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Microbiota diversity in nonalcoholic fatty liver disease and in drug-induced liver injury

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ABSTRACT

The gut microbiota could play a significant role in the progression of nonalcoholic fatty liver disease (NAFLD); however, its relevance in drug-induced liver injury (DILI) remains unexplored. Since the two hepatic disorders may share damage pathways, we analysed the metagenomic profile of the gut microbiota in NAFLD, with or without significant liver fibrosis, and in DILI, and we identified the main associated bacterial metabolic pathways. In the NAFLD group, we found a decrease in *Alistipes, Barnesiella, Eisenbergiella, Flavonifractor, Fusicatenibacter, Gemminger, Intestinimonas, Oscillibacter, Parasutterella, Saccharoferementans* and *Subdoligranulum* abundances compared with those in both the DILI and control groups. Additionally, we detected an increase in *Enterobacter, Klebsiella, Sarcina* and *Turicibacter* abundances in NAFLD, with significant liver fibrosis, compared with those in NAFLD with no/mild liver fibrosis. The DILI group exhibited a lower microbial bacterial richness than the control group, and lower abundances of *Acetobacteroides, Blautia, Caloramator, Coprococcus, Flavobacterium, Lachnospira, Natronincola, Oscillospira, Pseudobutyrivibrio, Shuttleworthia, Themicanus* and *Turicibacter* compared with those in the NAFLD and control groups. We found seven bacterial metabolic pathways that were impaired only in DILI, most of which were associated with metabolic biosynthesis. In the NAFLD group, most of the differences in the bacterial metabolic pathways found in relation to those in the DILI and control groups were related to fatty acid and lipid biosynthesis. In conclusion, we identified a distinct bacterial profile with specific bacterial metabolic pathways for each type of liver disorder studied. These differences can provide further insight into the physiopathology and development of NAFLD and DILI.

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1. Introduction

The gut microbiota is a very complex ecosystem of resident bacteria that plays a key role in body homeostasis and immunity. The liver is the site of the first metabolic step for all substances absorbed by the intestinal mucosa, and therefore, there may be a direct relationship between the gut microbiota and liver metabolism. When intestinal dysbiosis occurs, microbiota products can influence the pathophysiology and progression of different types of liver disease [1], mainly when intestinal permeability is impaired.

Idiosyncratic drug-induced liver injury (DILI) is a liver disorder caused by drugs or other nonpharmacological compounds [2], in which the interplay of genetic and nongenetic host and environmental factors in susceptible individuals favours disease development [3,4]. Recently, it has been described that bacterial metabolite production can lead to the accumulation of substrates, which could subsequently induce hepatotoxicity [3]. Some compounds, such as p-cresol produced by *Clostridium difficile* or 1-phenyl-1,2-propanedione (PPD) produced by *Escherichia coli* or *Citrobacter freundii,* have both been associated with alterations in liver metabolism [3,5]. P-Cresol is a substrate for human cytosolic sulfotransferases, which are related to the hepatotoxic effect of acetaminophen [5], and PPD is involved in the hepatotoxicity induced by acetaminophen through the depletion of hepatic glutathione levels [6]. However, the role of the gut microbiota in idiosyncratic hepatotoxic reactions induced by drugs is not well understood.

On the other hand, nonalcoholic fatty liver disease (NAFLD) has emerged as the leading cause of chronic liver disease in the western world. In some cases, this disease can progress to nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis, or even hepatocellular carcinoma. Several predisposing factors have been associated with the development of steatosis, including genetic predisposition, obesity, diabetes mellitus and abnormal lipid metabolism, among others [7]. The gut microbiota is also considered a critical factor associated with the development of NAFLD and NASH. Previous studies have associated NASH with a decreased abundance of *Faecalibacterium* and *Ruminococcus* and an increased abundance of *Lactobacillus* in patients with advanced fibrosis [8]. A further study showed that the abundance of *Ruminococcus* and *Bacteroides* increased in patients with significant fibrosis, while that of *Prevotella* was decreased [9]. Although it is known that the gut microbiota is altered in patients with NAFLD, there are no studies that compare the NAFLD-associated gut microbiota with the gut microbiota in DILI patients.

The relationship between NAFLD and DILI can be bidirectional [10, 11]. NAFLD could predispose to hepatotoxicity induced by certain drugs. Additionally, there are drugs that can induce fatty liver disease or may aggravate preexisting steatosis [10–13]. Steatosis is one of the most prevalent forms of hepatocellular injury, occurring in 64% of DILI cases [14], with hepatocellular liver injury being the most common form of DILI. Accordingly, there is growing evidence that NAFLD could increase the risk and/or severity of liver injury induced by different drugs. However, it remains a matter of ongoing debate. In subjects with a preexisting liver disease, such as NAFLD, aminotransferase levels can fluctuate as part of the natural course of the disease, and the detection of DILI signals may be challenging in this type of patient [15,16]. The potential relationship between NAFLD and DILI suggests that they may share pathogenic mechanisms. NAFLD and DILI are likely characterized by the induction of hepatic oxidative stress, the impairment of mitochondrial fatty acid beta-oxidation, the inhibition of mitochondrial respiration, damage to mitochondrial DNA and a drug-induced increase in the uptake of fatty acids [17,18]. One of the most prevalent mechanisms of these hepatic molecular alterations is the cytochrome P450 (P450)-dependent production of toxic and reactive metabolites that cause toxicity [19], which can ultimately lead to innate and adaptive immune responses [20,21]. These P450s are involved in the biotransformation of xenobiotics, such as drugs, dietary factors, and environmental chemicals.

However, although the underlying metabolic damage in these two liver diseases may be similar in some respects, there may be other genetic, immune or environmental factors, such as the gut microbiota, that could be exclusively associated with the development of each of these two liver diseases. When there is an alteration of intestinal permeability, the passage of bacterial endotoxins through the intestinal epithelium to the systemic circulation is allowed, reaching different organs [22]. These products, such as lipopolysaccharides, lipoteichoic acid, flagellins or structurally conserved motifs present on the surface of different types of pathogens (pathogen-associated molecular patterns), short-chain fatty acids and bacterial-derived metabolites released to the host, are recognized by the liver [18] and can initiate hepatotoxicity, inflammation, mitochondrial ROS production and altered nuclear gene expression [23]. Although previous studies have shown an association between gut microbiota composition and NAFLD [9,24], studies in DILI patients are much less numerous, and the interplay between the gut microbiota and the course of disease is still elusive. Regarding this, a change in the gut microbiota, produced by antibiotics or diet, could produce a modification in the secretion of metabolites, which can interact with the host receptors modulating hepatic signalling and the activity of xenobiotic-metabolizing enzymes. This could change the pharmacokinetics of drugs by altering xenobiotic-metabolizing enzyme activity and produce hepatotoxicity [25]. The prediction of the bacterial functional composition of the gut microbiota based on the relationships between phylogeny and its function can provide direct evidence of the abilities of the microbiota to modify host metabolism [26]. However, the data available are limited, and the specific roles of many bacterial groups in the progression of the disease remain unclear.

The aim of this study was to explore the gut microbiota in patients with NAFLD with different degrees of fibrosis and DILI to identify whether there were different microbial markers that may be associated with the development of these two liver diseases. Furthermore, bacterial predictive functional profiling of the microbiota was performed to investigate the significant shifts in functions of the gut microbiota, influencing the main bacterial metabolic pathways. These results will provide new insight regarding the specific role of the microbiota in the progression of these liver diseases.

2. Materials and methods

2.1. Study design

We investigated the gut microbiota in a cohort study that included 13 DILI patients, 29 NAFLD patients and 20 healthy volunteers. Samples from patients were processed and frozen immediately after their reception in the Virgen de la Victoria University Hospital Biobank (Andalusian Public Health System Biobank). All patients were of Caucasian origin. Their demographic features and clinical, biochemical and noninvasive test results are shown in Table 1. Other types of liver disorders, such as viral, autoimmune, genetic, or alcoholic hepatitis, were ruled out in healthy controls and NAFLD and DILI patients. All the participants gave their written informed consent, the study protocol was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki), and the study was approved by the Malaga Provincial Research Ethics Committee, Spain (PI-0285–2016).

2.2. Cohort of patients with DILI

DILI patients were recruited from the prospective Spanish DILI Registry. In-depth details of this registry have been described elsewhere [27]. Briefly, suspected DILI cases were assessed for (i) the compatibility of the time span between drug intake and the onset of signs, symptoms or blood test abnormalities, (ii) all biochemical, histological and imaging data to exclude alternative (liver) diseases and (iii) the outcome of the liver injury. Afterwards, the CIOMS/RUCAM (Council for International Organizations of Medical Sciences/Roussel Uclaf Causality

Table 1
Anthropometric and biochemical variables in the subjects included in the study.

	Control	F$\leq$ 1 NAFLD	F$\geq$ 2 NAFLD	DILI
N (men/women)	20 (12/8)	11 (8/3)	18 (11/7)	13 (8/5)
Age (years)	49 ± 10	45 ± 10	54 ± 10	52 ± 15
Weight (kg)	69 ± 10	81 ± 13[a]	87 ± 8[a]	78 ± 14
BMI (kg/m^2)	25 ± 3	29 ± 4[a]	30 ± 3[a]	27 ± 3[c]
Glucose (mg/dl)	87 ± 9	97 ± 6[a]	113 ± 25[a,b]	102 ± 19[a]
Cholesterol (mg/dl)	164 ± 36	191 ± 34	197 ± 38[a]	190 ± 44[a]
Triglycerides (mg/dl)	83 ± 41	113 ± 70	160 ± 92[a]	179 ± 75[a,b]
Insulin (μU/mL)	5.9 ± 1.8	14.0 ± 8.2	23.6 ± 21.8[a]	11.4 ± 9.8
HOMA-IR	1.4 ± 0.6	3.4 ± 2.0[a]	6.7 ± 6.0[a]	2.7 ± 2.3
AST (UI/L)	23 ± 8	40 ± 21	49 ± 21[a]	287 ± 248[a,b,c]
ALT (UI/L)	24 ± 9	76 ± 62[a]	84 ± 36[a]	503 ± 354[a,b,c]
GGT (UI/L)	30 ± 15	111 ± 105[a]	149 ± 151[a]	303 ± 538[a]
ALP (UI/L)	50 ± 20	80 ± 27[a]	87 ± 46[a]	181 ± 87[a,b,c]
Hepatic parameters Fibrosis				
FLI	22 ± 15	67 ± 29[a]	85 ± 16[a]	Nc
NAFLD FS	-2.64 ± 0.61	-2.32 ± 0.67	-1.10 ± 1.37[a,b]	Nc
FIB4	0.82 ± 0.32	0.91 ± 0.25	1.57 ± 1.13[a]	Nc
APRI	0.27 ± 0.45	0.51 ± 0.32[a]	0.72 ± 0.43[a]	Nc

Results are expressed as mean ± standard deviation.
BMI: body mass index, HOMA-IR: homeostasis model assessment of insulin resistance. AST: aspartate aminotransferase, ALT: alanine aminotransferase, GGT: gamma-glutamyltransferase, ALP: alkaline phosphatase; FLI: fatty liver index, NAFLD FS: NAFLD fibrosis score, FIB4: fibrosis-4, APRI: AST-to-platelet ratio index. Nc: Not calculated.
[a] $p < 0.05$: significant differences with regard to healthy control group.
[b] $p < 0.05$: significant differences with regard to F$\leq$ 1 NAFLD group.
[c] $p < 0.05$: significant differences with regard to F$\geq$ 2 NAFLD group.

