

Gut microbiome differences in youth with perinatally acquired HIV and fatty liver disease: a multicentre study

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Abstract

Background

Metabolic dysfunction-associated fatty liver disease (MAFLD) is increasingly prevalent in youth with perinatally acquired HIV (YPHIV). Gut dysbiosis has been proposed as a potential contributor to MAFLD, but its role in YPHIV remains poorly understood.

Methods

In this prospective multicentre study, 86 participants (34 YPHIV, 52 uninfected controls), with and without overweight-associated MAFLD underwent ultrasound liver steatosis assessment and stool 16S rRNA gene sequencing. Multivariable models evaluated microbial diversity, taxonomic abundance, and predicted functions..

Results

HIV infection was associated with reduced microbial diversity, distinct compositional shifts, and altered functional pathways, whereas MAFLD showed no significant overall microbiome signature. Within YPHIV, MAFLD correlated with decreased *Parabacteroides* abundance and subtle functional differences despite preserved diversity.

Conclusions

HIV infection predominantly influences gut microbiome composition in youth, with MAFLD-related microbial alterations being modest. These findings underscore the importance of accounting for HIV-associated dysbiosis in studies of liver disease and suggest potential microbial targets for further investigation in YPHIV with MAFLD.

Background

Metabolic dysfunction-associated fatty liver disease (MAFLD) has emerged as one of the leading causes of chronic liver disease worldwide, paralleling the increasing prevalence of obesity and metabolic syndrome. Current estimates indicate that MAFLD affects 20–40% of adults in Western countries and approximately 8–10% of children, making it the most common chronic liver disease in the pediatric population [1–4]. While most patients remain asymptomatic, a substantial proportion progress to steatohepatitis, fibrosis and, ultimately, cirrhosis, highlighting the need to better understand the mechanisms underlying disease development and children represent a special concern.

People living with HIV (PLWH) are at particularly high risk of developing MAFLD, with reported prevalences ranging from 28.8% to 48.0%, even in the era of effective antiretroviral therapy [5–7]. This burden is also observed among children and youth with perinatally acquired HIV (YPHIV), in whom MAFLD prevalence ranges from 8% to 30%, consistently exceeding that reported in their uninfected

peers. Unlike the general population, however, a substantial proportion of MAFLD cases in PLWH occur in the absence of obesity or other classical metabolic risk factors, suggesting that HIV related mechanisms contribute to disease pathogenesis beyond traditional metabolic dysfunction [8, 9].

The mechanisms underlying MAFLD among PLWH are likely multifactorial. Besides established contributors such as obesity, Western diet and genetic susceptibility, persistent immune activation, chronic inflammation, and microbial translocation have all been implicated in liver injury despite long-term viral suppression [9, 10] (ref). In addition, lifelong exposure to HIV infection and antiretroviral therapy may further contribute to metabolic dysfunction in YPHIV, making this population particularly vulnerable. Therefore, it is important to investigate non-classical pathways involved in MAFLD development in this group [11, 12].

Among these potential mechanisms, the gut microbiome has attracted growing attention. The gut-liver axis plays a central role in metabolic homeostasis, and intestinal dysbiosis has been associated with both MAFLD and non-alcoholic steatohepatitis in adults and children [13–16]. Increased abundance of bacterial pathways involved in lipopolysaccharide biosynthesis and other pro-inflammatory metabolites suggests that alterations in the intestinal microbiota may contribute to hepatic steatosis through immune and metabolic pathways [13, 17]. HIV infection itself is characterized by profound and persistent alterations of the gut microbiome, which have been linked to chronic immune activation and systemic inflammation [18–23]. Whether these HIV-associated microbial changes contribute to the development of MAFLD, particularly among lean PLWH without conventional metabolic risk factors, remains largely unknown.

Therefore, we aimed to characterize the gut microbiome of youth with and without perinatally acquired HIV, according to the presence or absence of MAFLD, and to explore the relative contribution of HIV infection and metabolic factors to microbial composition.

Methods

Study design and participants

We carried out a prospective study within the Spanish National Network of Children and Adolescents Living with HIV (CoRISpe). Youth with perinatally acquired HIV under follow-up in Hospital Universitario La Paz and Hospital General Universitario Gregorio Marañón in Madrid, Spain, were recruited from June 2018 to December 2020. Uninfected siblings, partners, and adolescents who attended for post-exposure prophylaxis were included as uninfected controls (UC), matched by gender and age (± 2 years). A group of adolescents diagnosed with MAFLD under follow-up at Vall d'Hebrón Hospital, Barcelona, Spain, were included for the comparison of microbiome. The recruitment of participants from different centers and geographical settings may have introduced residual confounding related to center-specific or environmental factors, including dietary habits, local exposures, sample handling and transport procedures, all of which may influence gut microbiome composition and could not be fully accounted for in the present analyses. As these participants were recruited on the basis of a pre-existing

diagnosis of overweight and MAFLD, this subgroup had, by design, a 100% prevalence of MAFLD. The control group therefore comprised two strata with structurally different expected MAFLD and metabolic risk factors prevalence; uninfected siblings/partners/post-exposure prophylaxis attendees, reflecting background population rates, and MAFLD participants. All participants underwent liver ultrasound, and met the diagnostic criteria for MAFLD based on a combination of two ultrasound- criteria: shear wave elastography (pSWE) and/or the Controlled Attenuation parameter (CAP) by transient elastography (TE), as defined in our previous study [24]. CAP values in dB/m were reported as the median of 10 acquisitions, and the cut-off point of 248 dB/m defined by Karlas et al was used to define the presence of steatosis (Karlas et al. 2017). Previously defined criteria (Dasarathy et al. 2009; Bril et al. 2015) were used for the diagnosis of steatosis by pSWE: increased parenchymal echogenicity of the liver, hepatic vein blurring, portal vein blurring, and visualization of the diaphragm. Liver biopsy was not performed, according to guidelines [25].

Exclusion criteria for participants and controls were: history of HBV or HCV infection or co-infection, chronic inflammatory diseases, acute infections or opportunistic diseases at the moment of inclusion, and reporting >30g in men and >20g in females of alcohol intake.

The study was approved by the Ethics Committee of Hospital La Paz (PI-3232) and all participating hospitals, and was conducted according to the Declaration of Helsinki. All participants and/or their legal guardians signed informed consent/assent before inclusion in the study.

Study variables

Epidemiological, anthropometric, and clinical characteristics were collected from medical records. Weight, height, waist, and hip measurements were taken according to a unified protocol. Body Mass Index (BMI) was automatically calculated and z-score adjusted. Standard deviations for BMI according to age and gender were calculated using the 2010 Spanish Growth charts (Carrascosa 2014). Overweight was defined in children and adolescents as a BMI z-score between +1 and +2 SD, and obesity as > 2 SD. For patients $\geq$ 18 years, the WHO definition for overweight (BMI>25) and obesity (BMI>30) was used (WHO 2024).

