SUPPLEMENTARY FILE

Combining amplicon sequencing and metabolomics in cirrhotic patients highlights distinctive microbiota features involved in bacterial translocation, systemic inflammation and hepatic encephalopathy.

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SUPPLEMENTARY MATERIALS AND METHODS

Subjects enrolled
Forty-six patients with liver cirrhosis (aged 60.3±11.5 years, sex ratio M/F 32/13) hospitalized at the Department of Gastroenterology, University Hospital Policlinico Umberto I, were included in the study (see online supplementary table S1). The diagnosis of cirrhosis was proven through liver biopsy or based on clinical, biochemical and ultrasonographic signs. Exclusion criteria were: diagnosis of infection (based on fever, leukocytosis, elevated C Reactive protein (CRP), Erythrosedimentation Rate (ESR), procalcitonin, clinical symptoms, and positive microbiological cultures when present), use of systemic antibiotics in the last 3 months, variceal bleeding within the last 4 weeks, alcohol or illicit drug intake within the last 3 months. Lactulose or Rifaximine therapies were not considered a cause of exclusion. Patients with any condition of immunodeficiency (HIV, immunosuppression) or with a diagnosis of hepatocellular carcinoma were excluded. Fourteen healthy age-matched individuals (aged 53.8±7.8 years, sex ratio M/F 7/6) were recruited among neighbours to serve as controls. In this group, those who were taking, or had taken in the last 3 months, medications that could potentially modify gut microbiota (antibiotics, probiotics) were excluded. Both patients and controls signed an informed consent and the protocol of the study was approved by the Hospital Ethics Committee (Protocol Number 2515/15, Rif. 3696). At the enrolment, general physical examination and vital signs were recorded. The origin of cirrhosis, previous and present complications of the disease, laboratory findings (hemogram, serum electrolyte levels, renal and liver function tests, inflammatory parameters) were collected. The severity of liver disease was evaluated by Child-Turcotte-Pugh (CTP) and model for end stage liver disease (MELD) scores. The chronic use of beta-blockers, lactulose, proton pump inhibitors (PPI) and other drugs possible influencing gut microbiota was recorded. Patients included in the study and healthy controls gave a fresh stool sample that was promptly stored at -80°C. Cirrhotic patients also underwent serum collection of peripheral vein blood samples (2 mL) for cytokines titration (TNF-α, IL1β, IL6) and blood microbiota assessment. Portal blood (2 mL) was taken from seven cirrhotic patients admitted for TIPS procedure, and underwent the same analyses of peripheral blood. Both peripheral and portal blood samples were immediately frozen upon collection at -80°C. In patients and controls undergoing a colonoscopy for prevention of colorectal carcinoma as indicated by clinical guidelines, or due to a general work up, before being admitted to liver transplant list, a mucosal biopsy (cecum) was obtained for the assessment of intestinal microbiota adherent to the mucosa, and immediately stored at -80°C. All retrieved demographic and clinical parameters were anonymously used to build a matrix employed for subsequent multivariate statistical analysis.

ELISA tests
Evaluation of peripheral and portal blood levels of IL-6, TNF-α, and IL1-β cytokines was carried out by Enzyme-Linked ImmunoSorbent Assay (ELISA). Briefly, 2 ml of peripheral and portal blood were collected as specified above in a test tube with anticoagulant, and centrifuged at 3000 rpm for 10 minutes. One-hundred μL of supernatant were used to evaluate cytokines level by enzyme immunoassays carried out with commercial kits (Human ELISA Ready-SET-Go!, cat# 88-7066-22 for IL-6, cat# 88-7346-22 for TNF-α, cat# 88-7261-22 for IL1-β, eBioscience, San Diego, CA, United States), and assays were performed in triplicate following the manufacturer’s instructions. Plates (96-well ELISA plate, Corning Costar 9018, included in the kit) were read at 450 nm (subtracted by 570 nm readings) and intensity measurements were analyzed with an online software (http://www.elisaanalysis.com/) to retrieve values expressed as pg/mL (a four-parameters logistic curve was used for standard curve interpolation). A matrix of ELISA data was
generated for subsequent multivariate statistical analysis. Mann-Whitney U test was used to assess significant comparisons ($P \leq 0.05$).

