

An altered fecal microbiota profile in patients with non-alcoholic fatty liver disease (NAFLD) associated with obesity

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ABSTRACT

Introduction: increasing evidence suggests a role of intestinal dysbiosis in obesity and non-alcoholic fatty liver disease (NAFLD). The advances in recent years with regard to the role of the gut microbiota raise the potential utility of new therapeutic approaches based on the modification of the microbiome.

Objective: the aim of this study was to compare the bacterial communities in obese patients with or without NAFLD to those of healthy controls.

Patients and methods: the fecal microbiota composition of 20 healthy adults, 36 obese patients with NAFLD and 17 obese patients without NAFLD was determined by 16S ribosomal RNA sequencing using the Illumina MiSeq system.

Results: the results highlighted significant differences in the phylum *Firmicutes* between patients with and without NAFLD, which was a determining factor of the disease and supported its possible role as a marker of NAFLD. At the genus level, the relative abundance of *Blautia*, *Alkaliphilus*, *Flavobacterium* and *Akkermansia* was reduced in obese patients, both with or without NAFLD, compared to healthy controls. Furthermore, the number of sequences from the genus *Streptococcus* was significantly higher in patients with NAFLD in comparison with individuals without the disease, constituting another possible marker. Comparison of bacterial communities at the genus level by a principal coordinate analysis indicated that the bacterial communities of patients with NAFLD were dispersed and did not form a group.

Conclusion: in conclusion, these results indicate the role of intestinal dysbiosis in the development of NAFLD associat-

ed with obesity. There was a differential microbiota profile between obese patients, with and without NAFLD. Thus, supporting gut microbiota modulation as a therapeutic alternative for the prevention and treatment of NAFLD.

Key words: 16S rRNA. Dysbiosis. High-throughput sequencing. Intestinal microbiota phylotypes. Non-alcoholic fatty liver disease. Obesity.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), the liver manifestation of the metabolic syndrome, is characterized by a variety of liver phenotypes ranging from simple steatosis to non-alcoholic steatohepatitis (NASH). The aggressive form of the disease can potentially progress to cirrhosis, liver failure and hepatocellular carcinoma (1,2). The pathophysiology of NAFLD remains unclear but the intestinal microbiome has recently gained great attention in metabolic diseases, as gut dysbiosis has been recognized in obesity (3,4), the metabolic syndrome (5), diabetes (6-8) and cardiovascular diseases (9). Recent studies in obese mice have identified the gut microbiota as a potentially important participant in the pathogenesis of NAFLD, with a different gut microbiota between obese and lean mice. This altered microbial

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composition can explain the response to a high-fat diet and whether these mice will develop hepatic steatosis (10-12). A small number of studies have been carried out in humans that broaden our understanding of the role of the microbiota in the pathogenesis and development of NAFLD (13-16). In this regard, no single bacteria has been shown to be responsible for the development of steatosis. The majority of studies have focused on identifying differences in gut microbiota composition between healthy individuals and patients with NAFLD (16-20). Most of them showed a dysbiotic gut microbiome, with particular shifts associated with obesity that correlated closely with the prevalence and progression of NAFLD. Thus, it may play a critical role in the development of this disease (15,21).

Therefore, the determination of the gut microbiota composition provides new information to the classical predictions of NAFLD and suggests novel strategies to manipulate the gut microbiota towards a healthier community structure. These include the use of pre/probiotics or even fecal microbiota transplant (20). Based on the accumulating evidence, we hypothesized that there could be a specific composition of the gut microbiota in obese patients with NAFLD that contributed to the pathogenesis of the disease. Therefore, the objective of this study was to compare the fecal gut microbiome community structure and composition in obese patients with and without NAFLD to those of healthy controls. Illumina MiSeq analysis was used to characterize microbial communities based on sequencing of the 16S ribosomal ribonucleic acid (rRNA) gene.

