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Gut microbiota characterization in ageing, mild cognitive impairment, and Alzheimer's disease in the context of mediterranean lifestyle in a Spanish population

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Abstract

Background Alzheimer's disease (AD) is a neurodegenerative disorder often preceded by a prodromal stage of Mild Cognitive Impairment (MCI). Previous research suggests that gut microbiota (GMB) dysbiosis may contribute to cognitive decline via the microbiota-gut-brain axis (MGBA). Notably, GMB composition patterns can vary across populations and stages of dementia. This study aimed to characterize the GMB in a cohort of older adults from Tarragona (Spain) diagnosed with AD or MCI, or presenting a healthy cognitive status (HC), all of whom follow a Mediterranean lifestyle (ML).

Methods The present cross-sectional, multicenter case–control study analyzed fecal samples from 99 individuals,including 31 with AD, 30 with MCI, and 38 HC,aged 60–85 years, recruited from seven hospitals and specialized cognitive centers in the province of Tarragona, Spain. Shotgun metagenomic sequencing was conducted with taxonomic profiling using Kraken2. APOE genotyping was performed from fecal DNA using TaqMan assays. Richness, alpha and beta diversity, differential abundance, multivariate linear modeling, and Jonckheere–Terpstra trend tests were conducted to identify GMB species signatures associated with MCI and AD.

Results Richness, alpha and beta diversity did not differ across groups. Differential abundance analysis identified 109 taxa, of which ten microbial species were shared across comparisons. Notably, several species, including *Coprococcus comes* and *Odoribacter splanchnicus*, emerged as replicable candidates, showing both discriminatory value and severity-related declines, alongside taxa with context-dependent or adverse associations.

Conclusions Overall GMB diversity did not differ across cognitive groups, but specific taxa, particularly short-chain fatty acid producers, showed consistent associations with cognitive decline in this ML cohort. These findings support a role for the GMB in AD pathology and suggest that targeting key microbial species may provide novel avenues for prevention and intervention.

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Keywords Alzheimer's disease, Mild cognitive impairment, Dementia, Aging, Gut microbiota, Dysbiosis, Mediterranean diet

Introduction

Alzheimer's Disease (AD) is a neurodegenerative disorder of the central nervous system, resulting in gradual cognitive decline. The hallmark neuropathological features of AD include extracellular amyloid-β plaque accumulation and intracellular neurofibrillary tangles of hyperphosphorylated tau protein in the brain [1]. Clinically, the onset of AD is often marked by a prodromal stage known as Mild Cognitive Impairment (MCI) [2]. While not all individuals with MCI progress to AD, those with the amnestic subtype (aMCI) are at a notably higher risk. The most common form of AD is sporadic late-onset AD (LOAD, onset age ≥ 65 years), and its pathogenesis is thought to involve a interaction of genetic, environmental, lifestyle and dietary factors [3].

In recent years, the microbiota–gut–brain axis (MGBA), a bidirectional communication pathway between the gut and the brain, has gained attention as a potential contributor to AD onset and progression [4]. Dysbiosis of the gut microbiota (GMB) can lead to increased intestinal permeability and systemic inflammation, which may in turn influence neurodegenerative processes through metabolic, endocrine, neural and immune pathways [5]. These processes include promoting amyloid-β aggregation, tau phosphorylation, neuroinflammation, metabolic dysfunction, and oxidative stress [5]. A recent systematic review and meta-analysis revealed that AD and MCI patients exhibit a higher degree of gut dysbiosis and significant differences in both alpha (α) and beta (β) diversity metrics compared to HC [6]. Alterations in specific microbial taxa have also been observed in AD, although findings have varied between studies and populations [7]. Transitional changes on GMB may become apparent years before the onset of AD and could be identified during the stage of MCI, suggesting their potential role as an early-accessible gut-derived biomarkers [8].

The aging process, a significant non-modifiable risk factor for AD, along most modifiable risk factors for AD, have been directly or indirectly linked to alterations in GMB, thereby playing a role to its pathogenesis [9]. Consequently, interventions focusing on diet and lifestyle have been proposed as strategies for dementia prevention [10]. In particular, adopting a Mediterranean lifestyle (ML), which includes adherence to a Mediterranean diet (MD) (e.g., rich in fruits, vegetables, whole grains, legumes, fish, and olive oil, along with culturally ingrained habits like regular physical activity, social engagement and adequate rest), has been associated with better cognitive health and lower dementia risk [11]. Adherence to the MD has been shown to promote greater gut microbial diversity and enrichment of beneficial short-chain fatty acids producing bacteria (SCFAs), which are linked to anti-inflammatory and neuroprotective effects [12, 13, 14]. However, the extent to which ML can counteract or modulate GMB changes in the context of AD and MCI remains unclear.

In the present study, a comprehensive analysis of GMB composition was conducted in HC older adults, MCI patients, and AD patients living in a Mediterranean region. All participants shared a similar dietary and lifestyle background, which helps minimize confounding by gross lifestyle differences. The study hypothesized that (1) individuals with MCI and AD would exhibit an altered GMB composition compared to HC; (2) GMB profiles would correlate with cognitive and clinical measures of impairment; (3) specific bacterial taxa would show progressive shifts in relative abundance across groups; and (4) adherence to an ML might have a protective role on cognitive health. This study is registered on ClinicalTrials.gov (ID: NCT05943925) and forms part of the DEMBIOTA Project (PID2019-103888RB-I00).

Methods

Study design and setting

This cross-sectional study was conducted at multiple centers in the province of Tarragona (Catalonia, Spain), including hospital neurology and geriatrics departments and memory clinics. The study protocol was approved by the Clinical Research Ethics Committee (CEIm) of the Institute of Health Research Pere Virgili (IISPV, Ref. CEIm: 183/2020) and carried out in accordance with the Declaration of Helsinki. All participants (or their legal representatives) provided written informed consent prior to enrollment.

Study participants

Participants aged 60–85 years were recruited between January 2021 and February 2024. A total of 150 individuals were screened for eligibility at Verge de la Cinta Hospital (Tortosa), Xarxa Santa Tecla Hospital (Tarragona), Sant Joan University Hospital of Reus (Reus), Lerín Neurocognitive Institute (Reus), Pius Hospital (Valls) and Santa Creu Hospital of Jesus (Tortosa). Figure 1 illustrates the recruitment flow: of the 150 individuals screened, 50 were excluded prior to enrollment due to administrative constraints ($n = 13$), refusal to participate ($n = 12$), caregiver-related issues ($n = 7$), alternative diagnoses identified ($n = 6$), not meeting inclusion criteria ($n = 5$), logistical or sample collection issues ($n = 2$),

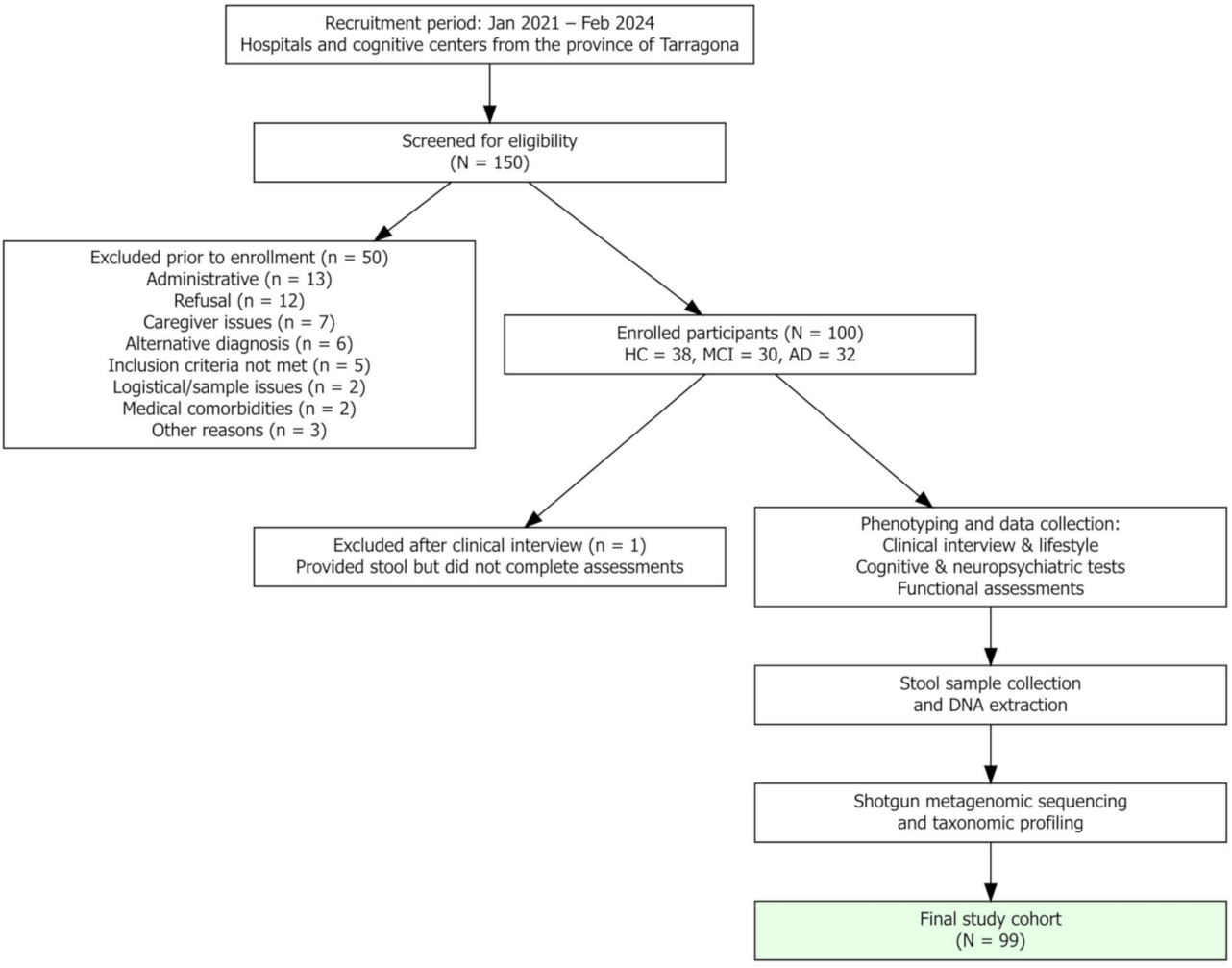

Fig. 1 Recruitment Flow. Flow diagram summarizing participant screening and enrolment from January 2021 to February 2024