**Microbiota characterization of biopsies, blood and stool samples**

Biopsies underwent a first wash (30s mid-speed vortex) with 0.016% dithioerythritol (DTT, cat#D0632, Sigma-Aldrich, Milan, Italy) in phosphate-buffered saline (PBS, cat#AU-L0615-500, Aurogene, Rome, Italy) to remove the mucus, then washed three more times in PBS. Total DNA from stool samples (200 mg / each), from biopsies (15 mg /each) and from portal/peripheral blood (200 μl / each) was automatically extracted with Maxwell® RSC Instrument (Promega. Wisconsin, USA, kit #AS1400). For all three kind of samples, manufacturer’s protocol was modified by incubating them with proteinase K at 56 °C, followed by a 4 hours incubation at 37 °C with 2 mg/ml (final concentration) of lysozyme (cat# L6876, Sigma-Aldrich, Milan, Italy) to ensure a proper disruption of Gram-positive bacterial species. Next Generation Sequencing (NGS) of 16S rDNA V3-V4 regions amplicons was thus carried out on a total of 109 samples so divided: i) cecum samples (17 HC, 6 controls); ii) stool samples (35 HC, 14 controls); iii) blood samples (30 HC peripheral, 7 HC portal). Samples underwent robotic library preparation and sequencing as per Illumina 16S metagenomics standardized operational workflow (16S Metagenomic Sequencing Library Preparation, Part # 15044223 Rev. B). Each 16s library was checked for size with Agilent 2200 Tapestation (Agilent Technologies, Santa Clara, CA, United States) and quantified with Qubit 2.0 fluorometer using Qubit dsDNA HS Assay Kit (cat# Q32851, Thermo Fisher Scientific, MA, United States). Sequencing was performed at Italian Institute of Technology (https://www.iit.it/it.centers/clns-sapienza) with Illumina MiSeq platform, Reagent Kit v3 (cat# MS-102-3003, Illumina, San Diego, CA, United States), 2x300 paired-end, 600-cycle. Raw FASTQ files were analyzed with Mothur pipeline v.1.38.1 [1] for quality check and filtering (sequencing errors, chimerae) on a Workstation CELSIUS R940 (Fujitsu, Minato-ku Tokyo, Japan). Filtered reads (9209053 in total, on average 84487 per sample, see online supplementary table S3) were clustered into Operational Taxonomical Units (OTUs), after singletons/doubletons elimination, by de novo OTU picking at 97% pairwise identity using standardized parameters and SILVA rDNA Database v.1.19 [2] for alignment. In all, 1990 OTUs were identified. Given the high heterogeneity of the six datasets (biop_cirr, biop_ctrl, feces_cirr, feces_ctrl, periph_blood, portal_blood) in terms of OTUs and filtered-quality reads numbers, all samples were normalized (rarefied) to the number of reads present in the less rich one (3101 reads for a portal blood sample). Sample coverage was computed with Mothur and resulted to be on average equal to 99% for all samples (mean±SDM, 99.1%±0.5%), thus meaning a suitable normalization procedure for subsequent analyses. Analysis Of Molecular Variance [3] (AMOVA, which represents the difference of datasets’ centroids), Homogeneity Of Molecular Variance (HOMOVA, representing the difference of datasets’ standard deviations), parsimony test, LEfSe [4], and Random Forest (RF) error rate were computed with Mothur v.1.38.1.

