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Multi-Omic Insights Into Mediterranean Diet-Associated Microbiota

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ABSTRACT

This study aimed to evaluate the gut microbiota and mycobiota composition, depending on the Mediterranean diet (MD) adherence, using metataxonomics. Combining metagenomics and metatranscriptomics, we also investigate the gene expression level in the bacterial community. Two groups of healthy subjects greatly differing in adherence were selected. Significant differences in microbiota composition were observed between individuals with high adherence (HAMD; mean 10.5 +/− 0.9 points) and low adherence (LAMD; 5.23 +/− 83 points). Notably, the olive oil, vegetable, and fruit consumption presented an important discriminant power between groups. *Saccharomyces*, *Penicillium*, and *Candida* were the most abundant genera. Mycobiota richness was higher in LAMD than in HAMD. *Aspergillus* was identified as a biomarker for LAMD, whereas *Yarrowia*, a potential probiotic, was a biomarker for HAMD. Metatranscriptomics indicated that Bacillota was the most metabolically active phylum in the gut microbiota. The low-abundant genus, *Methanobrevibacter*, showed high transcriptional activity, contributing to the crucial methanogenesis process. Gene expression analyses further highlighted functional differences. Overall, HAMD microbiota presented increased metabolic activity, protein synthesis, and cellular mobility. Overexpression of flagellin and urease genes may enhance immune response in HAMD. Further metatranscriptomic studies are necessary to deepen our understanding of intestinal microbiota transcriptional programs and their interactions with the diet and human health.

1 | Introduction

The human gut is inhabited by bacteria, archaea, viruses, and fungi, being the bacterial community (hereafter microbiota) the most abundant. The gut microbiota plays a fundamental role in nutrition by intervening in the degradation of complex polysaccharides and fiber, and in the metabolism of phenolic compounds and carotenoids from plant-based foods [1, 2]. These metabolic processes yield a wide variety of metabolites that participate in the development and regulation of the immune system, the proliferation, differentiation, and maintenance of the intestinal epithelium, protection against pathogens or regulation of the nervous system [3]. In turn, various intrinsic and extrinsic factors influence the structure and functions of the gut microbiota. In

Abbreviations: ANCOM-BC2, analysis of composition of Microbiome with bias correction; ASV, amplicon sequence variants; BH correction, Benjamini–Hochberg correction; HAMD, high adherence to Mediterranean diet; KEGG, Kyoto encyclopedia of genes and genomes; KO, gene family identifiers; ko, pathways; LAMD, low adherence to Mediterranean diet; MD, Mediterranean diet; MEDAS, Mediterranean diet adherence screener; MTG, metagenome; MTT, metatranscriptome; PCoA, principal coordinates analysis; PERMANOVA, permutational multivariate analysis of variance.

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fact, the diet is the major driving force shaping the composition and diversity of the gut microbiota [4].

The Mediterranean diet (MD) is a well-recognized healthy diet associated with a lower risk of many chronic diseases [5] and may also help to promote a diverse and rich gut microbiome. The impacts of MD modulation have mainly focused on changes in the composition of the bacterial population. However, the fungal community that also plays an important role in gut homeostasis remains understudied, and further research on the effect of diet on the mycobiome is needed. To date, numerous studies have been conducted on the effects of MD on the microbiota composition applying 16S ribosomal RNA gene sequencing [6–8]. However, only a few works address this issue through other omic approaches that provide more detailed information on bacterial species/strain, gene content, metabolites, or proteins [9–11]. Metaranscriptomics, an RNA-based approach, enables to relate genomic potential to the functional and metabolic capabilities of gut microbiota [12, 13]. In earlier work, we analyzed the metatranscriptome of healthy individuals, associating the functional profile with metabolically active bacteria [14]. Also, it has been determined that the metatranscriptome is more temporally dynamic than the metagenome, responding to environmental factors such as the diet [15, 16].

In this work, we applied, for the first time, metataxonomics, metagenomics, and metatranscriptomics, to elucidate the impact of the MD on the structure and metabolic activity of the bacterial community as well as its effects on the mycobiome composition. The integration of multi-omics techniques becomes crucial to greatly increasing our knowledge of the relationships between diet, host, and microbiome for the development of precision nutrition and microbiome-based interventions.

2 | Experimental Section

2.1 | Study Design and Subject Recruitment

We have carried out a cross-sectional study at baseline in a cohort of healthy individuals (men and women aged 18–75 years) recruited in a Mediterranean region (Valencia, Spain). Inclusion criteria were age, sex, being healthy, and the score reached on the screener for adherence to the MD (individuals were selected depending on the low or high adherence and paired by sex and age). To assess MD adherence of the participants, we used the 14-item questionnaire "Mediterranean Diet Adherence Screener" (MEDAS) [17] designed and validated by our group in the PREDIMED study (See the detailed 14-item questionnaire in Table S1). According to previous data on MD adherence [17], we predefined the scoring levels. Subjects with scores between 9 and 14 were included in the group of high adherence to the MD (HAMD, $n = 54$), and those with scores of 6 or less in the group with low adherence (LAMD, $n = 48$). In order to maximize the differences between groups (HAMD and LAMD), subjects with 7 or 8 points were not included in the study. Sample size was initially estimated at about 100 participants (about 50% in the group of HAMD and 50% in the LAMD group). Considering the complexity of the study, we selected a pooled estimator of the alpha diversity of the microbiota. Assuming a pooled SD of 10 units, the study would require a sample size of 44 per group of adherence, to achieve a power of 80% and a level of significance of 5% (two-sided), for detecting a difference in means between groups of 6 units. For other comparisons with less SD, the power will be greater. Exclusion criteria were: use of antibiotics, prebiotics or probiotics in the last 6 month; diseased; infectious diseases; liver disease or chronic renal failure; serious psychiatric disorders; any severe comorbid condition; alcohol abuse or addiction; history of major organ transplantation; concurrent therapy with immunosuppressive drugs or cytotoxic agents; current treatment with systemic corticosteroids; current use of weight loss medication; pregnant or breastfeeding women; any other condition that may interfere with the completion of the study protocol. Participants were recruited between November 2020 and July 2021 at the University of Valencia.

The study protocol was approved by the Ethics Committee of the Public Health Department and the Center for Public Health Research, Valencia, Spain (20200109/05 and 20220401/06) and the Ethics Committee of Research in Humans of the Ethics Commission in Experimental Research of University of Valencia (UV-INV_ETICA1206333). The study was conducted in accordance with the Helsinki Declaration and Good Clinical Practice Guidelines of the International Conference of Harmonization. The privacy rights of participants have been observed and all of them provided written informed consent prior to participate in this study.

2.2 | Dietary and Lifestyle Assessments

As indicated, adherence to the MD was assessed with the MEDAS questionnaire supervised by trained personnel. Validated questionnaires on physical activity, tobacco smoking, sleep quality, and chronotype were also completed.

2.3 | Sample Collection

Blood samples will be obtained through venous puncture after a fasting period of at least 8 h. Aliquots of buffy coat and plasma will be prepared from the collected blood and frozen at −80°C, using a blood collection tube for basic biochemical analyses.