PATIENTS AND METHODS

Patients and fecal samples

Seventy-three subjects were included in the study aged between 20 and 60 years old. Seventeen obese patients

without NAFLD, 36 obese patients with NAFLD and 20 healthy control subjects were included. According to clinical, analytical and ultrasonographic data, patients were classified as NAFLD or non-NAFLD (Table 1). All patients were asked about alcohol consumption in a clinical interview and this data was included in the medical records of every patient. In addition, all controls were healthy volunteers without steatosis and fibrosis or any diagnosed disease and intestinal symptoms at the time of sampling. The criteria of the Adult Treatment Panel (ATP) III were used for the diagnosis of metabolic syndrome (22), which considered at least three of the following issues: waist greater than 102 cm in males or 82 cm in females, triglycerides levels greater or equal to 150 mg/dl, HDL cholesterol levels less than 40 mg/dl in males or less than 50 mg/dl in females, blood pressure greater or equal to 130/85 mmHg and a fasting blood glucose level higher or equal to 100 mg/dl. None of the patients included in the study had been treated with antibiotics for at least one month prior to sampling. The study was conducted according to the guidelines outlined in the Declaration of Helsinki and all procedures involving human subjects were approved by the local ethics committee. All patients gave informed written consent before participating in the study.

Fresh stools were collected from the three groups of subjects. All samples were homogenized and aliquoted within three hours of defecation. The aliquots were stored at -80 °C until analysis.

DNA extraction

Total genomic DNA was isolated from fecal samples using the QIAamp DNA Stool Mini kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. Some modifications were made, the initial bead beating step and the lysis temperature was increased from the recommended

Table 1. Clinical characteristics of controls and patients

Parameters	Controls (n = 20)	Obese with NAFLD (n = 36)	Obese without NAFLD (n = 17)	p value*	p value [†]	p value [‡]
Sex (female/male)	13/7	22/14	12/5	0.659	0.424	0.506
Age	37.75 (26-59)	49.7 (31-64)	40.12 (25-56)	0.000	0.283	0.001
BMI (kg/m ²)	21.05 (15-27.7)	45.64 (38.9-61.1)	46.9 (39-63)	0.000	0.000	0.339
Glucose (mg/dl)	86.8 (77-105)	109 (80-255)	92.94 (76-136)	0.000	0.244	0.010
Insulin (mUI/ml)	3.5395 (2.23-5.23)	25.7 (4.8-81)	19.67 (3.8-42.3)	0.000	0.000	0.169
HOMA-IR	0.76 (0.16-0.44)	7.03 (1.03-23)	4.51 (0.81-8.99)	0.000	0.000	0.055
Triglycerides (mg/dl)	66.2 (43-127)	146.97 (46-344)	107.88 (56-220)	0.000	0.000	0.067
Total cholesterol (mg/dl)	176.05 (136-213)	184.36 (110-232)	175.76 (128-230)	0.199	0.940	0.226
HDL (mg/dl)	58.05 (45-85)	49.25 (32-76)	48.53 (34-70)	0.010	0.022	0.834
LDL (mg/dl)	107.6 (61-146)	106.72 (28-176)	105.6 (65-166)	0.837	0.641	0.600
ALT	22 (12-34)	34.22 (15-91)	27.12 (10-52)	0.007	0.390	0.185
AST	20.7 (12-29)	24.61 (11-85)	20.82 (12-29)	0.700	0.045	0.717
GGT	23 (13-63)	51.11 (12-295)	33.65 (13-100)	0.002	0.158	0.140

Data are expressed as the mean (SEM). ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: gamma glutamyl transferase. *Control vs obese with NAFLD. [†]Control vs obese without NAFLD. [‡]Obese with NAFLD vs obese without NAFLD.

70 to 90 °C in order to aid the recovery of DNA from bacteria that are difficult to lyse. The concentration of isolated DNA was determined using the NanoDrop ND-1000 spectrophotometer (Saveen & Werner, Limhamn, Sweden) and the extracted DNA was stored at -20 °C.

PCR amplification and sequencing

PCR amplification of the 16S rRNA was performed based on PCR primers in the literature (12) that were specific for V3-V4 regions with added Illumina adapter overhang nucleotide sequences. The PCR assays were carried out in triplicate as follows: 25 µl reaction solutions with 50 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 2x KAPA HiFiHotStart Ready Mix (Kapa Biosystems, Wilmington, MA, USA). The PCR conditions were as follows: three minutes at 95 °C, followed by 25 cycles of 30 seconds at 95 °C, 30 seconds at 55 °C and 30 seconds at 72 °C and a final extension at 72 °C for five minutes.