significant medical comorbidities ($n = 2$), or other reasons ($n = 3$). Of the 100 participants enrolled (HC = 38, MCI = 30, AD = 32), one was excluded after the clinical interview because they provided a stool sample but did not complete the neuropsychological assessments. The final study cohort therefore comprised 99 individuals (AD = 31, MCI = 30, HC = 38) who completed all study procedures. A simulation-based power analysis using Bray–Curtis PERMANOVA based on Human Microbiome Project (HMP) community data [15] indicated that a sample size of 30 subjects per group would provide approximately 80% power to detect community-level differences corresponding to a 4% group-unique effect at $\alpha = 0.05$. Given that our diagnostic groups exceeded this minimum size, the study was judged sufficiently powered to detect microbiome differences of this magnitude.

Inclusion criteria for the AD group comprised: (1) AD diagnosis by a neurology service in accordance with the National Institute on Aging Alzheimer's Association (NIA-AA) 2011 guidelines; (2) Aged 60–85 years; (3) Global Deterioration Scale (GDS) [16] and Functional Assessment Staging procedure (FAST) [17] (GDS-FAST) score of 4–5 points. Inclusion criteria for the MCI group comprised: (1) aMCI diagnosis by a neurology service in accordance with the NIA-AA 2011 guidelines; (2) Aged 60–85 years; (3) GDS-FAST score of 3 points. Inclusion criteria for the HC group comprised: (1) Healthy individuals; (2) Aged 60–85 years.

Participants were excluded if they had any of the following: (1) a diagnosis of, or comorbidity with, another significant neurological disease (e.g., cerebrovascular disease such as stroke or vascular dementia, Parkinson's disease, multiple sclerosis, epilepsy, traumatic brain injury, brain tumor, or other neurodegenerative disorders); (2) a major psychiatric disorder (including major depressive disorder); (3) use of oral antibiotics in the 6 months prior to stool sampling; (4) use of systemic corticosteroids in the 2 weeks prior to stool sampling; (5) use of immunosuppressants or immunostimulants in the 6 months prior; (6) active gastrointestinal disease; (7) high

consumption of commercial probiotics (≥ 10^8 CFU per organism per day); (8) or inability to provide informed consent (e.g., due to illiteracy or severe cognitive/communication impairments).

All participants were from the same geographic region and cultural background, ensuring generally similar diet and lifestyle patterns. No specific dietary or lifestyle exclusion criteria were applied beyond the medical exclusions listed. Instead, we assessed adherence to the ML in all participants, supporting the comparability of diet/lifestyle across the cohort.

Demographic, clinical, and lifestyle assessments

During clinical interviews, a comprehensive range of data was collected for each participant. Demographic information included age, sex, handedness, nationality, years of education, marital status, and household composition. Anthropometric data (height, weight) were recorded, and body mass index (BMI) was calculated as weight (kg)/[height (m)]^2. We also documented medical history and exposure to potential modifiable risk factors for dementia [10]. This included presence of hearing impairment, hypertension, dyslipidemia, type 2 diabetes mellitus, obesity, history of stroke, history of traumatic brain injury (TBI) and any significant occupational exposure to chemical or environmental toxins (e.g., pesticides, solvents, heavy metals). Medication use information was recorded in detail, including any regular medications (categorized by class, e.g., antihypertensives, antidiabetics, lipid-lowering agents, proton pump inhibitors (PPIs), cholinesterase inhibitors, analgesics/anti-inflammatories, antipsychotics/mood stabilizers) as well as dietary supplements (whether the participant was taking any vitamins, minerals, herbal products, or disease-specific nutritional supplements). If supplements were used, the type was noted (vitamin/mineral, mixed supplement, etc.). Lifestyle factors such as smoking habits (current, former, or never smoker and years of tobacco use) and alcohol consumption (current, former, or never drinker and years of alcohol use) were recorded. Dietary and lifestyle habits were assessed using the Mediterranean Lifestyle Index [18] (MEDLIFE) questionnaire. MEDLIFE is a 28-item validated self-report instrument capturing adherence to various aspects of the ML, including diet (food intake frequency and dietary habits) as well as physical activity, social habits, and rest. Higher MEDLIFE scores indicate greater adherence to an optimal ML. This measure allowed us to quantify and compare lifestyle habits across participants.

Neuropsychological, neuropsychiatric and functional measurements

Each participant underwent an extensive battery of standardized neuropsychological tests and questionnaires administered by trained researchers in a single session (~ 1.5 h). Standard administration protocols were followed, and breaks were provided as needed to maintain engagement. Raw test scores were converted to normalized scores using published norms for the Spanish population to account for age and education effects where applicable. Cognitive assessments, named according to their method of administration, included the Time, Spatial and Personal Orientation subtests of the BTII (TO-BTII, SO-BTII, PO-BTII, respectively) [19]; the Mini Mental Status Examination (MMSE) [20] and Mini-Examen Cognoscitivo (MEC) [21]; Memory Impairment Screen (MIS) test [22]; Verbal Span: Digit Span Forward and Backward (DSF, DSB, respectively) [19]; Free and Cued Selective Reminding Test (FCSRT) [23]; Trail Making Test A (TMTA) and B (TMTB) [24, 25]; Clock Drawing Test (CDT) [26]; Copy of simple and semi-complex construction praxis subtest of the BTII (CCPS-BTII) [19]; Frontal Assessment Battery (FAB) [27]; Cognitive Reserve Scale (CRS) [28]; C-form shortened-version of the Boston Naming Test [29], a 15-item adaptation validated for the Spanish population [31], The Categorial Fluency: Semantic (with 'animals') and Formal Fluency (initial letter 'P') subtests from the BTII [19, 30] (SF, FF, respectively); and Clinical Dementia Rating (CDR) [31, 32]. Emotional and neuropsychiatric assessments included the Goldberg Anxiety and Depression Scale [33]; Life Events Questionnaire (LEQ) [34], adapted from the PREDIMED-PLUS study [35]; and Neuropsychiatric Symptomatology (NPS) test from the BTII [19]. Functional assessment included the Daily-Life Activities (ADL) test from BTII [19]. Throughout the assessment process, researchers took steps to maximize participant cooperation and data authenticity: establishing rapport, conducting evaluations in a quiet and comfortable environment, allowing rest breaks, and involving caregivers for collateral information.

Stool sample collection and DNA isolation

Participants (or their caregivers) were provided with a stool collection kit (Norgen Biotek Corp., Canada) and instructions to collect a fecal sample at home within 3 days prior to their clinical visit. The kit included a nucleic acid preservation solution to stabilize microbial DNA at ambient temperature. On the day of the visit, samples were brought to the clinic, aliquoted, and immediately stored at − 80 °C at the Rovira i Virgili University biobank until analysis. Genomic DNA from the microbial community was extracted using the Fast Stool DNA Mini Kit (Qiagen, Germany), following the manufacturer's guidelines (Illumina, California, USA, catalog no. 20018705). DNA concentration and purity were assessed using a Qubit 4.0 fluorometer and Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Massachusetts, USA). This

quantification method relies on a fluorescent molecule that intercalates with double-stranded DNA, and fluorescence intensity is proportional to DNA concentration, measured against two standards. Approximately 200 ± 30 mg of stool was processed, with DNA eluted in 100–200 µL of nuclease-free water. DNA extraction included an initial lysis step at 95 °C to enhance yield.

Shotgun metagenomics and quality control

Shotgun metagenomic sequencing was conducted at the Center for Omic Sciences (COS), a Mixed Unit of the Rovira i Virgili University and Eurecat, considered a Unique Scientific and Technical Infrastructure (ICTS) dedicated to the study of omics technologies. Libraries were quantified using a Qubit 4.0 fluorometer with the Qubit dsDNA HS Assay Kit, and the library fragment length was assessed using an Agilent TapeStation with the Agilent High Sensitivity DNA kit (Agilent Technologies, California, USA). Libraries that did not meet the required quality standards (concentration ≤ 750 pM or length outside the 400–600 bp range) were excluded from further analysis. A 750 pM equimolar pool of the remaining libraries was prepared and loaded into Illumina P2 and/or P3 300-cycle sequencing reagents, followed by cluster formation on an Illumina flowcell. Sequencing was performed on the Illumina NextSeq2000 platform using 2 × 150 bp paired-end chemistry. Raw sequence data were converted to FASTQ format, generating two files per sample (R1 and R2, representing paired reads). Samples with fewer than 7.5 million reads or a median quality score below 30 were excluded to ensure a minimum sequencing yield of 1.125 Gb per sample. After quality control and filtering, the mean (median; range) sequencing depth per sample was 10.0 M (9.5 M; 2.7–20.0 M) reads for AD, 11.9 M (11.8 M; 3.7–35.9 M) for HC, and 9.0 M (6.0 M; 4.1–24.3 M) for MCI (see Supplementary Table S1). Detailed read quality data for all samples are provided in Supplementary Table S2. Taxonomic classification was performed using Kraken2 [36] with the k2_pluspfp_16gb_20220607's database, which includes bacteria, archaea, protozoa, fungi, viruses, plasmids, human sequences, and UniVec Core (vector sequences). Normalized taxonomic abundances were used for downstream statistical analyses in R, with unidentified reads systematically excluded.