**OTUs species assignment and multivariate statistical analyses**

Bioinformatic and statistical analyses on recognized OTUs were performed with Python v.2.7.11. The most representative and abundant read within each OTU (as evidenced in the previous step with Mothur v.1.38.1) underwent a nucleotide Blast using the National Center for Biotechnology Information (NCBI) Blast software (ncbi-blast-2.3.0) and the latest NCBI 16S Microbial Database (ftp://ftp.ncbi.nlm.nih.gov/blast/db/). Retrieved species (first 500
OTUs) had the following Blast parameters values (mean±SDM) (see online supplementary file): E-value (1.82*10^{-85}±3.59*10^{-85}), total score (703.5±103.6), percentage identity (94.9±4.0) and mismatches (21.2±16.3). A matrix of bacterial relative abundances was built at each taxon level (phylum, class, order, family, genus, species, OTUs) for subsequent multivariate statistical analyses. Measurements of a diversity (within sample diversity) such as observed_ots and Shannon index, were calculated at OTUs level using the SciKit-learn package v.0.4.1. Exploratory analysis of β-diversity (between sample diversity) was calculated using the Yue & Clayton measure of dissimilarity (θ) calculated with Mothur and represented in Principal Coordinate Analyses (PCoA), while for Hierarchical Clusterization Analysis (HCA) ‘Bray-Curtis’ metrics and ‘complete linkage’ method were used implementing in-house scripts (Python v.2.7.11). In order to compare the microbiota taxa (at mean relative abundance level ≥ 0.5%) with demographics/clinical, NMR metabolomics, and ELISA datasets, a multivariate statistical Pearson correlation analysis (and related P values) was performed with in-house Python scripts. Pearson correlation matrices (metric=Bray-Curtis, method=complete linkage) were generated also for intra- and inter-dataset (biop_cirr, biop_ctrl, feces_cirr, feces_ctrl, periph_blood, portal_blood, NMR metabolomics) cluster generation and positive/negative correlation coefficient discovery. Mann-Whitney U and Kruskall-Wallis tests were employed to assess significance for pair-wise or multiple comparisons, respectively, taking into account a P value less than or equal to 0.05 as significant. Cross-correlation Pearson matrices for network analysis (metric=Bray-Curtis, method=complete linkage) were generated with in-house scripts (Python v.2.7.11) and visualized with Gephi v.0.9.1 [5], taking into account OTUs having a mean relative abundance ≥ 0.5% and Pearson correlation coefficients -0.7 > r > 0.7 as previously reported [6], after Benjamini-Hochberg FDR at 10%. A network analysis was performed on each dataset (biop_cirr, biop_ctrl, feces_cirr, feces_ctrl, periph_blood, portal_blood, NMR metabolomics, and merged 16S/NMR) using co-occurrences and visual representation as proposed by current guidelines [7–11]. Degree value, measuring the in/out number of edges linked to a node, and betweenness centrality, measuring how often a node appears on shortest paths between pairs of nodes in a network, were computed with Gephi v.0.9.1. Networks’ density and modularity were also computed with Gephi v.0.9.1: density would reflect the ‘shrinkage’ of network itself, while modularity the capability of network in hosting different (and various) communities within itself. Intra-network communities (even for Functional Metagenomics Communities, or FMCs) were retrieved using the Blondel community detection algorithm [12], by means of randomized composition and edge weights, and a resolution equal to 1 [13]. Clustering validation (K-means++ [14], Birch [15], Affinity Propagation [16]) and performance measures (Silhouette score [17], Calinski-Harabasz score [18]) were employed to confirm intra-network clusterization into communities. To find correlations among bacterial species, genes (KOs, effect size ≥ 0.47), fecal metabolites and presence/absence of hepatic encephalopathy (HE), we used logistic regression (-∞ < β < ∞), randomized lasso (0 < β < 1), elastic net (-∞ < β < ∞), and SGD classifier (-∞ < β < ∞) within Python SciKit learn module [19,20] on mean-centered and unit-variance dataframes. Odds ratios (OR) were computed from logistic regression coefficients (β) with the formula OR=2^β [21].

**Metagenome prediction and pathway analysis**

Biom file was generated with Mothur v.1.38.1 using Greengenes database (v. 13_5_99), and used with PICRUSt 1.0.0 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) [22] with default parameters, in order to predict Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs (KOs) from 16S V3-V4 amplicon data. Specific KOs related to significant NMR metabolites were bioinformatically assigned to each sample by
means of KEGG online website (http://www.genome.jp/kegg/ko.html) and Integrated Database Retrieval System (http://www.genome.jp/dbget/), taking into account Orthology and Reactions databases for a refined search. STAMP [23] was then utilized employing two-sided Welch’s t-test and η² (effect size) to detect specific KOs having discriminant power ($P \leq 0.05$) and functional relationship to NMR data of cirrhotic/controls fecal samples. Mean relative KO abundances, normalized per sample number, were computed with in-house Python scripts, and those lower than a definite threshold ($2\times10^{-6}$ % for phylum, $5\times10^{-6}$ % for genus) were excluded from KO contribution graphical analysis. For peripheral and blood functional analysis, STAMP was used with Kruskal-Wallis H test, Tukey-Kramer post-hoc test (0.95), and 10% Benjamini-Hochberg FDR.

**Nuclear Magnetic Resonance (NMR)**

Fecal samples of controls and cirrhotics were investigated using NMR spectroscopy to solve spectra of complex mixtures and to recognize and quantify each component without chemical separation [24]. Briefly, NMR spectra of fecal samples were recorded at 27°C on a Bruker AVANCE 600 spectrometer operating at the proton frequency of 600.13 MHz and equipped with a Bruker multinuclear z-gradient inverse probehead capable of producing gradients in the z-directions with a strength of 55 G/cm. 1H spectra were referenced to methyl group signals of TSP (δ=0.00 ppm) and they were acquired by co-adding 64 transients with a recycle delay of 7 s. The residual HDO signal was suppressed using a pre-saturation. The experiment was carried out by using a 90° pulse of 11.75 μs, and 32K data points. 1H spectra were transformed with 0.5 Hz line broadening and zero filling, size 65K, manually phased, calibrated on methyl group signals of TSP (δ=0.00 ppm), and baseline corrected using the TOPSPIN v1.3 software. Spectra were prepared for statistical analysis by dividing the entire spectrum into small regions (0.02 ppm width), called “buckets”. Regions with only background noise, the water resonance, and the extreme regions of spectra were excluded from the buckets. The total integral (as the sum of all 418 buckets) for each spectrum was normalized to 1000. 2D NMR experiments, namely, 1H-1H TOtal Correlation SpectroscopY (TOCSY), and 1H-13C Heteronuclear Single Quantum Coherence (HSQC) were performed using the same experimental conditions previously reported [24]. The mixing time for 1H-1H TOCSY was 80 ms. HSQC experiments were performed using a coupling constant 1JC-H of 150 Hz. Diffusion Ordered SpectroscopY (DOSY) experiment was performed using bipolar LED sequence with sine-shaped gradient of different intensities. The gradient strength was incremented in 32 steps from 2 to 95% of the maximum gradient strength (55 G/cm). The following experimental settings were used: diffusion time, $\Delta$ was 100 ms, gradient duration, $\delta/2$ was 1.1 ms, the longitudinal eddy current delay was 25 ms, the gradient pulse recovery time was set to 100 μs. After Fourier transformation and baseline correction, the diffusion dimension was processed by means of the Bruker TOPSPIN software (version 1.3). NMR spectra were bioinformatically analyzed by in-house scripts made with Python v.2.7.11, employing probabilistic quotient normalization (PQN) [25,26], baseline removal (rolling-ball) and peak shifting (binning) correction. A matrix of normalized and corrected NMR peaks’ areas was generated for subsequent multivariate statistical analyses.
**Supplementary table S1.** Demographic and clinical characteristics of patients with liver cirrhosis.

