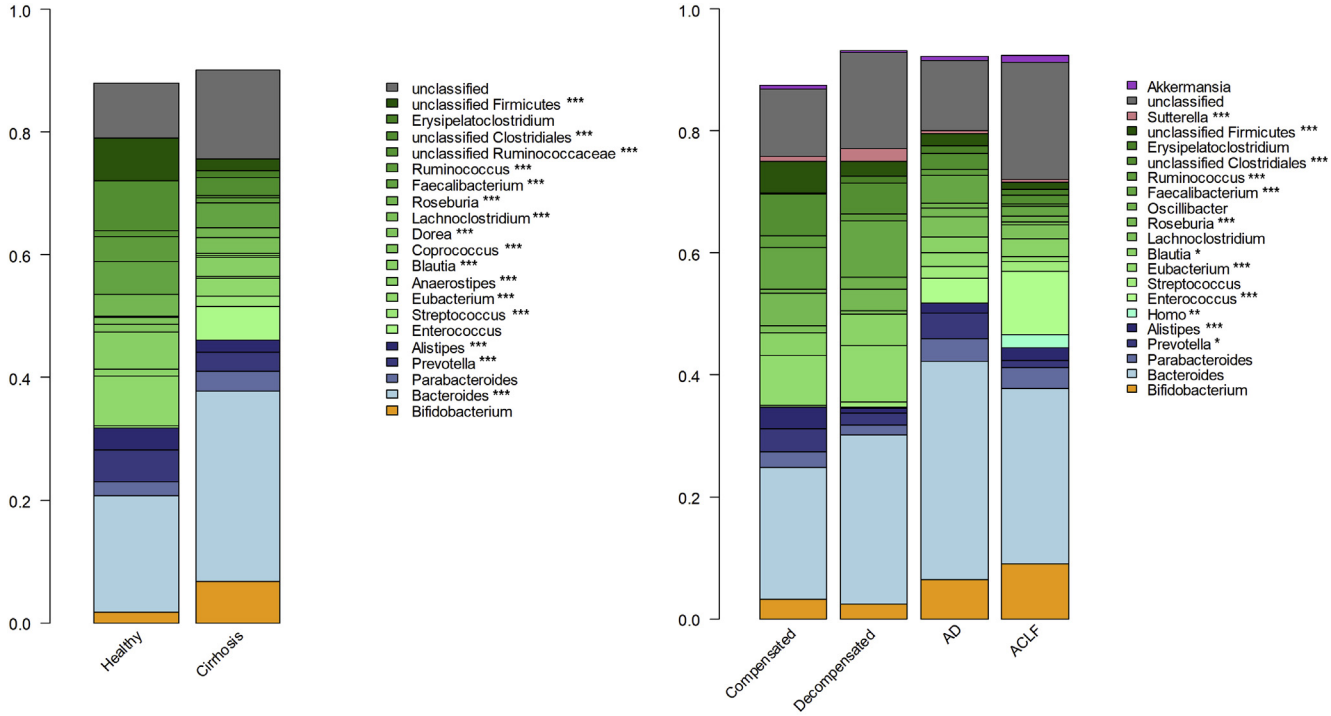
Supplementary Figure 7. (*Top*) Spearman correlation between clinical variables and gut microbiome in the group of patients not treated with antibiotics, global view. Metagenomic species were selected with at least 1 significant correlation ($q < 0.05$, FDR correction). (*Bottom*) Enlarged picture of the cluster in the *right* 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* denotes positive correlation; *red* denotes negative correlation; $P < .01$. *Blank spaces* indicated a nonsignificant correlation).