The participants were asked to collect the fecal sample in a sterile Falcon tube with 10 mL of RNA later storage solution (Sigma–Aldrich Quimica, Madrid, Spain) and were instructed to subsequently store the sample in the freezer until the visit. After delivery to the researchers, the stool samples were immediately stored at −80°C until DNA and RNA extraction.

2.4 | Anthropometric, Blood Pressure, and Biochemical Measures

Anthropometric measurements and blood pressure were determined by trained staff following standard procedures. Basic biochemical analyses included fasting plasma glucose, plasma lipids, uric acid, kidney function, and liver function.

2.5 | Microbiome Analysis

2.5.1 | Total DNA Extraction and Sequencing

Fecal samples were treated as previously described [18]. Then, the total DNA extraction was performed in the robotic workstation MagNA Pure LC Instrument (Roche) using the MagNA Pure LC DNA isolation kit III (bacteria and fungi) (Roche, Switzerland).

To analyze the mycobiome composition, we amplified the Internal Transcribed Spacer 2 (ITS2) region of the rRNA operon using the primers ITS3-F and ITS4-R, and the PCR conditions described elsewhere [18]. To assess the bacterial composition from the fecal samples, the V3–V4 region of the 16S ribosomal RNA gene was amplified by PCR with the forward and reverse primers following Illumina protocol. The 16S and ITS2 amplicon libraries were constructed following Illumina instructions and sequenced using the Kit v3 (2 × 230 cycles) in a MiSeq platform (Illumina, Netherlands). To construct the metagenomic libraries, we used the total DNA with the Illumina NexteraXT DNA Sample Preparation Kit, according to the manufacturer's instructions. The sequencing was performed using NextSeq2000 platform (300 bp, paired end), resulting in 712 million read pairs.

2.5.2 | Total RNA Extraction and Sequencing

Total RNA extraction will be carried out using the Rneasy PowerMicrobiome Kit (Quiagen, Germany). Illumina Stranded Total RNA Prep Ligation with Ribo-Zero Plus kit (Illumina, Netherlands) was used for ribosomal RNA depletion and construction of the libraries following the manufacturer's instructions. Sequencing was carried out on a NextSeq2000 sequencer with the NextSeq2000 P2 sequencing kit (2 × 250 bp) that approximately yields 263 million read pairs.

2.6 | Sequence Analysis and Statistics

2.6.1 | Amplicon Analysis

First, quality assessment was done to all the samples, where low quality and short reads were filtered out and trimmed. Then, the sequences were analyzed with the software DADA2 (v1.20.0) [19], in R (v4.1.0) [20], which infers the amplicon sequence variants (ASV) with their sample-wise abundances. Also, the ASVs with homology to the *Homo sapiens* genome were removed. Next, the taxonomic classification was obtained by BLAST comparison against the reference database SILVA (v138.1) for bacterial ASVs and against the UNITE ITS database (v9.0) for fungal ASVs [21, 22], obtaining contingency tables at the ASV and genus level.

The Shannon diversity and Pilou's evenness indexes, and Chao1 richness estimator at the ASV and genus level were obtained with vegan library from R package. Permutational multivariate analysis of variance (PERMANOVA) was performed using the adonis function from the vegan library with 900 permutations. To evaluate the potential confounding effects of different factors, such as age, gender, body mass index, smoking, walking habits, Epworth Sleepiness Scale (ESS), chronotype, and sleep hours, on the association between adherence of MD and microbiota composition, we used PERMANOVA test including fixed terms of interaction in the model. Analysis of Composition of Microbiome with Bias Correction (ANCOM-BC2) was used to identify differentially abundant taxa at the ASV level between HAMD and LAMD groups, and then, we applied Boruta algorithm to select relevant taxa [23, 24]. Beta-diversity, the Bray–Curtis dissimilarity index, and principal coordinates analysis (PCoA) were generated with in-house R scripts. We used envfit function from the vegan library in R platform to determine which foods from the MEDAS test presented higher discriminant power between HAMD and LAMD groups, and with which taxa are correlated.

2.6.2 | Metagenomic and Metatranscriptomic Analysis

Metagenome (MTG) and metatranscriptome (MTT) paired-end reads are filtered out by quality and length and trimmed. Also, human and ribosomal RNA reads were removed. Then, MTG and MTT sequences were analyzed with the SqueezeMeta pipeline with merged mode (v1.6.2) [25]. The SqueezeMeta pipeline used GenBank nr database and the lowest common ancestor algorithm for the taxonomic assignment. For functional annotation, the gene sequences were compared against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database obtaining the gene family identifiers (KO) [26]. This pipeline merges all the taxonomic and functional results and generates different tables such as "gene table" that contains information about taxonomy, function, contig, abundance in the samples, and amino acid sequence, allowing a taxon to be linked to a function and its contribution to be determined. The raw counts of KOs and pathways (ko) for each sample were normalized by gene length and sequencing depth giving transcripts per kilobase million (TPM) normalization. To assess significant differences of KOs, we applied the Wilcoxon rank-sum test using the Benjamini–Hochberg (BH) correction (adjusted p-value<0.05). Also, we performed an enrichment analysis that allowed us to identify statistically significant over/under-represented pathways. To detect over- or under-expressed genes, we calculated the log2 RNA/DNA abundance ratio (TPM) for each KO in all samples. We considered that a gene was over- or under- expressed if the log2 RNA/DNA abundance ratio was significantly different from zero by applying the Wilcoxon rank-sum test using the BH correction (adjusted p value < 0.05) and higher than 2 (absolute value).

2.7 | Statistical Analysis of the Sample Characteristics

To evaluate differences in the continuous variables between groups, we used the Wilcoxon rank-sum test. Student's t-test was used to compare normally distributed variables. The Chi-square test and Fisher's exact test were used for categorical variable comparisons. A nominal two-sided p-value < 0.05 was considered statistically significant.

3 | Results

3.1 | Cohort Characteristics

In this study, we analyzed 102 healthy participants, depending on the level of adherence to the MD. Fifty-four individuals were

included in the HAMD group (adherence from 9 to 14 points) and 48 in the LAMD group (adherence equal to or less than 6 points). Participants in both groups were paired by sex and age. Table 1 shows the main characteristics of these groups. Due to the study design, mean adherence to the MD in the HAMD group was very high (10.47 +/− 0.95 points) in comparison ($p = 6.9 \times 10^{-49}$) with the LAMD group (5.23 +/− 0.83 points). When analyzing the adherence to the specific items (See Table S1 for compliance), we detected strong differences in the adherence to the olive oil consumed, vegetables, and fruits. Also relevant were the differences for "sofrito", fish, nuts, sweets, legumes, and red meats. Less significant differences (< 0.05) were detected for wine and sugar-sweetened beverages. No differences were detected for butter. Interestingly, no significant differences in other lifestyle factors or basic biochemical parameters were observed (Table 1).

The proportions of normal weight, overweight, and obese subjects were: 59%, 29.6%, and 11.1% in the HAMD group versus 43.8%, 37.5%, and 18.8%, respectively, in the LAMD. The differences did not reach the statistical significance ($p = 0.110$ for trend).