| Characteristic                          | Value          |
|----------------------------------------|----------------|
| Number of Pts.                         | 46             |
| Age (years)                            | 60.3 ± 11.5    |
| Sex (M/F)                              | 32/13          |
| Aetiology of cirrhosis                 | 14 (31)/13 (28.9)/ 5 (11.1)/13 (28.9) |
| CTP Class (A/B/C) n° (%)               | 14 (32.5)/23 (53.5)/6 (14) |
| MELD score                             | 13.5 ± 4       |
| Presence of oesophageal varices, n° (%)| 38 (86.3%)     |
| Ascites, n° (%)                        | 24 (54.5%)     |
| Hepatorenal Syndrome, n° (%)           | 2 (4.5%)       |
| Hepatic Encephalopathy, n° (%)         | 8 (18.2%)      |
| Albumin (g/dl)                         | 3.1 ± 0.5      |
| Sodium (mEq/L)                         | 136 ± 4        |
| Bilirubin (mg/dl)                      | 1.7 ± 1        |
| Creatinine (mg/dl)                     | 1 ± 0.4        |
| INR                                    | 1.4 ± 0.3      |
| VES (mm/h)                             | 29 ±2          |
| CRP (mg/dl)                            | 1.6 ± 3        |
| Death during the recovery, n° (%)      | 5 (11.3)       |
| Hepatocellular Carcinoma, n° (%)       | 16 (36)        |
| Median Arterial Pressure (mmHg)        | 83±11          |
| Cardiac frequency (bpm)                | 72 ± 13        |
| White Blood Cells (n°/mm3)             | 3987 ± 2237    |
| GPT (UI/ml)                            | 38.6±33        |
| PLT (n/mm3)                            | 94098±10049    |
| Hemoglobin (g/dl)                      | 10.6±2         |
| B-blockers, n° (%)                     | 34 (74)        |
| Diuretic therapy, n° (%)               | 27 (61)        |
| Proton Pump Inhibitors, n° (%)         | 33 (74)        |
| Lactulose, n° (%)                      | 17 (38)        |
| Rifaximine, n° (%)                     | 2 (4.5)        |
**Supplementary table S2.** Reads and OTUs obtained from MiSeq runs for each dataset.

|                | biop_cirr | biop_ctrl | faeces_cirr | faeces_ctrl | periph_cirr | portal_cirr | Total (or avg/sample) |
|----------------|-----------|-----------|-------------|-------------|-------------|-------------|-----------------------|
| samples (n)    | 17        | 6         | 35          | 14          | 30          | 7           | 109                   |
| raw_reads      | 2270128   | 1254162   | 7861085     | 6444647     | 3575803     | 767706      | 22173531              |
| raw_reads/sample| 133537    | 209027    | 224602      | 460332      | 119193      | 109672      | 203427/sample         |
| FQ_reads\(^a\) | 936810    | 510400    | 3477986     | 2360223     | 1598877     | 324757      | 9209053              |
| FQ_reads/sample| 55106     | 85067     | 99371       | 168587      | 53296       | 46394       | 84487/sample          |
| OTUs\(^b\)    | 448       | 297       | 353         | 256         | 432         | 204         | 1990                  |
| OTUs/sample    | 26        | 49        | 10          | 18          | 14          | 29          | 146                   |

\(^a\) FQ, filter-quality with Mothur pipeline v.1.38.1  
\(^b\) OTUs, Operational Taxonomical Units