Assessment Method) scale was applied, and finally, cases were evaluated by three DILI experts before they were included in the abovementioned DILI Registry. The biochemical DILI criteria used were defined by an international DILI expert group [15]. Sixty-two percent of the included DILI patients had jaundice, and 85% required hospitalization. The type of liver injury (R=ALT/ULN/ALP/ULN) was hepatocellular (R≥5) in 69%, cholestatic (R≤2) in 15% and mixed (R>2 and R<5) in 15% of cases. DILI severity was mild in 31% of patients, 62% of patients showed a moderate injury, 0% of patients showed a severe injury, and 7% of patients underwent liver transplantation. Culprit agents responsible for DILI were dietary supplements (n = 2), nanodrol (n = 1), clenbuterol/amoxicillin-clavulanate (n = 1), terbinafine (n = 1), isoniazide (n = 1), levofloxacin (n = 1), ciprofloxacin (n = 1), amoxicillin-clavulanate (n = 2), ampicillin (n = 1), clindamycin (n = 1) and trabectedin (n = 1).

2.3. Cohort of patients with NAFLD

Patients fulfilling invasive and noninvasive criteria for the diagnosis of NAFLD were prospectively recruited from the Digestive Diseases Unit of the Virgen de la Victoria University Hospital. Inclusion criteria were histological diagnosis of NASH (presence of steatosis in ≥5% of the liver, hepatocellular damage, hepatocyte ballooning or presence of fibrosis in the liver biopsy) or noninvasive diagnosis of NAFLD (for those patients without liver biopsy, the diagnosis was assumed by excluding other causes of liver damage, noninvasive testing and the presence of steatosis on an abdominal ultrasound). The exclusion criteria were as follows [16]: alcohol intake > 20 g/day in men and > 10 g/day in women; secondary causes of NAFLD or other causes of chronic liver disease; consumption of drugs that can potentially induce NAFLD (steroids, amiodarone, methotrexate, tamoxifen, and sodium valproate); type 1

diabetes and severe psychiatric disorders; and the administration of antibiotics in the previous 3 months. We divided NAFLD patients into two groups according to the degree of liver fibrosis: no significant liver fibrosis (F≤1) (n = 11) and significant fibrosis (F≥2) (n = 18) measured by FibroScan®. This classification was chosen according to a previous study, which suggested that the risk of long-term overall mortality, liver transplantation, and liver-related events was present only after liver fibrosis progression to F2 [28].

2.4. Cohort of healthy controls

Healthy controls were recruited from among workers of the Virgen de la Victoria University Hospital and the University of Malaga. The inclusion criteria were the absence of a history of liver disease. The exclusion criteria were as follows: previous history of hepatotoxicity or any other chronic liver disease, altered liver profile at the time of inclusion, body mass index> 25 kg/m^2, diabetes mellitus, dyslipidaemia, metabolic syndrome or NAFLD, and the administration of antibiotics in the previous 3 months.

2.5. FibroScan examination

Participants were examined in a fasting state. Transient elastography (TE) was performed by a single experienced operator using an ECHOS-ENS FibroScan 402 (Echosens, Paris, France) with an M or XL probe on the right lobe of the liver. Liver stiffness measurement (LSM) was described by the median of 10 successful measurements. LSM was considered reliable only if IQR/med < 30% and success rate > 60%. Ten successful acquisitions were performed for all patients. NAFLD patients were included in two groups depending on the degree of fibrosis. No significant liver fibrosis (F≤1) and significant liver fibrosis (F≥2) based on TE were defined as LSM ≥ 7 kPa, ≥ 8.7 kPa and ≥ 10.3 kPa (≥F2, ≥F3 and F4, respectively) for the M probe and LSM ≥ 6.2 kPa, ≥ 7.2 kPa and ≥ 7.9 kPa (≥F2, ≥F3 and F4, respectively) for the XL probe [29].

2.6. Biochemical measurements

Faeces and blood samples were collected from all patients. Faecal samples were collected and immediately stored at −80 °C until analysis. Blood samples were collected in a fasting state, and serum was separated and immediately frozen at − 80 °C. In DILI patients, blood samples were collected between Days 1–11 after DILI recognition. Serum biochemical variables were measured in duplicate in a modular analytics E170 analyser (Roche Diagnostics GmbH, Mannheim, Germany). HOMA-IR was calculated with the following equation: HOMA-IR=fasting insulin (μIU/mL) × fasting glucose (mmol/L)/22.5.

2.7. Steatosis, NASH, and fibrosis noninvasive tests

Abdominal ultrasonography was performed on the DILI, NAFLD and control groups. The fatty liver index (FLI) was used to assess hepatic steatosis [30]. To evaluate liver fibrosis, the NAFLD fibrosis score (NAFLD FS) [31], the fibrosis-4 index (FIB4) [32] and the AST-to-platelet ratio index (APRI) [33] were used.

2.8. Statistical analysis for biochemical and anthropometric variables

All data were analysed with GraphPad Software (Prism 8.1.1) (GraphPad Software, San Diego, CA). The differences between the groups were compared using Kruskal–Wallis tests followed by post hoc analyses using Dunn's test. Values were considered statistically significant when $p < 0.05$.

2.9. DNA extraction and 16S rDNA metagenomic sequencing library preparation

Total DNA extraction from faeces was performed using a QIAamp Power Faecal Pro DNA (Qiagen ES) following the manufacturer's recommendations. The DNA was eluted into DNase/RNase-free water, and its concentration and purity were evaluated by absorbance measurement (NanoDrop ND-1000 spectrophotometer, Isogen). Amplification of the 16 S rRNA targeting the V3-V4 hypervariable region was performed using the primers 16S-V3–314 F forward (5′TCGTCGGCAGCGTCA GATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG3′) and V4–805 reverse (5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTAC HVGGGTATCTAATCC3′) with added Illumina adapter overhang nucleotide sequences. PCR conditions used were 3 min at 95 °C, followed by 25 cycles of 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C, with a final extension at 72 °C for 5 min. Each reaction mixture (25 µl) contained 5 ng of genomic DNA, 0.5 µl of amplicon PCR forward primer (0.2 µM), 0.5 µl of amplicon PCR reverse primer (0.2 µM) and 12.5 µl of 2 × KAPA HifiHotStart Ready Mix (Roche). According to the manufacturer's protocol, PCR products were purified with Agencourt AMPure XP beads (Beckman Coulter Genomics, Danvers, MA, USA) to remove excess primers and primer dimers. In a second index PCR, 5 µl of each amplicon was used as a template. Dual indices and Illumina sequencing adapters for each amplicon were attached in the second PCR using a Nextera XT Index Kit v2 (Illumina Inc.). In this case, amplification was carried out under the following conditions: 3 min at 95 °C, followed by 8 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C, with a final extension at 72 °C for 5 min. Constructed 16 S rDNA metagenomic libraries were purified with Agencourt AMPure XP beads. Quantification of the library, quality control and average size distribution were determined with an Agilent Tapestation 4200. Libraries were normalized and pooled to 40 nM based on quantified values. Pooled samples were denatured and diluted to a final concentration of 6 pM with a 30% PhiX (Illumina) control. Amplicons were subjected to sequencing using a MiSeq Reagent Kit V3 in the Illumina MiSeq System.

2.10. Bioinformatic, ordination and statistical analysis

Sequence reads were processed using Mothur v1.44.3 and VSearch for alignment, clustering and chimaera detection [34,35]. Sequences were clustered into operational taxonomic units (OTUs) at 97% identity. The SILVA 138 database of full-length 16 S rDNA gene sequences was used for alignments of unique sequences and taxonomical assignations [36]. Finally, cleaned sequences were rarefied to 10,000 reads per sample. Original libraries are publicly available under the Bioproject ID (PRJNA808210) at the National Center for Biotechnology Information (NCBI). All statistical analyses were performed at the genus level. Regarding alpha diversity (reciprocal Simpson diversity index and Simpson evenness), Good's coverage and population richness (Chao1 estimator of richness) were calculated using Mothur v1.44.3 and compared between groups using Kruskal–Wallis multiple testing with Benjamini–Hochberg FDR corrections (PRISM 8). Bacterial community structure was visualized with nonparametric dimensional scaling (k = 4, model stress=0.089) based upon the Bray–Curtis dissimilarity matrix using the vegan and vegan3d packages in R. Beta diversity clustering was assessed using the AMOVA test with Mothur v1.44.3. Adonis analysis (permutational multivariate analysis of variance using distance matrices) was performed using the vegan package implemented in R. Significant population differences were assessed with the ALDEX2 package in R. Briefly, population abundance was first derived from counts using a Dirichlet-multinomial model, and then differences were identified with Kruskal–Wallis tests using Benjamini–Hochberg FDR correction [37]. Post hoc pairwise differences between groups were assessed with the DESeq2 package in R using Benjamini–Hochberg FDR for multitest corrections.

2.11. Prediction of the bacterial functional composition

A prediction of the bacterial functional composition of the metagenome of the gut microbiota from its 16 S profile was inferred for each stool sample using PICRUST (Phylogenetic Investigation of Communities by reconstruction of Unobserved States). This bioinformatic software package represents a computational approach designed to predict the bacterial functional composition of a metagenome using the marker 16 S rRNA and a database of reference genomes [26]. For metagenome prediction, PICRUST takes an input OTU (taxonomic unit) table that contains identifiers that match tips from the marker gene, with corresponding abundances for each of those OTUs across one or more samples. The software works in a two-step process. In the initial gene content inference step, the gene content is precomputed for each organism in a reference phylogenetic tree. The subsequent metagenome inference step combines the resulting gene content predictions for all microbial taxa with the relative abundance of 16 S rRNA genes in one or more microbial community samples. This predictive metagenomic approach provides a useful tool to link phylogeny and function in uncultivated microbial communities. All details about this computational approach have already been described in the work of Langille et al. [26] Subsequently, filtered sequences were clustered into operational taxonomic units (OTUs) using the nearest MOTHUR neighbour algorithm with a 0.03 distance unit cut-off. OTU sequences were aligned against the 16 S reference database SILVA. The resulting OTU table was then normalized by 16 S rRNA gene copy number, and the predicted bacterial gene family abundance was inferred for each sample [26]. Significantly different pathways between grouped patients were assessed using the Kruskal–Wallis test using Benjamini–Hochberg FDR correction implemented in R. The differences were considered significant for a p value less than 0.05.

