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Original article

Impacts of an antioxidant-rich diet and lifestyle factors on gut microbiota diversity and brain health: An exploratory analysis from the NutBrain study

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SUMMARY

Background and aims: Healthy diet and lifestyle have been linked to improved gut microbiota diversity and neurocognitive outcomes. However, few human studies have simultaneously examined an antioxidant-rich diet (ARD) in combination with other lifestyle factors and their effects on gut microbiota diversity, brain morphometry, and cognitive function. Our aim was to investigate how the dietary antioxidant capacity and a healthy lifestyle profile influence gut microbiota diversity and composition, brain morphometry, and global cognitive function in older adults.

Methods: In a cross-sectional analysis of the NutBrain study (2019–2023), a cohort of 246 dementia-free individuals aged ≥65 years, completed a 3-day food diary to estimate the total dietary antioxidant capacity (Oxygen Radical Absorbance Capacity - ORAC). Global cognitive function was assessed using the Mini-Mental State Examination (MMSE). Gut microbiota α- and β-diversities and taxa abundances were derived by 16S rRNA amplicon-based sequencing of stool samples. Brain morphometry - including total brain, white matter, grey matter, and ventricular cerebrospinal fluid volumes - was assessed using magnetic resonance imaging. Multiple linear regression models, accounting for many potential confounders (i.e.: socio-demographics, use of drugs, energy intake, inflammatory and anthropometric markers, and APOE genotyping) examined how ORAC, both alone and combined with smoking and physical activity (devising a healthy lifestyle score, Hscore), affected microbiota diversity, MMSE scores, and brain volumes.

Results: Higher ORAC adherence was associated with greater gut microbiota diversity ($p \leq 0.05$). Several taxa, such as *Barnesiella*, *Coprococcus*, *Ruminococcus*, *Parabacteroides*, *Lachnospiraceae* NK4A136 group, and *Clostridia* UCG-014 group exhibited increased abundances within the highest ORAC and Hscore

Abbreviations: ARD, Antioxidant-rich diet; NutBrain, Nutrition, gUT microbiota, and BRain AgINg Study; MRI, Magnetic Resonance Image; MMSE, Mental State Examination; GDPR, General Data Protection Regulation; EDTA, Ethylenediaminetetraacetic acid; APOE, Apolipoprotein E; CRP, C-reactive protein; IL-6, Interleukin-6; TNFα, Tumour necrosis factor-alpha; ORAC, Oxygen Radical Absorbance Capacity; TBV, Total Brain Volume; GMV, Gray Matter Volume; WMV, White Matter Volume; VCSF, Ventricular Cerebrospinal Fluid; IPAQ, International Physical Activity Questionnaire; SD, Standard deviation; CI, Confidence intervals.

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tertiles, as compared to the lowest ones. The highest tertile of total ORAC was also positively and significantly associated with greater total brain, white matter, and grey matter volumes ($p \leq 0.05$). These associations were stronger in participants classified as having a favourable lifestyle profile (regular physical activity, non-smokers), with notable correlations observed for total brain volume, gut α-diversity, white matter volume and MMSE ($p \leq 0.05$).

Conclusions: ARD is associated with increased gut microbiota diversity and enrichment of specific taxa, better cognitive function and brain morphometry outcomes. These associations were stronger in individuals with a healthy lifestyle profile.

Clinical Trial Registry number and website where it was obtained: NCT04461951, https://clinicaltrials.gov/.