APOE genotyping

To determine the APOE ε2/ε3/ε4 genotype from fecal samples, SNP genotyping of rs429358 and rs7412 was performed using TaqMan® allele discrimination assays. DNA was extracted from 200 ± 30 mg of fecal material using the QIAamp Fast DNA Stool Mini Kit (QIAGEN, ref. 51604), incorporating a 95 °C lysis step and eluting in 50–200 µL of nuclease-free water. DNA concentration was measured using a Qubit 4.0 fluorometer with the Qubit dsDNA High Sensitivity Assay Kit (Invitrogen, ref. Q32851). For each sample, 15 ng of DNA were dried and used in a 5 µL qPCR reaction containing 2.5 µL of TaqMan™ Fast Advanced Master Mix (Thermo Fisher Scientific, ref. 4444557), 0.25 µL of 20× SNP assay and 2.25 µL of H_2O. The assays employed were C___3084793_20 (rs429358: C/VIC vs. T/FAM) and C____904973_10 (rs7412: C/VIC vs. T/FAM). Reactions were run on a QuantStudio 6 system with fast-cycling conditions (95 °C for 20 s, then 40 cycles of 95 °C for 1 s and 60 °C for 20 s). Genotyping calls were made using Design & Analysis Software v2.6.0, and APOE genotype was determined from the combination of the two SNPs according to the coding chart (ε2/ε2 = TT/TT, ε3/ε3 = TT/CC, ε4/ε4 = CC/CC, ε2/ε3 = TT/TC, ε2/ε4 = TC/TC, ε3/ε4 = TC/CC). A total of 99 fecal DNA samples were analyzed: successful APOE genotype calls were obtained in 94 (94.95%) participants. Among these, the distribution was 53.2% ε3ε3 (50/94), 35.1% ε3ε4 (33/94), 3.2% ε4ε4 (3/94), and 8.5% ε2ε3 (8/94) (see Supplementary Table S3 for full sample-level results). Five samples failed due to no amplification or inconclusive signals, likely reflecting the low proportion of human DNA and the presence of PCR inhibitors in fecal material. For downstream analyses, ε3/ε4 and ε4/ε4 genotypes were classified as APOE ε4-carriers, whereas ε3/ε3 and ε2/ε3 were classified as non-carriers.

Statistical analysis

Data analyses were conducted using R software (v. 4.3.2) and IBM SPSS Statistics (v. 29.0). The dataset initially contained 24 missing values scattered across some clinical and questionnaire variables. We assumed these were Missing at Random and applied multiple imputations to handle missing data. Specifically, we performed five imputations using Fully Conditional Specification with 10 iterations each. Continuous variables were imputed via linear regression models and categorical variables via logistic regression models. One cognitive test score, TMT-B, had > 30% missing values (due to many participants not completing it); following best practices, TMT-B was excluded from analyses rather than imputed, to maintain robustness. The imputation process yielded pooled estimates that did not significantly differ from observed values, indicating that the missing data treatment did not introduce bias. The Shapiro-Wilk test was used to assess normality, and Levene's test evaluated homogeneity of variances. Normally distributed continuous variables were summarized as mean and standard deviation, while non-normally distributed variables were presented as median and interquartile range. Categorical variables were summarized as counts (n) and percentages (%). For normally distributed variables, one-way ANOVA was performed. Post-hoc comparisons were conducted

using Tukey's Honestly Significant Difference test when variances were equal or Tamhane's T2 test when variances were unequal. Non-normally distributed variables were analyzed using the Kruskal-Wallis test, with pairwise comparisons conducted using the Mann-Whitney U test. Categorical variables were compared using Pearson's chi-squared test. When the assumptions were not met, Pearson's chi-squared test with Monte Carlo simulations was employed. Microbiome Multivariable Association with Linear Models (MaAsLin2) was applied independently for each variable of interest to explore associations between the relative abundance of specific GMB taxa and clinical data. The analysis parameters included no abundance or prevalence filtering (minimum abundance: –inf, minimum prevalence: 0.000), no normalization, and log transformation. A linear model was employed, with fixed effects including diagnostic group, sex, age, education, BMI, APOE ε4 status, and medication use. Other collected variables (e.g., comorbidities, lifestyle factors) were not included in the multivariable model to avoid overfitting, especially given that they did not significantly differ between groups or were collinear with the included covariates. The BH correction for FDR was applied to adjust for multiple comparisons, with significance set at $q < 0.05$. Data standardization was enabled, and results were generated using a single computational core.

Microbial diversity, LEfSe analysis and abundance trend assessment

Richness, α and β diversity analyses were conducted using QIIME2 [37], excluding unidentified reads. The Chao1 and Shannon diversity indices were calculated at species level after rarefaction (10 iterations of random subsampling to 1,788 reads, matching the lowest sequence count from a single participant). Statistical differences in α-diversity between groups were assessed using the Kruskal-Wallis test, with pairwise comparisons conducted using the Mann-Whitney U test, applying the Holm-Šidák (HS) correction to control for multiple testing. Given that age and education differed between groups, a rank-based ANCOVA was performed to adjust α-diversity comparisons for these covariates. Separate models were fitted for Chao1 and Shannon indices with Diagnosis (Group) as the main factor and age and education as covariates. Group *p*-values were obtained based on Type III sums of squares from the rank-transformed models. For β-diversity, we calculated Bray–Curtis dissimilarity between samples based on species level relative abundances. We visualized β-diversity via non-metric multidimensional scaling (NMDS) and principal coordinates analysis (PCoA) on Bray–Curtis distances. To test for overall community differences, we used PERMANOVA (999 permutations) and ANOSIM in vegan. We ran PERMANOVA models both unadjusted and adjusted for covariates (adding age and education as covariates in the adonis2 function) to account for potential confounders. ANOSIM (which does not accommodate covariates) was used as a secondary, non-parametric test of group separation. Comparisons of the relative abundance of taxa at the phylum, order, class, family, genus, and species levels were performed on normalized data using Kruskal-Wallis test and Mann-Whitney U test, with both tests adjusted using BH correction for FDR. To identify potential biomarkers for colonic bacteria across groups, the linear discriminant analysis effect size (LEfSe) [38] method was applied. The analysis involved a Kruskal-Wallis sum-rank test to detect statistically significant differences among the groups, followed by pairwise comparisons (AD vs. HC, MCI vs. HC, AD vs. MCI) to identify group-specific biomarkers. We modified the LEfSe approach to incorporate multiple comparison correction: the *p*-values from the Kruskal–Wallis tests were adjusted using the BH correction for FDR procedure, and only taxa with $q < 0.05$ proceeded to logarithmic linear discriminant analysis (LDA). LDA was subsequently performed to estimate the effect size of each taxon exhibiting differential abundance, using an LDA score > 3.0 to select discriminative features. Overlaps of differentially abundant taxa were evaluated at the species-level using Venn diagram, as recent evidence indicates that genus-level analyses may obscure critical species-specific differences in the context of MCI and AD [39]. To refine the identification of species significantly associated with group differences in abundance, we applied a non-parametric rank-sum test with BH correction for FDR. In addition, to explore directional trends in the relative abundance of these taxa across ordered clinical severity scales, we employed the Jonckheere–Terpstra trend test, also incorporating BH correction for FDR.

Results

Demographic and clinical outcomes

The demographic and clinical characteristics of the HC, MCI, and AD groups are summarized in Table 1. HC participants were younger (68.21 ± 5.99 years) than MCI (72.13 ± 5.18 years) and AD (74.61 ± 5.60 years) ($p < 0.001$) and had higher education levels and CRS scores ($p = 0.048$ and $p = 0.025$, respectively). APOE ε4 prevalence was markedly higher in MCI (57.1%) and AD (65.5%) than in HC (10.8%; $p < 0.001$). Regarding medication use, PPIs were more common in MCI (26.7%) and AD (22.6%) compared with HC (2.6%; $p = 0.011$), cholinesterase inhibitors were used exclusively in AD (34.4%; $p < 0.001$), and antiplatelet/anticoagulant use differed across groups ($p = 0.040$) (all $p > 0.05$; Table 1).