3. Results

3.1. Analysis of the faecal microbiota of patients by barcoded pyrosequencing

The 16 S amplicon sequencing yielded 10,000 identifications per sample after corrections for multiple tests. Bacterial populations with a median value of zero among the entire group of patients were dismissed. The identity and relative abundance of the microbial populations present in the four groups of patients were investigated at the phylum, family (Supplementary Figure 1 A and 1B) and genus levels (Fig. 1). Overall, at the phylum level, the most abundant taxa included Firmicutes, Bacteroidetes, Proteobacteria and Verrucomicrobia for all groups of samples (Supplementary Figure 1A). At the family level, we found *Bacteroidaceae, Ruminococcaceae*, and *Lachnospiraceae* to be dominant in all types of samples (Supplementary Figure 1B). The mean alpha diversity (inverse Simpson index) and evenness (derived from the Simpson index) were variable among patients, although no significant differences were found between groups (Supplementary Figure 2). We found a significant difference (p = 0.0032) in only bacterial richness (Chao1 richness index) between the DILI and control groups, showing an increase in the number of species in the control samples (Supplementary Figure 2).

3.2. Link between DILI and the gut microbiota composition

At the phylum level, DILI patients presented significant differences compared with NAFLD patients. DILI patients showed a reduction in the mean cumulative counts of Firmicutes compared with those in NAFLD patients (Fig. 2). In addition, there was also a significant increase in the abundance of the Bacteroidetes phylum when the DILI group was compared with the control (p = 0.026), F≤ 1 NAFLD (p = 0.034) and F≥ 2 NAFLD (p = 0.001) groups (Supplementary Figure 1).
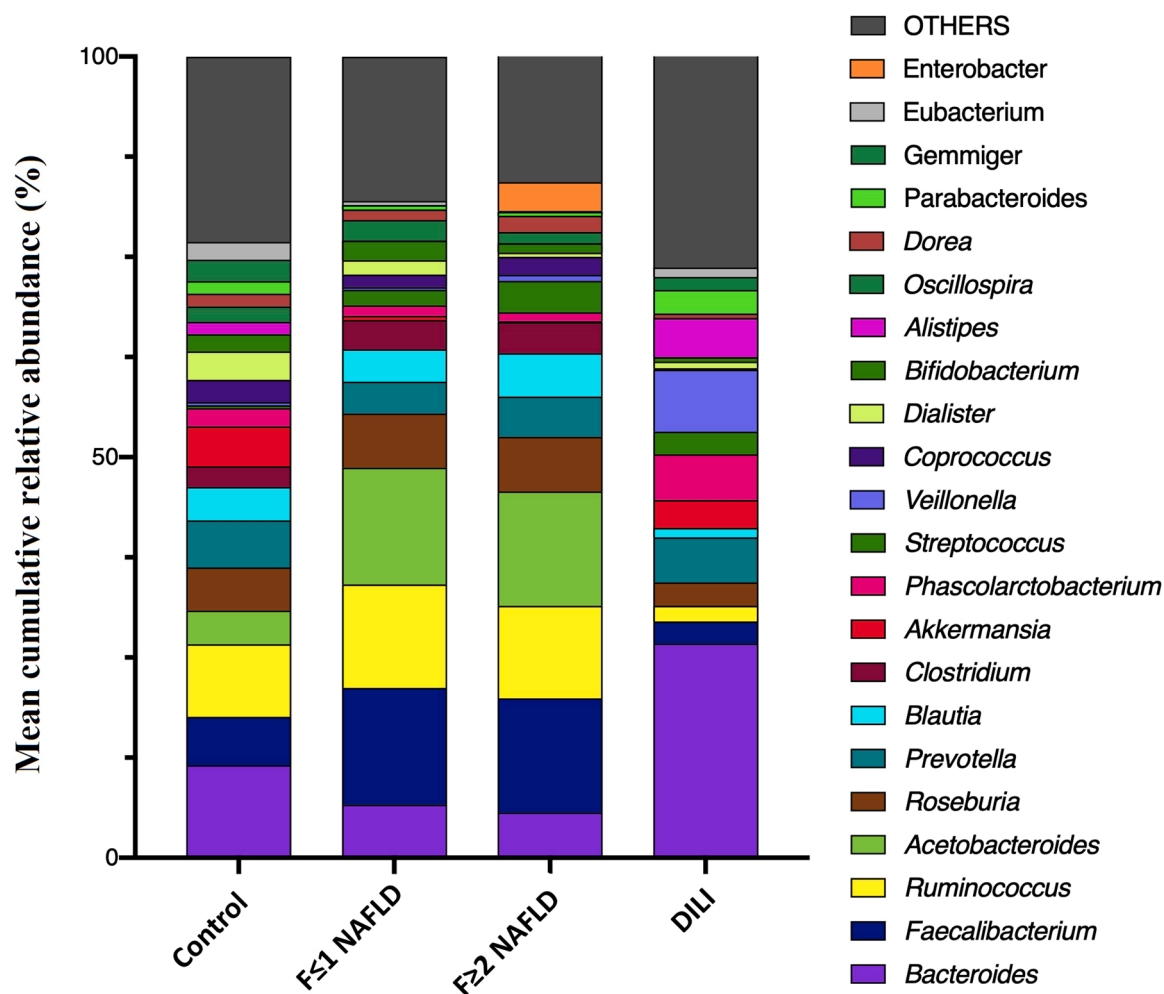
Additional differences between groups were found at lower

Fig. 1. Gut microbiota composition for control, F≤ 1 NAFLD, F≥ 2 NAFLD and DILI patients. Composition at the genus level shown in a bar chart detailing the mean cumulative relative abundance (%) of the 22 core genera common to the four groups of patients. Only genera with relative abundance > 1% were plotted.

taxonomic levels (Table 2). At the genus level, we found that the DILI group presented a statistically significant decrease in the abundances of 39 bacterial groups when compared with those in the control and both NAFLD groups. Of these 39 genera, those that presented a relative abundance greater than 1% were considered (35 genera) (Fig. 3). A significant decrease in abundance was detected for *Acetobacteroides*, *Blautia*, *Caloramator*, *Coprococcus*, *Flavobacterium*, *Lachnospira*, *Natronincola*, *Oscillospira*, *Pseudobutyrivibrio*, *Shuttleworthia*, *Themicanus* and *Turicibacter* (Table 2).

At the genus level, we found that the DILI group presented a statistically significant decrease in the abundances of 39 bacterial groups when compared with the control and both NAFLD groups. Of these 39 genera, those that presented a relative abundance greater than 1% were considered (35 genera) (Fig. 3). A significant decrease in abundance when compared with the control and both NAFLD groups was detected for *Acetobacteroides*, *Blautia*, *Caloramator*, *Coprococcus*, *Flavobacterium*, *Lachnospira*, *Natronincola*, *Oscillospira*, *Pseudobutyrivibrio*, *Shuttleworthia*, *Themicanus* and *Turicibacter* (Table 2).

The abundances of these 12 genera in the four groups studied showed a similar profile (Table 3). Regarding the DILI group versus each NAFLD group (F≤1 and F≥2), we observed a decrease in the abundances of 5 more genera in DILI patients (*Alkaliphilus*, *Dorea*, *Faecalibacterium*, *Roseburia* and *Sarcina*), in addition to those already described.

Further comparisons between the DILI and F≥ 2 NAFLD groups revealed a decrease in the abundances of *Anaerofilum*, *Enterobacter* and *Klebsiella* in DILI patients (Fig. 4 A). Post hoc pairwise differences only

with the control group also revealed a decreased abundance of the *Anaerofilum* genus in the DILI group (Table 2).

In contrast, we did not find a significant increase in the abundance of bacteria at the genus level in the DILI group when compared with that in the control group. However, differences with only the NAFLD groups were identified. An increase in the abundances of 13 genera was observed in DILI patients compared with those in F≤ 1 NAFLD and F≥ 2 NAFLD patients (Table 2). Furthermore, an increase in *Bacteroides* and *Eubacterium* abundances was identified in the DILI group after pairwise comparison only with the F≥ 2 NAFLD group (Fig. 4B).

Using three-dimensional dynamic ordination implemented in RStudio and beta diversity analysis, we observed the sample distribution as a function of pathology, with a clear spatial separation between the DILI and NAFLD groups, while the control group appeared more scattered in the different planes (Fig. 5).

3.3. Link between NAFLD and the gut microbiota composition

As previously described, both types of NAFLD patients presented an increase in mean cumulative counts of Firmicutes when compared with DILI patients. In addition, there was also a significant increase in the abundance of the phylum Firmicutes in the F≤ 1 NAFLD group compared with that in the control group (Fig. 2).