**Supplementary figure S1. Distribution of OTUs.** Venn diagram reports distribution of all OTUs (enclosed numbers) among the six datasets: biop_cirr, biop_ctrl, feces_cirr, feces_ctrl, periph_cirr, portal_cirr (panel A). The ‘core’ microbiota for all datasets is composed by 52 OTUs (yellow circle).
Supplementary table S3. Mean relative abundances (≥ 0.5%) of bacterial Phyla. In bold are significant comparisons (P≤0.05).

| Phylum       | Biopsies cirr | Biopsies ctrl | P value (MW) biopsies | Feces cirr | Feces ctrl | P value (MW) faeces | Peripher blood | Portal blood | P value (MW) blood |
|--------------|---------------|---------------|-----------------------|------------|------------|---------------------|---------------|-------------|-------------------|
| Actinobacteria | 2.88          | 1.04          | 0.278                 | 1.64       | 0.52       | 0.357               | 3.62          | 4.69        | 0.698             |
| Bacteroidetes | 30.19         | 38.96         | 0.310                 | 42.78      | 51.71      | 0.432               | 9.12          | 17.43       | 0.393             |
| Firmicutes    | 34.42         | 41.30         | 0.100                 | 34.87      | 39.39      | 0.104               | 5.43          | 11.08       | 0.404             |
| Fusobacteria  | 1.21          | 3.20          | 0.273                 | 0.63       | 0.00       | **0.005**           | 0.04          | 0.00        | 0.110             |
| Proteobacteria| 28.26         | 11.34         | 0.074                 | 12.10      | 6.42       | 0.199               | 81.19         | 65.11       | 0.427             |
| Verrucomicrobia| 2.26          | 3.57          | 0.401                 | 7.18       | 1.41       | **0.013**           | 0.36          | 0.97        | 0.397             |
| unclassified  | 0.54          | 0.44          | 0.916                 | 0.48       | 0.37       | 0.127               | 0.09          | 0.38        | **0.043**        |

MW = Mann-Whitney U test

Supplementary table S4. Mean relative abundances of representative bacterial species described in the manuscript. In bold are significant P≤0.05. MW = Mann-Whitney U test