3.2 | Composition and Diversity of the Microbiota in LAMD and HAMD Groups

After adjusting by confounding variables such as age, sex, BMI, among others (Table S2), the diet, as adherence to MD (HAMD and LAMD), still explains significantly the microbiota composition variability at the ASV and genus level ($p = 0.0011$, $p = 0.0022$, respectively) (Figures 1A,B). Moreover, the interaction between BMI and diet had an effect on the microbiota composition ($p = 0.0044$). Thus, we grouped the participants in normal weight (18.5 kg/m^2 < BMI < 24.9 kg/m^2) and overweight/obese (BMI > 25 kg/m^2) groups, in which the overall microbiota composition was still significantly different between HAMD and LAMD individuals (Figure S1). The richness estimator, Chao1, was significantly higher in the HAMD group ($p = 0.0006$) at the ASV level but no differences were found in Shannon diversity and Pilou's evenness indexes, indicating an evenly homogeneity in the microbiota composition in each group. The microbiota was composed of bacteria from the phyla of Bacillota (formerly, Firmicutes) (54.96%), Bacteroidota (Bacteroidetes) (33.93%), Actinobacteriota (Actinobacteria) (5.63%), Verrucomicrobiota (Verrucomicrobia) (2.71%), and Pseudomonadota (Proteobacteria) (2.14%). We observed that the relative abundance of Verrucomicrobiota was higher in HAMD-associated microbiota ($p = 0.0127$, adj. $p = 0.0951$) while Actinobacteriota was higher in the LAMD group ($p = 0.0253$, adj. $p = 0.1268$). Additional prokaryotes found were archaea, being *Methanobrevibacter* (0.57%) the most abundant genus (Figure S2). *Methanobrevibacter* did not show a significant difference between HAMD and LAMD groups after ANCOM-BC2 normalization. Differently abundant the ASVs between HAMD and LAMD were identified using ANCOM-BC2 and Boruta analysis (Figure 1C, Table S3). Thus, 10 gut commensal genera were significantly more abundant in the HAMD microbiota, highlighting *Prevotella9, Roseburia, Oscillospiraceae UCG-002, Lachnospiraceae ND3007 group*, or *Blautia* among others. In addition, 5 taxa, corresponding to *Ruminococcus gnavus*, *Ruminococcus torques, Ruminococcaceae incertae sedis, Bacteroides uniformis*, and *Anaerostipes hadrus*, represented the biomarkers for the LAMD group (Figure 1C).

Through the MEDAS questionnaire, we determined that the daily consumption of olive oil, vegetables, and fruits was significantly higher in the HAMD group (p = 2.e−15, p = 4.43e−14, and p = 1.03e−08, respectively). Furthermore, according to the consumption pattern, we observed, by applying envfit program at the ASV and genus level, that the daily consumption of olive oil (ASV $p = 0.024$, genus $p = 0.001$), vegetables (ASV $p = 0.035$, genus $p = 0.007$), and to a lesser extent fruits (ASV $p = 0.075$, genus $p = 0.027$) had an important discriminant power between LAMD- and HAMD-associated microbiota. Moreover, the consumption of olive oil, vegetables, and fruits, presented a high positive correlation (> 0.9) with the taxa enriched in HAMD-microbiota such as *Oscillospiraceae UCG-002*, *Blautia, Alistipes, Prevotella9*, *Roseburia*, and *Lachnospiraceae ND3007 group* (Table S4). Although there was a small significant difference in soft drink consumption between the two groups ($p = 0.033$), these drinks presented a significant discriminant power between HAMD and LAMD microbiota at both the ASV ($p = 0.048$) and genus ($p = 0.017$) levels, being positively correlated mainly with the LAMD biomarkers (Table S4).

3.3 | Composition and Diversity of the Mycobiota in LAMD and HAMD Groups

Regarding the alpha diversity of the mycobiota and unlike what was found for the bacterial community, Chao1 estimator was significantly higher in LAMD group at the ASV and genus level ($p = 0.02$ and $p = 0.01$). Moreover, the mycobiota was significantly less diverse and rich than the microbiota (p = 2.2e−16) in both groups. No significant difference was found in terms of beta-diversity at genus level. *Saccharomyces* was the most abundant genus in both groups (12.4% in HAMD and 12.3% in LAMD), followed by *Penicillium* (7.1% and 7.7%, respectively) and *Candida* (6.4% and 7.1%, respectively), whereas that *Aspergillus* was significantly more abundant in LAMD ($p = 0.05$) and the yeast *Yarrowia* in HAMD ($p = 0.049$) (Figure 2).

3.4 | MD-Associated Metagenome and Metatranscriptome

We analyzed the bacterial MTG and MTT for fecal samples of 101 individuals, belonging 53 individuals to the HAMD group and 48 to the LAMD group.

To assess the functionally active microbiota, we determined the taxonomic composition of each sample from the MTG and MTT (Figure 3). The microbiota composition retrieved from MTT, regardless of the adherence to MD of the individuals, was significantly dominated by Bacillota (p = 5.5305e−07, adj. p-value = 8.3e−07) and it also presented a higher relative abundance of the minor phyla Euryarchaeota (p = 2.7053e−18, adj. p-value = 2.4348e−17), Actinomycetota (p = 2.0916e−05, adj. p-value = 2.6893e−05), and Synergistota (p = 4.6940e−15, adj. p-value = 1.4082e−14) than those obtained from MTG (Figure 3). Also, we calculated the contribution of each species to the metatranscriptome as the ratio of the relative abundance in the MTT divided by the relative abundance in MTG (Table S5). We observed that several genera were over-represented in the MTT

TABLE 1 | General characteristics of the study population.

	HAMD ($N = 54$) [a]	LAMD ($N = 48$) [b]	*p*-value
Woman gender	50.00%	52.00%	0.99
Age (years)	40.61 (SD:16.39)	38.48 (SD:13.12)	0.44
MEDAS-14			
Mean MEDAS score	10.47 (SD: 0.95)	5.23 (SD: 0.83)	$6.7x10^{-49}$
Olive oil amount (% adherence)	87.00%	7.8%	2.1x10–[15]
Vegetable	70.40%	0,02	$4.4x10^{-14}$
Fruit	70.40%	14.60%	$1.0\ x10^{-8}$
Nuts	75.90%	33.30%	$1.0\ x10^{-5}$
Fish	50.00%	14.60%	$1.2\ x10^{-4}$
Legumes	40.70%	12.50%	$1.1\ x10^{-3}$
Red meat	87.00%	54.20%	0
Sugar-sweetened beverage	90.70%	75.00%	0.03
Anthropometry			
BMI (Kg/m^2)	24.6 (SD:3.99)	26.1 (SD:5.09)	0.19
Fat mass (%)	25.58 (SD:9.53)	26.94 (SD:9.09)	0.51
Waist circumference (cm)	84.90 (SD:15.01)	86.16 (SD:13.11)	0.54
Blood pressure (mmHg)			
Systolic BP [c]	127.75 (SD:14.33)	125.37 (SD:14.31)	0.45
Diastolic BP	75.25 (SD:9.11)	73.64 (SD:9.22)	0.19
Blood chemistry (mg/dL)			
Total cholesterol	191.79 (SD:44.43)	197.33 (SD:29.68)	0.13
Glucose	95.5 (SD:13.62)	92.37 (SD:10.21)	0.34
Triglycerides	96.79 (SD:13.62)	94.58 (SD:45.19)	0.38
Uric acid	5.36 (SD:1.49)	5.33 (SD:1.29)	0.98
Total bilirubin	0.93 (SD:1.16)	0.89 (SD:0.57)	0.90
Creatinine	0.81 (SD:0.15)	0.79 (SD:0.16)	0.65
ALT (U/L) [d]	20.24 (SD:7.64)	24.79 (SD:22.27)	0.32
Lifestyle			
Smoking (No)	87.00%	75.00%	0.19
Walk 20 min/day (Yes)	88.80%	85.42%	0.82
Sleep			
Epworth sleepiness scale			
Severe excessive sleepiness	1.85%	2.08%	0.72
Excessive sleepiness	14.82%	20.83%	
Normal sleepiness	83.33%	77.08%	
Sleep hours	6.43 (SD:1.07)	6.6 (SD:1.04)	0.54
Chronotype			
Morning_more_evening	29.63%	47.92%	0.75
Evening_more_morning	18.52%	10,.42%	
Clearly morning	38.89%	29.16%	
Clearly evening	12.96%	12.50%	