At the genus level, we found some changes in the gut microbiota for both NAFLD groups (the F≤1 and F≥2 groups) between them and with regard to the control and DILI groups (Fig. 3). With a cut-off greater than
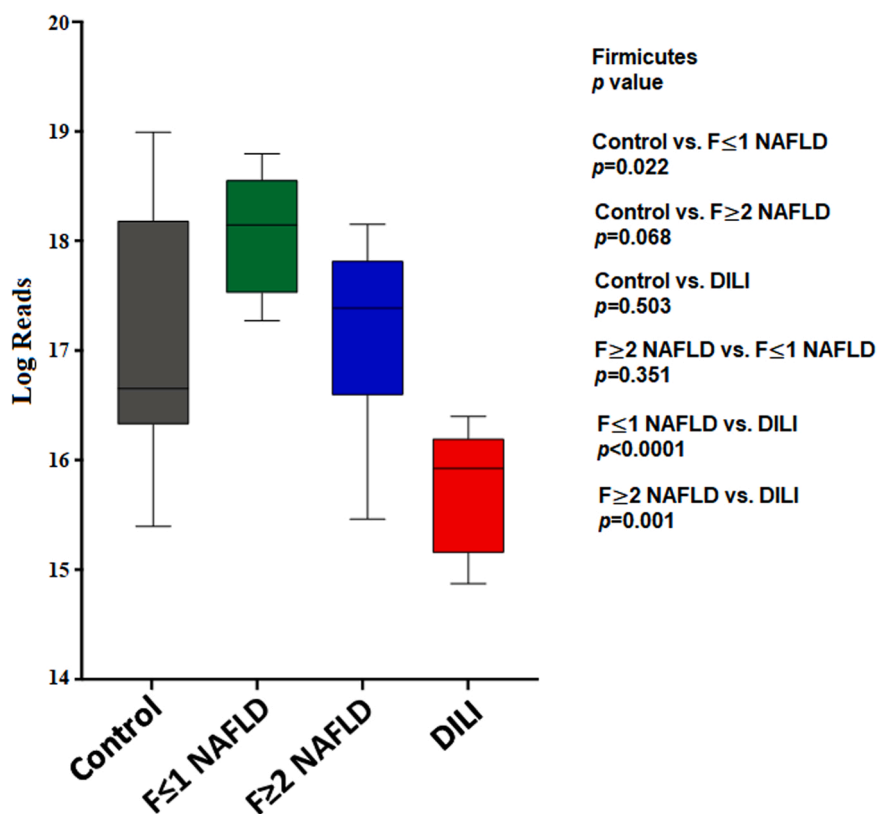
Fig. 2. Box plot showing the phylum Firmicutes and its abundance in the different patient groups. The results of DESeq2 and Aldex analysis after multiple comparisons testing with Benjamini–Hochberg corrections shown in box and whiskers, with whiskers from the minimum to maximum. Log Reads: Log2 relative normalized abundance.

1% of relative abundance, a decreased abundance was observed for 11 genera in both groups of NAFLD patients, including *Alistipes, Barnesiella, Eisenbergiella, Flavonifractor, Fusicatenibacter, Gemminger, Intestinimonas, Oscillibacter, Parasutterella, Saccharoferementans* and *Subdoligranulum* (Table 2). The abundance of these genera in the four groups studied showed a similar profile (Table 3). Further comparisons between the F≤ 1 NAFLD and control group revealed a reduction in the abundance of the genera *Romboutsia* and *Coprobacter* and an increase in the abundance of *Faecalibacterium* in the F≤ 1 NAFLD group (Supplementary Figure 3). The abundance of the *Faecalibacterium* genus in the four groups studied showed a specific profile (Table 3). Similarly, we compared the F≥ 2 NAFLD group with the control group. Further differences in the abundances of some genera were found, including a reduction in *Eubacterium, Odoribacter* and *Ruminococcus* abundances and an increase in *Enterobacter, Klebsiella* and *Sarcina* abundances in the F≥ 2 NAFLD group (Supplementary Figure 4).

Regarding F≤ 1 NAFLD and F≥ 2 NAFLD patients, the two groups presented a similar profile, with a few significant differences among them in the abundances of the genera *Enterobacter, Klebsiella, Sarcina* and *Turicibacter* (increased in F≥2 NAFLD patients) (Fig. 6). The abundance of the *Enterobacter* and *Klebsiella* genera in the four groups studied showed a specific profile (Table 3).

3.4. Bacterial metabolic pathways and gut microbiota composition

We found different bacterial metabolic pathways associated with the gut microbial composition altered in DILI and NAFLD patients (Table 4). Regarding the DILI group, a total of 7 pathways were found to be altered only in the DILI group when compared with all other groups (Table 3). Six out of 7 pathways were enriched in these patients, while the remaining pathway was diminished and corresponded with sucrose degradation III (sucrose invertase). Only two of these six enriched

pathways in DILI were associated with metabolic degradation (reactive tricarboxylic acid cycles and pyruvate fermentation to propanoate I). All the other enriched pathways were associated with metabolic biosynthesis. They included carbohydrate biosynthesis (CMP-3-deoxy-D-manno-octulosonate biosynthesis); cofactor, carrier, and vitamin biosynthesis (6-hydroxymethyl-dihydropterin diphosphate biosynthesis I); nucleotide and nucleoside biosynthesis (superpathway of pyrimidine deoxyribonucleotides de novo biosynthesis); and fatty acid and lipid biosynthesis (unsaturated fatty acid biosynthesis).

In NAFLD, most of the differences in the metabolic pathways were found in relation to DILI and affected only one of the two NAFLD groups, most of which were related to the F≤ 1 NAFLD group (Table 4). However, there was another group of pathways altered in the DILI group compared with both NAFLD groups, mainly related to amino acid, nucleoside and nucleotide biosynthesis (Table 4).

Regarding the differences found between the NAFLD and control groups, the most significant differences were found with the F≥ 2 NAFLD group and were mainly related to glycogen biosynthesis from ADP-D-glucose, UDP-N-acetyl-D-glucosamine biosynthesis, and fatty acid and lipid biosynthesis (Table 4).

We also found several differences between the F≤ 1 and F≥ 2 NAFLD groups (Table 4). Our results revealed that most of the pathways were enriched in the F≥ 2 NAFLD group and corresponded with lipid and fatty acid biosynthesis, generation of precursor metabolites and energy and menaquinol biosynthesis. Moreover, the main pathways enriched in the F≤ 1 NAFLD group were mainly related to amino acid biosynthesis (L-aspartate, L-asparagine and L-histidine biosynthesis).

4. Discussion

High-resolution 16 S rRNA gene profiling provides an indispensable method to investigate complex gut microbiota ecosystems [38],

Table 2

Results from post-hoc pairwise differences between genera from control, F≤ 1 NAFLD, F≥ 2 NAFLD and DILI groups with Deseq2 package (p-values corrected for multi-testing using Benjamini-Hochberg FDR method are shown).

Increase in DILI compared with:	F≤ 1 NAFLD	F≥ 2 NAFLD	Control	Reduction in DILI compared with:	F≤ 1 NAFLD	F≥ 2 NAFLD	Control
Alistipes	< 0.0001	< 0.0001	0.966	*Acetobacteroides*	< 0.0001	**< 0.0001**	**< 0.0001**
Bacteroides	0.172	< 0.0001	0.297	*Anaerofilum*	0.076	**< 0.0001**	**< 0.0001**
Barnesiella	< 0.0001	< 0.0001	0.926	*Alkaliphilus*	0.018	**0.014**	0.201
Eisenbergiella	< 0.0001	< 0.0001	0.998	*Blautia*	< 0.0001	**< 0.0001**	**0.001**
Eubacterium	0.877	0.004	0.998	*Caloramator*	< 0.0001	**< 0.0001**	**< 0.0001**
Flavonifractor	< 0.0001	< 0.0001	0.998	*Coprococcus*	< 0.0001	**< 0.0001**	**< 0.0001**
Fusicatenibacter	< 0.0001	< 0.0001	0.998	*Dorea*	0.012	**0.005**	0.096
Gemmiger	< 0.0001	< 0.0001	0.998	*Enterobacter*	0.969	**< 0.0001**	0.056
Intestinimonas	< 0.0001	< 0.0001	0.998	*Faecalibacterium*	< 0.0001	**< 0.0001**	0.343
Odoribacter	0.004	< 0.0001	0.32	*Flavobacterium*	< 0.0001	**< 0.0001**	**< 0.0001**
Oscillibacter	< 0.0001	< 0.0001	0.998	*Klebsiella*	0.877	**< 0.0001**	0.463
Parasutterella	< 0.0001	< 0.0001	0.998	*Lachnospira*	< 0.0001	**< 0.0001**	**< 0.0001**
Ruminococcus	< 0.0001	< 0.0001	0.998	*Natronincola*	< 0.0001	**< 0.0001**	**< 0.0001**
Saccharofermentans	< 0.0001	< 0.0001	0.6	*Oscillospira*	< 0.0001	**< 0.0001**	**< 0.0001**
Subdoligranulum	< 0.0001	< 0.0001	0.391	*Pseudobutyrivibrio*	< 0.0001	**< 0.0001**	**< 0.0001**
				Roseburia	0.007	**0.046**	0.517
				Sarcina	< 0.0001	**< 0.0001**	0.803
				Shuttleworthia	< 0.0001	**< 0.0001**	**0.016**
				Thermicanus	< 0.0001	**< 0.0001**	**< 0.0001**
				Turicibacter	0.004	**< 0.0001**	**< 0.0001**
Increase in F≤ 1 NAFLD compared with:	**F≥ 2 NAFLD**		**Control**	**Reduction in F≤ 1 NAFLD compared with:**	**F≥ 2 NAFLD**		**Control**
Faecalibacterium	0.968		0.003	*Alistipes*	0.998		**< 0.0001**
				Barnesiella	0.998		**< 0.0001**
				Coprobacter	0.998		**< 0.0001**
				Eisenbergiella	0.998		**< 0.0001**
				Enterobacter	< 0.0001		0.595
				Flavonifractor	0.998		**< 0.0001**
				Fusicatenibacter	0.998		**< 0.0001**
				Gemmiger	0.998		**< 0.0001**
				Intestinimonas	0.998		**< 0.0001**
				Klebsiella	< 0.0001		0.513
				Oscillibacter	0.998		**< 0.0001**
				Parasutterella	0.998		**< 0.0001**
				Romboutsia	0.998		**< 0.0001**
				Saccharofermentans	0.998		**< 0.0001**
				Sarcina	0.005		0.177
				Subdoligranulum	0.998		**< 0.0001**
				Turicibacter	0.026		0.48
Increase in F≥ 2 NAFLD compared with:	**Control**			**Reduction in F≥ 2 NAFLD compared with:**	**Control**		
Enterobacter	< 0.0001			*Alistipes*	< 0.0001		
Klebsiella	< 0.0001			*Barnesiella*	< 0.0002		
Sarcina	< 0.0001			*Eisenbergiella*	< 0.0001		
				Eubacterium	0.004		
				Flavonifractor	< 0.0001		
				Fusicatenibacter	< 0.0001		
				Gemmiger	< 0.0001		
				Intestinimonas	< 0.0001		
				Odoribacter	< 0.0001		
				Oscillibacter	< 0.0001		
				Parasutterella	< 0.0001		
				Ruminococcus	< 0.0001		
				Saccharofermentans	< 0.0001		
				Subdoligranulum	< 0.0001		

Significant p-values (<0.05) are written in bold.

allowing the identification of associated microbial markers for a wide range of diseases. In this study, we found an increase in Bacteroidetes abundance and a decrease in the abundance of the Firmicutes phylum in DILI patients. This profile could be significant due to the different contents of P450 enzymes in both phyla [25]. Bacteroidetes species have a higher diversity of P450s and produce a greater quantity and diversity of secondary metabolites than Firmicutes species [39]. This could favour a greater absorption of these secondary metabolites in DILI and produce an indirect regulation of host xenobiotic metabolism, which would cause a direct toxic effect on hepatocytes and affect human health [25,40].