| Species                        | Biop Cirr | Biop Ctrl | P value (MW) biopsies | Feces Cirr | Feces Ctrl | P value (MW) faeces | Peripheral blood | Portal blood | P value (MW) blood |
|--------------------------------|-----------|-----------|-----------------------|------------|------------|---------------------|---------------|-------------|-------------------|
| Acinetobacter guillouiae       | 0.011     | 0.011     | 0.527                 | 0.002      | 0.000      | 0.383               | 25.909        | 14.364      | 0.561             |
| Akkermansia muciniphila        | 2.261     | 3.569     | 0.401                 | 7.179      | 1.387      | **0.015**           | 0.356          | 0.967       | 0.397             |
| Alistipes onderdonkii          | 0.006     | 0.102     | **0.017**             | 0.603      | 0.723      | **0.014**           | 0.003          | 0.000       | 0.679             |
| Bacteroides coprocola          | 0.044     | 0.000     | 0.621                 | 1.284      | 0.689      | 0.844               | 0.057          | 0.765       | **0.001**         |
| Bacteroides coprophilus        | 0.269     | 0.193     | **0.042**             | 1.284      | 0.297      | 0.264               | 0.084          | 0.143       | 0.922             |
| Bacteroides plebeius           | 0.021     | 1.403     | 0.720                 | 0.322      | 2.741      | 0.112               | 0.226          | 0.138       | 0.980             |
| Bacteroides stercoris          | 0.085     | 0.161     | 0.410                 | 1.182      | 0.403      | 0.332               | 0.016          | 0.000       | 0.520             |
| Bacteroides uniformis          | 0.051     | 1.129     | **0.021**             | 2.066      | 5.609      | **0.005**           | 0.024          | 0.332       | 0.151             |
| Bacteroides vulgatus           | 3.908     | 2.601     | 0.806                 | 16.977     | 8.566      | **0.048**           | 0.285          | 1.216       | 0.218             |
| Barnesiella intestinhibominis  | 7.910     | 8.685     | 0.834                 | 0.309      | 1.963      | **1.46E-07**        | 0.964          | 1.907       | 0.497             |
| Organism                          | 8.341 | 8.444 | 0.834 | 0.006 | 0.000 | 0.146 | 1.521 | 2.916 | 0.426 |
|----------------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Barnesiella viscericola          | 0.577 | 0.000 | 0.621 | 0.270 | 0.000 | 0.040 | 0.000 | 0.000 | 1     |
| Bifidobacterium breve            | 4.921 | 1.123 | 0.599 | 0.000 | 0.000 | 1     | 0.055 | 0.000 | 0.215 |
| Brevibacillus brevis             |       |       |       |       |       |       |       |       |       |
| Campylobacter concisus           | 0.027 | 0.005 | 0.495 | 0.156 | 0.000 | 0.078 | 0.000 | 0.115 | 0.045 |
| Clostridium clostridiforme       | 0.121 | 0.097 | 0.858 | 0.427 | 0.044 | 0.016 | 0.001 | 0.000 | 0.679 |
| Clostridium xylanolyticum        | 0.091 | 0.537 | 0.087 | 0.081 | 4.457 | 1.66E-06 | 0.272 | 1.037 | 0.112 |
| Elizabethkingia miricola         | 0.956 | 0.124 | 0.005 | 0.002 | 0.000 | 0.557 | 2.312 | 3.068 | 0.669 |
| Enterobacter cloacae             | 1.167 | 0.124 | 0.010 | 1.559 | 0.000 | 0.003 | 5.475 | 3.695 | 0.394 |
| Escherichia fergusonii           | 7.690 | 3.628 | 0.195 | 6.627 | 0.039 | 0.003 | 0.377 | 1.281 | 0.047 |
| Faecalibacterium prausnitzii     | 0.317 | 4.294 | 0.003 | 1.756 | 5.471 | 0.005 | 0.200 | 0.378 | 0.094 |
| Gemmiger formicilis              | 0.028 | 0.537 | 0.002 | 0.818 | 2.230 | 0.008 | 0.169 | 0.088 | 0.478 |
| Oscillobacter ruminantium        | 0.347 | 0.951 | 0.019 | 0.717 | 2.948 | 2.60E-05 | 0.177 | 0.783 | 0.019 |
| Propionibacterium acnes          | 1.444 | 0.306 | 0.019 | 0.002 | 0.000 | 0.383 | 2.856 | 3.515 | 0.641 |
| Pseudomonas fluorescens          | 1.969 | 0.382 | 0.012 | 0.002 | 0.002 | 0.873 | 31.966 | 20.731 | 0.342 |
| Roseburia faecis                 | 0.163 | 1.580 | 0.107 | 0.814 | 1.734 | 0.008 | 0.049 | 0.000 | 0.173 |
| Ruminococcus gnavus              | 2.969 | 2.268 | 0.362 | 0.648 | 0.007 | 0.001 | 0.199 | 0.217 | 0.686 |
| Ruminococcus torques             | 0.757 | 1.811 | 0.049 | 0.401 | 0.230 | 0.458 | 0.011 | 0.000 | 0.520 |
| Sphingomonas paucimobilis         | 10.602 | 2.370 | 0.441 | 0.001 | 0.005 | 0.143 | 8.177 | 14.581 | 0.185 |
| Veillonella dispar                | 0.943 | 0.204 | 0.342 | 9.684 | 0.101 | 8.68E-05 | 0.180 | 0.723 | 0.118 |
Supplementary figure S2. Beta-diversity of all samples divided by etiology. PCoA analysis was performed on a distance matrix based on Yue & Clayton measure of dissimilarity, dividing all samples (n=109) by etiology. PCoA classified patients by etiology (Amova $P=0.003$, Bonferroni pair-wise error rate: 0.0033, RF error rate: 0.577), even if not so indicative due to the heterogeneous origin of samples. PCoA evidenced significant differences for the following comparisons: Alcohol-Ctrl ($P<0.001$), Alcohol_Viral-Ctrl ($P=0.003$), Crypto_Auto_Wilson-Ctrl ($P=0.001$), Viral-Ctrl ($P<0.001$) and NASH-Ctrl ($P<0.001$).

Supplementary figure S3. Hierarchical Clusterization Algorithm (HCA) of cirrhotic and control samples. HCA was performed on all samples (x axis) taking into account the bacterial species having a mean relative abundance equal to or higher than 0.5% (y axis). Both dendrograms were generated with Bray-Curtis similarity and complete-linkage agglomeration. Colors on x axis (samples) refer to the different samples’ origin, cirrhosis etiology and drug treatments (beta-blockers, diuretic, lactulose, PPI, rifaximine).
Supplementary figure S4. Beta-diversity of all samples divided by drug treatment. PCoA analysis was performed on a distance matrix based on Bray-Curtis measure of dissimilarity, dividing fecal and peripheral blood cirrhotic samples by five drug treatments (beta-blockers, diuretic, lactulose, PPI, rifaximine). R statistic and P values were retrieved from Anosim analysis.
**Supplementary table S5.** Significant KEGG orthologues with positive effect size (higher proportion) in cirrhotics