Note: Values are means (SD) for continuous variables and % for categorical variables.

[a]HAMD, High Adherence Mediterranean Diet.

[b]LAMD, Low Adherence Mediterranean Diet.

[c]BP, Blood Pressure.

[d]ALT, Alanine transformamiento.

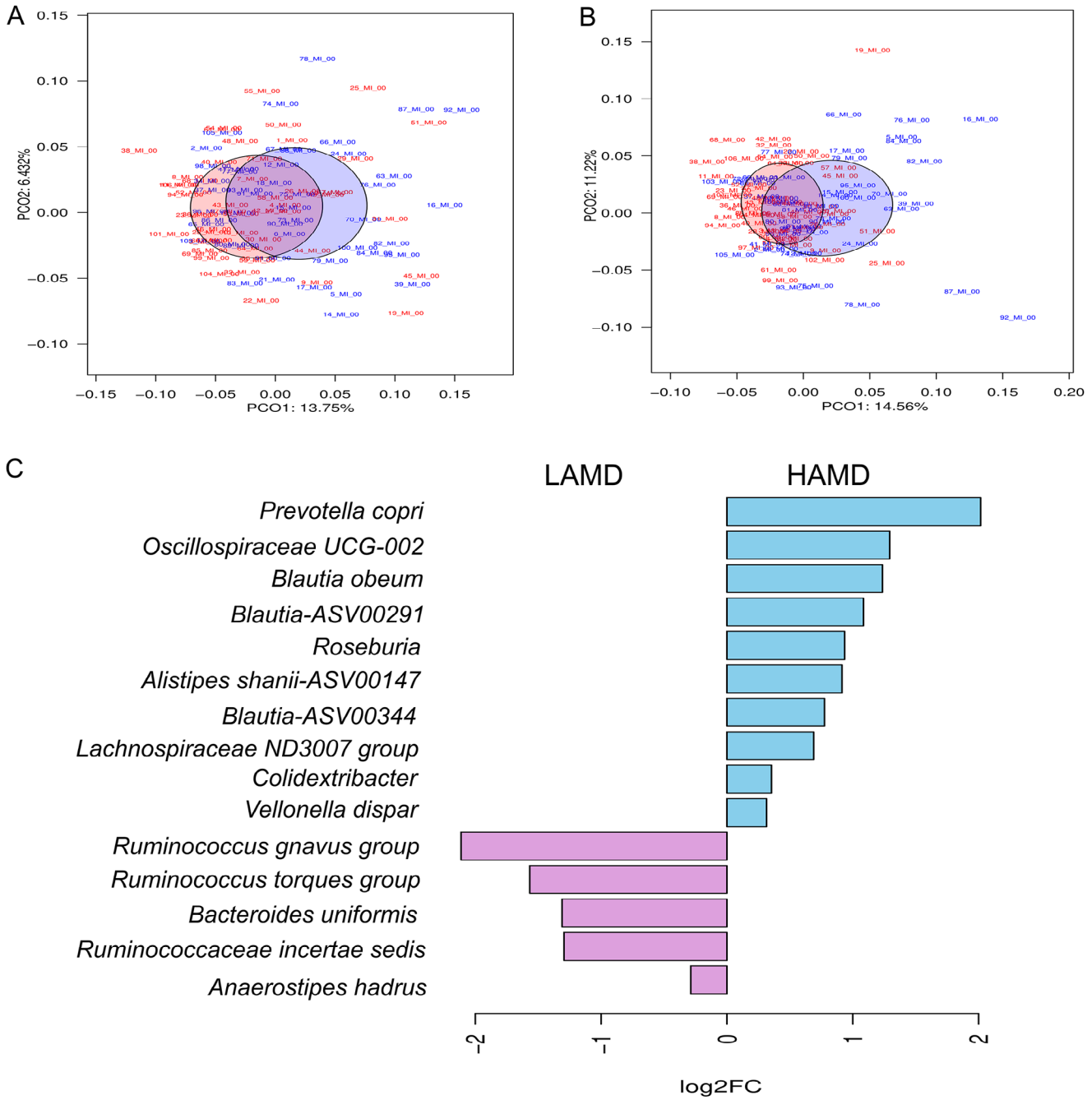

FIGURE 1 | Analysis of overall microbiota composition. (A) Principal coordinates analysis (PCoA) based on the Bray–Curtis dissimilarity index at the ASV level. (B) PCoA based on the Bray–Curtis dissimilarity index at the genus level. Adjustment variables include age, gender, body mass index, smoking and walking habits, Epworth Sleepiness Scale, chronotype, and sleep hours. Red represents the high adherence to Mediterranean diet group (HAMD) and blue represents the low adherence to the Mediterranean diet group (LAMD). (C) Differential microbiota composition of HAMD and LAMD groups. Fold change (FC) for the significant differential ASVs identified by ANCOM-BC2 and Boruta analysis.

regardless of their low relative abundance in the MTG, such as *Methanosphaera* and *Methanobrevibacter* from Euryarchaeota phylum (Figure S3).