Each reaction was cleaned up using the AgencourtAMPure XP beads (Beckman Coulter Genomics, Danvers, MA, USA) following the instructions of the manufacturer to remove excess primers and primer dimers. In a second index PCR, 5 µl of each amplicon were used as template. Dual indices and Illumina sequencing adapters to each amplicon were attached in the second PCR using the Nextera XT Index kit (Illumina Inc.). The amplification was performed with the 2x Kappa HiFiHotStart Ready Mix (KappaBiosystems, Boston, MA) and the cycling conditions were: 95 °C for three minutes, followed by eight cycles of 95 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 30 seconds and a final step of 72 °C for five minutes. The indexed product was cleaned with AMPure XP beads and quantified with Quant-iTPicoGreen (Thermo Fisher Scientific). The agilent 2100 Bioanalyzer was used to determine the library quality control and the average size distribution. Libraries were normalized and pooled to 40 nM based on quantified values. The library pool was then denatured, diluted to 6 pM and a PhiX control was added. The pool was sequenced using the Illumina MiSeq system via pair end 300 bp reads with MiSeq V3 reagents and the data were processed using the MiSeq Reporter software.

Bioinformatic and statistical analysis

Samples were analyzed using the BaseSpace Application 16S Metagenomics v1.0 (<https://basespace.illumina.com/home/index>) to determine the bacterial taxonomical composition. This platform uses the Illumina-curated version of the GreenGenes taxonomic database and RDP Classifier (Ribosomal Database Project) described by Wang Q et al. (23). Moreover, the Quantitative Insights into Microbial Ecology software (QIIME version 1.9.0) was used to verify the results (24,25). These sequences were clustered into operational taxonomic units (OTUs) using UCLUST, with a similarity threshold of 0.97 (26,27), and were aligned using PyNast (28) against 16S reference database GreenGenes (version 13.8) using default parameters. These OTUs were analyzed with the Vegan package (29) in the R software (R Development Core Team, 2011) to estimate the alpha and beta diversity of the samples. Significant differences were tested by the Mann-Whitney U test ($p < 0.05$).

RESULTS

Clinical characteristics

An NAFLD diagnosis was established by clinical, analytical criteria (liver function test) and from ultrasonographic data when steatosis was detected. According to NAFLD fibrosis score, 27.78% of patients with NAFLD did not have significant fibrosis (F0-F2), 24.07% of patients had advanced fibrosis (F3-F4) and 48.15% were indeterminate. The characteristics and clinical data for all participants in the study are summarized in table 1. There were statistical differences between obese patients with and without NAFLD in comparison to healthy controls in the main parameters associated with obesity, metabolic syndrome and NAFLD. These included body mass index (BMI), liver damage markers, glucose, insulin and the homeostatic model assessment of insulin resistance (HOMA-IR), triglycerides and high and low density lipoprotein cholesterol (HDL and LDL) levels ($p < 0.05$). However, there were only significant differences in glucose levels when obese patients with NAFLD vs non-NAFLD patients were compared ($p < 0.05$). Around 64.81% of all obese patients were diagnosed with the metabolic syndrome, 25.93% were considered as diabetic patients and 74.47% had insulin resistance.

Differences in fecal microbial composition patterns between healthy and obese patients with and without NAFLD

A total of 19,683,256 bacterial sequences were obtained from the analysis of the 73 fecal samples. The number of reads per sample ranged from 41,604 to 999,815. Analysis of the sequences showed that this niche was colonized by bacteria from mainly two phyla, *Firmicutes* (45%) and *Bacteroidetes* (46%). A smaller proportion represented bacteria of the phylum *Proteobacteria* (5.8%). These three phyla represented more than 90% of the sequences analyzed. Figure 1A shows the relative bacterial composition at the phylum level for each group. Statistical analysis revealed that the number of reads of the phylum *Proteobacteria* was higher in obese patients both with and without NAFLD compared to healthy controls ($p < 0.05$). There were also significant changes in the phylum *Firmicutes* ($p < 0.05$). Obese patients with NAFLD had a higher number of reads of this phylum compared to non-NAFLD obese patients (Fig. 1B) and is therefore a possible marker of this pathology. Nevertheless, no significant differences in the *Firmicutes/Bacteroidetes* ratio were found between healthy and obese patients, with and without NAFLD (data not shown).