For YPHIV, HIV and ART-related variables were collected from the CoRISpeS database. The CDC 2014 classification was used to categorize HIV disease stages. HIV viral load was considered suppressed when < 50 copies/mL.

We use MAFLD rather than the more recent MASLD terminology, as this study was designed and diagnostic criteria applied prior to the 2023 nomenclature update. As MAFLD is an emerging comorbidity specifically of clinical concern in youth with perinatally acquired HIV, the primary analysis of this study was the comparison of gut microbiome composition and function according to MAFLD status within the YPHIV subgroup. Analyses comparing participants by HIV status and by MAFLD status across the full sample were conducted first to establish the extent to which HIV infection itself shapes the gut microbiome, and are presented as contextual analyses.

Sample processing

Fasting blood was drawn for hemogram and biochemistry, including a complete lipid profile. Liver enzymes (AST, ALT, and GGT) were considered elevated according to laboratory ranges: ALT > 35UI/L, AST and GGT when > 40 UI/L. Fasting total cholesterol was considered elevated when > 200 mg/dL, and triglycerides when >150 mg/dL. Real-time measurements of plasma viral load (VL) were quantified using the Cobas TaqMan assay (Roche Diagnostics Systems Inc., Branchburg, NJ) with a detection limit of 50 copies/ml. Absolute and percentage of CD4 and CD8 T-cell (measured with standard flow cytometric methods) were performed for PLWH at the participating centers. For the two comparison groups, HIV, HBV, and HCV infection were excluded.

Stool samples were collected in regular stool collection tubes at inclusion, processed, and cryopreserved at -80°C until use. Microbiome analysis was performed at Ramón y Cajal Research Institute (IRYCIS). For sequencing, DNA extraction was performed in the robotic workstation MagNA Pure LC Instrument (Roche) using the MagNA Pure LC DNA isolation kit III (Bacteria, Fungi) (Roche). Total DNA was quantified with a Qubit Fluorometer (ThermoFisher). For each sample, the V3-V4 regions of the 16S rRNA gene were amplified, and the amplicon libraries were constructed following Illumina instructions (Illumina, San Diego, CA, USA). The sequencing was performed using the kit V3 (2 × 300 cycles) with the MiSeq sequencer (Illumina) at the IRYCIS Sequencing Service, Madrid, Spain. We obtained an average of 48,795 (removing outliers) 16S rRNA gene joined sequences per sample.

Bioinformatic and statistical analysis

Raw sequencing reads were processed using the nf-core/ampliseq pipeline (v2.9.0). Briefly, reads underwent quality assessment with FastQC, adapter and quality trimming using Cutadapt, denoising and chimera removal with DADA2, and taxonomic assignment against the SILVA reference database (release 138) using QIIME2. To minimize biases related to sequencing depth, samples were rarefied to 26089 reads, a threshold at which rarefaction curves reached a plateau (Supplementary Figure S1).

Microbial analyses were performed at the genus level. Alpha diversity, reflecting within-sample microbial diversity, was estimated using the Shannon index and compared between groups by analysis of covariance (ANCOVA). Beta diversity, reflecting differences in overall microbial community composition between samples at the genus level, was assessed using Bray-Curtis dissimilarity, visualized by principal coordinates analysis (PCoA), and compared using PERMANOVA (ADONIS test from the R package vegan v2.6-7 correcting for co-variables, by="margin" parameter with function betadisper). Homogeneity of group dispersions was assessed using betadisper, and ANOSIM was performed when appropriate to exclude dispersion-driven differences. For non-significant comparisons, effect sizes with 95% confidence intervals (partial η^2 [or ω^2] for ANCOVA-adjusted Shannon diversity; R^2 from PERMANOVA) are additionally reported to aid interpretation of null findings.

Differential taxonomic abundance, aimed at identifying taxa associated with HIV or MAFLD status after covariate adjustment, was assessed using ANCOM-BC2 (Bioconductor release v3.19) at the genus level,

on the non-rarefied genus-collapsed count table (275 genus-level taxa), since ANCOM-BC2 models sampling fractions internally and does not require rarefaction. Of the genus-level taxa analyzed, 40/275 (14.5%) could not be confidently assigned to a genus against SILVA 138; these are reported at their lowest resolved rank and explicitly labelled as unclassified (e.g. 'unclassified Ruminococcaceae'). To analyze gut microbiome composition while controlling for confounders, baseline covariates (HIV status, MAFLD, overweight status, and age) were incorporated into models, dropping invariant factors within subgroup analyses. Low-prevalence taxa (present in <10% of samples) were excluded, and taxonomic resolution was restricted to the genus level due to 16S rRNA gene sequencing limits. Differential abundance was evaluated using the ANCOM-BC2 framework with Hochberg directional FWER correction. To ensure rigor, primary candidate taxa were identified at adjusted significance thresholds ($p < 0.05$ cohort-wide; exploratory $p < 0.10$ within YPHIV), while conservative calls additionally required passing pseudo-count sensitivity testing and exhibiting an absolute log fold change of 1 or greater [26].

Predicted microbial metabolic functions were inferred from 16S rRNA gene data using PICRUSt2 (v.2.5.3), using default parameters, applied to the non-rarefied ASV table, as PICRUSt2 normalises by sequencing depth internally. Predictions were restricted to sequences with an NSTI below the default threshold of 2; the mean weighted NSTI across samples was 0.074 (range 0.031–0.153), indicating adequate representation of the study sequences in the reference database. In addition, although Weighted Gene Correlation Network Analysis (WGCNA, v1.73) was originally developed for gene expression analysis, its underlying framework is data-type agnostic and has been increasingly applied to microbial abundance data to characterize co-abundance network structure and identify hub taxa [27]. Genera were retained if present in >5 samples and if their total count exceeded the 10th percentile of taxon totals, yielding 149 genera across 86 samples. Counts (rarefied to 26,089 reads, seed 13) were converted to per-sample relative abundances; a pseudocount equal to 1/100 of the smallest non-zero relative abundance was added to all values prior to centered log-ratio (CLR) transformation. A signed adjacency matrix was built using Pearson correlations with a soft-thresholding power of $\beta = [3/5]$, selected as the lowest power achieving a scale-free topology fit $R^2 \geq 0.8$. Modules were identified from the topological overlap dissimilarity by dynamic hybrid tree cut (deepSplit = 4, minClusterSize = 20, pamRespectsDendro = FALSE); no module merging was applied / modules with eigengene dissimilarity < 0.25 were merged, yielding three modules. Hub genera were defined as |module membership| > 0.6 with Bonferroni-corrected $P < 0.05/149$; the network was visualized in Python using NetworkX (v3.4.2). Formal module stability or bootstrap resampling analyses were not performed, since the number of samples exceeded the recommended for WGCNA, however, the findings should be interpreted as descriptive of co-abundance patterns within this cohort.