With respect to the functional annotation, we detected 8023 KOs and 491 pathways across all the samples at either MTG or MTT level. We observed that 17 KOs from MTT (MTT-KO) were missing in the MTG. These MTT-KOs would represent transcripts of low-abundant but transcriptionally active bacteria. Moreover, the whole functional composition tended to be different between HAMD and LAMD, for both MTG (gene content) and MTT (transcript content) (PERMANOVA, $p = 0.057$ and $p = 0.051$, respectively) (Figure S4). Next, we evaluated the gene expression level by means of log2 RNA/DNA abundance ratio for each KO in both groups. Thus, we found 5042 and 5089 KOs differentially (over or under) expressed in HAMD and LAMD groups, respectively. We then used these KOs as input in a KEGG pathways enrichment analysis, selecting those that presented an adjusted p-value < 0.05. Thus, we analyzed the expression level of the selected pathways in each group. Figure 4 showed that the pathway "RNA transport"

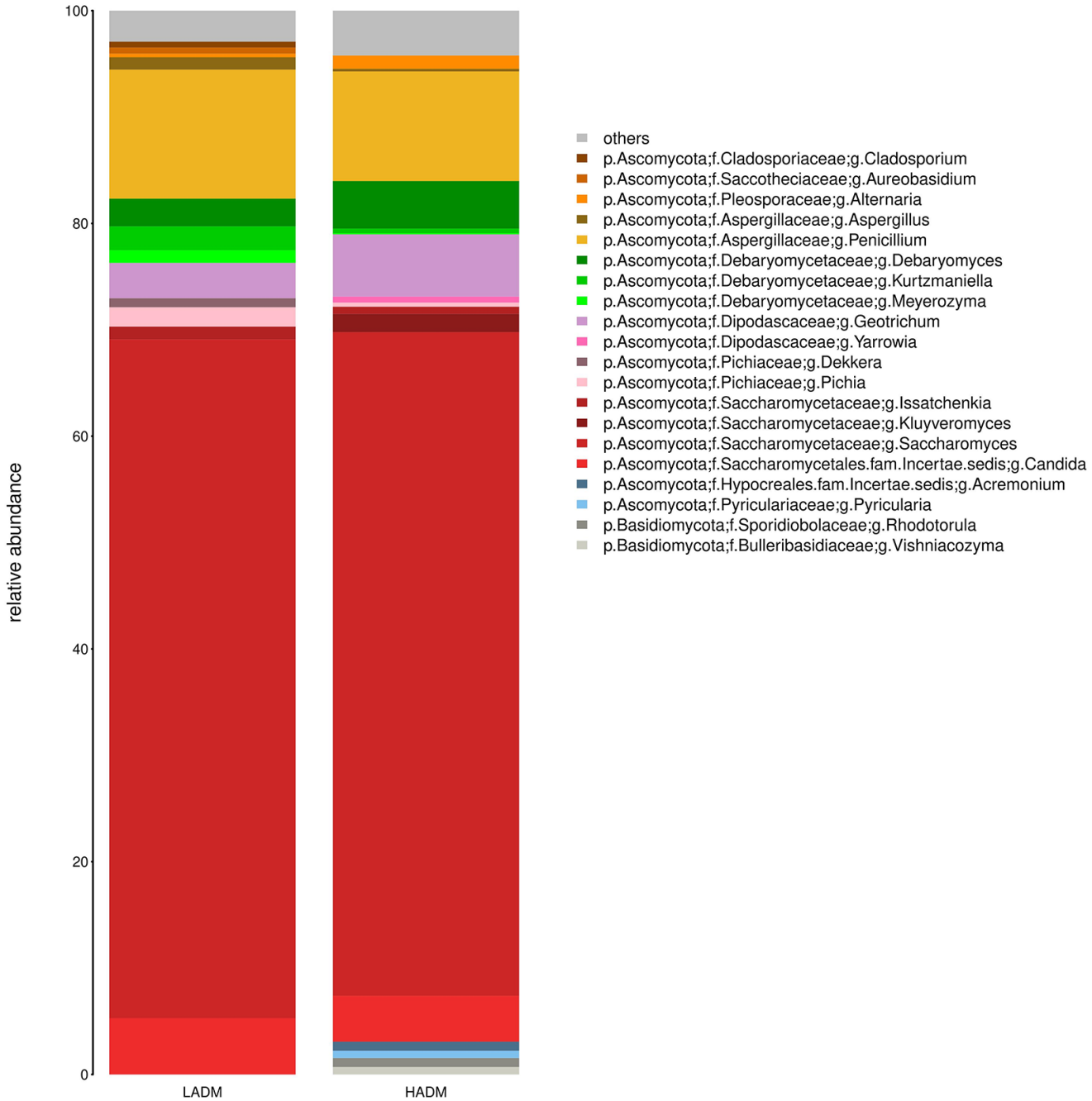

FIGURE 2 | Mycobiota composition. Relative abundance of the fungal genera in "high adherence to Mediterranean diet" (HAMD) and "low adherence to Mediterranean diet" (LAMD) groups. Each taxon represents the mean of the percentages of that taxon in all samples in the group. "Others" represents the genera that have a relative abundance of less than 2% in all samples.

presented the highest overexpression level. Also, "chaperones and folding catalysts" and "methane metabolism" presented a high expression level in both groups. On the other hand, pathways related to "signal transduction", "protein signaling and cellular processes", "protein metabolism", and "cellular community" were under-expressed. Next, we search for pathways with differential expression levels between HAMD and LAMD (Figure 5). The pathways, "RNA transport", "messenger RNA biogenesis", "RNA degradation", "translation factors", "spliceosome", "exosome", "legionellosis", and "arginine biosynthesis", presented a significant higher overexpression in HAMD than in LAMD, while "fatty acid degradation" and "pyruvate metabolism" were differentially overexpressed in LAMD. We also identified the taxa which contributed to each of the KOs assigned to the differentially expressed pathways (Figure S5). In the HAMD group, the KOs included in the functional pathways belonging to the category "protein families: genetic information processing" exhibited a RNA-based microbial activity and protein folding. Thus, these KOs were mainly attributed to the genera *Bacteroides*, *Prevotella*, *Alistipes*, *Parabacteroides*, and *Odoribacter* from Bacteroidota; *Streptococcus* and *Faecalibacterium* from Bacillota; *Collinsella* and *Bifidobacterium* from Actinomycetota; and *Akkermansia* from Verrucomicrobiota. The K02406 (flagellin gene), which was assigned to the pathway "legionellosis", was related to bacterial