At the class level, changes were also detected when the microbiota were compared between control subjects and obese patients with and without NAFLD. The number of reads of *Gammaproteobacteria* and *Erysipelotrichi* class (classified within *Proteobacteria* and *Firmicutes* phyla, respectively) were significantly different between controls and obese patients with NAFLD (Fig. 1C), whereas the relative abundance of *Bacilli* (classified within the *Firmicutes* phylum) was significantly increased in obese patients with NAFLD compared to healthy controls and obese patients without NAFLD. On the contrary, there was a reduction in the *Betaproteobacteria* class (*Proteobacteria* phylum) in these patients compared to obese patients without NAFLD. Thus, again indicating the existence of a specific microbiota profile associated with NAFLD (Fig. 1C).

Phylogenetic analysis of the sequences determined the presence of 617 genera of known bacteria from fecal samples from healthy controls and obese patients with and without NAFLD. Most of the sequences were classified within six genera: *Bacteroides*, *Prevotella*, *Blautia*, *Faecalibacterium*, *Clostridium* and *Oscillospira*. The hierarchical heatmap (Fig. 2) is based on the 48 most abundant genera present in the different samples and there were no distinct clusters based on the disease. In contrast, significant changes at genus level have been found to be related to obesity and NAFLD. As shown in figure 1D, the number of reads of *Oscillospira* and *Eubacterium* were significantly lower in both groups of obese patients than in healthy controls ($p < 0.05$). In addition, the *Akkermansia* genus reduction was also observed

in both obesity groups, although these differences were not statistically significant. The *Megasphaera*, *Lactobacillus* and *Acidaminococcus* genus showed the opposite pattern ($p < 0.05$) (Fig. 1D). Furthermore, *Blautia*, *Alkaliphilus* and *Flavobacterium* were reduced in patients with NAFLD in comparison to controls ($p < 0.05$) (Fig. 1D). Interestingly, *Streptococcus* was more frequent in patients with NAFLD than in patients without fatty liver associated with obesity ($p < 0.05$). This supports its possible role as a marker of this disease (Fig. 1D). Considering the NAFLD fibrosis score, *Megasphaera* and *Velionella* genera were significantly lower in patients with advanced fibrosis compared to patients without significant fibrosis ($p = 0.037$ and $p = 0.046$, respectively), while *Lactobacillus* and *Natronincola* showed the