Clinical characteristics were compared using the χ^2 test for categorical variables and the Wilcoxon rank-sum test for continuous variables. Statistical analyses were performed in R and Python, and two-sided p-values <0.05 were considered statistically significant.

Results

General characteristics of the study population

A total of 86 participants were included (48.8% women) with a median age of 18.2 years (IQR: 6.10 – 35), from which 34 were YPHIV, and 52 were UC. All YPHIV participants were on ART at the moment of inclusion, and 86.8% were virologically suppressed. 11/34 YPHIV (32.4%) and 18/52 controls (34.6%) had a diagnosis of MAFLD (11/86, 12.8%, and 18/86, 20.9%, of the overall cohort, respectively) by non-invasive imaging. Main clinical characteristics (including HIV/ART related variables) are shown in Table 1. No differences were noticed between patients with and without MAFLD. Overweight was common among UC with MAFLD, who were younger and had significantly higher BMI than YPHIV—consistent with recruitment criteria. In contrast, MAFLD in YPHIV occurred also in the absence of overweight/obesity; the association between overweight and MAFLD was non-significant among YPHIV. UC with MAFLD were significantly younger than YPHIV participants and their siblings/partners and PREP users; age was included as a covariate in all adjusted analyses, minimizing its potential influence on the reported associations.

Table 1. Clinical, anthropometric and related characteristics of the study population, by HIV and MAFLD status.

Variable	YPHIV MAFLD+ (N=11, 32% of YPHIV)	YPHIV MAFLD- (N=23, 68% of YPHIV)	UC MAFLD+ (N=18, 35% of UC)	UC MAFLD- (N=34, 65% of UC)	Overall (N=86, 100%)	p-value YPHIV vs UC in MAFLD+	p-value MAFLD+ vs MAFLD-
Age (years)						0.714	0.037 *
Mean (SD)	22.0 (9.14)	18.6 (6.28)	14.5 (3.98)	21.4 (7.60)	19.3 (7.30)		
Sex						0.963	0.224
Female	6 (54.5%)	10 (43.5%)	5 (27.8%)	21 (61.8%)	42 (48.8%)		
Male	5 (45.5%)	13 (56.5%)	13 (72.2%)	13 (38.2%)	44 (51.2%)		
Ethnicity						0.029 *	0.563
Caucasian	9 (81.8%)	13 (56.5%)	15 (83.3%)	28 (82.4%)	65 (75.6%)		
Latin American	2 (18.2%)	2 (8.7%)	1 (5.6%)	4 (11.8%)	9 (10.5%)		
African Sub- Saharian	0 (0%)	7 (30.4%)	1 (5.6%)	0 (0%)	8 (9.3%)		
Other	0 (0%)	1 (4.3%)	1 (5.6%)	2 (5.9%)	4 (4.7%)		
BMI (kg/m2)						0.004 **	< 0.001 ***
Mean (SD)	24.2 (6.89)	20.3 (2.97)	29.7 (6.80)	21.8 (3.12)	23.4 (5.73)		
BMI category						0.105	< 0.001 ***

Healthy	7 (63.6%)	20 (87.0%)	3 (16.7%)	27 (79.4%)	57 (66.3%)		
Overweight	3 (27.3%)	3 (13.0%)	10 (55.6%)	7 (20.6%)	23 (26.7%)		
Obesity	1 (9.1%)	0 (0%)	5 (27.8%)	0 (0%)	6 (7.0%)		
Overweight						0.064	< 0.001 ***
no	7 (63.6%)	20 (87.0%)	3 (16.7%)	27 (79.4%)	57 (66.3%)		
yes	4 (36.4%)	3 (13.0%)	15 (83.3%)	7 (20.6%)	29 (33.7%)		
CD4 nadir (cells/uL)						-	-
Mean (SD)	309 (138)	384 (255)	-	-	359 (223)		
Missing	1 (9.1%)	3 (13.0%)	-	-	56 (65.1%)		
ART Families						-	-
II	2 (18.2%)	12 (52.2%)	-	-	14 (16.3%)		
Unknown	4 (36.4%)	2 (8.7%)	-	-	6 (7.0%)		
Other	4 (36.4%)	4 (17.4%)	-	-	8 (9.3%)		
PI	1 (9.1%)	5 (21.7%)	-	-	6 (7.0%)		
Missing	0 (0%)	0 (0%)	-	-	52 (60.5%)		

Data are presented as n (%) for categorical variables and as mean (SD) for continuous variables. * p<0.05; ** p<0.01; *** p<0.001. BMI, body mass index; SD, standard deviation.

HIV is the major driver of gut microbiome composition

HIV and gut microbiota diversity

To investigate the relative contribution of HIV infection and MAFLD to gut microbiome composition, we first analyzed the complete study cohort.

HIV status had a significant effect on alpha diversity (Figure 1A and Supplementary Table 2A, $p=0.033$), with YPHIV showing significantly lower Shannon diversity than UC. In the same multivariable model, MAFLD, overweight status, and age were not independently associated with alpha diversity (Supplementary Table 2A).

Similarly, beta diversity analysis demonstrated significant differences according to HIV status (ADONIS: $p=0.005$, $R^2=0.028$; ANOSIM $R=0.129$, $p=0.002$), whereas no significant clustering was observed according to MAFLD status (Figure 1B). Although statistically significant, the effect size associated with HIV status was modest ($R^2=0.028$), consistent with the multifactorial determinants typically underlying gut microbial community composition. Together, these findings indicate that HIV infection, rather than MAFLD, explained the observed differences in gut microbial diversity.

HIV shapes gut microbial composition and community structure independently of MAFLD diagnosis.

Differential abundance analysis using ANCOM-BC2 identified several taxa associated with HIV infection after adjustment for MAFLD, overweight and age (Figure 2A). Specifically, *Oscillospiraceae* UCG-002, *Adlercreutzia* and one unclassified *Ruminococcaceae* ASV were significantly depleted in YPHIV compared with UC. According to MAFLD status, 67 ASVs met the criterion of $q < 0.05$, but none passed the sensitivity analysis for pseudocounting; therefore, they were not considered significant. (Figure 2B).