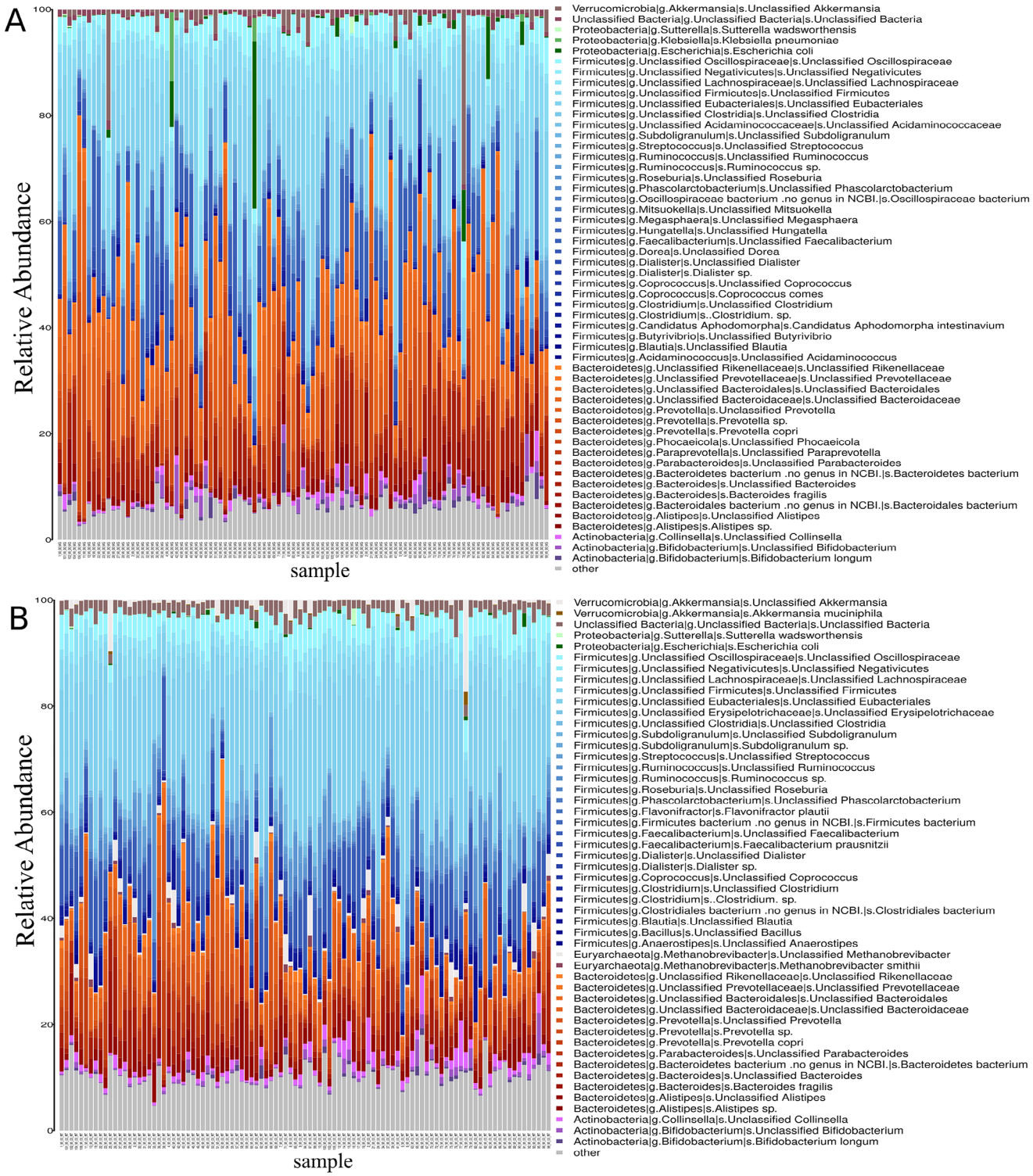

FIGURE 3 | Microbiota composition. (A) Relative abundance of the most abundant taxa from metagenomics (MTG) and (B) from metatranscriptomics (MTT). Each taxon represents the mean of the percentages of that taxon in all samples in the group. "Others" represents the taxa that have a relative abundance of less than 2% in all samples. Grey gradient represents Euryarchaeota; pink, Actinobacteriota; brown, Bacteroidota; blue, Bacillota; and green gradient, Pseudomonadota.

motility and was mainly expressed by Bacillota genera such as *Roseburia*, *Clostridium*, *Eubacterium, Flavonifactor*, *Anaerotruncus*, and *Butyrivibrio*. The K01428, K01430, and K14048 related to the pathway "arginine biosynthesis" represented a urease activity, and they were mainly mediated by bacteria belonging to *Clostridiaceae* and *Lachnospiraceae* families. However, K00262 (glutamate dehydrogenase), also assigned to "arginine biosynthesis" pathway, originated by Bacteroidota genera such as *Bacteroides*, *Prevotella*, *Alistipes*, *Parabacteroides*, and *Paraprevotella* in addition to *Clostridium, Megasphaera*, *Streptococcus*, *Ruminococcus*, and *Faecalibacterium* from Bacillota phylum. Moreover, the KOs related to protein synthesis and signaling

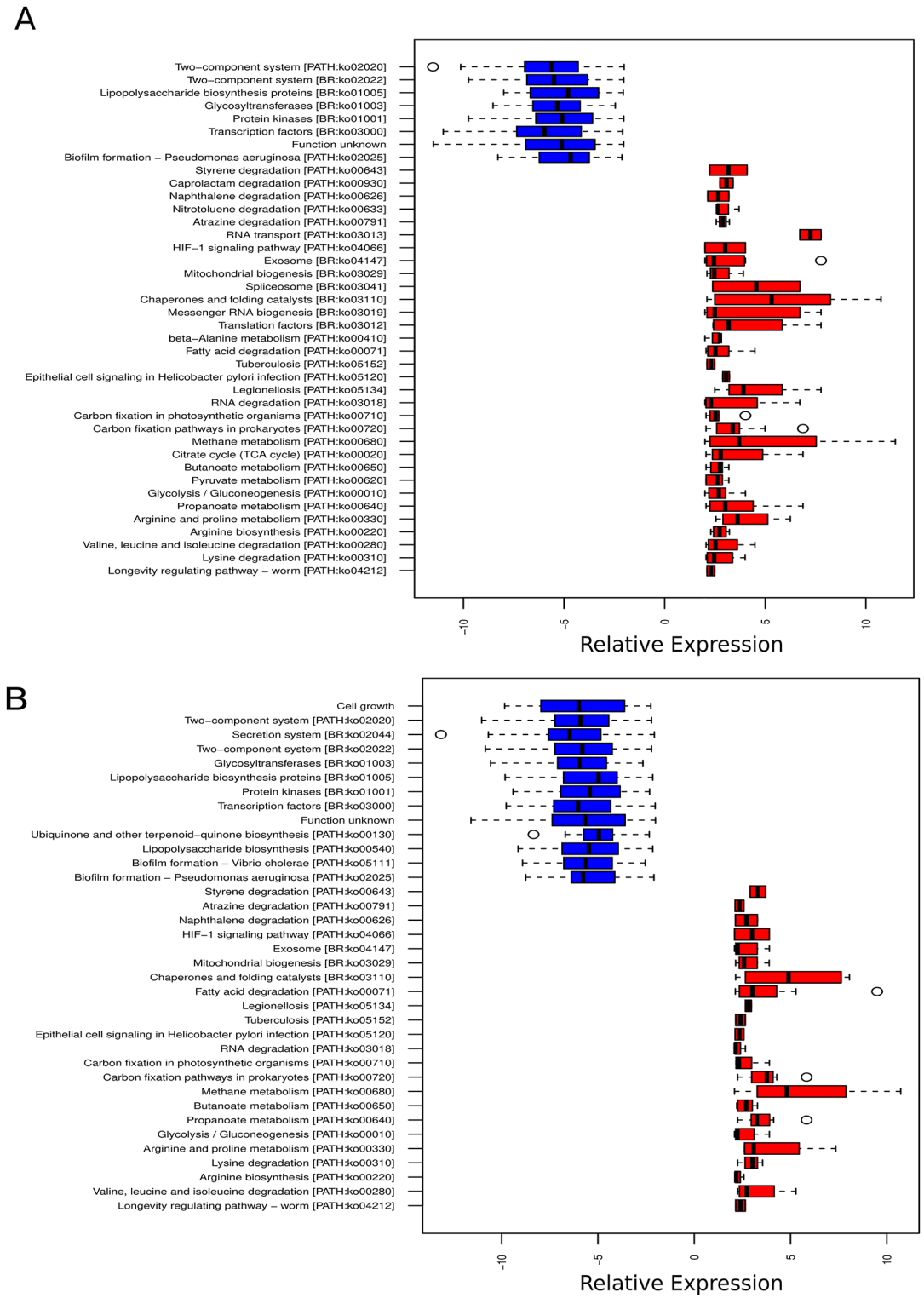

FIGURE 4 | Relative expression of the enriched metabolic pathways. (A) In "high adherence to Mediterranean diet" (HAMD) group. (B) In "low adherence to Mediterranean diet" (LAMD) group. The relative expression of the pathways corresponded to the mean of the relative abundance of the KOs assigned to each pathway in the metatranscriptome respect to that in metagenome (log2 RNA/DNA). In blue, under-expressed pathways, in red overexpressed pathways in each group.