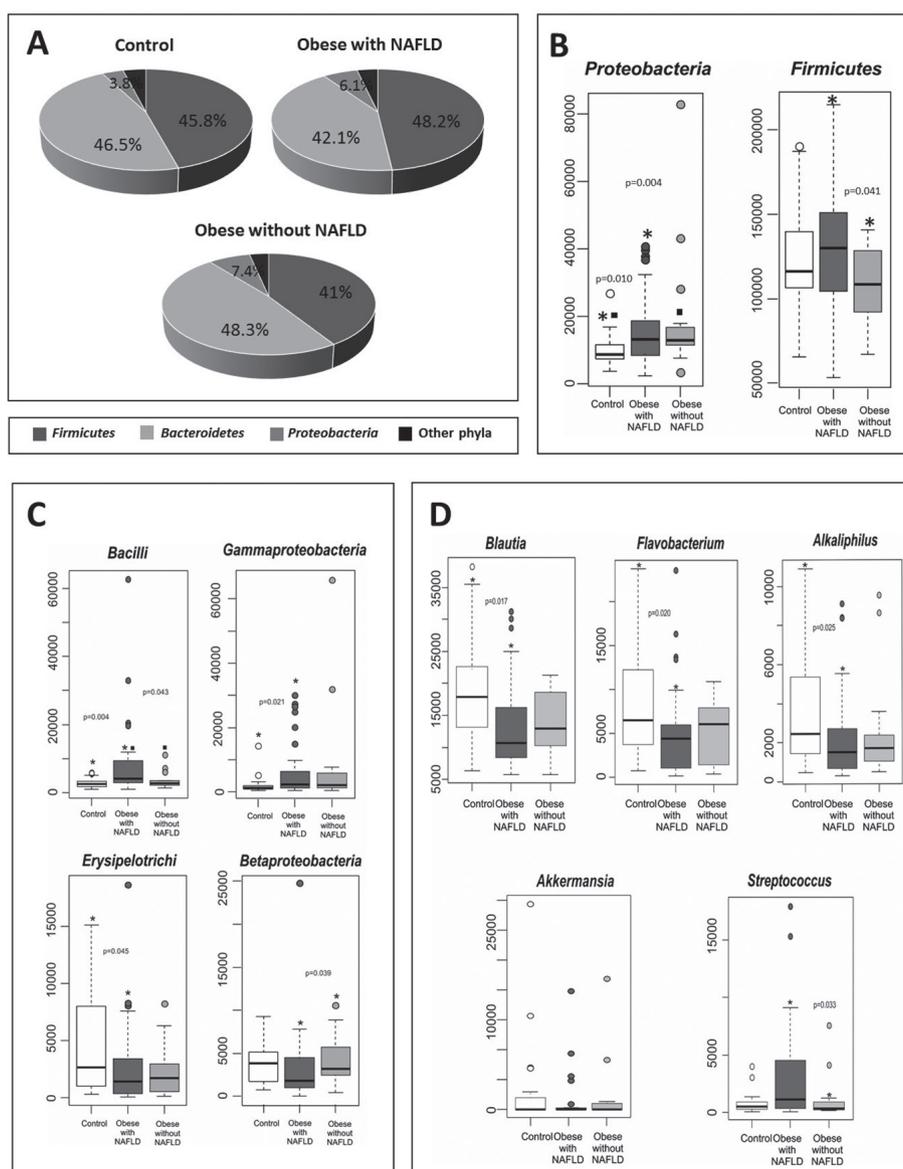


Fig. 1. Comparison of the phylotypes between healthy and obese patients with and without NAFLD at the phylum, class and genus levels. A. Pie charts showing the bacterial community composition in controls and obese patients with and without NAFLD at the phylum level. B. Box plot summarizing the significant differences at the phylum level using the Mann-Whitney U test ($p < 0.05$). C. Box plot representing the differences between controls and obese patients with and without NAFLD at the class level using the Mann-Whitney U test ($p < 0.05$). D. Box plot showing significant differences between controls, obese patients with NAFLD and obese patients without NAFLD at the genus level using the Mann-Whitney U-test ($p < 0.05$).