Analysing the microbiota in more depth, we found a unique microbiota signature in the DILI group, with a lower abundance of 12 genera compared with that in the NAFLD and control groups (Table 3). Most of these depleted genera in DILI have rarely been related to hepatic diseases, with none have been associated with acute liver injury. Among

them, we found a decreased abundance of *Oscillospira, Coprococcus* and *Blautia*. These genera were previously found to be depleted in the faecal microbiota of patients with autoimmune hepatitis [41]. Regarding *Oscillospira,* its low abundance could be involved in the decrease in secondary bile acid levels found in patients with severe DILI [42]. A previous study showed that its abundance in stool was positively correlated with changes in the levels of secondary bile acids (deoxycholic acid and conjugated deoxycholic acid) [43]. However, there are notably few studies on the association between the gut microbiota and DILI. We hypothesize that these genera could be associated with DILI and be used as a microbiota-derived signature related to the possible development of this liver disease.

However, we found another microbial signature that differentiates DILI only from both types of NAFLD but not from the control group and includes genera enriched or depleted in DILI. In fact, this microbial
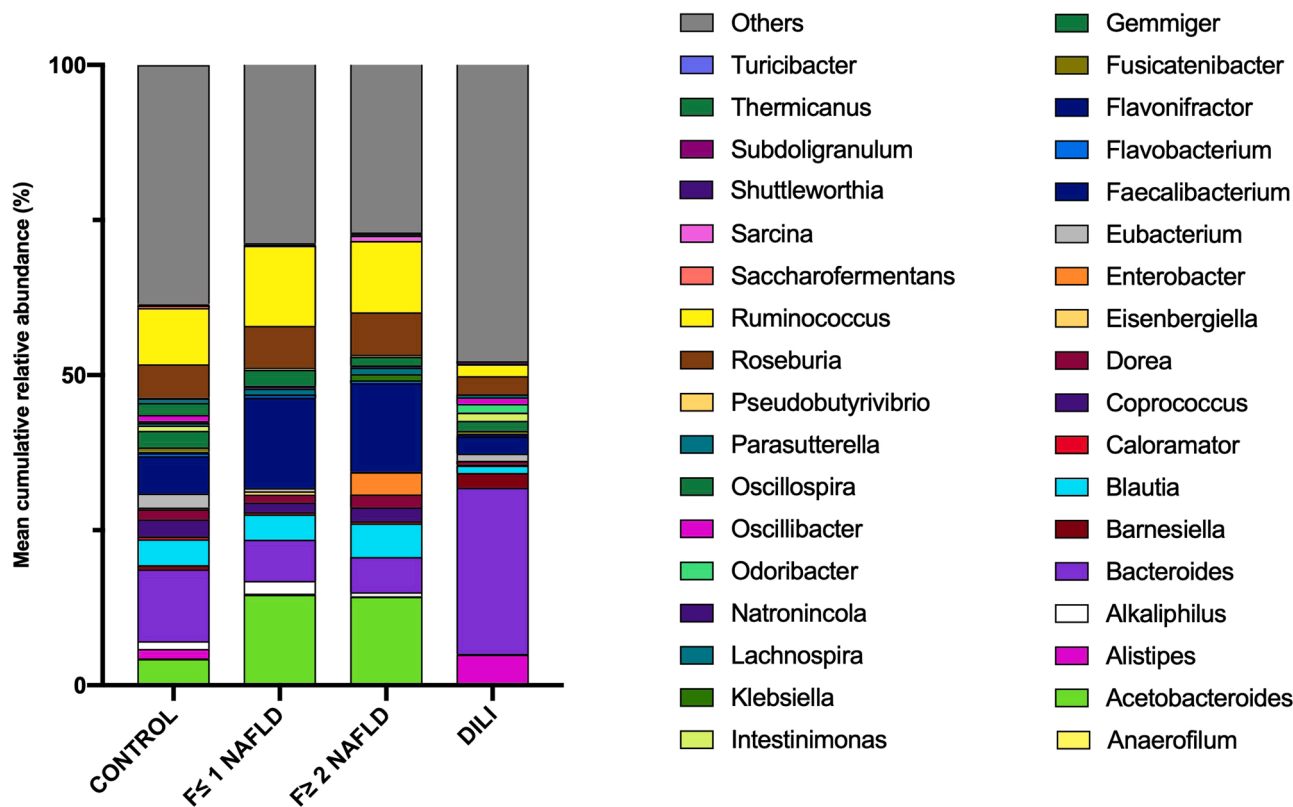
Fig. 3. Bar chart detailing the 35 genera with a relative abundance greater than 1% that were found to be significantly differentially abundant for DILI patients when compared with the other 3 groups.

Table 3
Schema of the four main profiles found for the abundance of genera with significant differences between the different groups studied (control, F≤1 NAFLD, F≥2 NAFLD and DILI patients).

| Control	F≤ 1 NAFLD	F≥ 2 NAFLD	DILI	Genera
Normal	Normal	Normal	Decreased	*Acetobacteroides, Blautia, Caloramator, Coprococcus, Flavobacterium, Lachnospira, Natronincola, Oscillospira, Pseudobutyrivibrio, Shuttleworthia, Themicanus, Turicibacter*
Normal	Decreased	Decreased	Normal	*Alistipes, Barnesiella, Eisenbergiella, Flavonifractor, Fusicatenibacter, Gemminger, Intestinimonas, Oscillibacter, Parasutterella, Saccharoferementans, Subdilogranulum*
Normal	Highly increased	Increased	Normal	*Faecalibacterium*
Normal	Normal	Increased	Normal	*Enterobacter, Klebsiella*

The abundance of the genera from the F≤ 1 NAFLD, F≥ 2 NAFLD and DILI groups is referred to the control group, whose abundance is considered as "normal".

signature includes genera whose abundance is altered in NAFLD, since their abundance in DILI is similar to that found in the control group. We found two profiles in this microbial signature. The first includes those genera depleted in both types of NAFLD groups compared with those in the DILI and control groups (Table 3). Regarding this microbial profile, we found genera that have already been previously related to different liver diseases. For example, the results for *Alistipes* indicate that its abundance changes as a function of the type of liver disease [44–46]. In

autoimmune hepatitis, a decrease in the *Alistipes* proportion was found when compared with that in a healthy control group [44]. Previously, *A. finegoldii* was found to be depleted in individuals with NASH compared with that in healthy controls, and *A. onderdonkii* was depleted in the F≥ 2 vs. F≤ 1 NAFLD groups [45]. There is evidence indicating that some species of *Alistipes* may have a protective effect against liver fibrosis: a general reduction has been associated with an advanced liver fibrotic status and with a decrease in the levels of short-chain fatty acids (SCFAs), such as acetate and propionate [46]. With regard to other genera depleted in NAFLD in the DILI group, we found *Barnesiella, Fusicatenibacter* and *Ruminococcus.* These genera are associated with the production of SCFAs [47], and their depletion could have negative effects on liver function since microbial SCFAs seem to exert ameliorative effects on NAFLD progression [48]. The abundance of *Fusicatenibacter*, which was reduced in NAFLD groups, was also found to be decreased in NAFLD patients in another study [49]. Moreover, there was a decrease in *Fusicatenibacter* and *Ruminococcus* abundance in stool samples from cirrhotic patients [49]. However, while some previous studies described a high abundance of *Ruminococcus* in NAFLD patients but low proportions in alcoholic liver disease, autoimmune hepatitis and liver cirrhosis patients [50], other studies found a decreased abundance of this genus in NAFLD [51]. This group of genera seems to be involved in the regulation of bacterial SCFA levels. Therefore, further studies are needed to confirm whether this bacterial pathway affects the course of NAFLD or whether there are other bacterial pathways involved that have not been studied to date.

There was a small second microbial profile including those genera that were depleted in the DILI group compared to the F≥ 2 NAFLD group. In other words, our results showed that these genera were enriched in the F≥ 2 NAFLD group compared with the DILI, F≤ 1 NAFLD and control groups (Table 3). Therefore, this finding mainly suggests a different profile according to the fibrosis levels in NAFLD patients. In this second microbial signature, we found *Enterobacter* and *Klebsiella.*
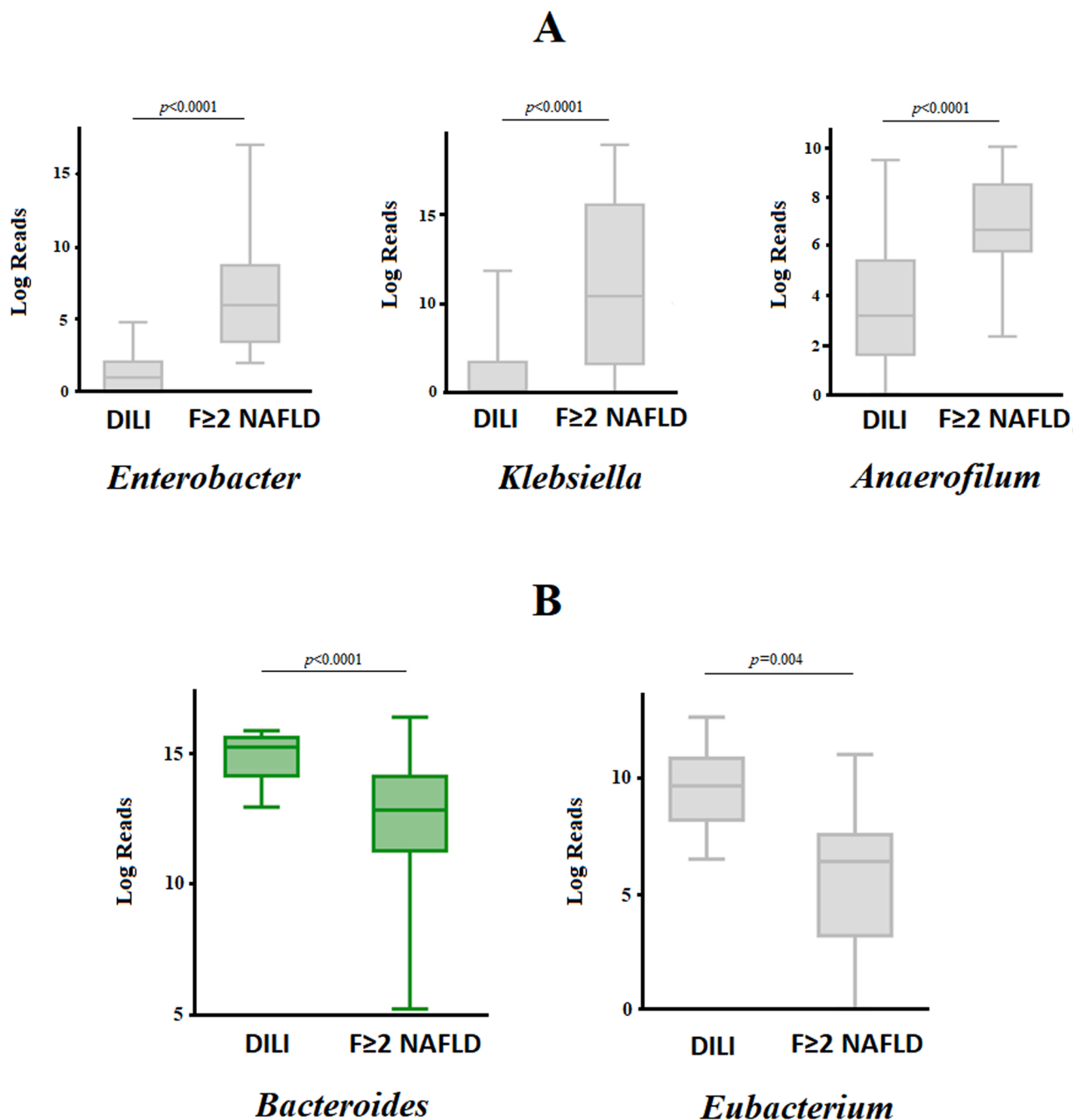
Fig. 4. (A) Increase and (B) decrease in the abundance of bacterial genera additionally identified in DILI patients after pairwise comparison only with the F≥ 2 NAFLD group. The results of DESeq2 with Benjamini–Hochberg FDR corrections. Only genera with a relative abundance > 1% were plotted. *Bacteroides* appears in green since it is one of the 10 genera with the highest relative abundance in the analysed samples. Log Reads: Log2 relative normalized abundance.