Neuropsychological and functional outcomes are summarized in Table 2. All cognitive and functional measures showed robust group differences, except for

Table 1 Demographic and clinical outcomes among groups

	Total (*n* = 99)	HC (*n* = 38)	MCI (*n* = 30)	AD (*n* = 31)	*p* value
Sex, female, n (%)	47 (47.5)	19 (50)	13 (43.3)	15 (48.4)	0.857
Age, mean (SD)	71.33 (6.23)	68.21 (5.99)	72.13 (5.18)*	74.61 (5.60)"	**< 0.001**
APOE ε4 carrier, n(%)	39 (41.5)	4 (10.8)	16 (57.1)*	19 (65.5)"	**< 0.001**
Education, median [Q1, Q3]	9 [7, 12]	11 [8.50, 14.25]	9 [6.75, 11]	9 [6, 12]	**0.048**
BMI score, mean (SD)	25.92 (3.70)	26.89 (4.22)	25.29 (2.88)	25.33 (3.58)	0.119
CRS score, median [Q1, Q3]	11 [8, 16]	13.50 [9, 17.75]	11 [7.75, 13.25]	10 [7, 14]	**0.025**
Medications, n (%)					
None, n(%)	12 (12.1)	9 (23.7)	2 (7.1)	1 (3.2)	0.628
Any, n(%)	85 (85.9)	29 (76.3)	26 (92.9)	30 (96.8)	0.855
Type of medication (if any), n (%)					
Lipid-lowering agents, n(%)	27 (27.3)	9 (23.7)	9 (30)	9 (29)	0.738
Antiplatelets/Anticoagulants, n(%)	9 (9.1)	0 (0)	4 (13.3)*	5 (16.1)"	**0.040**
Antidiabetics, n(%)	9 (9.1)	3 (7.9)	5 (16.7)	1 (3.2)	0.143
Proton pump inhibitors, n(%)	16 (16.2)	1 (2.6)	8 (26.7)*	7 (22.6)"	**0.011**
Cholinesterase inhibitors, n(%)	11 (11.1)	0 (0)	0 (0)	11 (35.5)"#	**< 0.001**
Analgesics/Anti-inflammatories, n(%)	11 (11.1)	2 (5.3)	5 (16.7)	4 (12.9)	0.265
Antipsychotics/Mood stabilizers, n(%)	3 (3)	0 (0)	1 (3.3)	2 (6.5)	0.301
Hearing impairment, n(%)	4 (4)	1 (2.6)	1 (3.3)	2 (6.5)	0.705
Medical History Variables, n(%)					
Hypertension, n(%)	38 (38.4)	17 (44.7)	10 (35.7)	11 (35.5)	0.667
Dyslipidemia, n(%)	35 (35.4)	12 (31.6)	12 (42.9)	11 (35.5)	0.639
Diabetes mellitus (T2), n(%)	11 (11.1)	5 (13.2)	5 (17.9)	1 (3.2)	0.188
Obesity, n(%)	11 (11.1)	7 (18.4)	2 (7.1)	2 (6.5)	0.210
History of stroke, n(%)	2 (2)	0 (0.0)	0 (0.0)	2 (6.5)	0.114
History of TBI, n(%)	4 (4)	1 (2.6)	2 (7.1)	1 (3.2)	0.630

AD: Alzheimer's Disease; APOE: Apolipoprotein E; BMI: Body Mass Index; HC: Healthy Controls; MCI: Mild Cognitive Impairment; CRS: Cognitive Reserve Scale; SD: Standard Deviation; Q1 and Q3: 25th and 75th percentiles, respectively. *: $p < 0.05$ for the comparison between HC and MCI; ": $p < 0.05$ for the comparison between HC and AD; #: $p < 0.05$ for the comparison between MCI and AD

GADS anxiety, depression, and total scores (all $p > 0.05$). Post-hoc comparisons confirmed graded impairment from HC to MCI to AD across global cognition (MMSE, MEC), memory (MIS, FCSRT subscales), orientation (PO-, SO-, TO-BTII), executive function (FAB, TMTA, fluency tasks), visuospatial ability (CDT, CCPS-BTII), and language (BNT). Functional outcomes (Activities of Daily Living, GDS-FAST, CDR) also differed significantly across groups, consistent with increasing clinical severity (all $p < 0.001$; Table 2).

Lifestyle-related characteristics are reported in Table 3. Most variables, including smoking status, years of tobacco/alcohol consumption (YTC, YAC), LEQ, household size, multilingualism, toxin exposure, and MEDLIFE total and subdomain scores, did not differ significantly between groups (all $p > 0.05$). Exceptions included alcohol consumption status (HC = 25.0%, MCI = 10.0%, AD = 9.7%; $p = 0.006$) and CRS scores, which were significantly higher in HC than in MCI or AD ($p = 0.025$). A trend toward lower physical and social activity scores in AD was observed ($p = 0.072$), but this did not reach statistical significance (Table 3).

Overall GMB composition

Prior to diversity analysis, overall GMB composition was compared across diagnostic groups. Figure 2 depicts the relative abundances at the genus level in HC, MCI, and AD. The most prevalent genera across all groups were *Bacteroides*, *Alistipes*, *Faecalibacterium* and *Phocaeicola*. After correction for multiple comparisons, no significant group differences were observed in the relative abundance of any taxonomic level. Thus, the broad composition of the GMB was generally similar among the three groups. Several genera showed nominal differences ($p < 0.05$), but none remained significant following FDR correction ($q < 0.05$).

Microbial diversity

Diversity measures of the GMB were subsequently evaluated. As shown in Fig. 3A, a rank-based ANCOVA adjusting for age and education revealed no significant group effects for either richness (Chao1; $F(2,94) = 0.33$, $p = 0.720$) or α-diversity (Shannon; $F(2,94) = 1.64$, $p = 0.200$), with small effect sizes (partial $\eta^2 < 0.03$). PERMANOVA on Bray–Curtis distances, adjusted for age and education, revealed no significant group differences

Table 2 Neuropsychological and functional profiles among groups

	Total (*n* = 99)	HC (*n* = 38)	MCI (*n* = 30)	AD (*n* = 31)	*p* value
MMSE, median [Q1, Q3]	27 [23, 30]	30 [28.75, 30]	26 [24.75, 28]*	21 [18, 26]″#	**< 0.001**
MEC, median [Q1, Q3]	31 [26.50, 35]	35 [33, 35]	30 [28, 32]*	23 [20, 30]″	**< 0.001**
PO-BTII, median [Q1, Q3]	25 [25, 25]	25 [25, 25]	25 [24.75, 25]	25 [23, 25]″	**< 0.001**
SO-BTII, median [Q1, Q3]	25 [23, 25]	25 [25, 25]	25 [23, 25]*	23 [17, 25]″#	**< 0.001**
TO-BTII, median [Q1, Q3]	70 [54, 70]	70 [70, 70]	62.50 [38.75, 70]*	57 [25, 63]″	**< 0.001**
MIS, median [Q1, Q3]	4 [1, 7]	8 [6, 8]	2 [0.75, 4]*	1 [0, 2]″	**< 0.001**
DSF, median [Q1, Q3]	5 [5, 6]	6 [5, 6]	5 [5, 6]	5 [4, 6]″	**0.007**
DSB, median [Q1, Q3]	4 [3, 4]	4 [3, 4.25]	4 [3, 4]	3 [3, 4]″	**0.028**
FCSRT, median [Q1, Q3]					
1st free recall, median [Q1, Q3]	2 [0, 6]	6 [5, 8]	1.50 [0, 3]*	0 [0, 2]″	**< 0.001**
Total free recall, median [Q1, Q3]	8 [0, 22]	23.50 [17.50, 30]	0 [0, 9]*	0 [0, 0]″	**< 0.001**
Total recall, median [Q1, Q3]	22 [0, 40]	41 [36, 44.25]	0 [0, 31.25]*	0 [0, 0]″	**< 0.001**
Delayed free recall, median [Q1, Q3]	1 [0, 9]	10 [7.50, 12]	0 [0, 5]*	0 [0, 0]″	**< 0.001**
Total delayed recall, median [Q1, Q3]	7.92 [0, 15]	15 [12, 16]	0 [0, 12]*	0 [0, 0]″	**< 0.001**
TMTA, median [Q1, Q3]	63 [45, 93]	48.5 [32.75, 63.75]	69 [52.75, 90.75]*	88.21 [62, 170]″	**< 0.001**
CDT, median [Q1, Q3]	9 [7, 10]	10 [9, 10]	9 [7, 10]	6.5 [3, 8.50]″#	**< 0.001**
FAB, median [Q1, Q3]	16 [14, 18]	18 [16.75, 18]	15 [14, 17]*	12 [9, 15]″	**< 0.001**
CCPS-BTII, median [Q1, Q3]	30 [29, 30]	30 [30, 30]	30 [29, 30]	30 [25, 30]″	**0.007**
BNT, median [Q1, Q3]	11 [9, 12]	12.50 [11, 14]	10 [9, 11]*	9 [7, 12]″	**< 0.001**
SF, mean (SD)	14.63(5.86)	18.67 (4.9)	14.23 (4.20)*	10.06 (4.75)″#	**< 0.001**
FF, median [Q1, Q3]	11 [6, 15]	13.50 [9, 17]	9.50 [6.75, 14]	6 [4, 12]″	**< 0.001**
GADS					
Anxiety Score, median [Q1, Q3]	1 [0, 3]	1 [0, 3]	2.50 [0, 5]	1 [0, 3]	0.067
Depression Score, median [Q1, Q3]	0 [0, 2]	0 [0, 1]	0 [0, 2]	0 [0, 3]	0.490
Total Score, median [Q1, Q3]	1 [0, 5]	1 [0, 3]	3.50 [0.75, 6]	1 [0, 4]	0.069
NPS, median [Q1, Q3]	2 [0, 7]	0 [0, 0]	4 [2, 9]*	6 [3, 10]″	**< 0.001**
NPS-C, median [Q1, Q3]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 0]″	**0.010**
Activities of Daily-Living					
Adv. and Instrumental Activities, median [Q1, Q3]	10 [0, 28]	0 [0, 0]	13 [8.50, 21.75]*	40 [24, 51]″#	**< 0.001**
Basic activities, median [Q1, Q3]	0 [0, 0]	0 [0, 0]	0 [0, 0]	1 [0, 3]″#	**< 0.001**
GDS-FAST, median [Q1, Q3]	3 [1, 4]	1 [1, 1]	3 [3, 3]*	4 [4, 5]″#	**< 0.001**
CDR, median [Q1, Q3]	0.5 [0, 1]	0 [0, 0]	0.5 [0.5, 0.5]*	1 [1, 2]″#	**< 0.001**