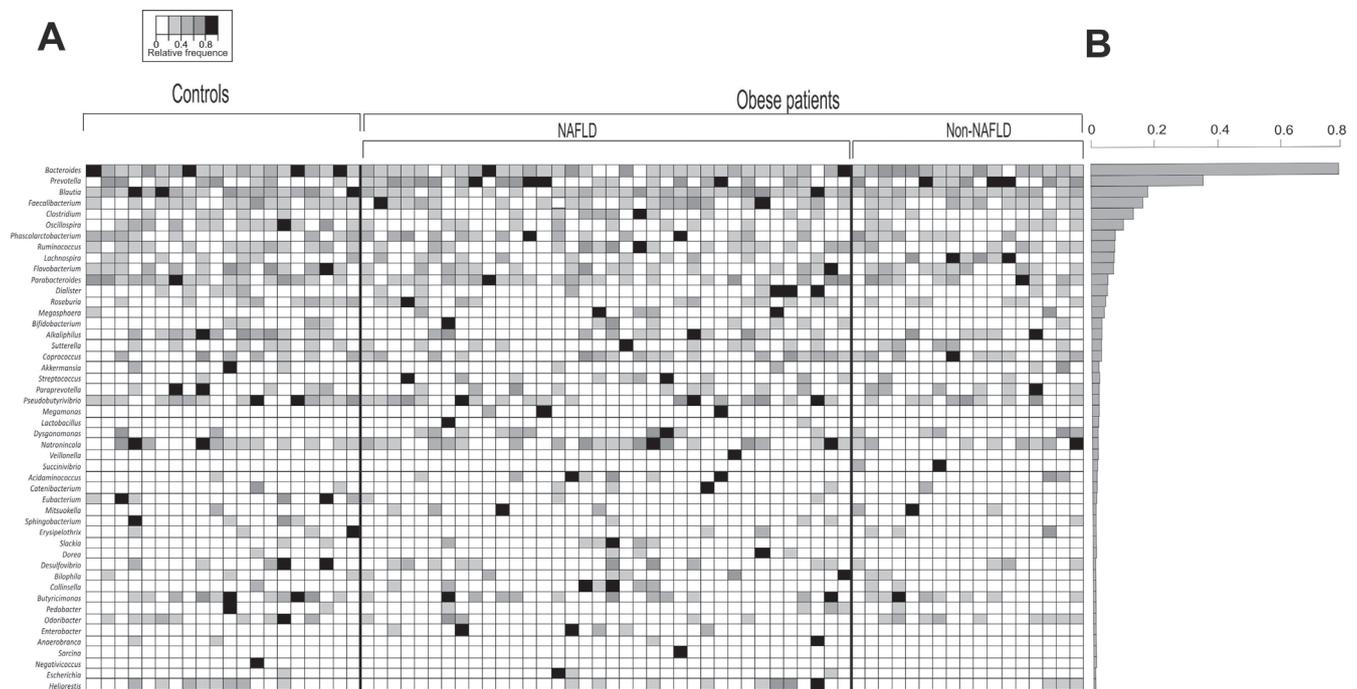


Fig. 2. Microbiota heatmap at the genus level. A. The heatmap plot depicts the log of the relative frequency of each OTU (right vertical axis) within each sample (horizontal axis). The log of the relative values for OTU are indicated by the gray level scale intensity according to the legend in the top left corner. The assignment of each OTU (left vertical axis) was performed using the naïve Bayesian classifier provided by the Ribosomal Database Project (RDP). B. OTU abundance distribution histogram.

opposite pattern ($p = 0.033$ and $p = 0.025$, respectively). On the contrary, the *Akkermansia* genus was also significantly lower in patients with advanced fibrosis in relation to patients with indeterminate fibrosis ($p = 0.021$). Similar results were observed for the *Roseburia*, *Natronincola* and *Paraprevotella* genera, which were lower in patients with NAFLD without significant fibrosis versus patients with indeterminate fibrosis ($p = 0.028$, $p = 0.038$ and $p = 0.04$, respectively). In addition, there was a greater frequency of the *Megasphaera* genus in the microbiota composition of patients without significant fibrosis compared to NAFLD with indeterminate fibrosis ($p = 0.037$).

Study of bacterial richness

Bacterial richness was also assessed in fecal samples in the three groups based on rarefaction curve analysis: healthy controls and obese patients with and without NAFLD. Bacterial richness analysis showed the presence of an average of 180.62 ± 26.73 OTUs per sample in healthy controls, 240.37 ± 46.1 OTUs in obese patients with NAFLD and 246.5 ± 30.1 OTUs in non-NAFLD obese patients (Fig. 3A). The statistical analysis showed a significantly greater bacterial richness in obese patients than in healthy controls, regardless of non-alcoholic fatty liver disease. Thus, highlighting obesity as the main factor responsible for these differences (Fig. 3A). To better understand the richness observed among the three groups, a Venn diagram was developed to display the overlaps between groups (Fig. 3B). This diagram showed that 482 OTUs of the total (617) were shared among all the samples. Thirty-seven and 25 OTUs were exclusive to fecal samples from controls and obese patients with NAFLD,

respectively. By contrast, fewer OTUs (9) were exclusive to fecal samples from obese patients without NAFLD. The number of common OTUs between the samples of obese patients with and without NAFLD was 34. However, a smaller number of OTUs was shared between controls and obese patients with NAFLD and between controls and obese non-NAFLD (22 and 8 OTUs, respectively) (Fig. 3B).

Bacterial community comparison between healthy controls and obese patients with and without NAFLD

Beta diversity measurements were performed on the fecal content to assess the differences in global bacterial composition among the three groups using Principal Coordinates Analysis (PCoA), based on the Chao dissimilarity index. This analysis revealed that the bacterial community of healthy controls grouped separately from the bacterial communities of obese patients without NAFLD according to the first Axis (Fig. 3C), whereas the communities of obese patients with NAFLD were dispersed along this axis. The first Axis score plot (3.75%) revealed a clear separation of bacterial communities according to obesity. Nevertheless, NAFLD was not a determinant factor which led to bacterial communities grouping together.