| KEGG ortholog | Metabolic pathways | Gene name | Description | Difference in mean % proportion (cirrhotic vs ctrls) | P value |
|---------------|--------------------|-----------|-------------|-----------------------------------------------------|---------|
| K02770        | ko02060 Phosphotransferase system (PTS) | PTS-Fru-EIIIC/FruA | PTS system, fructose-specific IIC component | 0.005 | 0.042 |
| K02822        | ko02060 Phosphotransferase system (PTS) | PTS-Ula-EIIB/ulaB/sgaB | PTS system, ascorbate-specific IIB component [EC:2.7.1.194] | 0.004 | 0.044 |
| K01447        | ko01000 Enzymes | cwIA/xlyA/xlyB | N-acetylmuramoyl-L-alanine amidase [EC:3.5.1.28] | 0.004 | 0.032 |
| K00852        | ko00030 Pentose phosphate pathway | rbsK | ribokinase [EC:2.7.1.15] | 0.004 | 0.048 |
| K06193        | ko01120 Microbial metabolism in diverse environments | phnA | protein PhnA | 0.003 | 0.040 |
| K07341        | ko02048 Prokaryotic defense system | doc | death on curing protein | 0.002 | 0.042 |
| K10563        | ko03410 Base excision repair | mutM/fpg | formamidopyrimidine-DNA glycosylase [EC:3.2.2.23 4.2.99.18] | 0.002 | 0.034 |
| K00940        | ko00230 Purine metabolism ko00240 Pyrimidine metabolism | ndk/NME | nucleoside-diphosphate kinase [EC:2.7.4.6] | 0.002 | 0.037 |
| K04564        | ko04211 Longevity regulating pathway | SOD2 | superoxide dismutase, Fe-Mn family [EC:1.15.1.1] | 0.001 | 0.028 |
| K03637        | ko00790 Folate biosynthesis | moa/CNX3 | cyclic pyranopterin monophosphate synthase [EC:4.6.1.17] | 0.001 | 0.039 |

a) In bold are the orthologues within the first 20 KOs.
# Supplementary table S6. Significant KEGG orthologues related to NMR results, all with negative effect size (lower proportion) in cirrhotics

| KEGG ortholog\(a\) | Metabolic pathways | Gene name | Description | Difference in mean % proportion (cirrhotic vs ctrls) | \(P\) value |
|---------------------|--------------------|-----------|-------------|------------------------------------------------------|-------------|
| K00043  | ko00650 Butanoate  | gbd       | 4-hydroxybutyrate dehydrogenase [EC:1.1.1.61] | < -0.001 | 0.033 |
| K00109  | ko00650 Butanoate  | E1.1.99.2  | 2-hydroxyglutarate dehydrogenase [EC:1.1.1.99.2] | < -0.001 | 0.043 |
| K01028  | ko00650 Butanoate  | scoA      | 3-oxoacid CoA-transferase subunit A [EC:2.8.3.5] | -0.001  | 0.039 |
| K01666  | ko00360 Phenylalanine metabolism | mhpE     | 4-hydroxy2-oxovalerate aldolase [EC:4.1.3.39] | < -0.001 | 0.041 |
| K02688  | ko03000 Transcription; Transcription factors | prpR     | transcriptional regulator, propionate catabolism operon regulatory protein | < -0.001 | 0.026 |
| K03416  | ko00640 Propanoate metabolism | E2.1.3.1-5S methylmalonyl-CoA carboxyltransferase 5S subunit [EC:2.1.3.1] | < -0.001 | 0.043 |
| K15036  | ko00640 Propanoate metabolism ko01200 Carbon metabolism | K15036 | acetyl-CoA/propionyl-CoA carboxylase [EC:6.4.1.36.1.2] | < -0.001 | 0.035 |
| K01844  | ko00310 Lysine degradation | kamD     | beta-lysine 5,6-aminomutase alpha subunit [EC:5.4.3.3] | < -0.001 | 0.044 |
| K00306  | ko00310 Lysine degradation | PIPOX    | sarcosine oxidase / L-pipecolate oxidase [EC:1.5.3.71.5.3.1] | < -0.001 | 0.043 |
| K00831  | ko00680 Methane metabolism ko00260 Glycine, serine and threonine metabolism ko01200 Carbon metabolism | serC/PSAT1 | phosphoserine aminotransferase [EC:2.6.1.52] | -0.002  | 0.031 |
| K02203  | ko00680 Methane metabolism ko00260 Glycine, serine and threonine metabolism | thrH     | phosphoserine / homoserine phosphotransferase [EC:3.1.3.3 2.7.1.39] | -0.004  | 0.046 |
| K03390  | ko00680 Methane metabolism ko01200 Carbon metabolism | hdrC     | heterodisulfide reductase subunit C [EC:1.8.98.1] | < -0.001 | 0.046 |
| K06034-K13039 | ko00680 Methane metabolism | comD-comE | sulfopyruvate decarboxylase subunit alpha and beta [EC:4.1.1.79] | < -0.001 | 0.043 |
| K11780- | ko00680 cofG-cofH | FO synthase subunit 1 and 2 | < -0.001 | 0.043 |
| K11781  | Methane metabolism | [EC:2.5.1.77] |
|---------|-------------------|--------------|
| K14940  | ko00680 Methane metabolism | cofF | gamma-F420-2-alpha-L-glutamate ligase [EC:6.3.2.32] | < -0.001 | 0.030 |
| K14028-K14029 | ko00680 Methane metabolism ko00010 Glycolysis / Gluconeogenesis ko01200 Carbon metabolism | mdh1/mxaF - mdh2/mxaF | methanol dehydrogenase (cytochrome c) subunit 1 and 2 [EC:1.1.2.7] | < -0.001 | 0.045 |
| K14081  | ko00680 Methane metabolism ko01200 Carbon metabolism | mtaC | methanol corrinoid protein | < -0.001 | 0.024 |
| K00138  | ko00010 Glycolysis / Gluconeogenesis | aldB | aldehyde dehydrogenase [EC:1.2.1.-] | < -0.001 | 0.039 |
| K06859  | ko00010 Glycolysis / Gluconeogenesis ko01200 Carbon metabolism | pgI1 | glucose-6-phosphate isomerase, archaeal [EC:5.3.1.9] | < -0.001 | 0.043 |
| K00029  | ko01200 Carbon metabolism | maeB | malate dehydrogenase (oxaloacetate-decarboxylating)(NADP+) [EC:1.1.1.40] | -0.002 | 0.045 |
| K00031  | ko01200 Carbon metabolism | IDH1/IDH/icd | isocitrate dehydrogenase [EC:1.1.1.42] | -0.003 | 0.048 |
| K00051  | ko01200 Carbon metabolism | E1.1.1.82 | malate dehydrogenase (NADP+) [EC:1.1.1.82] | < -0.001 | 0.041 |
| K00282-K00283 | ko01200 Carbon metabolism | gcvPA - gcvPB | glycine dehydrogenase subunit 1 and 2 [EC:1.4.4.2] | -0.001 | 0.039 |
| K14451  | ko01200 Carbon metabolism | mcl2 | (3S)-malyl-CoA thioesterase [EC:3.1.2.30] | < -0.001 | 0.047 |
| K14472  | ko01200 Carbon metabolism | smtB | succinyl-CoA:(S)-malate CoA-transferase subunit B [EC:2.8.3.22] | < -0.001 | 0.041 |
| K15016  | ko01200 Carbon metabolism | K15016 | enoyl-CoA hydratase / 3-hydroxyacyl-CoA dehydragenase [EC:4.2.1.17 1.1.1.35] | < -0.001 | 0.043 |
| K15019  | ko01200 Carbon metabolism | K15019 | 3-hydroxypropionyl-coenzyme A dehydratase [EC:4.2.1.116] | < -0.001 | 0.041 |