AD: Alzheimer's Disease; Adv: Advanced; BNT: Boston Naming Test; CCPS-BTII: Copy of Simple and Semi-complex Construction Praxis subtest of the Barcelona Test II; CDR: Clinical Dementia Rating; CDT: Clock Drawing Test; DSB: Digit Span Backward; DSF: Digit Span Forward; FAB: Frontal Assessment Battery; FCSRT: Free and Cued Selective Reminding Test; FF: Formal Fluency; GADS: Goldberg Anxiety and Depression Scale; GDS-FAST: Global Deterioration Scale; HC: Healthy Controls; MCI: Mild Cognitive Impairment; MEC: Lobo's Mini-Examen Cognoscitivo; MIS: Memory Impairment Screen; MMSE: Mini-Mental State Examination; NPS: Neuropsychiatric Symptomatology subtest of the Barcelona Test II; NPS-C: Complementary Neuropsychiatric Symptomatology subtest of the Barcelona Test II; PO-BTII: Personal-orientation subtest of the Barcelona Test II; SF: Semantic Fluency; SO-BTII: Spatial-orientation subtest of the Barcelona Test II; TMTA: Trail Making Test-A; TO-BTII: Time-orientation subtest of the Barcelona Test II; Q1 and Q3: 25th and 75th percentiles; SD: Standard deviation. *: $p < 0.05$, comparison HC vs. MCI; ″: $p < 0.05$, comparison HC vs. AD; #: $p < 0.05$, comparison MCI vs. AD

($F(2,94) = 1.29$, $R^2 = 0.026$, $p = 0.219$) (Fig. 3A). ANOSIM results were consistent, showing minimal between-group dissimilarity ($R = 0.037$, $p = 0.059$).

Differential abundance analysis

LEfSe analyses were conducted to detect individual taxa with differential abundance between diagnostic groups, recognizing that specific microbial changes may be biologically relevant even in the absence of significant shifts in broad community metrics. Using a $q < 0.05$ and LDA > 3.0 threshold, LEfSe identified numerous taxa with significantly different relative abundances in pairwise comparisons. Figure 4A–C summarizes the discriminatory taxa by taxonomic rank. Across all comparisons, 109 unique taxa exceeded the LDA threshold: 53 differed between HC and AD, 42 between MCI and AD, and 27 between HC and MCI. Overlap of differentially abundant taxa at the species level (LEfSe, $q < 0.05$; LDA > 3) (Fig. 4D) revealed that five taxa were shared between AD vs. HC and MCI vs. HC, four between AD vs. HC and AD vs. MCI, and one between MCI vs. HC and AD vs. MCI (see Supplementary Table S4 for details). Rank-based comparisons (Fig. 4E) examined the ten overlapping species: *Coprococcus comes*, *Odoribacter splanchnicus*, *Bacteroides faecis*, *Alistipes communis*, *Alistipes sp. dk3624*, *Bacteroides eggerthii*, *Clostridioides*

Table 3 Lifestyle-related characteristics among groups

	Total (*n* = 99)	HC (*n* = 38)	MCI (*n* = 30)	AD (*n* = 31)	*p* value
Smoke Status					
Current smoker, n(%)	7(7.2)	4(11.1)	2(6.7)	1(3.2)	0.680
Former smoker, n(%)	35(36.1)	11(30.6)	13(43.3)	11(35.5)	
Non-smoker, n(%)	55(56.7)	21(58.3)	15(50)	19(61.3)	
YTC, median [Q1, Q3]	0 [0, 20]	0 [0, 21]	1 [0, 20]	0 [0, 20]	0.866
Alcohol					
Current consumer, n(%)	15(15.5)	9(25)	3(10)	3(9.7)"	**0.006**
Former consumer, n(%)	5(5.2)	0(0)	0(0)	5(16.1)"	
Non-consumer, n(%)	77(79.4)	27(75)	27(90)	23(74.2)"	
YAC, median [Q1, Q3]	0 [0, 0]	0 [0, 0.75]	0 [0, 0]	0 [0, 0]	0.197
LEQ, mean (SD)	12.67 (5.70)	13.63(6.19)	13.33(5.13)	10.85(5.33)	0.097
Household Size, mean (SD)	2.16(0.72)	2.06(0.71)	2.2(0.61)	2.26(0.82)	0.493
Number of languages					
Bilingual, n(%)	71	28(73.7)	23(76.7)	20(64.5)	0.347
Monolingual, n(%)	17	4(11.1)	6(20)	7(22.6)	
Multilingual, n(%)	11	6(15.8)	1(3.3)	4(12.9)	
Exposure to toxins, n(%)	74	29 (76.3)	21 (70.0)	24 (77.4)	0.769
MEDLIFE					
Food consumption, median [Q1, Q3]	9 [8, 11]	9.50 [8, 11]	9 [7.75, 10]	10 [9, 11]	0.075
Dietary habits, median [Q1, Q3]	5 [4, 5]	5 [4, 5]	5 [4, 5]	4 [3, 6]	0.721
Physical and social activity, median [Q1, Q3]	4 [3, 4]	4 [3, 5]	4 [3, 4]	3 [2, 4]"	0.072
Total Score, median [Q1, Q3]	18 [16, 19]	18 [16, 19.25]	17 [16, 19]	17 [16, 20]	0.269

APOE, Apolipoprotein E; AD: Alzheimer's Disease; HC: Healthy Controls; MCI: Mild Cognitive Impairment; BMI: Body Mass Index; LEQ: Lifetime of Experiences Questionnaire; MEDLIFE: Mediterranean Lifestyle Index Test; CRS: Reserve Cognitive Scale; YAC: Years of Alcohol Consumption; YTC: Years of Tobacco Consumption. Q1 and Q3 indicate the 25th and 75th percentiles, respectively; SD: Standard Deviation. *: $p < 0.05$ for comparison between HC and MCI; ": $p < 0.05$ for comparison between HC and AD; #: $p < 0.05$ for comparison between MCI and AD

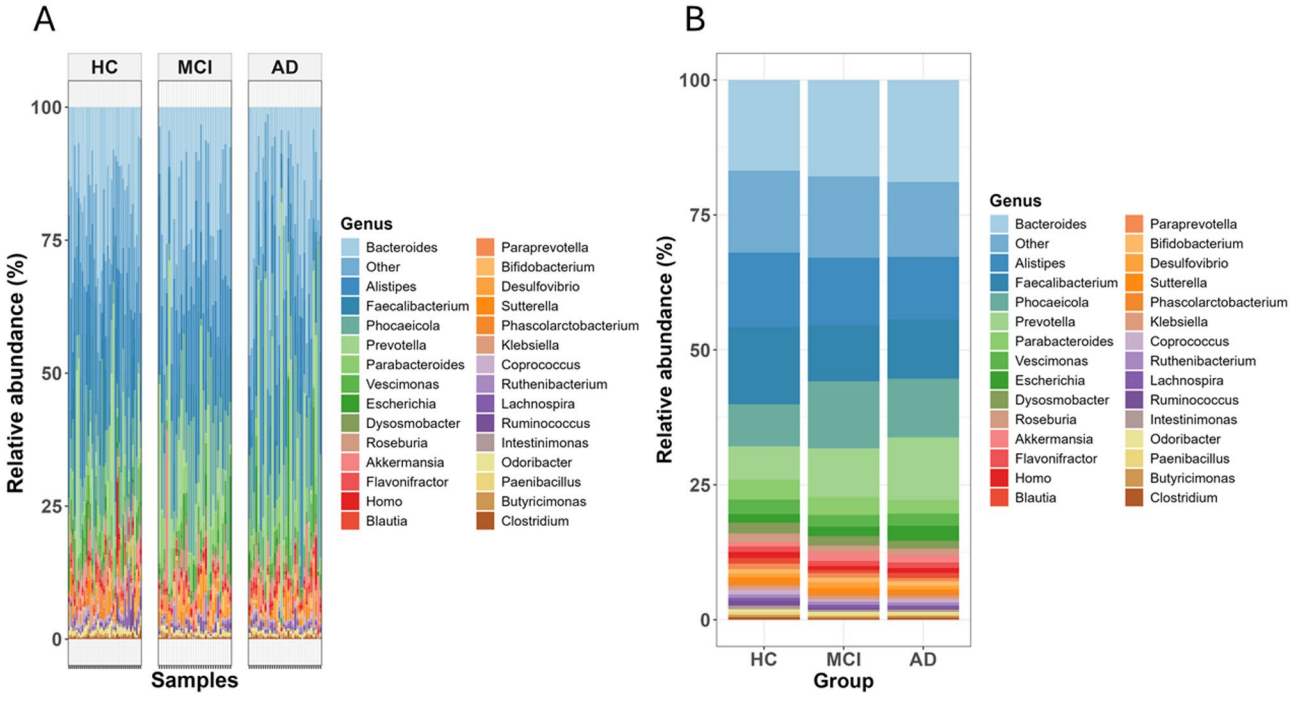

Fig. 2 GMB composition of HC, MCI and AD groups at genus level. **(A)** Stacked bar plots showing the relative abundance of genera in individual samples, grouped by diagnostic category, **(B)** Average relative abundance of genera across groups. HC, Healthy Controls; MCI, Mild Cognitive Impairment; AD, Alzheimer's Disease