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1. Introduction

At a time when non-communicable diseases represent a growing global health challenge, adherence to a healthy diet and lifestyle is emerging as a cornerstone of prevention and well-being. Optimal nutrition contributes to the prevention of cardiovascular diseases, metabolic disorders and cancer and plays a critical role in improving mental health and mitigating cognitive decline in older adults [1,2]. The mechanisms underlying these associations are complex and multifaceted and include among the others inflammation, oxidative stress, mitochondrial dysfunction, and epigenetic changes [3]. Diet exerts its influence not only through its direct effects on human cells, but, also, by shaping the gut microbiota, which plays a crucial role in maintaining overall health. The gut microbiota is actively involved in metabolism and immune function, and its effects extend to susceptibility to infectious diseases, obesity, diabetes, chronic gastrointestinal diseases, cancer, and neurodegenerative disorders. Its composition is influenced by several factors, including host genetics, health status, medication use, geographic location, and lifelong dietary habits. Among these, diet stands out as a key determinant, highlighting the significant role of habitual dietary patterns in shaping the gut microbiota and, in turn, influencing health outcomes [4,5]. In addition, diet may exert a protective effect on mental and cognitive health, possibly through macro- and microstructural changes in brain volume (total brain volume - TBV, grey matter volume - GMV, hippocampal volume, cortical thickness, white matter volume - WMV, white matter microstructure), increased glucose metabolism and reduced Aβ deposition (i.e., a marker of Alzheimer's Disease) [6]. Recent advances have highlighted the bidirectional communication between dietary patterns, gut microbiota and brain health – the *diet–gut–brain axis* – as a promising framework for understanding the interplay between diet, gut microbiota and neurocognitive outcomes. This axis links nutrition to changes in the gut microbiota composition and function that affect metabolism, immunity, cognition, emotional well-being and neuropsychiatric disorders [7]. Given the multifaceted interplay between diet, gut microbiota and brain health, emerging evidence suggests that specific plant-based dietary components, such as polyphenols, carotenoids, and vitamins with antioxidant properties, may be particularly promising in mitigating cognitive decline and maintaining brain integrity. However, although the influence of such components on cognitive function has been investigated [8–11], the results are rather heterogeneous. Furthermore, to our knowledge, few studies have investigated their association with brain structural metrics [12] and gut microbiota composition [13]. To address these gaps, this study examines: i) the effect of an antioxidant-rich diet (ARD) on gut microbiota diversity, brain morphometric correlates, and global cognitive function; ii) the combined effect of the diet and other lifestyle factors, such as smoking habits and physical activity, in a single score; and iii) the potential mechanisms involved in a subsample of the Italian population-based Nutrition, gUT microbiota, and BRain AgINg Study (NutBrain) Study [14].

2. Methods

2.1. Study design, setting, and sample selection

This study is part of the cross-sectional, population-based NutBrain study, which involved community-dwelling adults aged 65 and older residing in the Lombardy region of Italy who underwent cognitive assessments, and whose socio-demographic characteristics, health status, lifestyle, and anthropometric measures were collected (phase I). The present study was performed in a sub-sample that participated in a comprehensive clinical examination, including blood pressure and heart rate measurements, neurological examination, and magnetic resonance imaging (MRI) (phase II). Stool and blood samples were also collected from all participants. Individuals who were willing and able to undergo all testing procedures, including neuroimaging and blood and stool sampling, were selected as described previously [14]. Briefly, the inclusion/exclusion criteria were as follows: i) Cognitive assessment protocol: exclusion of subjects with severe psychiatric disorders, other neurological conditions (including dementia), or severe sensory disorders; ii) Gut microbiota protocol: exclusion of subjects on total parenteral nutrition, with gastrointestinal disorders, previous major gastrointestinal surgery, use of antibiotics, or undergoing radiotherapy or chemotherapy; iii) Brain MRI protocol: exclusion of subjects with metal fragments in the body, surgically implanted metal devices, severe claustrophobia, or inability to lie down in the MRI scanner for the study duration. Of the 821 subjects presented to the research facility, 816 completed the screening evaluation. Based on cognitive status, eligibility criteria, and the project budget, approximately one third of these individuals were invited to participate in phase II. A total of 255 subjects underwent clinical assessment and those with severe dementia were excluded. This resulted in a final analytical sample of 246 participants. Blood samples were collected from all participants to determine inflammatory markers and APOE genotyping. Of these, 203 met the eligibility criteria for brain MRI, and 178 had a viable microbiota sample (Supplementary Fig. S1). A comparison of the baseline characteristics of phase II participants and non-participants (Supplementary Table S1) revealed no significant differences in terms of sex, educational level, BMI, polypharmacy, physical activity or cognitive performance. However, phase II participants were slightly older and smoked less ($p \leq 0.05$).