DISCUSSION

Classic culturing techniques have been used for a long time to determine the composition of the intestinal microbiota, selecting specific bacterial communities. However, the currently available high-throughput sequencing (using

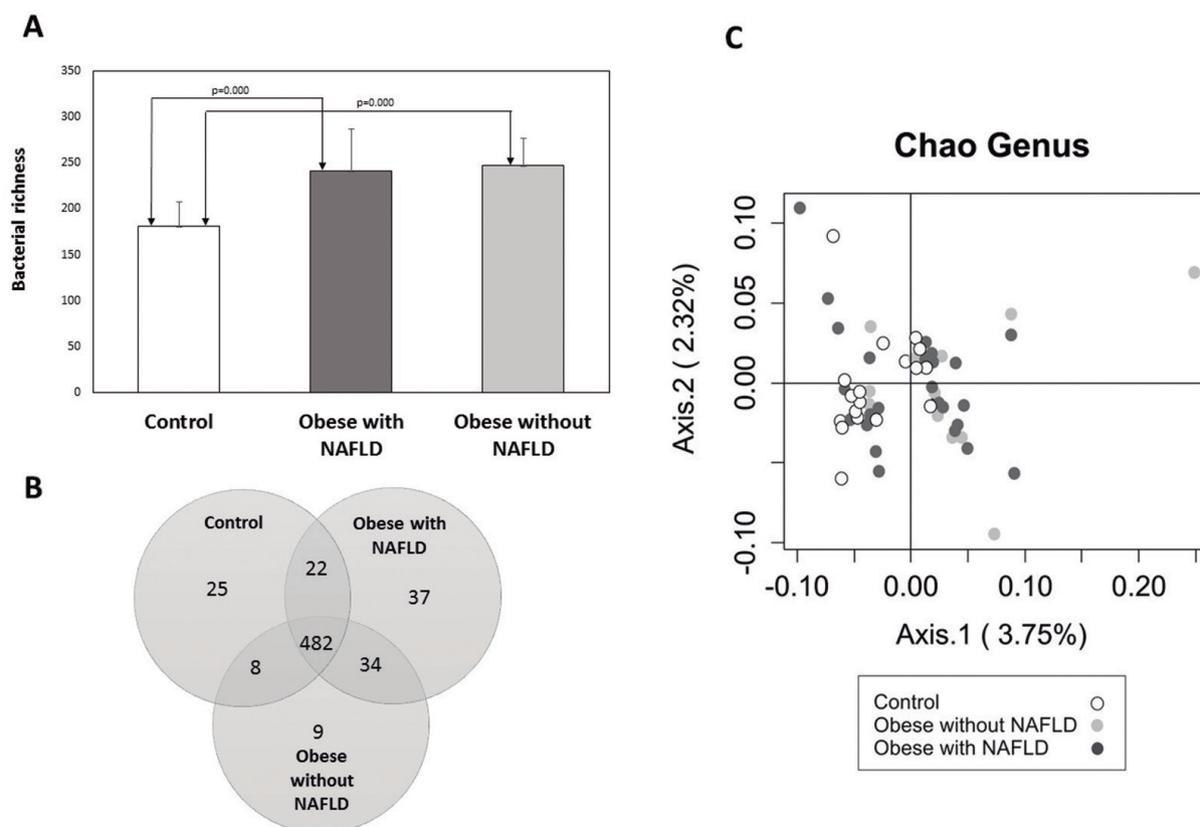


Fig. 3. Comparison of bacterial communities between controls and obese patients with and without NAFLD. **A.** Bacterial richness is expressed as an average of the number of OTUs. The arrows between the bars represent significant differences detected by the Mann-Whitney U test ($p < 0.05$). **B.** Venn diagram comparing the OTUs in fecal samples of healthy control and obese patients with and without NAFLD. **C.** Principal coordinate analysis (PCoA) plot derived from the Morisita-Horn dissimilarity index at the genus level between the three groups. The percentage of the total variance explained is indicated in parenthesis in each axis.