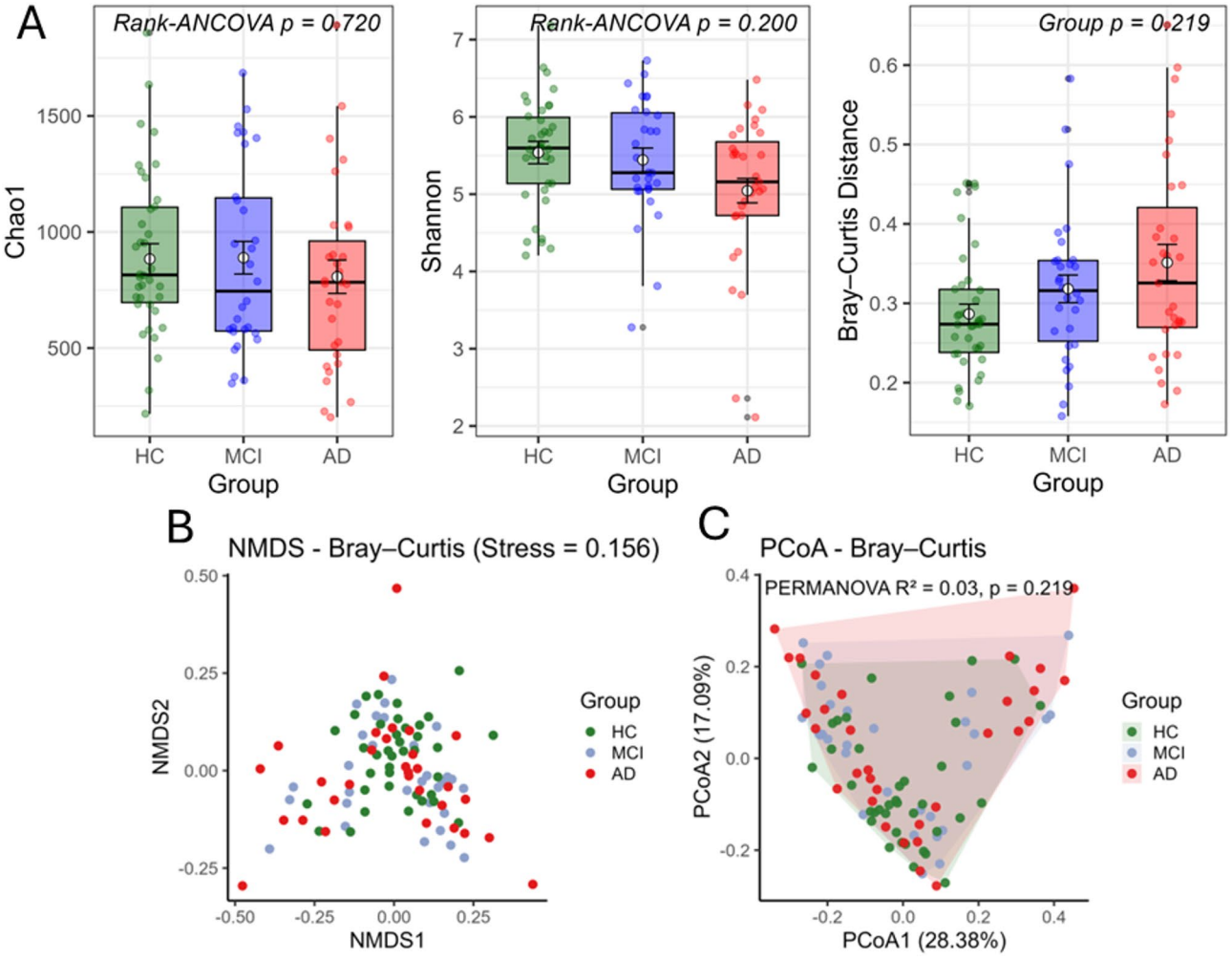

Fig. 3 Diversity of the GMB among study groups and multivariate ordination analyses. **(A)** Covariate-adjusted richness (Chao1), α-diversity (Shannon) and β-diversity (Bray-Curtis), **(B)** NMDS plot of Bray–Curtis dissimilarities, **(C)** PCoA of Bray–Curtis distances. Convex hulls indicate the 95% confidence intervals. AD: Alzheimer's Disease; HC: Healthy Controls; MCI: Mild Cognitive Impairment; NMDS, Non-metric Multidimensional Scaling; PCoA, Principal Co-ordinates Analysis

difficile, *Coprococcus sp. ART55/1*, *Roseburia intestinalis*, and *Parabacteroides distasonis.* Among these, *Coprococcus comes* ($q = 0.0498$) and Odoribacter splanchnicus ($q = 0.0498$) reached statistical significance (Table S5).

Taxon-clinical correlations

Multivariate models adjusted for diagnostic group, education, age, sex, BMI, APOE ε4 status, and medication use; Fig. 5B) revealed additional statistically significant associations. Across all participants, *Parabacteroides distasonis* was positively associated with depression scores (GADSD; coef = 0.47, $q = 0.001$), while *Roseburia intestinalis* (coef = 0.55, q = 0.002), *Coprococcus comes* (coef = 0.42, $q = 0.011$), and *Clostridioides difficile* (coef = 0.32, $q = 0.012$) were positively associated with orientation (OBTII). In the AD subgroup, *Parabacteroides distasonis* was positively associated with both depression scores (GADSD; coef = 0.96, $q = 0.002$) and neuropsychiatric symptoms (NPS; coef = 1.04, $q = 0.003$), while *Alistipes sp. dk3624* (coef = 0.96, $q = 0.005$) and Bacteroides eggerthii (coef = 0.82, $q = 0.010$) also showed significant associations with NPS and GADSD, respectively. Within the HC subgroup, *Roseburia intestinalis* showed significant positive associations with executive function (CCPS-BTII; coef = 0.51, $q = 0.002$), semantic fluency (SF; coef = 0.60, $q = 0.003$), and orientation (OBTII; coef = 0.45, $q = 0.008$), while *Coprococcus comes* was also positively associated with semantic fluency (SF; coef = 0.65, $q = 0.013$). It should be noted that lower scores obtained in TMTA and ADL indicate better performance. No significant associations were identified in the MCI subgroup. The detailed correlation coefficients, standard errors, *p*-values, and q-values for each significant taxon-clinical pair for all groups, AD, MCI and HC groups independently, are summarized in Table 4. Details

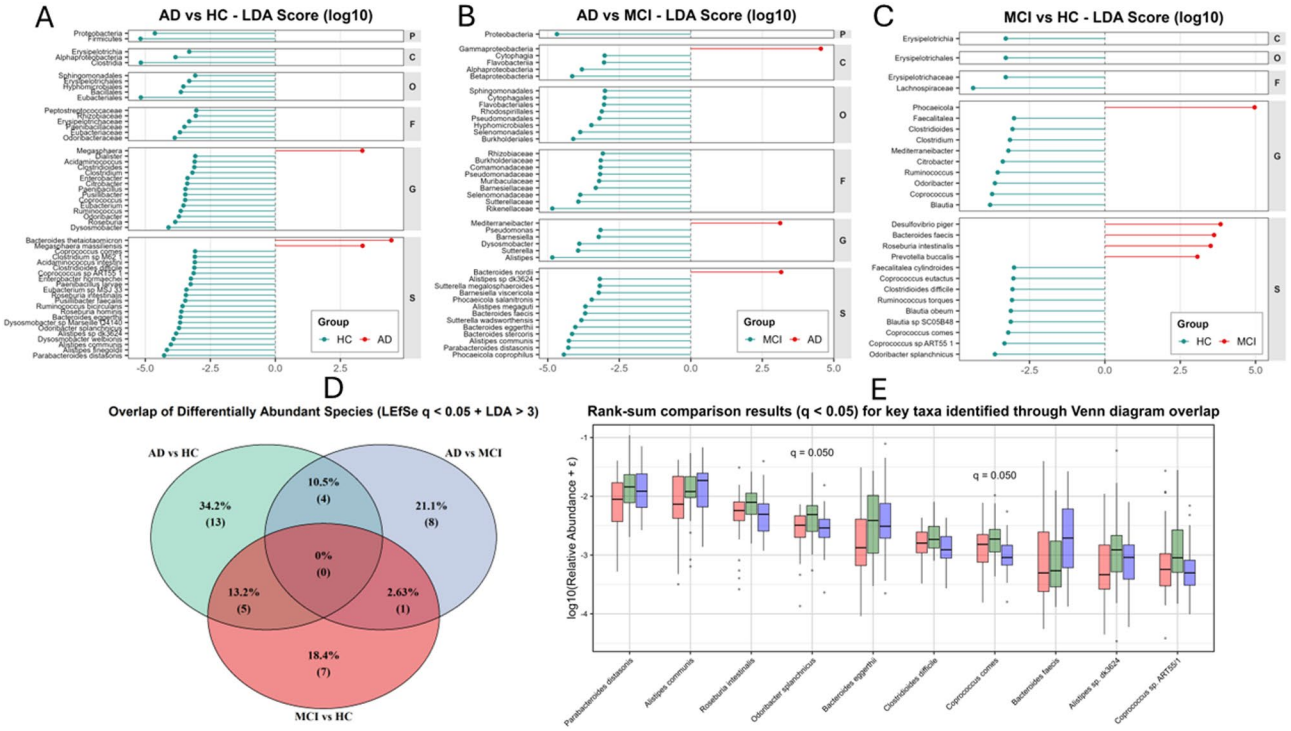

Fig. 4 Hierarchical Overlap Reduction and Taxon Prioritization. **A-C** LEfSe LDA plot (q < 0.05, LDA > 3.0) between (**A**) AD vs. HC, (**B**) AD vs. MCI, and (**C**) MCI vs. HC, **D.** Venn diagram of taxa identified by LEfSe across pairwise group comparisons. The numbers indicate the total number of significant taxa detected (species level: LDA > 3.0, q < 0.05) in each comparison. Overlapping regions represent taxa shared between comparisons., **E.** Rank-sum comparison results (q < 0.05) for the 10 key taxa identified through the Venn diagram overlap. AD: Alzheimer's Disease; HC: Healthy Controls; LDA: Linear Discriminant Analysis; MCI: Mild Cognitive Impairment

for total correlations are provided in Table S6, S7, S8 and S9, respectively.