2.2. Ethics

All participants provided formal written informed consent to participate in the study. For those unable to provide full written

informed consent, a caregiver or guardian was identified to provide guidance regarding participation. The study protocol and amendments were reviewed and approved by the Medical Ethics Committee of Pavia, Italy (approval ref. prot. 20180036036, 20/04/2018; amendment ref. prot. 20190045757, 21/05/2019). The study was conducted in accordance with the tenets of the Declaration of Helsinki, as revised in 2013. Data were handled, monitored, computerized, and stored in compliance with the European General Data Protection Regulation (EU) 2016/679 (GDPR), with data transmission secured through encryption and password protection.

2.3. Cognitive outcome

During phase I, experienced neuropsychologists administered well-established neuropsychological tests to evaluate global cognitive function and specific cognitive domains. For the present analysis, we considered global cognitive function assessed using the Mini-Mental State Examination (MMSE) [15], with scores ranging from 0 to 30, with higher scores indicating better cognitive function. The assessment adhered to a standardized procedure for both administration and scoring.

2.4. Blood sample collection, processing, and biomarker determination

Qualified nurses collected 27 ml of blood in ethylenediaminetetraacetic acid (EDTA) and 6 ml in sodium citrate tubes and transported it to the Cellular Models and Neuroepigenetics Section of the IRCCS Mondino Foundation within 2 h of phlebotomy for processing and storage. All the subjects underwent venous sampling in the early afternoon of recruitment (between 14:00 and 15:00). The samples were processed on the same day and stored at 4 °C until extraction.

Apolipoprotein E (APOE) genotyping: DNA extraction was performed from whole blood in EDTA tubes using the Maxwell® Clinical Sample Concentrator 48 Instrument (Promega Corporation, Madison, WI, USA) and the Maxwell® CSC Blood DNA Kit (Promega Corporation) according to the manufacturer's instructions. DNA quality and quantity were assessed using a Nanodrop One C spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). Samples were tested for APOE genotype by real-time PCR by analysing two different polymorphisms, located in the fourth exon of APOE. APOE genotyping was performed as previously described [16]. Real-time PCR was performed using the CFX384 Touch Real-Time PCR Detection System (Biorad, Hercules, CA, USA). Fluorescence intensity was assessed at cycles 42 and 32 for single nucleotide polymorphisms rs7412 and rs429358, respectively.

Inflammatory markers quantification: C-reactive protein (CRP) levels in plasma samples were assessed using the CRP Human ELISA Kit (ab99995) (Abcam, Cambridge, UK) according to the manufacturer's instructions. The amount of bound CRP was estimated by measuring colour intensity at 450 nm by an EnSight® Multimode Plate Reader (PerkinElmer, High Wycombe, UK) and Kaleido Data Acquisition Software (PerkinElmer). The concentration of CRP in the samples was compared with the standard curve Plasma concentrations of interleukin-6 (IL-6) and tumour necrosis factor-α (TNFα) were assessed using the Ella automated immunoassay system and Simple Plex kits (Bio-techne Corporation, Minneapolis, MN, USA), according to the manufacturer's instructions. IL-6 and TNFα concentrations, corrected for dilution, were calculated against a lot-specific standard curve using Simple Plex Runner software (Bio-techne Corporation) (Supplementary Fig. S2A).

2.5. Stool sample preparation, processing, and gut microbiota analysis

2.5.1. Gut microbial composition: stool collection, libraries preparation and metabarcoding 16S rRNA gene sequencing

Participants selected for phase II of the study were instructed to collect approximately 250 mg of stool sample in a sterile stool specimen container provided by the research team during phase I and to deliver the samples to the hospital on the same day as the clinical assessment, within 12 h from excretion. Samples were immediately frozen at −20 °C and later stored at −80 °C in the biorepository until processing. Total microbial DNA was extracted from the stool samples using a modified protocol [17] specifically designed for efficient and unbiased bacterial DNA extraction from human stool. The quality of the genomic DNA was assessed using the TapeStation 2200 system (Agilent, Santa Clara, CA, USA), and only samples with a DNA Integrity Number (DIN) $\geq$ 4 were used for downstream analyses. For each sample, the V3–V4 hypervariable regions of the 16S rRNA gene were PCR amplified according to the Illumina 16S Metagenomic Sequencing Library Preparation protocol (Illumina, San Diego, CA, USA, https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf), using primers with overhanging adapter sequences (primer selection described in [18]) and barcoded using the Illumina dual indexing strategy. Indexed libraries were pooled at equimolar concentrations and sequenced on an Illumina MiSeq sequencing platform, generating 2 × 300 bp paired-end reads according to the manufacturer's instructions (Supplementary Fig. S2B).