To determine whether these taxonomic changes reflected coordinated alterations in the gut ecosystem rather than isolated bacterial differences, we performed weighted gene correlation network analysis (WGCNA) (Figure 3). Three microbial co-abundance modules were identified showing a strong association between HIV infection and the turquoise module, enriched in *Blautia*, *Anaerostipes*, *Ruminococcus gnavus* group, *Sellimonas* and *Bacteroides*. Conversely, HIV infection was negatively associated with the blue module, mainly composed of *Oscillospiraceae* UCG-002, *Oscillospiraceae* UCG-005, *Christensenellaceae* R7-group, *Ruminococcus* and Family XIII AD3011 group (Figure 3). These findings support the concept that HIV is associated with coordinated restructuring of microbial communities rather than isolated changes in individual taxa.

HIV is associated with predicted functional alterations of the gut microbiome

To explore whether taxonomic differences translated into functional changes, predicted KO abundances were inferred using PICRUSt2. Functional analysis identified several KO gene families differentially represented according to HIV status (Figure 4A and Supplementary Table 3), whereas no biologically relevant functional differences were observed according to MAFLD (Figure 4B, and Supplementary Table 3) status in the overall cohort (although two significant KO gene families reached significance).

Overall, diversity, taxonomic, network and functional analyses consistently identified HIV infection as more strongly associated with gut microbiome variation than MAFLD in this cohort.

Differential microbiome signatures associated with MAFLD within YPHIV

Because HIV represented the major source of microbiome variation, subsequent analyses were restricted to YPHIV to explore microbial signatures specifically associated with MAFLD, minimizing the confounding effect of HIV status. Given the limited sample size of the YPHIV subgroup (n=34), the analysis remains statistically exploratory

Microbial diversity according to MAFLD status

Within the YPHIV subgroup (n=34), neither alpha nor beta diversity differed according to MAFLD status (Figure 5; Supplementary Table 2B) in the adjusted multivariate model. Thus, the presence of MAFLD was not associated with major changes in overall microbial diversity among YPHIV.

Differential abundance and predicted microbial functions

Although no significant differences in global diversity were detected, exploratory differential abundance analysis ($q < 0.1$) performed across the 148 genera tested within the YPHIV subgroup, identified a reduced abundance of *Parabacteroides* in YPHIV with MAFLD compared with those without MAFLD (Figure 6A). Furthermore, predicted functional analysis identified differences in KO gene families associated with MAFLD within YPHIV (Figure 6B). The most relevant altered KOs predicted included two biologically relevant processes, such as "DNA ligase 1" and "sirohdrochlorin cobaltochelataase", although they should be tested experimentally.

Although exploratory due to the limited sample size, these findings suggest that specific microbial taxa and predicted functions may be associated with MAFLD within YPHIV, despite the absence of major changes in overall microbial diversity. Furthermore, although overweight has previously been characterized as an important factor for microbiota composition, an exploratory analysis restricted to YPHIV with MAFLD (n=11) reporting 3 taxa with abundance statistically significant (Supplementary Figure 2) should be taken cautiously before making assumptions about overweight role.

Discussion

This study primarily aimed to characterize gut microbiome alterations associated with MAFLD in youth with perinatally acquired HIV (YPHIV), a population in which MAFLD is an emerging and incompletely understood comorbidity. To contextualize this comparison, we first examined the relative contribution of HIV infection and MAFLD to gut microbiome composition in the overall cohort, providing one of the first comprehensive characterizations of the gut microbiome in this specific population according to MAFLD status. These analyses showed that HIV infection, rather than MAFLD, was more strongly associated with differences in gut microbial diversity, composition, and predicted function. Once this major source of variation was accounted for, only subtle and exploratory microbial signatures associated with MAFLD were observed within YPHIV, suggesting that HIV-associated dysbiosis may overshadow or interact with the microbial alterations traditionally attributed to metabolic liver disease.

These findings are consistent with the well-established impact of HIV infection on the intestinal ecosystem. Previous studies have demonstrated persistent gut dysbiosis in PLHIV despite effective antiretroviral therapy, characterized by depletion of beneficial commensals, expansion of pro-inflammatory taxa, microbial translocation and chronic immune activation (Vujkovic-Cvijin et al., 2013; Dillon et al., 2014; Serrano-Villar et al., 2016), although it is well known that the specific compositional changes observed vary according to age, geographic region, and other host characteristics. Our results extend these observations to the understudied population of YPHIV, who face lifelong HIV and antiretroviral exposure. Prior work in this population, including the Pediabiota cohort and other pediatric HIV/HEU cohorts, has focused on individual taxa and diversity metrics [28–31]. In this study, network analysis demonstrates that HIV infection is also associated with coordinated restructuring of the gut microbial ecosystem. This finding addresses a gap identified in recent conceptual frameworks, which call for a more ecological, network-informed approach to studying HIV-associated gut dysbiosis in children [32].

In contrast, we observed only limited microbiome differences associated with MAFLD after accounting for HIV infection, age and overweight. This finding differs from several previous studies reporting distinct microbial signatures in MAFLD, but may reflect important methodological and biological differences [33–35]. Most published studies have evaluated MAFLD in the general population, where obesity and metabolic syndrome are the predominant drivers of disease. In our cohort, a substantial proportion of YPHIV with MAFLD were not overweight, suggesting that mechanisms other than classical metabolic dysfunction may contribute to HIV associated hepatic steatosis. Furthermore, the use of ANCOM-BC2 with adjustment for relevant covariates provides a more conservative assessment of differential abundance than approaches frequently used in earlier studies, reducing the likelihood of confounding by age or adiposity [36].

Although overall microbial diversity remained similar within YPHIV according to MAFLD status, exploratory analyses identified reduced abundance of *Parabacteroides* together with differences in predicted microbial functions. These findings should be interpreted cautiously as hypothesis-generating, given the modest sample size (n = 34) and the relaxed significance threshold. However, they may represent early microbial alterations associated with MAFLD in this specific population. Interestingly, *Parabacteroides* has been linked to bile acid metabolism, short-chain fatty acid production and maintenance of intestinal barrier integrity, suggesting plausible biological mechanisms through which gut dysbiosis could contribute to liver injury independently of obesity [37, 38].

Network analysis supported these taxon-level findings, showing that among the factors examined, only HIV infection was associated with coordinated, module-level restructuring of the gut microbiota rather than isolated taxonomic shifts. We observed a concurrent gain of a module enriched in *Blautia*, *Anaerostipes*, *Ruminococcus gnavus* group, *Sellimonas* and *Bacteroides*, and loss of a module enriched in *Oscillospiraceae*, *Ruminococcus*, *Christensenellaceae* and Family XIII AD3011 group. Both modules are dominated by short-chain-fatty-acid-producing taxa, and depletion of *Ruminococcaceae* and *Oscillospiraceae* together with expansion of *Lachnospiraceae* has previously been reported in MAFLD and

in HIV infection (Shen et al., 2017; Korobeinikova et al., 2023; San-Juan-Vergara et al., 2018), suggesting that HIV-associated network restructuring may converge on lipid-metabolism-relevant microbial guilds through shared short-chain fatty acid pathways.