The increase in the abundance of most of these genera has been previously associated with the induction of NAFLD, NASH and related metabolic disorders [52–54]. As we showed, the abundance of the *Enterobacter* genus was greater in the NAFLD group than in the control group [53]. The relationship between these genera and NAFLD could be mediated through the endotoxins or bacterial-derived metabolites they produce [55]. Further studies are needed to investigate the effects of bacteria-derived components on the development and progression of liver diseases, especially DILI.

However, we found a genus enriched in both types of NAFLD compared with DILI but also enriched in the F≤ 1 NAFLD group compared with the control group (Table 3): the *Faecalibacterium* genus. In a previous study, Duarte et al. [51] found a decreased abundance of

Faecalibacterium in NAFLD patients. *Faecalibacterium* has gained attention due to its potential as a probiotic for the treatment of NAFLD, obesity and diabetes [56]. However, inconsistent results have been reported on the association between *Faecalibacterium* and obesity, suggesting that several factors, including sex, diet, geographic localization, or gut transit times, among others, must be considered to determine the role of this genus in the disease [57]. In this context, an increased abundance of *F. prausnitzii* has been associated with NAFLD comorbidities [57]. In our study, we found no differences between the two NAFLD groups, as in a previous study in which no differences in *Faecalibacterium* levels between NAFLD and NASH were reported [58]. Therefore, the findings in our study concerning the increased levels of *Faecalibacterium* in NAFLD patients in comparison with DILI and control patients deserve
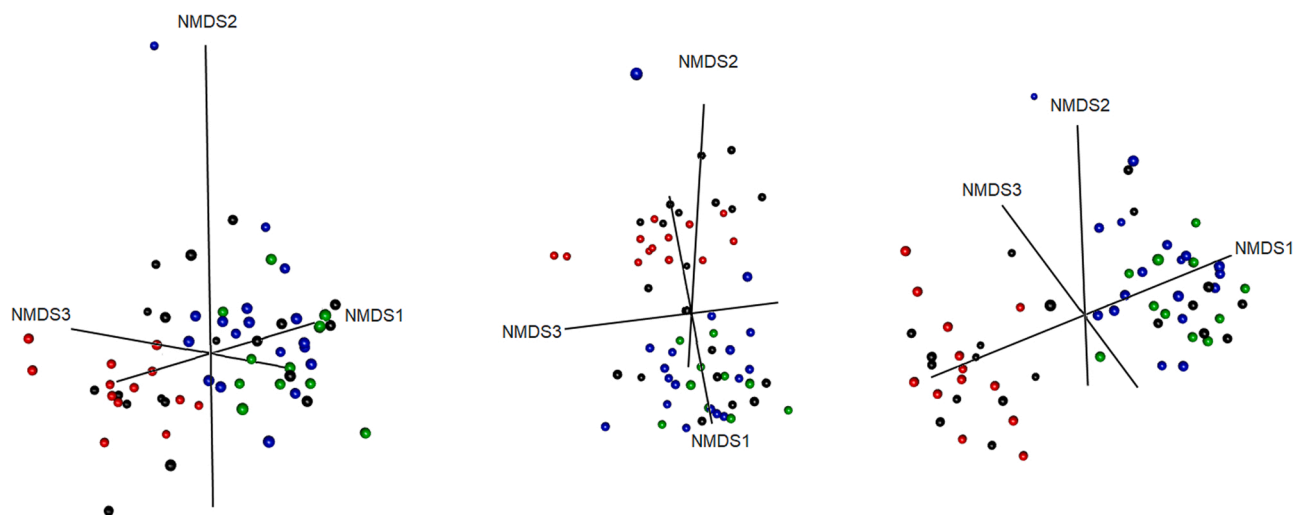
Fig. 5. Sample distribution as a function of liver disease using three-dimensional dynamic ordination. Beta-diversity analysis using a Bray–Curtis-based NMDS model of beta diversity (N dim K=3, stress=0.1173701). Statistical differences for group clustering were assessed with the AMOVA test (p < 0.05). Black circle: control; green circle: F≤ 1 NAFLD; blue circle: F≥ 2 NAFLD; red circle: DILI.

further investigation. As in the case of *Faecalibacterium*, other minor variable signatures were detected in our study when compared with the data available in the literature. In Supplementary Table 1, we have summarized the results of our study compared with the data available in the literature at the genus taxonomical level, highlighting the main microbial signatures associated with NAFLD. In addition to *Faecalibacterium,* as previously discussed, we found differences between our study and other studies regarding the *Barnesiella, Parasutterella* and *Romboutsia* genera. However, these other studies were all performed in mouse experimental models, which could explain the variations observed. Furthermore, we observed in this study a decrease in the abundance of the genus *Saccharofermentans*, which, to the best of our knowledge, has not been previously described in human patients with NAFLD.

We used PICRUST to obtain bacterial function information using the metagenomic profiles of the different groups. We observed an increase in the abundance of bacterial pathways associated with metabolic biosynthesis in the DILI group compared with that in the NAFLD and control groups. In relation to carbohydrate biosynthesis, the analysis showed that CMP-3-deoxy-D-manno-octulosonate biosynthesis I, which is a component of bacterial lipopolysaccharides (LPS), was enriched in DILI. It has been shown that increased LPS production from gram-negative bacteria contributes to metabolic alterations, including obesity or insulin resistance [59]. Moreover, portal-derived LPS and LPS pathway components may play a role in immune responses to DILI [60]. We also found bacterial pathways related to sugar degradation (pyruvate and sucrose), the tricarboxylic acid cycle and 6-hydroxymethyldihydropterin diphosphate biosynthesis pathways. These bacterial pathways could be involved in DILI regulating the immune response and immune checkpoints PD-1 and CTL-4. Previously, it was suggested that the sugar degradation and 6-hydroxymethyldihydropterin diphosphate pathways were associated with shorter progression-free survival in response to immunotherapy (anti-PD-1 and/or anti-CTLA-4) in patients with melanoma [61] and that the gut microbiota affects the response to anti-PD-1 immunotherapy in patients with hepatocellular carcinoma [62]. In addition, we have shown in a previous study that the immune response and immune checkpoints PD-1 and CTL-4 in peripheral blood mononuclear cells were associated with the presence of DILI [63]. Together, our findings suggest a possible association between changes in the gut microbiota and the host immune response, with the involvement of immune checkpoint molecules. Accordingly, in a previous study, we found a different immune response between NAFLD and DILI patients [64]. However, the causal relationship remains unknown. Another

pathway affected by the gut microbiota and associated with DILI was involved in bacterial pyrimidine synthesis. Inhibitors of dihydroorotate dehydrogenase, the rate-limiting enzyme step in de novo bacterial pyrimidine synthesis, elicit adverse hepatic outcomes through mitochondrial dysfunction [65]. The alteration of mitochondrial DNA homeostasis is a mechanism by which some DILI-associated drugs, such as ciprofloxacin and antiretroviral nucleoside reverse-transcriptase inhibitors, elicit their hepatotoxic effects [66].

In addition, there was a group of bacterial pathways enriched in the DILI group regarding both NAFLD groups, which are mainly related to amino acid, nucleoside and nucleotide biosynthesis. Moreover, there was a group of bacterial pathways enriched in both the DILI and F≥ 2 NAFLD groups compared to the F≤ 1 NAFLD group. They included menaquinol and fatty acid biosynthesis. In NASH animal models, an increase in CD36 palmitoylation has been observed with localization on the plasma membrane of hepatocytes, and its inhibition protected mice from the disease [67]. The deposition of long-chain fatty acids such as 3-keto stearic acids has been previously associated with the inhibition of beta-oxidation and therefore with abnormal fat deposition metabolism [68]. Additionally, stearic acid has a proinflammatory effect with the promotion of cytokine secretion, impacting liver diseases [69]. This alteration of bacterial fatty acid biosynthesis was also found when we compared the NAFLD and control groups. Our results agree with previous studies showing that alterations of gut microbiota composition, which occur during NAFLD development, can interfere and/or be associated with the lipid metabolism of the liver through both circulating bacterial products and free fatty acids. This phenomenon favours fat accumulation, endoplasmic reticulum and mitochondrial stress, and lipotoxicity [70]. Overall, these results, together with previous studies, suggest that bacterial lipid modifications could act as an early event in mitochondrial dysfunction and NAFLD progression [71]. On the other hand, menaquinones function as a reversible redox component of the electron transfer chain in bacteria and mitochondria but also include vitamin K, which facilitates the carboxylation of vitamin K-dependent proteins, such as growth arrest-specific protein 6 (Gas6) and periostin [72]. Both proteins seem to play an important role in the progression to chronic liver fibrosis [73,74]. However, Gas6 appears to have a "two--faced" role depending on clinical contexts and alleviates acute liver injury in mice [75]. However, this possible link between bacterial menaquinones and liver fibrosis must be confirmed both in vitro and in long-term studies in patients with NAFLD.