2.5.2. Bioinformatic data analysis

Raw sequencing data were processed by merging the two overlapping pairs for each amplified fragment using Pandaseq [19] resulting in a single fragment of approximately 460 bp. Fragments were quality-filtered by trimming down stretches of three or more low quality bases (phred score <3) from the 3′- end with the "split_libraries_fastq.py" utility in QIIME (v 1.9.0, [20]) and discarding reads whose trimmed length was <75 % of the original fragment. High-quality fragments were clustered into zero-radius Operational Taxonomic Units (zOTUs) by vsearch (utility: "cluster_unoise", [21]), discarding those with fewer than 3 supporting reads and likely chimeras (utility: "uchime3_denovo"). Taxonomies were assigned using RDP classifier [22] with the SILVA 16S database (release 138) [23] and default parameters. α-diversity was estimated according to the Chao1 metric, whereas β-diversity evaluations were based on the weighted and unweighted UniFrac distances [24]. After evaluating the rarefaction curves on sample α-diversity, the dataset was normalised to 10′000 reads per sample, and samples with a lower number of associated sequences were discarded.

2.6. MRI acquisition and image processing

2.6.1. MRI data acquisition

High-resolution T1-weighted (T1w) structural brain images were obtained for all participants using a 3 T MAGNETOM MRI scanner (Siemens Skyra, Erlangen, Germany) at the IRCCS Mondino Foundation in Pavia, Italy. The imaging protocol employed a 3D magnetization-prepared rapid gradient echo (MPRAGE) sequence, designed to deliver high-quality structural data suitable for volumetric and morphometric analyses. The acquisition parameters were as follows: repetition time (TR) = 2300 ms, echo time (TE) = 2.98 ms, inversion time (TI) = 900 ms, flip angle = 9°, field of view (FOV) = 256*256*176 mm^3, voxel size = 1.0 × 1.0 × 1.0 mm^3, and slice thickness = 1 mm. The imaging protocol

incorporated parallel imaging with GRAPPA (GeneRalized Autocalibrating Partial Parallel Acquisition) at an acceleration factor of 2, enabling efficient data acquisition while maintaining image quality. The total acquisition time for each scan was 5 min and 20 s. These parameters were selected to ensure compatibility with standardized imaging protocols, including those established by the Alzheimer's Disease Neuroimaging Initiative [25], facilitating cross-study comparisons and robust quantitative analyses.

2.6.2. MRI preprocessing and volumetric analysis

T1w images were processed using SIENAX (Structural Image Evaluation, using Normalization, of Atrophy Cross-sectional), part of the FSL software suite [26,27]. SIENAX estimates brain tissue volumes normalized for head size. The first step involved brain extraction using the BET (Brain Extraction Tool) algorithm to isolate the brain and skull images from the full-head MRI data [28]. Next, the extracted brain image was affinely registered to the standard MNI152 space [29], with the skull image utilized to calculate a volumetric scaling factor for normalization. Tissue types were then segmented into grey matter, white matter, and cerebrospinal fluid compartments using partial volume estimation. Finally, volumetric measurements were calculated, including TBV as the sum of GMV and WMV, and ventricular cerebrospinal fluid (VCSF) volume as a proxy of brain atrophy [30] (Supplementary Fig. S2C).