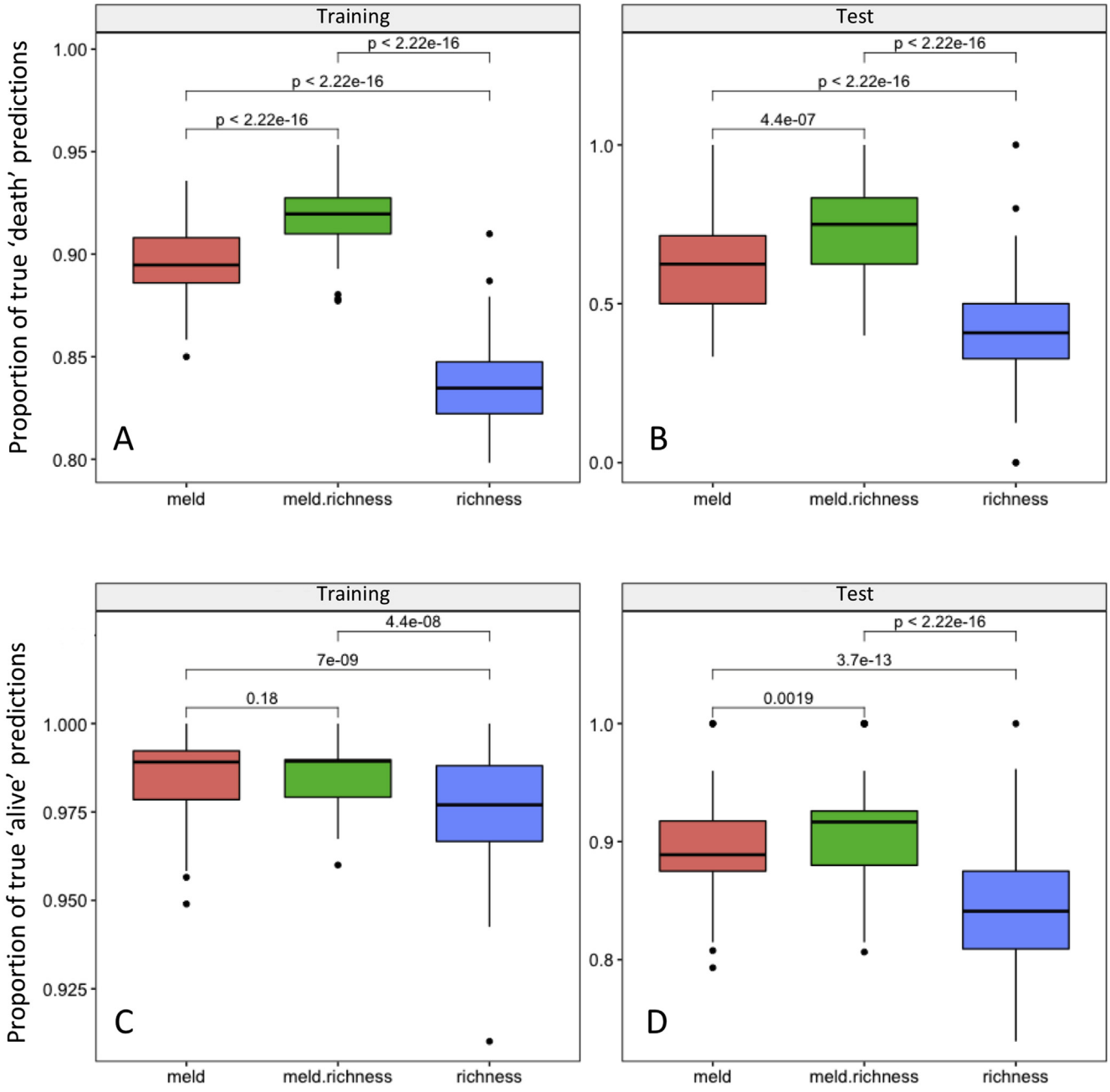
Supplementary Figure 8. The MGS with highest correlation with MELD score. Metagenomic species are in *rows*; MGS identification, genes number, and taxonomy (species name and genus) are indicated on the *left*. Abundance is indicated by color gradient from *white*, not detected, to *red*, most abundant. Individuals ordered by increased severity (MELD) are in *columns*. Significance of the correlation (q value, FDR adjusted) and direction of the correlation are given on the *right*.



Supplementary Figure 9. Network representation of MGS in patients with liver cirrhosis. MGS are grouped in MGS communities based on species abundance. The abundance of 1 community is the sum of the abundance of each MGS that composed the community. The name of each MGS communities is obtained by the name of the most abundant species.



Supplementary Figure 10. (*Left*) Taxonomy at the genus level of the gut microbiome for the healthy subjects and patients with cirrhosis. Significance of Wilcoxon test is denoted: ***q $<$ 0.001. (*Right*) Taxonomy at the genus level of the gut microbiome for compensated, decompensated outpatient, decompensated inpatients without ACLF (AD), and ACLF. Significance of Kruskal-Wallis test is denoted: *q $<$ 0.05; **q $<$ 0.01; ***q $<$ 0.001.



Supplementary Figure 11. Accuracy for predicting 3-month survival based on 100 random forest models. MELD is represented in *red*, MELD and richness are represented in *green*, and richness is represented in *blue*. (*Top*) Proportion of true death predictions. Results are divided according to model training with 80% of the data (*A*) and model testing with 20% of the data (*B*). (*Bottom*) Proportion of true alive predictions. Results are divided according to model training with 80% of the data (*C*) and model testing with 20% of the data (*D*).

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)	Patients With ACLF (n = 38)	*P* value
Site of infection			.461
Urinary tract infection	13 (28)	13 (34)	
Spontaneous bacterial peritonitis	5 (11)	6 (16)	
Pneumonia	6 (13)	9 (24)	
Skin and soft tissue	7 (15)	4 (11)	
Spontaneous bacteriemia	3 (6)	1 (3)	
Other[a]	13 (28)	5 (13)	
Systemic inflammatory response syndrome	12 (25)	26 (70)	<.001
Septic shock	0 (0)	17 (45)	<.001
Positive cultures	25 (53)	24 (51)	.273
Type of strain isolated			.278
Gram positive	14 (56)	10 (42)	
Gram negative	10 (40)	11 (46)	
Fungi	1 (4)	3 (8)	
Blood leukocytes ($\times 10^9$/L)	5 (4-8)	8 (5-15)	.001
C-reactive protein (mg/dL)	4 (1-6)	3 (1-6)	.834
Antibiotic type[b]			.02
Cephalosporin	26	6	
Carbapenem	15	23	
Antibiotics against gram positive	15	23	
Other	12	5	
Infection resolution	42 (91)	21 (57)	.001

NOTE. Values are n (%) or mean (interquartile range) for quantitative variables.

[a]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.

[b]Antibiotic treatment was categorized in 4 groups: only cephalosporins, carbapenem, antibiotics against gram-positive bacteria (including vancomycin, teicoplanin, tigecyclin, ampicilin), and others (levofloxacin, cirpofloxacin, amikacin, piperacilin-tazobacatm, linezolid). Patients could receive more than 1 antibiotic.