One limitation of this study is the relatively small sample size of the groups, which is a general challenge for all microbiota studies, meaning
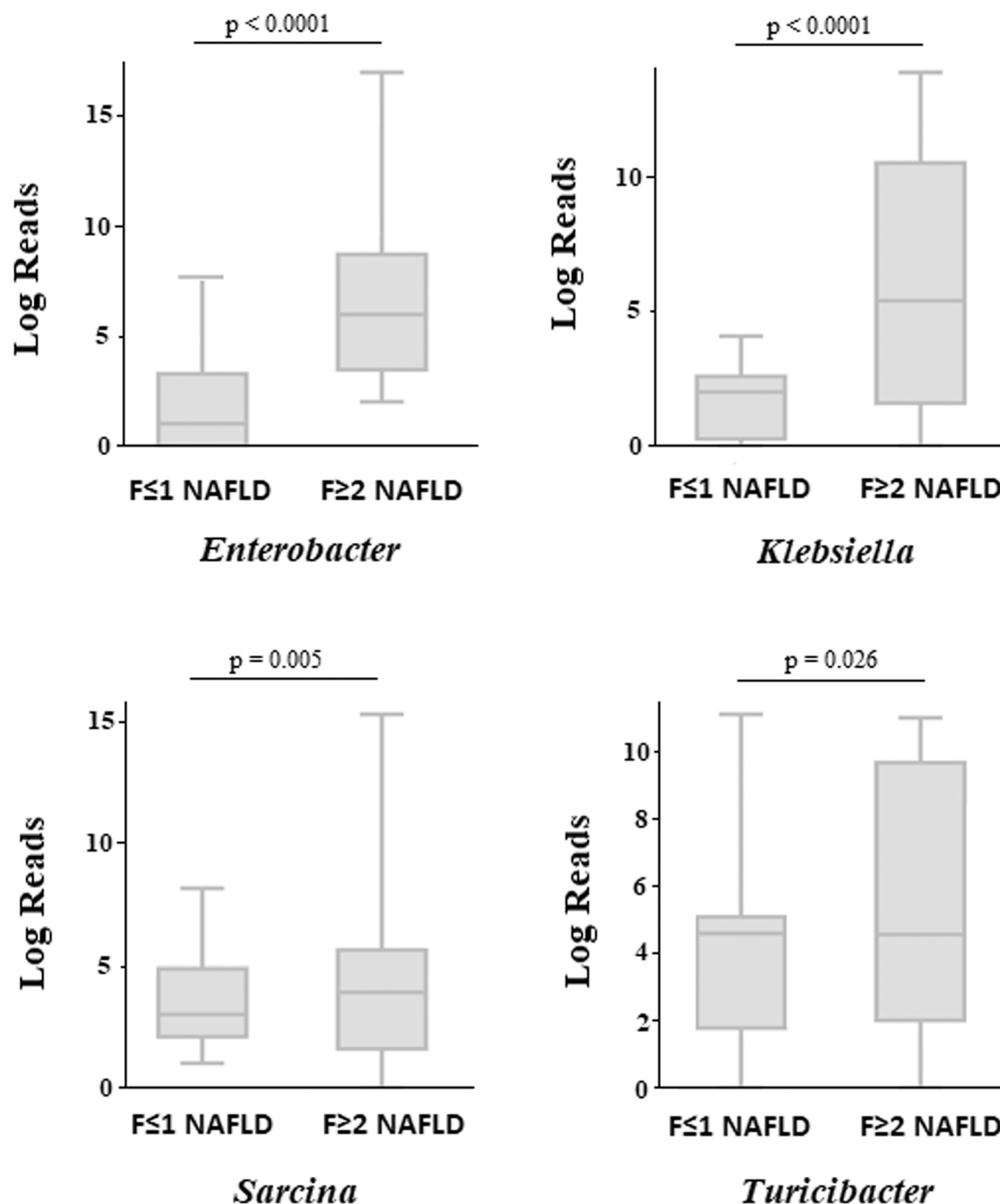
Fig. 6. Box plot showing bacterial genera whose relative abundance was > 1% and was significantly different between the F≤ 1 NAFLD and F≥ 2 NAFLD groups. The results of DESeq2 analysis with Benjamini–Hochberg FDR corrections. Log Reads: Log2 relative normalized abundance.

that the conclusions must be considered with caution. Despite DILI being a rare event and the recruitment of DILI patients for research being very limited, we used very selective exclusion criteria to avoid likely interferences among liver diseases. Accordingly, it is known that patients with other chronic liver diseases, including alcoholic liver disease, chronic hepatitis virus infection, primary sclerosing cholangitis and liver cirrhosis, have an altered specific microbiota, but the complex interaction between the gut microbiota and the liver is unclear. This alteration in the gut microbiota may affect the pathogenesis of these chronic liver diseases [76]. This issue must be confirmed in subsequent studies. Additionally, the use of antibiotics was noted for all patients enrolled in the study. The interaction between drugs, mainly antibiotics, and the gut microbiota has

been identified, and antibiotics may have the most important effect on gut microbiota homeostasis. However, due to the small size of the study, we were not able to establish further relations between the type of antibiotics/drugs and their role in the microbiota composition of patients. In addition, it is difficult to determine whether the alteration of the microbiota is caused by the drug itself or DILI. Another limitation of this study is the possible presence of obesity as a confounding factor, since it is known that the gut microbiota composition is altered in obesity [77]. Some of the changes observed in the gut microbiota are common to both diseases, obesity and NAFLD, although others are specific [78]. To avoid this possible effect, it would have been advisable to include a group of patients with obesity but without NAFLD, which could not be done for this study.

Table 4
Main metabolic pathways associated with the gut microbial composition altered in DILI or NAFLD patients.

Amino Acid Biosynthesis				
Pathway	Superpathway	Increase	Decrease	P value
ASPASN-PWY	L-aspartate and L-asparagine biosynthesis	Dili	F$\geq$ 2 NAFLD	0.004
		F$\leq$ 1 NAFLD	F$\geq$ 2 NAFLD	0.0138
HISTSYN-PWY	L-histidine biosynthesis	Dili	F$\geq$ 2 NAFLD	0.0003
		F$\leq$ 1 NAFLD	F$\geq$ 2 NAFLD	0.0077
ILEUSYN-PWY	L-isoleucine biosynthesis I (from threonine)	Dili	F$\leq$ 1 NAFLD	0.0053
		Dili	F$\geq$ 2 NAFLD	0.0019
PWY-2941	L-lysine biosynthesis II	F$\leq$ 1 NAFLD	Dili	0.0007
		F$\geq$ 2 NAFLD	Dili	0.0182
VALSYN-PWY	L-valine biosynthesis	Dili	F$\leq$ 1 NAFLD	0.0053
		Dili	F$\geq$ 2 NAFLD	0.0019
Carbohydrate and Glycan biosynthesis				
Pathway	**Superpathway**	**Increase**	**Decrease**	**P value**
PWY-1269	CMP-3-deoxy-D-manno-octulosonate biosynthesis	Dili	F$\leq$ 1 NAFLD	0.0027
		Dili	F$\geq$ 2 NAFLD	0.0013
		Dili	Control	0.0019
GLYCOGENSYNTH-PWY	Glycogen biosynthesis I (from ADP-D-Glucose)	Control	F$\geq$ 2 NAFLD	0.0228
UDPNAGSYN-PWY	UDP-N-acetyl-D-glucosamine biosynthesis I	Control	F$\geq$ 2 NAFLD	0.0195
DTDPRHAMSYN-PWY	dTDP-β-L-rhamnose biosynthesis	Dili	F$\leq$ 1 NAFLD	0.0428
		Dili	F$\geq$ 2 NAFLD	0.0047
Cell structure biosynthesis				
Pathway	**Superpathway**	**Increase**	**Decrease**	**P value**
PWY-6467	Kdo transfer to lipid IVA III	Dili	F$\leq$ 1 NAFLD	0.0077
		Dili	Control	0.0196
KDO-NAGLIPASYN-PWY	(Kdo)$_2$-lipid A biosynthesis	F$\geq$ 2 NAFLD	F$\leq$ 1 NAFLD	0.019
NAGLIPASYN-PWY	Lipid IV$_A$ biosynthesis	Dili	F$\leq$ 1 NAFLD	0.0071
		Dili	Control	0.0025
Macromolecule modification, nucleic acid modification				
Pathway	**Superpathway**	**Increase**	**Decrease**	**P value**
PWY-6700	Queuosine biosynthesis I (de novo)	Dili	Control	0.0314
Other Biosynthesis				
Pathway	**Superpathway**	**Increase**	**Decrease**	**P value**
PWY-6519	8-amino-7-oxononanoate biosynthesis I	Dili	F$\geq$ 2 NAFLD	0.0036
		Control	Dili	0.0354
POLYISOPRENSYN-PWY	Polyisoprenoid biosynthesis	Dili	F$\geq$ 2 NAFLD	0.0351
Generation of precursor metabolites and energy				
Pathway	**Superpathway**	**Increase**	**Decrease**	**P value**
PWY-6703	preQ$_0$ biosynthesis	Dili	F$\geq$ 2 NAFLD	0.0048
		Dili	Control	0.0056
P105-PWY	TCA cycle IV (2-oxoglutarate decarboxylase)	F$\geq$ 2 NAFLD	F$\leq$ 1 NAFLD	0.0266
GLYCOLYSIS-TCA-GLYOX-BYPASS	Superpathway of glycolysis, pyruvate dehydrogenase, TCA, and glyoxylate bypass	F$\geq$ 2 NAFLD	F$\leq$ 1 NAFLD	0.0183
P108-PWY	Pyruvate fermentation to propoanoate I	Dili	F$\leq$ 1 NAFLD	0.0055
		Dili	F$\geq$ 2 NAFLD	< 0.0001
		Dili	Control	< 0.0001
		F$\leq$ 1 NAFLD	Control	0.0461
Incomplete reductive TCA cycle				
Pathway	**Superpathway**	**Increase**	**Decrease**	**P value**
P42-PWY	C1 compound utilization and assimilation, CO$_2$ fixation, autotrophic CO$_2$ reductive TCA cycles	Dili	F$\leq$ 1 NAFLD	0.0141
		Dili	F$\geq$ 2 NAFLD	< 0.0001
		Dili	Control	< 0.0001
Inorganic nutrient metabolism				
Pathway	**Superpathway**	**Increase**	**Decrease**	**P value**
PWY-621	sucrose degradation III (sucrose invertase)	F$\leq$ 1 NAFLD	Dili	< 0.0001
		F$\geq$ 2 NAFLD	Dili	0.0031
		Control	Dili	< 0.0001
PWY-7456	β-(1,4)-mannan degradation	Dili	F$\geq$ 2 NAFLD	0.0163
PWY490–3	Nitrate reduction VI (assimilatory)	Dili	F$\geq$ 2 NAFLD	0.0141
SULFATE-CYS-PWY	sulfate assimilation and cysteine biosynthesis	Dili	F$\leq$ 1 NAFLD	0.0046
Cofactor, carrier, and vitamin biosynthesis				
Pathway	**Superpathway**	**Increase**	**Decrease**	**P value**
BIOTIN-BIOSYNTHESIS-PWY	Biotin biosynthesis from 8-amino-7-oxononanoate I, 8-amino-7-oxononanoate biosynthesis I	Dili	F$\leq$ 1 NAFLD	0.0022
PANTO-PWY	Phosphopantothenate biosynthesis I	Dili	F$\geq$ 2 NAFLD	0.0231
		Dili	Control	0.0398
FOLSYN-PWY	Tetrahydrofolate biosynthesis and salvage	Dili	F$\leq$ 1 NAFLD	0.009
HEMESYN2-PWY	Heme b biosynthesis from uroporphyrinogen-III	Dili	F$\leq$ 1 NAFLD	0.0066
		Dili	Control	0.0426
PWY-6892	Thiazole component of thiamine diphosphate biosynthesis I	Dili	F$\geq$ 2 NAFLD	0.0005
		Dili	Control	0.0209
PWY-5509	Adenosylcobalamin biosynthesis from adenosylcobinamide-GDP I	Dili	F$\leq$ 1 NAFLD	0.0151
		Dili	F$\geq$ 2 NAFLD	0.0043
PWY-5837	2-carboxy-1,4-naphthoquinol biosynthesis	F$\leq$ 1 NAFLD	Dili	0.0045
PWY-5861	Demethylmenaquinol-8 biosynthesis I	Dili	F$\leq$ 1 NAFLD	0.0002
		Dili	Control	0.0036