2.7. Dietary assessment and dietary antioxidant capacity determination

Participants were trained by a dietitian to complete a prospective food diary three days prior to the clinical assessment at the hospital. They recorded each food or drink consumed, along with the quantity, weighed before cooking whenever possible. Macro- and micronutrient intakes were estimated from the three-day food diary using the Metadieta 4.0 software (Me.Te.Da. S.r.l., San Benedetto Del Tronto, Italy). Recipes for local or mixed dishes were created if they were not available in the database. The average of the three-day dietary recalls was used to determine the mean intakes of energy, nutrients, and Oxygen Radical Absorbance Capacity (ORAC) [31]. The ORAC index, which indicates the total antioxidant capacity of the diet, was calculated using data from the USDA Foods Table [32]. The ORAC values of selected foods were expressed as μmol Trolox equivalents (TE) per 100 g of food (μmol TE/100 g) and categorized into tertiles as low, moderate, and high. To understand the contribution of the single nutrients to the ORAC values, we calculated the Spearman's correlation coefficient and considered the most significant associations ($r > 0.2$).

2.8. Covariates

For the present analysis, we considered the following covariates: age (continuous), sex, education (categorized as primary school or less, middle school, high school or higher), polypharmacy (calculated by summing participants' self-reported daily medications), and the presence of at least one ε4 allele in the apolipoprotein E (APOE) genotyping. In addition to diet, other lifestyle factors included smoking habits, categorized as never, former, or current smoker, and physical activity, assessed using the International Physical Activity Questionnaire (IPAQ) [33]. Physical activity was classified as follows: none or low if participants did not engage in physical activity or only performed one activity, such as slow walking; moderate if they engaged in more than one light or moderate activity, such as slow swimming, dancing, or cycling to work; and high if they participated in more than one vigorous activity, such as hiking, skiing, fast swimming, or running.

2.9. Development of the healthy lifestyle score

The healthy lifestyle score (Hscore), was created as a composite score by summing the following categorical variables: smoking habits (0: current smoker; 1: former smoker; 2: non-smoker), ORAC (according to tertiles, 0: low; 1: medium; 2: high), and physical activity (0: none or low; 1: moderate; 2: high). The total possible score ranged from 0 to 6, with higher scores indicating greater adherence to a healthy lifestyle. Hscores were categorized as low (i.e.: $\leq$2 healthy factors), medium (i.e.: 3), and high (i.e.: $\geq$4), corresponding to the first, second, and third tertiles, respectively. Equal weighting of each component was chosen to provide a simple, interpretable composite that reflects the multidimensional nature of lifestyle behaviours. The ORAC score was analysed both individually and as part of the Hscore, to compare the explanatory power of diet alone with that of the integrated lifestyle score regarding the outcomes considered.

2.10. Statistical analysis

Continuous variables were summarized as means and standard deviations (SD), while categorical variables were presented as counts and percentages. Statistical analyses on microbiome data were performed using non-parametric Monte Carlo-based tests and analysis of variance with partitioning among sources of variation (the “adonis” function) in the R package “vegan” (version 2.0–10) for α- and β-diversity, respectively. Differences in the abundances of the 30 most abundant bacterial genera were analysed using the non-parametric Kruskal–Wallis test, followed by *post-hoc* Dunn's test for pairwise comparisons. The Benjamini-Hochberg correction was applied to compensate for multiple testing over microbiota data. For clarity, within the text, non-corrected p-values were reported. Spearman's correlation analysis was used to identify nutrients contributing to the total ORAC score and to examine the relationships between gut microbiota α-diversity, brain and inflammatory markers, global cognitive function, total ORAC, and healthy lifestyle scores. Linear regression models were used to calculate β-coefficients and 95 % confidence intervals (CIs) for associations between total ORAC and Hscore with brain measures, MMSE, and gut microbiota α-diversity. Models included age, sex, education level, polypharmacy, physical activity, smoking, MMSE, energy intake (Kcal), TNFα, BMI, and APOEε4 carrier status, as covariates.

All analyses were performed using the statistical software packages STATA (version 15.0, StataCorp LP, College Station, TX, USA) and SPSS (release 25.0, IBM Corp., Armonk, NY, USA). Throughout the manuscript, a two-sided p-value of $\leq$0.05 was considered statistically significant, whereas p-values up to 0.1 were reported as trends. However, given the exploratory nature of the analysis, an adjusted p-value of 0.2 was considered as the threshold for statistical significance for microbiota data.