While most patients remain asymptomatic, a substantial proportion progress to steatohepatitis, fibrosis, and ultimately cirrhosis. This progression highlights the need to better understand the mechanisms underlying disease development, with particular attention to children, who represent a special concern.

An important strength of our work is the inclusion of both YPHIV and UC participants, with and without MAFLD, allowing the relative contribution of HIV and MAFLD to be explored within the same analytical framework. In addition, the combination of diversity analyses, differential abundance testing with adjustment for covariates, network analysis, and functional prediction provides complementary perspectives on gut microbiome alterations. The use of ANCOM-BC2 with adjustment for relevant clinical covariates also reduces the risk of identifying spurious associations driven by confounding factors.

However, our study has several limitations. The relatively small sample size, particularly within the subgroup of YPHIV with MAFLD, limited statistical power to detect subtle microbial differences and prevented extensive subgroup analyses. The cross-sectional design precludes causal inference, and predicted function as derived from 16S rRNA gene sequencing require confirmation using metagenomic or metabolomic approaches. Finally, dietary habits, location and other environmental factors that may influence microbiome composition were not systematically evaluated. The UC group is heterogeneous, which may introduce residual variation, though not into the primary within-YPHIV analysis. Additionally, although the overall sample size ($n = 86$) exceeds the minimum typically recommended for WGCNA (≥ 15 –20 samples) to yield a non-noisy correlation structure, module composition—and particularly hub taxon identification—can be less stable at moderate sample sizes; formal module stability or bootstrap resampling analyses were not performed, and the co-abundance network reported here should be interpreted as descriptive of this cohort rather than as a definitively stable structure.

Conclusions

In conclusion, HIV infection was more strongly associated with gut microbiome composition in YPHIV, whereas MAFLD was associated with more subtle taxonomic and predicted functional changes once HIV-related dysbiosis was taken into account. These findings suggest that future studies investigating MAFLD in YPHIV should carefully consider the dominant effect of HIV on the intestinal microbiome and support further longitudinal and multi-omic studies to clarify the contribution of microbial alterations to liver disease in this unique population.

Abbreviations

MAFLD, metabolic dysfunction-associated fatty liver disease; HIV, human immunodeficiency virus; YPHIV, youth with perinatally acquired HIV; UC, Uninfected Controls; ART, antiretroviral therapy; BMI, body

mass index; CAP, controlled attenuation parameter; TE, transient elastography; pSWE, point shear wave elastography; ANCOM-BC2, analysis of compositions of microbiomes with bias correction 2; WGCNA, weighted gene correlation network analysis; PICRUST2, phylogenetic investigation of communities by reconstruction of unobserved states 2; PCoA, principal coordinates analysis; VL, viral load; IQR, interquartile range; SD, standard deviation; CoRISpe, Spanish National Network of Children and Adolescents Living with HIV.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Hospital Universitario La Paz (reference PI-3232) and by the ethics committees of all participating hospitals, and was conducted in accordance with the Declaration of Helsinki. All participants and/or parents/legal guardians signed informed consent/assent before inclusion in the study.

Consent for publication

Not applicable.

Availability of data and materials

The 16S rRNA gene sequencing data generated and analyzed during the current study are available in the European Nucleotide Archive (ENA) repository under project accession number PRJEB123866 (<https://www.ebi.ac.uk/ena/browser/view/PRJEB123866>). The custom code used for data processing and statistical analysis is available at https://github.com/GERMAN00VP/MAFLD_ANALYSIS and will be archived with a persistent identifier at Zenodo repository. The manuscript and additional files will be also uploaded to this repository.

Competing interests

Authors declare that they have no competing interests

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Authors' contributions

Conceptualization, TS and SSV; methodology, TS, GV, EM; software, GV, EM; formal analysis, GV; investigation, EM.; resources, TS.; data acquisition, LE, MM, AO, MLN; data curation, TS, GV; writing—original draft preparation, GV, EM, TS; writing—review and editing, all authors; project administration, TS; funding acquisition, TS. All authors read and approved the final manuscript.