processes, were mostly attributed to the low-abundant genera of Euryarchaeota, *Methanobrevibacter*, and *Methanoesphaera*. In addition, these two archaea genera were responsible for the over-expression of genes involved in methanogenesis in both HAMD and LAMD groups. Besides, fatty acid degradation and pyruvate metabolism pathways, which were differentially overexpressed in LAMD, were attributed to a diverse array of genera from Bacillota phylum.

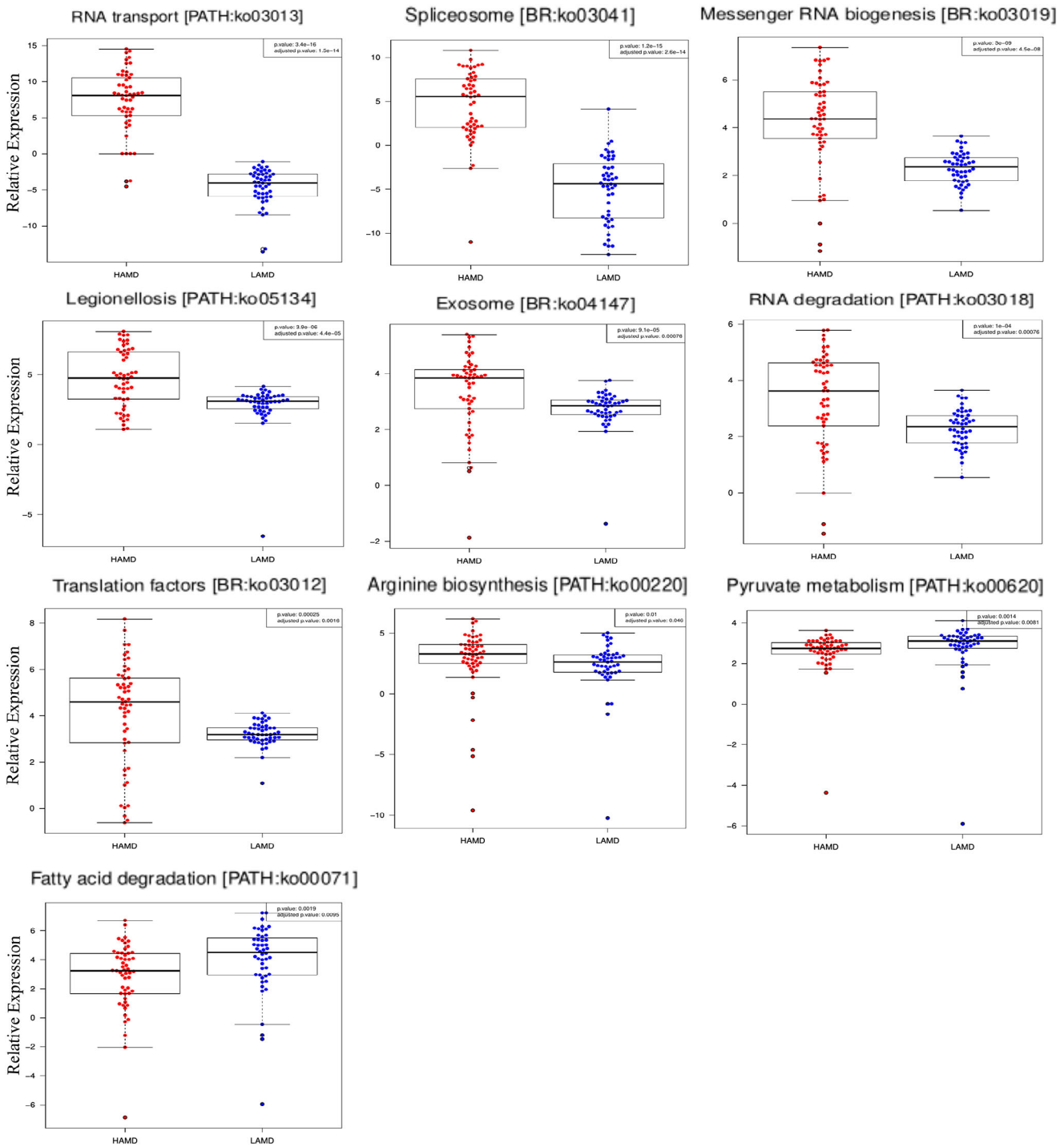

FIGURE 5 | Boxplots of the differential expression level of the enriched KEGG pathways. Differences in the expression level of the enriched metabolic pathways were assessed by Wilcoxon rank-sum test between "high adherence to Mediterranean diet" (HAMD, in red) group and "low adherence to Mediterranean diet" (LAMD, in blue) group. The relative expression of each pathway was calculated as the mean of the relative abundance of the KOs assigned to this pathway in the metatranscriptome with respect to that in metagenome (log2 RNA/DNA).

4 | Discussion

The interplay between diet and gut microbiota is a complex and dynamic relationship with a central role in human health. The MD characterized by high consumption of fruits, vegetables, whole grains, legumes, nuts, olive oil, moderate fish intake, and limited red meat, has consistently been associated with increased abundance of fiber-degrading and SCFA-producing bacteria [6, 7, 27–30]. In our study, carried out in healthy subjects from a Mediterranean population greatly differing in the MEDAS-14 score, adherence to the MD clearly shaped gut microbial structure, with distinct bacterial genera emerging as microbiota biomarkers for the HAMD and LAMD groups. Among the HAMD-associated genera, *Prevotella9* was significantly enriched. This commensal genus, known for its polysaccharide-degrading capacity, is characteristic of plant-rich diets and agrarian popu-

lations [7]. In support of this, envfit analysis identified a strong positive correlation between fruit and vegetable consumption and *Prevotella9* abundance. Additionally, other HAMD microbiota biomarkers have the ability to degrade plant fiber and to produce SCFA, such as *Oscillospiraceae UCG-002*, *Lachnospiraceae ND3007* group, or *Roseburia*. The strong association between Lachnospiraceae genera and fruit consumption further reinforces the pivotal role of dietary fiber in shaping the HAMD microbiota. Although the olive oil is a hallmark of the MD, few studies have addressed the effect of fatty acids on the microbiota [31–34]. Our results demonstrate a robust association between olive oil consumption and several HAMD biomarkers, including Oscillospiraceae UCG-002, A. shahii, Blautia, Prevotella9, and Roseburia. These findings align with both in vitro and in vivo studies showing that oleic acid-rich environments selectively promote the growth of beneficial bacterial genera [33, 34]. The genus Alistipes is of particular interest due to its ability to incorporate medium- and long-chain fatty acids from the gut lumen into membrane lipids such as phosphatidylethanolamine and sulfonolipids [35]. Walker et al. [36] demonstrated that sulfonolipids, including sulfobacin B, were markedly elevated in mice fed a high-fat diet enriched in oleic acid, and identified Alistipes and Odoribacter as major sulfonolipid producers. Recent evidence further suggests that sulfobacins A and B dampen inflammation by disrupting LPS-mediated TLR4 signaling [37]. Moreover, the LAMD group presented significantly higher intake of soft drinks, being the LAMD microbiota biomarkers strongly related to the consumption of these beverages, which are typically high in simple sugars.