3. Results

3.1. General characteristics of the sample

A total of 246 subjects were clinically assessed, with a mean age of 75.4 years (SD 6.2). Among the participants, 58.5 % were female, and 55.7 % had a high level of education (i.e.: high school diploma or higher). On average, participants took 3 medications per day and had a mean MMSE score of 28.1 (SD 1.6). The APOEε4 allele was present in 17.5 % of the sample. Most participants were non-smokers (57.3 %) and engaged in little or no physical activity (78.5 %) (Table 1).

Table 1
Descriptive characteristics of the whole cohort (n = 246).

		Mean or number	SD, range or %
Socio-demographic and health status			
Age (mean, range)		75.4	(63.9–93.9)
Sex	Males	102	41.5 %
	Females	144	58.5 %
Educational level	Primary school or less	40	16.3 %
	Middle school	69	28.1 %
	High school or high	137	55.7 %
MMSE	(Mean, SD)	28.1	1.6
Polypharmacy	(Mean, SD)	3	2.4
APOEε4 carrier status		43	17.5 %
Lifestyle			
Total ORAC, continuous	(Mean, SD)	9786.7	6520.1
Total ORAC, categorical	Low (mean value 4674,91)	82	33.5 %
	Moderate (mean value 8881,20)	82	33.5 %
	High (mean value 15878,32)	81	33.1 %
Smoking habits	Current	18	7.3 %
	Former	87	35.4 %
	No smokers	141	57.3 %
Physical exercise	None or low	193	78.5 %
	Moderate	42	17.1 %
	High	11	4.5 %
Hscore (continuous)	(Mean, SD)	2.8	1.2
Hscore (categorical)	Low (mean value 1,57)	102	41.5 %
	Medium (mean value 3,00)	79	32.1 %
	High (mean value 4,31)	65	26.4 %

SD (standard deviation), MMSE (Mini-mental State Examination), APOE (Apolipoprotein E), ORAC (Oxygen Radical Absorbance Capacity); Hscore (Healthy lifestyle score).

The mean ORAC value over the 246 subjects was 9786.7 μmol TE/100 g (SD 6520.1), with mean values of 4674.9 in the low tertile, 8881.2 in the moderate tertile, and 15878.3 in the high tertile. By evaluating the Spearman's coefficients with single nutrients, we found that the majority of the variation in the dietary ORAC score was explained by the following nutrients: total fibre, potassium, soluble and insoluble fibres, vitamin C, magnesium, iron, soluble carbohydrates, copper, folic acid, manganese, vitamin B6, vitamin K, vitamin E, biotin, plant proteins, beta-carotene, polyunsaturated fatty acids (PUFAs), and omega-3. The primary dietary sources of these nutrients were plant-based foods, such as fruits, vegetables, olive and seed oils, cereals, and legumes (Supplementary Fig. S3).

Finally, with regards to the Hscore, the average of the distribution was 2.8 (SD 1.2), with 102 (41.5 %), 79 (32.1 %) and 65 (26.4 %) patients belonging to the first, the second and the third tertile, respectively. Notably, only 6 subjects (2.4 %) had a score of 0 and only 2 (0.8 %) reached the maximum Hscore of 6.

Mean values for brain and inflammatory markers are presented in Supplementary Table S2.