Table 4 (*continued*)

Amino Acid Biosynthesis				
Pathway	Superpathway	Increase	Decrease	P value
PWY-5840	Menaquinol-7 biosynthesis	Dili	F≤ 1 NAFLD	< 0.0001
		Dili	Control	0.0012
PWY-5838	Menaquinol-8 biosynthesis I	Dili	F≤ 1 NAFLD	< 0.0001
		Dili	Control	0.0008
PWY-5897	Menaquinol-11 biosynthesis	Dili	F≤ 1 NAFLD	< 0.0001
		Dili	Control	0.0011
		F≥ 2 NAFLD	F≤ 1 NAFLD	0.047
PWY-5898	Menaquinol-12 biosynthesis	Dili	F≤ 1 NAFLD	0.0014
		F≥ 2 NAFLD	F≤ 1 NAFLD	< 0.0001
		F≥ 2 NAFLD	Control	0.0071
PWY-5899	Menaquinol-13 biosynthesis	Dili	F≤ 1 NAFLD	< 0.0001
		F≥ 2 NAFLD	F≤ 1 NAFLD	0.047
PWY-5863	Phylloquinol biosynthesis	Dili	F≤ 1 NAFLD	0.0023
PWY-5918	Heme b biosynthesis	Dili	F≤ 1 NAFLD	0.0381
PWY-6269	Adenosylcobalamin salvage from cobinamide II	Dili	F≤ 1 NAFLD	0.0128
		Dili	F≥ 2 NAFLD	0.0031
PWY-6147	6-hydroxymethyl-dihydropterin diphosphate biosynthesis I	Dili	F≤ 1 NAFLD	0.0176
		Dili	F≥ 2 NAFLD	< 0.0001
		Dili	Control	0.0059
PWY-7539	6-hydroxymethyl-dihydropterin diphosphate biosynthesis III	Dili	F≥ 2 NAFLD	0.0352
THISYN-PWY	Thiamine diphosphate biosynthesis I	Dili	F≥ 2 NAFLD	0.0031
		Dili	Control	0.0133
Fatty acid and lipid biosynthesis				
Pathway	Superpathway	**Increase**	**Decrease**	**P value**
FASYN-ELONG-PWY	Fatty acid biosynthesis I	Dili	F≤ 1 NAFLD	0.0045
		Dili	Control	0.0023
FASYN-INITIAL-PWY	Fatty acid biosynthesis initiation	F≤ 1 NAFLD	F≥ 2 NAFLD	0.0006
PWY-7663	Gondoate biosynthesis (anaerobic)	Dili	F≤ 1 NAFLD	0.0003
		Dili	F≥ 2 NAFLD	< 0.0001
		Dili	Control	0.0099
PWY-6282	Palmitoleate biosynthesis I (from (5Z)-dodec-5-enoate)	Dili	F≤ 1 NAFLD	0.0176
		F≥ 2 NAFLD	F≤ 1 NAFLD	< 0.0001
		F≥ 2 NAFLD	Control	0.0059
PWY-5971	Palmitate biosynthesis (type II fatty acid synthase)	F≤ 1 NAFLD	F≥ 2 NAFLD	< 0.0001
		F≥ 2 NAFLD	Control	0.0133
PWY-5973	Cis-vaccenate biosynthesis	Dili	F≤ 1 NAFLD	0.0115
		Control	F≤ 1 NAFLD	0.0445
PWY-5989	Stearate biosynthesis II	Dili	F≤ 1 NAFLD	0.0014
		F≥ 2 NAFLD	F≤ 1 NAFLD	< 0.0001
		F≥ 2 NAFLD	Control	0.0071
PWY-7664	Oleate biosynthesis IV (anaerobic)	Dili	F≤ 1 NAFLD	0.0006
		F≥ 2 NAFLD	F≤ 1 NAFLD	< 0.0001
		Control	F≤ 1 NAFLD	0.0339
		Control	F≥ 2 NAFLD	0.0037
PWY0–862	(5Z)-dodecenoate biosynthesis I	Dili	F≤ 1 NAFLD	0.0004
		Dili	Control	0.0349
		F≥ 2 NAFLD	F≤ 1 NAFLD	< 0.0001
		F≥ 2 NAFLD	Control	0.0025
PWYG-321	Mycolate biosynthesis	Dili	F≤ 1 NAFLD	0.0068
		F≥ 2 NAFLD	F≤ 1 NAFLD	< 0.0001
		F≥ 2 NAFLD	Control	0.006
Nucleoside and nucleotide biosynthesis				
Pathway	Superpathway	**Increase**	**Decrease**	**P value**
DENOVOPURINE2-PWY	Purine nucleotides de novo biosynthesis II	Dili	F≤ 1 NAFLD	0.0027
		Dili	F≥ 2 NAFLD	0.0283
PWY-7211	Pyrimidine deoxyribonucleotides de novo biosynthesis	Dili	F≤ 1 NAFLD	0.0006
		Dili	F≥ 2 NAFLD	0.0344
		Dili	Control	0.0147
PWY-5686	UMP biosynthesis I	Dili	F≥ 2 NAFLD	0.0144
PRPP-PWY	Histidine, purine, and pyrimidine biosynthesis	Dili	F≤ 1 NAFLD	0.025
PWY-6125	Guanosine nucleotides de novo biosynthesis II	Dili	F≤ 1 NAFLD	0.0002
		Dili	F≥ 2 NAFLD	0.003
PWY-6126	Adenosine nucleotides de novo biosynthesis II	Dili	F≤ 1 NAFLD	0.0078
		Control	F≤ 1 NAFLD	0.0215

However, the association and simultaneous presence of NAFLD and obesity/insulin resistance is almost always present [79]. These two conditions (obesity and insulin resistance) are the main factors that define the presence of metabolic syndrome, which is key in the appearance and development of NAFLD. From this point of view, more than confounding factors, they could be considered as factors that are clearly associated with NAFLD: as our study shows, the greater the BMI and insulin resistance, the greater the severity of NAFLD. Finally, all statistical analyses were performed at the genus level, as identification at the species level based on 16 S rRNA sequencing targeting the V3-V4 hypervariable region in human faeces should be considered only with caution. Further studies more deeply investigating the bacterial metabolic functions of gut microbial markers will decipher their future applications in the detection, prevention and treatment of these diseases.

In conclusion, our findings provide relevant insight concerning the potential role of the gut microbiota in DILI and NAFLD. These results are

essential to decipher new mechanisms by which bacteria can interact with host metabolism. In this study, we successfully provided a distinctive microbiota-based profile for the DILI, NAFLD and control groups, with a higher proportion of the Bacteroidetes phylum and a lower proportion of Firmicutes and different genera in the DILI group. Moreover, we identified potential microbial markers that differentiate NAFLD patients with different grades of fibrosis, such as *Enterobacter, Klebsiella, Sarcina* and *Turicibacter*. We also identified possible links between bacterial functionality and DILI and NAFLD, some already known and others yet to be confirmed in subsequent studies. These functional capacities of the microbiota could be associated with the cellular and molecular alterations described for DILI and NAFLD diseases. However, both the alteration in the abundance of certain genera, as well as the bacterial functional capacities of these genera found in these liver diseases, mainly DILI, merit further investigation.

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CRediT authorship contribution statement

Cristina Rodriguez-Diaz: Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Bernard Taminiau**: Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Alberto García-García**: Investigation, Resources. **Alejandro Cueto**: Investigation. **Mercedes Robles-Díaz**: Investigation, Resources. **Aida Ortega-Alonso**: Investigation, Resources. **Flores Martín-Reyes**: Investigation. **Georges Daube**: Formal analysis, Investigation, Writing – review & editing, Visualization. **Judith Sanabria-Cabrera**: Investigation. **Miguel Jimenez-Perez**: Investigation, Resources. **M. Isabel Lucena**: Conceptualization, Methodology, Resources, Writing – review & editing, Visualization, Funding acquisition. **Raúl J Andrade**: Conceptualization, Methodology, Resources Writing – review & editing, Visualization, Funding acquisition. **Eduardo García-Fuentes**: Conceptualization, Methodology, Formal analysis, Resources, Writing – original draft, Writing – review & editing, Visualization. **Miren García-Cortes**: Conceptualization, Methodology, Formal analysis, Resources, Writing – review & editing, Visualization, Funding acquisition.

Declaration of interest

None.

Data Availability

The data that support the findings of this study are deposited in the Bioproject (National Center for Biotechnology Information (NCBI)) at https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA808210, reference number [PRJNA808210]. The data that support the findings of this study are available from the corresponding author, [EGF], upon reasonable request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phrs.2022.106348.

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