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Figures

Figure 1

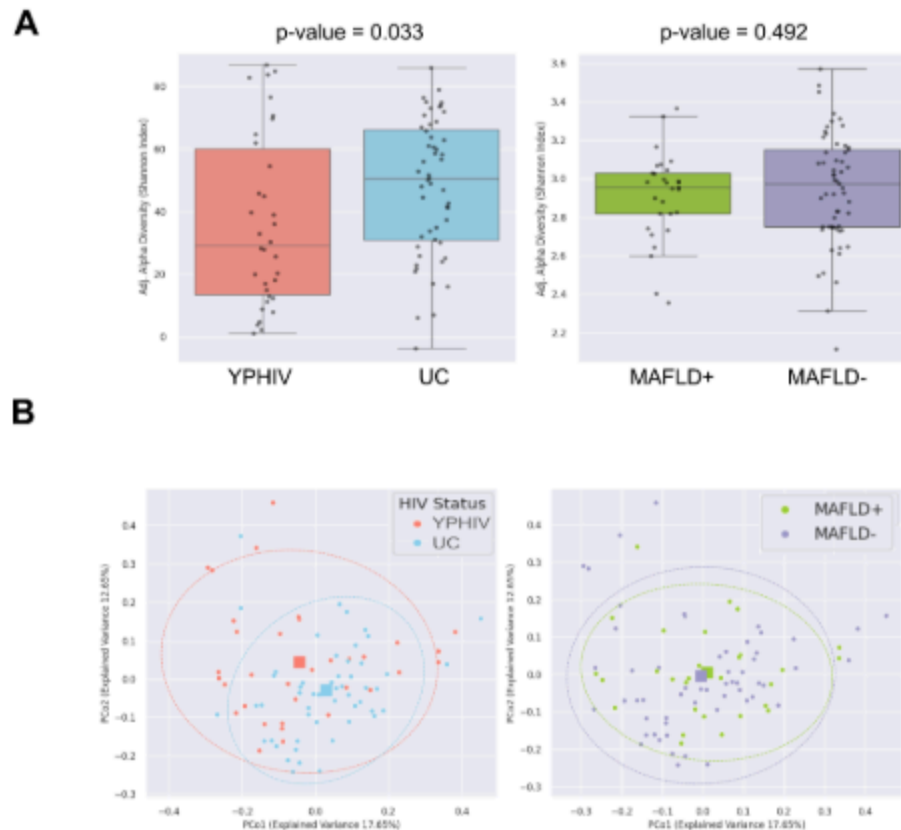


Figure 1

Microbial diversity indexes in all patients according to HIV infection and MAFLD diagnosis. A: Boxplots showing the adjusted alpha diversity (Shannon index) distribution comparison between different statuses in HIV (left: UC, blue / YPHIV, red) and MAFLD (right: MAFLD-, purple / MAFLD+, green). Significance is denoted by the p-value at the top of each plot (ANCOVA, N = 86). **B:** PCoA plots showing Beta diversity. The plots show the distribution of samples (N = 86) along the two principal components based on the Bray-Curtis distance metric. Large squares and dotted lines represent the centroid and the covariance ellipse for each group, respectively. The colors indicate different statuses for HIV (left: UC, blue / YPHIV, red) and MAFLD (right: MAFLD-, purple / MAFLD+, green). The proportion of variance explained by each principal coordinate (PCo) is indicated on the corresponding axis.

Figure 2

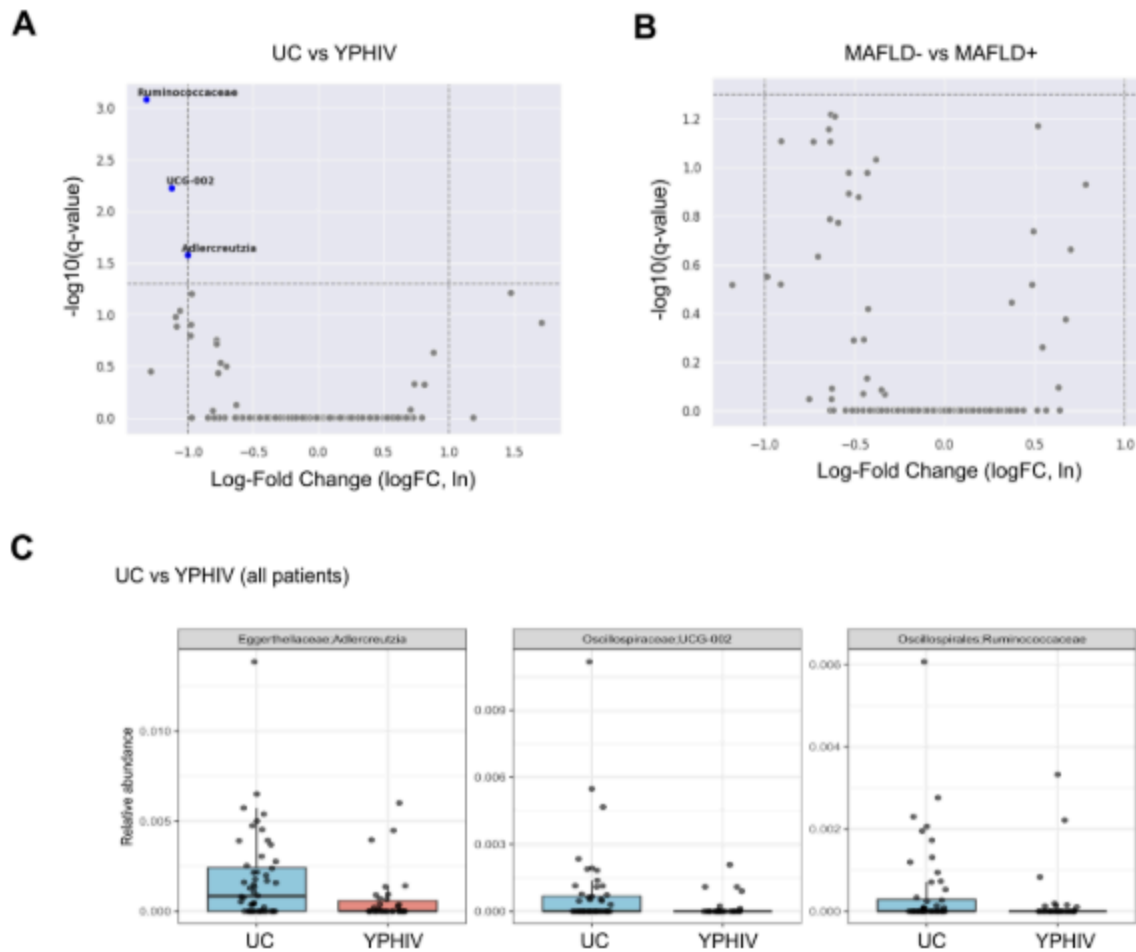
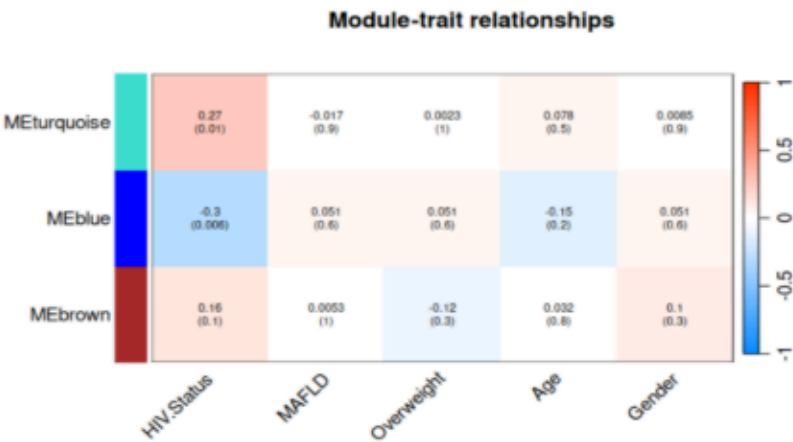


Figure 2

volcano plots of the differential abundance analysis using ANCOM-BC2 and comparing different statuses. For HIV infection (**A**) and MAFLD diagnosis (**B**) while correcting for co-variables and using the negative status as the reference. The y-axis represents the negative log10-transformed q-value, while the x-axis shows the natural logarithm of the fold change (LogFC). Significance thresholds are indicated by dotted lines (q-value threshold = 0.05; LogFC threshold = ± 1). Significantly increased taxa are colored in red, while blue dots represent a significant decrease in taxa abundance. **C.** Boxplots showing the relative abundance of the three significant taxa identified in A (*Eggerthellaceae*; *Adlercreutzia*, *Oscillospiraceae*; UCG-002, and *Oscillospirales*; *Ruminococcaceae*) by HIV status (UC, light blue; YPHIV, salmon).