Trend analysis

To determine whether specific taxa change monotonically with clinical progression, participants were stratified by CDR and GDS-FAST levels (Fig. 5B). Across increasing CDR scores, significant decreasing trends in abundance were observed for *Odoribacter splanchnicus* (statistic = 1300, $q = 0.026$), *Alistipes sp. dk3624* (statistic = 1380, $q = 0.029$), *Coprococcus sp. ART55/1* (statistic = 1367, $q = 0.029$), *Roseburia intestinalis* (statistic = 1353, $q = 0.029$), and *Coprococcus comes* (statistic = 1413, $q = 0.040$). Using GDS-FAST staging, a similar pattern was observed, with significant monotonic decreases for *Odoribacter splanchnicus* (statistic = 1187, $q = 0.009$), *Coprococcus sp. ART55/1* (statistic = 1294, $q = 0.039$), *Alistipes sp. dk3624* (statistic = 1337, $q = 0.041$), *Bacteroides eggerthii* (statistic = 1326, q = 0.041), *Coprococcus comes* (statistic = 1379, $q = 0.045$), *Parabacteroides distasonis* (statistic = 1378, q = 0.045), and *Roseburia intestinalis* (statistic = 1378, $q = 0.045$). The detailed statistics and *p*-values for each significant trend in CDR and GDS-FAST levels are summarized in Table S10 and S11, respectively.

Discussion

Clinical outcomes

The clinical characteristics of our study population revealed significant group differences, consistent with established patterns of disease progression. Participants in the HC group had higher education and cognitive reserve scores, which are thought to confer resilience against cognitive decline, consistent with the cognitive reserve hypothesis [40]. As expected, neuropsychological, neuropsychiatric and functional outcomes worsened progressively from MCI to AD [41, 42]. Emotional outcomes did not differ significantly among groups; however, higher scores were observed in the MCI group compared with AD. This trend may be explained by reduced self-awareness in AD, which can limit the ability to perceive or report emotional symptoms [43]. The generally homogeneous lifestyle profile of our cohort reduces the confounding impact of life-style variables and provides a suitable framework to isolate the potential contribution of GMB to cognitive decline. Importantly, these findings align with the 2024 Lancet Commission report on dementia prevention, intervention, and care [10], which emphasized education, cognitive reserve, cardiovascular and metabolic health and lifestyle factors as central modifiable risk factors influencing dementia trajectories.

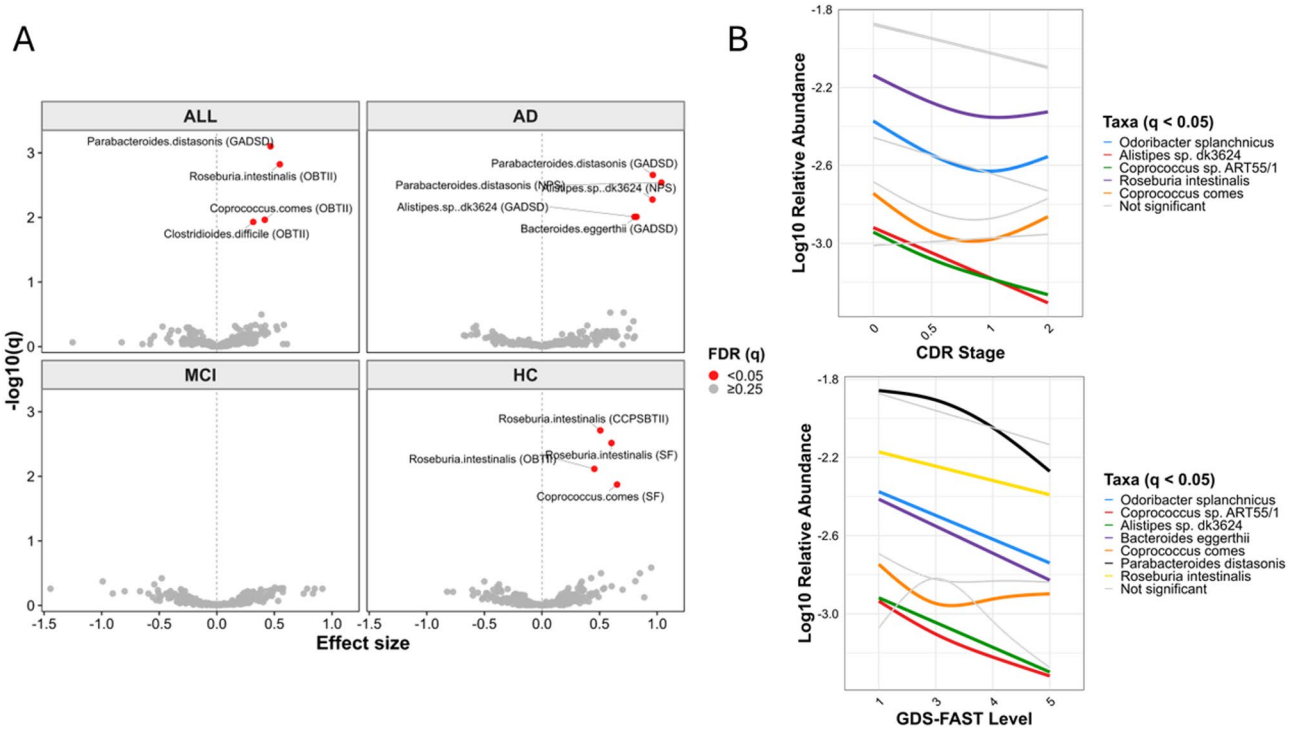

Fig. 5 Microbiome–clinical associations. **(A)** Volcano plot of MaAsLin2 multivariable associations ($q < 0.05$) adjusted for diagnostic group, sex, age, education, BMI, APOE ε4 status, and medication use, depicting coefficients (x-axis) versus −log10(q) (y-axis). **(B)** Jonckheere–Terpstra trend plots across CDR stages (0 → 2) and GDS-FAST levels (1 → 5) ($q < 0.05$). CDR, Clinical Dementia Rating; GDS-FAST, Global Deterioration Scale

Table 4 Significant associations between bacterial taxa and clinical outcomes identified by multivariate MaAsLin2 models adjusted for sex, age, education, BMI, APOE ε4 status and medication use (all $q < 0.05$)

feature	metadata	value	coef	stderr	*N*	*N*.not.0	pval	qval
All groups								
Parabacteroides distasonis	GADSD	GADSD	0.46631765	0.1338453	99	99	0.00079132	0.00079132
Roseburia intestinalis	OBTII	OBTII	0.54715894	0.15104635	99	99	0.0005014	0.00150421
Coprococcus comes	OBTII	OBTII	0.41648519	0.1512849	99	99	0.00725233	0.0108785
Clostridioides difficile	OBTII	OBTII	0.31629479	0.12275245	99	99	0.01174458	0.01174458
AD group								
Parabacteroides distasonis	GADSD	GADSD	0.96312479	0.24184361	31	31	0.00073303	0.00219909
Parabacteroides distasonis	NPS	NPS	1.03724166	0.28090522	31	31	0.0014422	0.00288439
Alistipes sp. dk3624	NPS	NPS	0.95975991	0.3067265	31	31	0.00528477	0.00528477
Bacteroides eggerthii	GADSD	GADSD	0.82128803	0.28758199	31	31	0.00976937	0.00976937
Alistipes sp. dk3624	GADSD	GADSD	0.80385261	0.2800659	31	31	0.00946163	0.00976937
HC group								
Roseburia.intestinalis	CCPSBTII	CCPSBTII	0.50679527	0.14926468	38	38	0.00194763	0.00194763
Roseburia.intestinalis	SF	SF	0.60371159	0.17307805	38	38	0.00152423	0.00304846
Roseburia.intestinalis	OBTII	OBTII	0.45498812	0.15919578	38	38	0.00767687	0.00767687
Coprococcus.comes	SF	SF	0.65223225	0.24821674	38	38	0.01341678	0.01341678

AD: Alzheimer's Disease; BMI: Body Mass Index; CCPS-BTII: Copy of Simple and Semi-complex Construction Praxis subtest of the Barcelona Test II; DSB: Digit Span Backward; GADS: Goldberg Anxiety and Depression Scale; HC: Healthy Controls; MCI: Mild Cognitive Impairment; NPS: Neuropsychiatric Symptomatology subtest of the Barcelona Test II; OBTII: Orientation subtest of the Barcelona Test II; CRS: Cognitive Reserve Scale; SF: Semantic Fluency. Coef: coefficient; pval: *p*-value; qval: q-value; stderr: standard error. No significant associations were detected in the MCI subgroup

Given their established effects, medication exposures and APOE ε4 status were included in our models, as both are known to influence the MGBA [44, 45].