16S rRNA) of environmental DNA allows the rapid analysis of microbial communities at a much higher rate than was previously possible (30). Several recent studies using these techniques have tried to interpret the structure and functionality of the human intestinal microbiota, as well as its association and involvement in obesity and NAFLD (18-20,31,32). In the present study, fecal samples were used to evaluate the differences of the fecal microbiota between healthy controls and obese patients with and without NAFLD using next-generation sequencing methods. Previous studies have shown that the gut microbiome of obese patients is distinct from healthy-weight individuals, with a lower proportion of *Bacteroidetes* and a higher proportion of *Firmicutes* (33-35). Other groups have also demonstrated dysbiosis of the distal gut in obese patients (36), although the exact findings have been unpredictable. No significant difference was found in the ratio of *Firmicutes/Bacteroidetes* between obese and healthy controls and similar results were shown in children (17). These discordant results may be due to a different methodology or differences in the patient populations such as the degree of the disease, ethnicity, diet, the environment, or associated comorbidities such as the metabolic syndrome and treatment. Nevertheless, we observed that the number of reads of the phylum *Proteobacteria* was significantly higher in obese patients compared to healthy controls. Similar results in relation to this phylum have been obtained in both humans and mice

(12,15,37,38). On the other hand, we also observed that the relative abundance of the *Firmicutes* phylum was significantly higher in obese patients with NAFLD compared to the obese group without NAFLD. This could indicate that obese NAFLD and obese non-NAFLD patients have a different fecal microbiome, which could be a possible determinant factor associated to disease development.

In our study, six genera predominant sequences were found: *Bacteroides*, *Prevotella*, *Blautia*, *Faecalibacterium*, *Clostridium* and *Oscillospira*. In addition, we found other genera that seem to be part of the gut microbiota in these patients, but have been detected less frequently. Furthermore, a substantial inter-individual heterogeneity in the gut microbial composition was also identified and there were no shifts based on the disease. These findings were similar to those of previous studies (39).

A considerable number of studies support the role of the gut microbiota in the pathogenesis of NAFLD (14,20,40,41). Compared with previous reports, our study presents evidence to support these findings. For example, we observed a significantly higher bacterial richness in obese patients, significant differences between the gut bacterial populations in healthy and obese patients with and without NAFLD. In addition, differences were found in the fecal microbiota composition among obese patients with NAFLD

with advanced and indeterminate fibrosis and those without significant fibrosis. For example, *Oscillospira* and *Eubacterium* were significantly lower in both groups of obese patients than in healthy controls. Recent studies have shown that the *Oscillospira* genus appears to be diminished in patients with NALFD and is positively associated with weakness (42,43). The *Akkermansia* genus is reduced in obese patients with and without NAFLD in comparison to healthy individuals. Various studies have shown that *Akkermansia muciniphila* improves the metabolic markers of obesity and NAFLD, suggesting that *A. muciniphila* could be a potential probiotic (44,45), whereas *Megasphaera*, *Lactobacillus* and *Acidominococcus* are significantly increased in obese patients with NAFLD. The elevation of the number of bacteria of the *Lactobacillus* genus has already been described in obese patients, which might indicate a possible role of this genus in weight gain and obesity (46,47). The *Streptococcus* genus has also shown similar results in different studies and could be associated with the pathogenesis of NAFLD (15). There was a greater detection in patients with NAFLD than in patients without fatty liver associated with obesity, justifying its possible role as a marker of this disease. In contrast, *Blautia*, *Alkaliphilus* and *Flavobacterium* were reduced in patients with NAFLD in comparison to controls. Other studies have also shown a decrease of the *Blautia* genus in obese and NASH patients compared to healthy subjects. In addition, it has been reported that this genus is able to reduce inflammation levels and increases intestinal peristalsis (48).

On the other hand, our results also showed that NAFLD was not a determinant factor that conditioned how the bacterial communities grouped together. It could be explained when considering NAFLD as an independent disease spectrum, with a different role of fecal microbiota (16). Likewise, the limitations of this study include the sample size of patients and the different location of the samples, which makes it difficult to generalize to other populations. However, we have found common differences in the gut microbiota composition in our patients from different geographical locations, reaffirming the existence of a specific microbiota profile involved in the development of NAFLD. Conversely, further evidence is required before the exact nature of the relationship between dysbiosis and NAFLD can be definitely resolved. Indeed, it remains unclear whether only dysbiosis contributes to NAFLD or also NAFLD can favor dysbiosis. Furthermore, whether dysbiosis is associated specifically with NAFLD or more generally with the metabolic disorders which lead to NAFLD development needs to be determined.

There is intestinal dysbiosis, essentially observed at the phylum and genus level, a different microbiota profile between obese patients with and without NALFD and a potential association with the development of NAFLD related to obesity. Thus, we conclude that this justifies the importance of modulating the gut microbiota as a possible therapeutic approach for this common and cureless disorder.

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