Figure 3

A



B

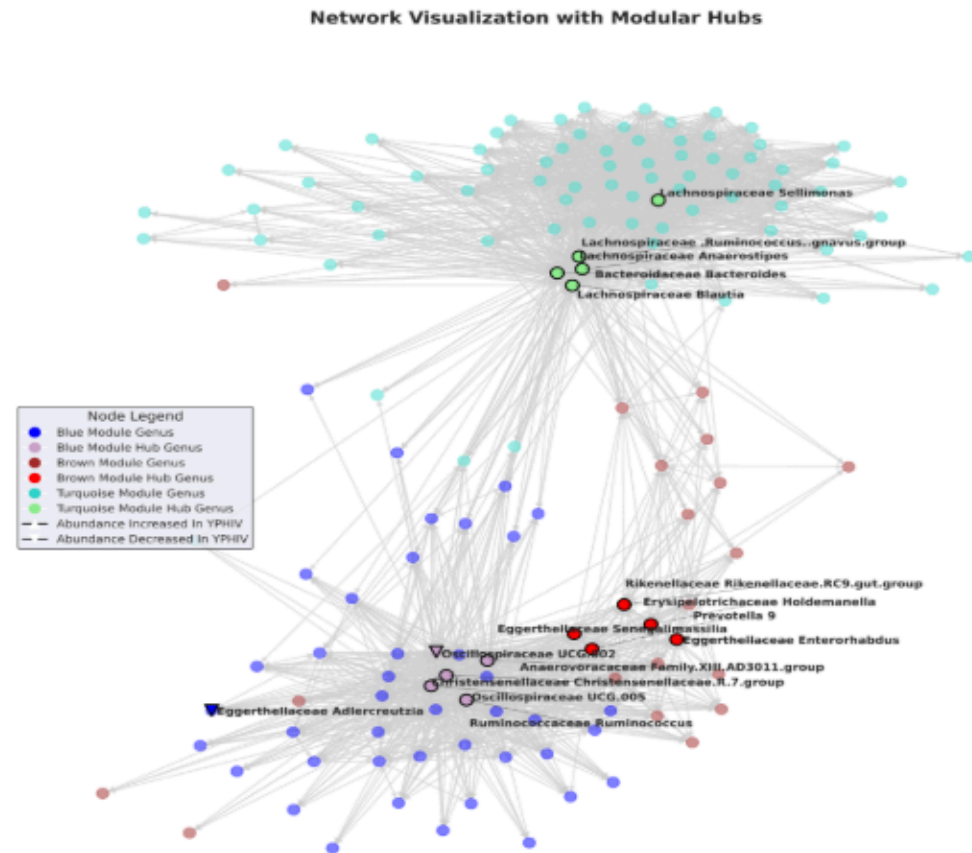


Figure 3

Weighted Gene Correlation Network Analysis (WGCNA). (A) Module (PC1)–trait correlation heatmap; each cell shows the correlation coefficient with the corresponding p-value in parentheses (turquoise, blue and brown denote the three co-abundance modules identified). (B)WGCNA network: nodes represent genera, colored by module (turquoise, blue, brown); edges represent co-abundance, with solid lines indicating genera with increased abundance in YPHIV and dashed lines indicating decreased

abundance. Brighter, black-outlined nodes are hub genera; triangular nodes indicate genera also differentially abundant between YPHIV and UC (Figure 2).

Figure 4

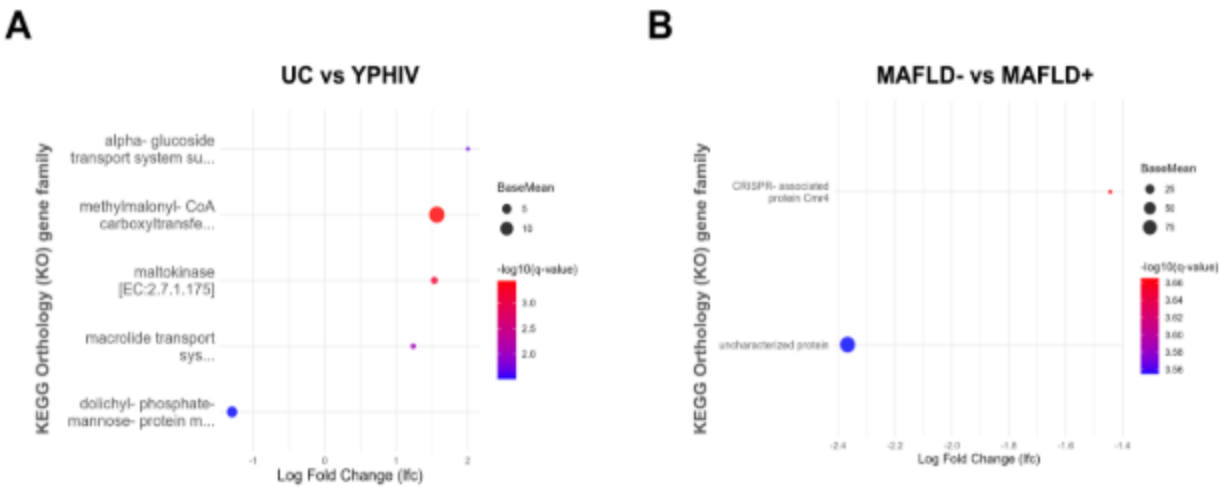


Figure 4

Bubble plots showing functional analysis comparing different statuses for HIV infection (A) and MAFLD diagnosis (B). The x-axis shows the natural-log fold change (LFC) in predicted KO abundance (in A, positive values indicate enrichment in YPHIV relative to UC; in B, positive values indicate enrichment in MAFLD+ relative to MAFLD-). Point size indicates the mean normalized abundance (BaseMean) across all samples, and point color indicates the statistical significance as $-\log_{10}(q\text{-value})$ from the differential abundance analysis after multiple-testing correction.