Overall GMB composition did not differ significantly between HC, MCI, and AD, though a trend toward higher *Bacteroidetes* relative to *Firmicutes* in MCI and AD echoed reports from Asian [46] and American cohorts [4]. The *Firmicutes/Bacteroidetes* ratio, often linked to gut dysbiosis, has shown inconsistent associations with AD, likely reflecting dietary and lifestyle influences [47]. In our Mediterranean cohort, the predominance of *Bacteroidetes* (particularly *Bacteroides* and *Prevotella*) may reflect diet-driven effects, with *Bacteroides uniformis* highlighting a potential neuroprotective role via SCFAs production. Prevotella, commonly linked to fiber-rich diets and anti-inflammatory activity, was also higher in MCI and AD, consistent with Guo, Peng [48], although other studies have reported reductions in AD compared with HC [49]. These findings suggest that adherence to a fiber-rich MD may buffer or reshape disease-related microbial shifts observed in other populations [12, 13, 14]. In our cohort, richness, α-diversity, and β-diversity (Bray–Curtis) did not differ between groups, aligning with Ueda, Shinkai [50] but contrasting with most reports of reduced α-diversity or distinct β-diversity in Asian [5, 48, 49, 51] and American [4] cohorts. These discrepancies may reflect methodological and population-specific factors, while the relatively homogeneous ML of our participants may have helped preserve overall community diversity [12, 13, 14].

MGBA axis

Given the absence of clear community-level differences, we next applied LEfSe to identify specific taxa with discriminatory potential across groups. SCFAs, including butyrate, acetate, and propionate, are key microbial metabolites produced through the fermentation of dietary fibers and resistant starches. In our study, several SCFAs-producing taxa identified by LEfSe and multivariate MaAsLin2 models *(Coprococcus comes*, *Odoribacter splanchnicus*, *Roseburia intestinalis*, *Parabacteroides distasonis*, *Bacteroides eggerthii and Alistipes sp. dk3624)* showed decreasing abundances with increasing CDR and GDS-FAST severity levels. These monotonic reductions suggest that advancing cognitive decline is accompanied by a loss of beneficial SCFAs producers. Functionally, *Coprococcus comes* was positively associated with orientation, semantic fluency, and activities of daily living in HC, while *Roseburia intestinalis* correlated with executive function, semantic fluency, and orientation. These findings align with previous reports of reduced *Coprococcus* and *Roseburia spp.* in AD patients [52, 53]. Similarly, *Odoribacter splanchnicus*, a propionate producer, was associated with improved cognitive outcomes [54]. Interestingly, not all SCFAs producers showed protective patterns. *Parabacteroides distasonis* and *Bacteroides eggerthii* were positively associated with depressive symptoms and neuropsychiatric burden in AD. While both are capable of SCFAs production, prior studies suggest their roles are highly context-dependent. Experimental work demonstrated that *Parabacteroides distasonis* can induce depressive-like behavior in Crohn's disease models [55], and *Bacteroides eggerthii* has been implicated in inflammation and metabolic dysregulation [56]. Conversely, metagenomic analyses have identified *Parabacteroides distasonis* as enriched in healthy controls vs. AD patients [57]. These results suggest that SCFAs production alone is not uniformly beneficial, and that the functional impact of these taxa may depend on host condition, comorbidities, and broader community context. Overall, our results suggest that cognitive decline is linked to a dysbiotic shift in the MGBA characterized by the loss of protective SCFAs producers alongside the emergence of taxa with context-dependent or adverse associations. This imbalance may compromise gut barrier integrity, promote systemic inflammation, and heighten neuroinflammatory signaling, thereby accelerating neurodegeneration [5]. Some associations, however, were unexpected. *Clostridioides difficile*, despite its well-established pathogenic role in gut infections and toxin production [58], was positively associated with orientation performance across groups. This finding diverges from prior literature and should be interpreted with caution, as it may reflect population-specific dynamics or secondary effects rather than a true protective association. While these findings highlight candidate microbial taxa for further investigation, the molecular mechanisms underlying their influence on the MGBA remain to be clarified. Future studies integrating metagenomics with metabolomic and immunological approaches will be critical to delineate these pathways and evaluate the therapeutic potential of microbiota-targeted interventions.

Mediterranean lifestyle and diet

Our taxa-level results converge with a substantial body of work reporting reductions in SCFAs-producing bacteria in cognitive impairment alongside positive associations with cognitive performance [4, 54]. While several recent meta-analyses have described group-level diversity and compositional differences in AD/MCI versus HC [6], our Mediterranean cohort showed no global α/β-diversity shifts, yet still exhibited directional declines of SCFAs producers across CDR and GDS-FAST and multivariable associations with cognition. This pattern supports the notion that functionally meaningful, rank-specific changes can occur even when community-wide metrics are stable, and it is compatible with a diet and region-modulated microbiome, where high fiber/polyphenol

intake may buffer large ecological displacements while still permitting subtle, clinically relevant taxon shifts [12, 13, 14]. Importantly, recent work has identified mechanistic links between gut microbial metabolites and cognitive decline that are independent of dietary influences [59], further underscoring the biological plausibility of our findings. Discrepancies with studies reporting stronger group separations likely reflect differences in sequencing modality (shotgun vs. 16 S), statistical adjustment (e.g., BMI, medications, APOE ε4), geography/dietary background and case-mix. Notably, *Coprococcus comes* and *Odoribacter splanchnicus* emerged as replicable candidates, showing both discriminatory value and severity-related decreases, underscoring their potential as biologically plausible biomarkers within the MGBA framework. These findings underscore the need for further research into the interaction between diet, microbiome functionality, and cognitive health. Future studies should compare the impact of the MD with other dietary patterns and evaluate multicomponent interventions that not only promote dietary adherence but also address lifestyle and environmental factors that shape the MGBA.

Limitations and strengths

Several limitations should be acknowledged. The cross-sectional design precludes causal inference, underscoring the need for longitudinal studies to determine whether GMB alterations precede cognitive impairment. The cohort's limited ethnic diversity may affect generalizability. Differences in age and education between groups could also have influenced findings, despite covariate adjustment. Moreover, while shotgun metagenomics allowed high-resolution profiling of taxonomic composition, functional and metabolic outputs were not directly assessed, limiting insight into underlying microbial pathways. In addition, rarefaction to 1,788 reads, although chosen to maximize sample retention and reduce sequencing-depth bias, may have excluded low-abundance taxa with potential biological relevance [60]. Despite these limitations, the study has important strengths. The use of shotgun metagenomic sequencing enabled precise taxonomic resolution and the identification of taxa associated with clinical outcomes. The well-characterized population, with comprehensive clinical, neuropsychological, and lifestyle data, provided a robust framework to assess gut–brain interactions. Furthermore, the relative uniformity of diet and lifestyle across participants minimized common sources of bias in microbiome studies. Finally, the identification of taxa showing consistent associations across cognitive stages highlights potential microbial biomarkers for early detection and therapeutic targeting in cognitive decline.

Conclusion

Overall GMB diversity did not differ across cognitive groups, but specific taxa, particularly short-chain fatty acid producers, showed consistent associations with cognitive decline in this Mediterranean lifestyle cohort. These findings support a role for the GMB in AD pathology and suggest that targeting key microbial species may provide novel avenues for prevention and intervention.

Abbreviations

AD	Alzheimer's Disease
ADL	Activities of Daily Living
APOE	Apolipoprotein E
aMCI	Amnestic Mild Cognitive Impairment
BMI	Body Mass Index
BNT	Boston Naming Test
BTII	Barcelona Test II
CCPS-BTII	Copy of Simple and Semi-complex Construction Praxis subtest of the BTII
CDR	Clinical Dementia Rating
CDT	Clock Drawing Test
CRS	Cognitive Reserve Scale
DSB	Digit Span Backward
DSF	Digit Span Forward
FAB	Frontal Assessment Battery
FAST	Functional Assessment Staging
FCSRT	Free and Cued Selective Reminding Test
FDR	False Discovery Rate
FF	Formal Fluency
GADS	Goldberg Anxiety and Depression Scale
GDS-FAST	Global Deterioration Scale – Functional Assessment Staging
GMB	Gut Microbiota
HC	Healthy Controls
LDA	Linear Discriminant Analysis
LEfSe	Linear Discriminant Analysis Effect Size
LEQ	Lifetime of Experiences Questionnaire
LOAD	Late-Onset Alzheimer's Disease
MCI	Mild Cognitive Impairment
MD	Mediterranean Diet
MEC	Mini-Examen Cognoscitivo (Spanish version of MMSE)
MEDLIFE	Mediterranean Lifestyle Index
MGBA	Microbiota-Gut-Brain Axis
MIS	Memory Impairment Screen
ML	Mediterranean Lifestyle
MMSE	Mini Mental Status Examination
NMDS	Non-metric Multidimensional Scaling
NPS	Neuropsychiatric Symptomatology
NPS-C	Complementary Neuropsychiatric Symptomatology
OTU	Operational Taxonomic Unit
PCA	Principal Component Analysis
PCoA	Principal Coordinates Analysis
PO-BTII	Personal Orientation subtest of the BTII
Q1, Q3	25th and 75th percentiles
SCFA	Short-Chain Fatty Acid
SD	Standard Deviation
SF	Semantic Fluency
SO-BTII	Spatial Orientation subtest of the BTII
TMTA	Trail Making Test A
TMTB	Trail Making Test B
TO-BTII	Time Orientation subtest of the BTII
YAC	Years of Alcohol Consumption
YTC	Years of Tobacco Consumption

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13195-025-01862-z.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

Supplementary Material 8

Supplementary Material 9

Supplementary Material 10

Supplementary Material 11

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Data availability
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
This study complied with the Declaration of Helsinki (as revised in Tokio 2004) and its subsequent updates and was approved by the Clinical Research Ethics Committee (CEIm) of the Institute of Health Research Pere Virgili (IISPV, Ref. CEIm: 183/2020). All participants, or their legal representatives, provided informed consent for the patient's involvement in the study prior to the patient's enrollment.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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