Beyond bacteria, the gut mycobiome, despite representing only 0.01–0.1% of gut microbial diversity, has emerged as an important contributor to intestinal homeostasis [18, 38, 39]. Likewise, the mycobiome composition is influenced by diet, though few studies have explored the effect of dietary habits on its structure [18, 38, 39]. In our analysis, the LAMD group exhibited a greater number of fungal genera compared to HAMD. The high consumption of fruits and vegetables in the HAMD group likely led to greater production of SCFAs through fiber fermentation, which have known antifungal effects [39]. Hoffmann et al. [40] also found a negative correlation between SCFA levels and *Aspergillus*, which we identified as a biomarker of the LAMD group. Unlike Mitsou et al. [27], we did not find a greater abundance of *Candida albicans* in the HAMD group. Instead, *Yarrowia* was identified as a HAMD mycobiota biomarker. This yeast has been isolated from olive oil and characterized as a potential probiotic [41], showing antimicrobial and antioxidant activity as well as cholesterol-lowering ability. Thus, according to Carlino et al. [42], *Yarrowia* would be acquired from the olive oil with subsequent intestinal colonization.

Integrating metagenomic and metatranscriptomic data allowed us to characterize dietary influences on microbial gene expression. Notably, two low-abundance archaeal genera were highly transcriptionally active, overexpressing methanogenesis-related genes in agreement with Vázquez-Castellanos et al. [43]. These two archaea genera maintain bacterial fermentation and energy extraction efficiency by removing hydrogen excess and converting it into methane. Molecular chaperones assist in various bacterial processes, including *de novo* protein folding and stabilization, secretion, and damage repair under stress [44]. Moreover, their expression can be induced by various stimuli, including dietary compounds [45]. We detected an overexpression of different chaperones (DnaK, GroEL, IbpB, and ClpL) regardless of the diet, though their specific expression inducers may vary with dietary environments. In fact, Abulizi et al. [46] indicated, by metataxonomics and metaproteomics, that the type of fatty acids alters the relative microbial abundance and the predicted functions. In HAMD-associated microbiota, the overexpression of chaperone genes occurred as part of a burst of other differently overexpressed functions involved in the synthesis of proteins and information processing, suggesting higher metabolic activity and growth rate of the gut bacteria. We also detected overexpression of the flagellin gene in HAMD-associated microbiota. This result may imply enhanced cell motility, consistent with findings from the fecal metaproteome of vegetarians and vegans [9]. Furthermore, the immune system recognizes flagellin through Toll-like receptor (TLR)5, initiating an immune response. Recently, Clasen et al. [47] described a class of "silent" flagellins that are enriched in non-Western populations and mainly produced by *Lachnospiraceae* genera, such as *Roseburia* or *Lachnospira*. Thus, unlike the flagellin from pathogenic bacteria, which potently activates the immune system, silent flagellins bind to the immune receptor TLR5 but fail to trigger a strong inflammatory response, allowing the host immune system to tolerate beneficial commensal bacteria and to maintain gut homeostasis. Moreover, a recent study has identified a positive correlation between silent flagellin-producing bacteria, like Roseburia, and higher levels of high-density lipoprotein cholesterol, which is considered beneficial for cardiovascular health [48]. Given that flagellin expression is environmentally regulated, our findings suggested that MD, rich in fiber, would induce the expression of silent flagellin in HAMD *Lachnospiraceae* biomarkers, which, along with other proteins, would have a beneficial impact on human health. The HAMD microbiota also exhibited overexpression of urease genes. Because humans lack urease, colonic urea hydrolysis depends entirely on microbial activity. Thus, we found that this hydrolytic capability was mediated by commensal bacteria belonging to *Clostridiaceae* and *Lachnospiraceae* families. Although urease activity is often associated with pathogenicity, it also contributes to nitrogen recycling and amino acid biosynthesis. Studies in children on low-protein diets indicate that urease gene enrichment supports ammonia reuse for microbial and host amino-acid synthesis [49–51]. In line with this, we observed overexpression of glutamate dehydrogenase in the HAMD microbiota. This enzyme, broadly distributed in all living organisms, catalyzes the reversible conversion of glutamate to α-ketoglutarate and ammonia, generating ATP and contributing to nitrogen metabolism. Recently, Wang et al. [52] showed that reduced microbial ammonia production impairs brain glutamine availability and increases susceptibility to stress-related behaviors, underscoring the physiological relevance of this pathway. Finally, the overexpression of "fatty acid degradation" and "pyruvate metabolism" pathways in LAMD-associated microbiota suggested enhanced energy extraction, which may contribute to weight gain in individuals with low adherence to the MD.

In conclusion, the MD is associated not only with a beneficial microbial composition enriched in fiber-degrading and SCFA-producing bacteria but also with functional transcriptional signatures indicative of improved gut health and immune

modulation. The enrichment of probiotic-associated taxa such as Yarrowia and the selective induction of beneficial microbial activities such as silent flagellin expression and enhanced nitrogen recycling, underscore the multidimensional impact of the MD on gut ecosystem function. As the first study combining metagenomics and metatranscriptomics to assess the metabolic effects of MD adherence on the gut microbiota, our work highlights the importance of linking microbial identity with gene expression. Such integrative approaches are essential to understand individualized microbial responses to diet and to guiding the development of personalized nutritional interventions.

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The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding authors (maria.jose.gosalbes@uv.es and dolores.corella@uv.es), upon reasonable request. The sequences generated and analyzed during the current study are available in the EBI database under the project number PRJEB89354: for the 16S amplicons the accession numbers are from ERS24439591 to ERS24439692, for the ITS2 from ERS24439693 to ERS24439790, for metagenomic sequences from ERS24439791 to ERS24439791, and for metatranscriptomic sequences from ERS24439892 to ERS24439992.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting File 1: mnfr70450-sup-0001-TableS1.pdf.

Supporting File 2: mnfr70450-sup-0002-TableS2.pdf.

Supporting File 3: mnfr70450-sup-0003-TableS3.pdf.

Supporting File 4: mnfr70450-sup-0004-TableS4.pdf.

Supporting File 5: mnfr70450-sup-0005-TableS5.pdf.

Supporting File 6: mnfr70450-sup-0006-FigureS1-S5.pdf.