Figure 5

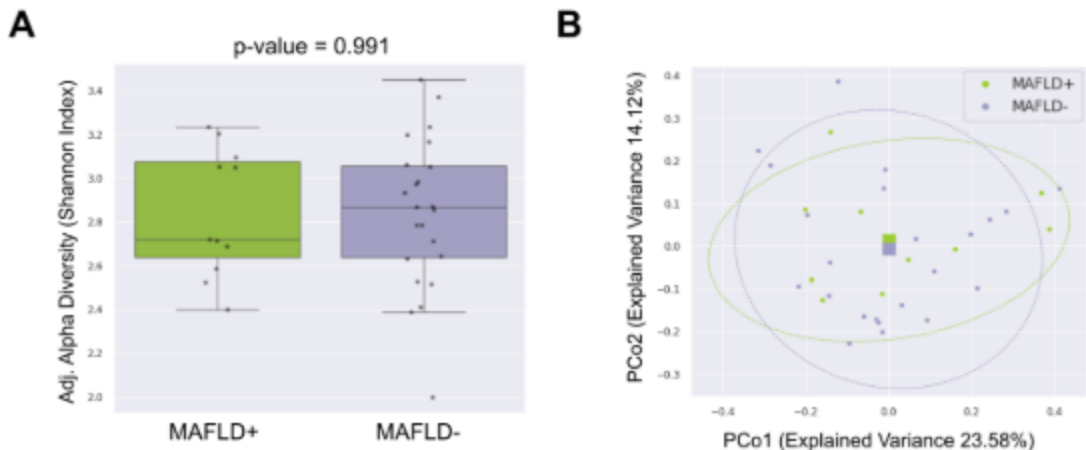


Figure 5

Microbial diversity indexes according to MAFLD diagnosis within YPHIV. A: Boxplot showing the co-variables adjusted distribution of alpha diversity measured by the Shannon index among the YPHIV patients (N = 34, ANCOVA, P = 0.991, partial $\eta^2 < 0.001$) divided according to MAFLD status. **B.** Beta diversity: Principal coordinate analysis (PCoA) of the Bray-Curtis resemblance matrix (PERMANOVA $R^2 = 0.015$, $P = 0.944$; betadisper $P = 0.766$). The colors indicate different statuses for MAFLD status, with purple representing negative and green representing positive. The proportion of variance explained by each principal coordinate (PCo) is indicated on the corresponding axis.

Figure 6

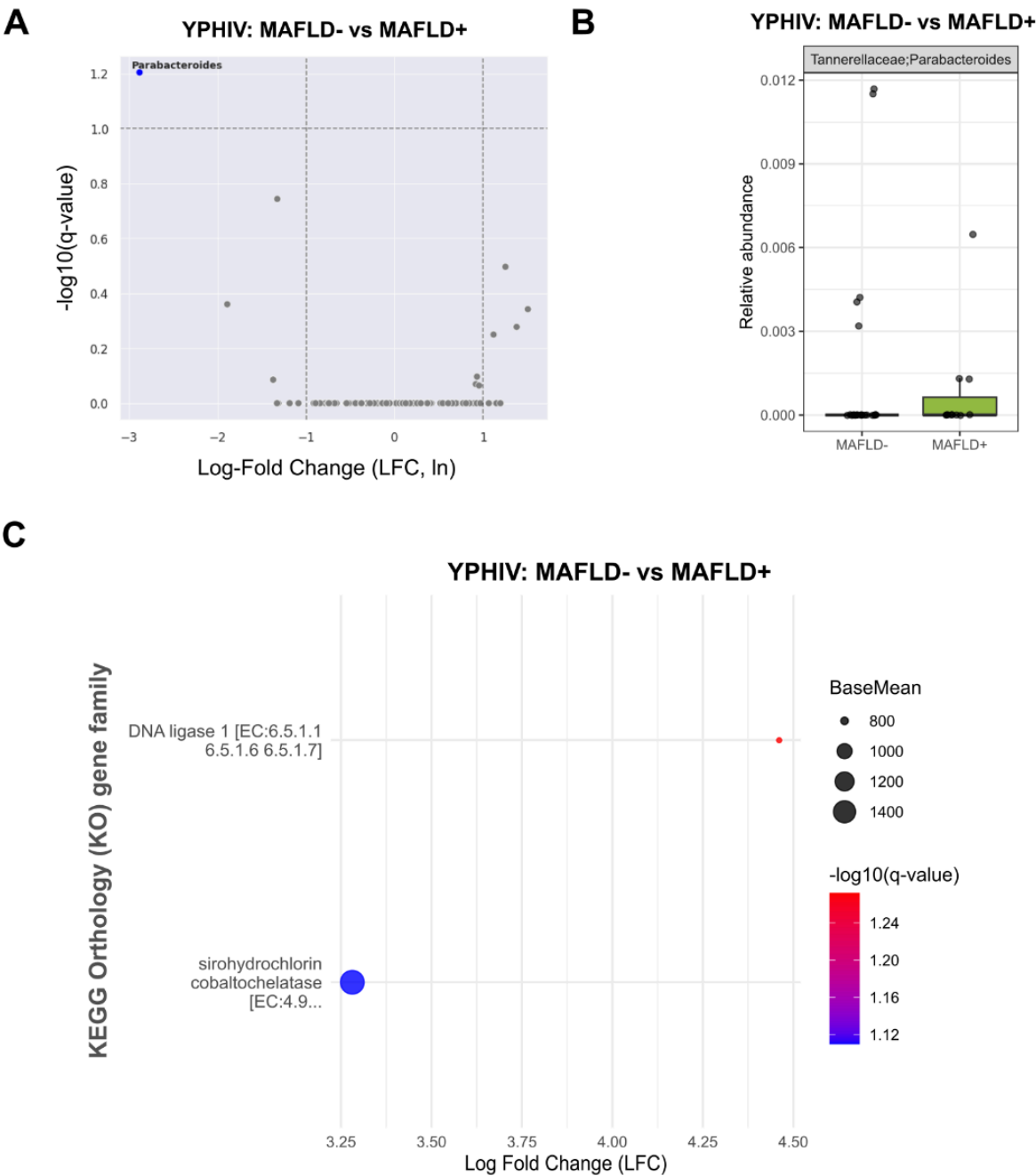


Figure 6

Microbial abundance and functions among YPHIV according to MAFLD diagnostic status. **A.** Volcano plot of the differential abundance analysis using ANCOM-BC2 comparing different statuses for MAFLD (while correcting for co-variables) among YPHIV patients, using the negative status as the reference. The y-axis represents the negative log₁₀-transformed q-value, while the x-axis shows the natural logarithm of the fold change (LogFC). Significance thresholds are indicated by dotted lines (q-value threshold = 0.1; LogFC threshold = ± 1). Significantly increased taxa are colored in red, while blue dots represent a significant decrease in taxa abundance. **B.** Boxplot of relative abundance of Parabacteroides, the only significant taxon identified in A, by MAFLD status (MAFLD-, white; MAFLD+, teal, N=34). **C.** Bubble plot showing functional analysis. The x-axis shows the natural-log fold change (LFC) in predicted KEGG Orthology (KO) gene family abundance (positive values indicate enrichment in MAFLD+ relative to MAFLD-), and each point corresponds to a KO gene family on the y-axis. Point size indicates the mean normalized abundance (BaseMean) across all samples, and point color indicates the statistical significance as $-\log_{10}(\text{q-value})$ from the differential abundance analysis after multiple-testing correction.

Supplementary Files

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