a) In bold are the orthologues within the first 20 KOs.
Supplementary figure S5. Picrust analysis for phyla and genera contributions. Picrust analysis (panel C) was used to compute the average relative contributions of phyla and genera for selected KOs. The 10 KOs with higher percentage contribution in cirrhotics (positive effect size - overrepresented, see online supplementary table S4) were reported in descendent order of effect size, while 8 KOs with lower percentage contribution in cirrhotics (negative effect size - underrepresented, see online supplementary table S5), involved in carbon (pathway ko01200) and methane (pathway ko00680) metabolism, were reported in numerical ascending order.
**Supplementary table S7.** Clustering validation and performance measures of controls and cirrhotics FMNs into FMCs

| AP     | Control feces (16S + NMR) | Cirrhotics feces (16S + NMR) |
|--------|---------------------------|-----------------------------|
|        |                          |                             |
| #_clust| Silhouette_score (K-means)| Silhouette_score (Birch)    |
| _ers   | Calinski_score (K-means)  | Calinski_score (Birch)      |
| 2      | 0.257                     | 0.255                       |
|        | 22.16                     | 20.05                       |
| 3      | 0.331                     | 0.320                       |
|        | 25.33                     | 23.74                       |
| 4      | 0.408                     | 0.408                       |
|        | 32.09                     | 32.56                       |
| 5      | **0.457**                 | **0.448**                   |
|        | **41.16**                 | **39.32**                   |
| 6      | 0.444                     | 0.441                       |
|        | 39.17                     | 36.23                       |

AP = Estimated number of clusters calculated by Affinity Propagation

**Supplementary figure S6.** Correlation heatmaps of fecal bacteria and metabolites. Correlation heatmaps were performed on fecal 16S and NMR merged datasets, both for controls (left) and cirrhotics (right). Pearson coefficient ($r$), ranging from positive (blue) to negative (red) values, was used to build correlation heatmaps (metric=Bray-Curtis, method=complete linkage), while cluster coloring was based on FMCs derived from network analysis (figure 2C, 2D).
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