3.2. Gut microbiota analysis

The microbiota profiling was based on a total of 178 samples, with an average composition typical of a Western elderly population. At the phylum level, the microbiota was dominated by *Firmicutes* (46.4 %), *Bacteroidetes* (40.2 %), *Verrucomicrobiota* (4.6 %), *Proteobacteria* (4.0 %), and *Actinobacteriota* (1.5 %). At the genus level, *Bacteroides* was the predominant taxon, accounting for an average relative abundance of 22.2 %, followed by *Alistipes*, *Akkermansia*, *Subdoligranulum*, *Faecalibacterium*, *Oscillospiraceae* UCG-002, *Prevotella_9*, and *Parabacteroides,* which were present at an average relative abundance ranging from 2.6 % to 4.5 %. Minor contributors, with an average relative abundance between 1 % and 2 %, included *Dialister*, *Escherichia-Shigella*, *Barnesiella*, *Christensenellaceae* R-7 group, *Phascolarctobacterium*, *Clostridia* UCG-014 group, *Roseburia*, *Eubacterium eligens* group, *Lachnospiraceae* NK4A136 group, and *Coprococcus* (Supplementary Fig. S4). Figure 1 presents the relationship between gut microbiota diversity and composition across tertiles of total ORAC. Subjects in the high tertile exhibited greater α-diversity than those in the low tertile ($p = 0.015$) (Fig. 1A). Additionally, microbial composition differed between the high and low tertiles (trend of $p = 0.069$ and significant difference $p = 0.045$, unweighted and weighted UniFrac distances, respectively) (Fig. 1B). In terms of relative bacterial abundance (Fig. 1C), we observed a significant decrease in *Streptococcus* ($p = 0.012$) when comparing high vs. low ORAC tertiles. Conversely, several genera, including *Barnesiella* ($p = 0.007$), *Coprococcus* ($p = 0.036$), *Ruminococcus* ($p = 0.007$), *Parabacteroides* ($p = 0.012$), *Lachnospiraceae* NK4A136 group ($p = 0.024$), and *Clostridia* UCG-014 group ($p = 0.011$), were significantly increased in high ORAC tertile as compared to low ORAC tertile. Notably *Ruminococcus* was also significantly higher in high ORAC tertile when compared to moderate ORAC tertile, and *Coprococcus* was significantly higher also comparing moderate versus low ORAC tertiles. The relationship between tertiles of the Hscore is shown in Fig. 2. Examining the α-diversity rarefaction curves, subjects in the high tertile had greater α-diversity than those in the low tertile ($p = 0.012$) (Fig. 2A). A significant difference in microbial composition (unweighted UniFrac, $p = 0.022$) was observed between subjects with high and low Hscores tertiles (Fig. 2B). In terms of relative bacterial abundances (Fig. 2C), *Ruminococcus* ($p = 0.017$), *Clostridia* UCG-014 ($p = 0.001$), *Barnesiella* ($p = 0.019$), *Parabacteroides* ($p = 0.033$), *Prevotella_9* ($p = 0.048$), *Eubacterium eligens* ($p = 0.003$), *Lachnospiraceae* NK4A136 group ($p = 0.016$), *Coprococcus* ($p = 0.017$) were increased in the high tertile compared to the low tertile. *Clostridia* UCG-014 and *Parabacteroides* were also significantly higher in the high vs medium comparison ($p = 0.006$ and $p = 0.002$, respectively).

3.3. Correlation analysis

The heatmap in Fig. 3 displays the statistically significant Spearman's correlations ($p \leq 0.05$) between gut microbiota α-diversity, brain measures, inflammatory markers, MMSE, total ORAC, and Hscore across the entire cohort. As expected, CRP showed a positive correlation with IL-6 and TNFα (r between 0.28 and 0.40), and TBV was correlated with WMV and GMV (r between 0.78 and 0.88). These latter three parameters were inversely related to VCSF (r between −0.29 and −0.43). Given how they were obtained, total ORAC and Hscore were also positively correlated ($r = 0.73$). The microbiota α-diversity was positively correlated with WMV ($r = 0.17$) and negatively correlated with IL-6/TNFα ($r = 0.15$). Similarly, WMV, GMV, and TBV were negatively correlated with IL-6 and TNFα (r between −0.16 and −0.20). Notably, total ORAC was positively correlated with MMSE ($r = 0.16$), TBV ($r = 0.15$), WMV ($r = 0.17$), and the α-diversity ($r = 0.25$). Similarly, Hscore was positively correlated with GMV ($r = 0.16$), TBV ($r = 0.17$), and the α-diversity ($r = 0.21$) and inversely correlated with IL-6 ($r = -0.15$).

Moreover, among bacterial genera, *Barnesiella* was positively correlated with TBV and GMV ($r = 0.196$ and $r = 0.174$, respectively) and MMSE ($r = 0.153$) and positively correlated with ORAC/Hscore. Meanwhile, it showed a negative correlation with VCSF ($r = -0.279$), IL-6 ($r = -0.233$), and TNFα ($r = -0.192$). Similarly, the *Eubacterium eligens* group showed positive correlations with TBV and GMV ($r = 0.166$ and $r = 0.162$, respectively), and negative

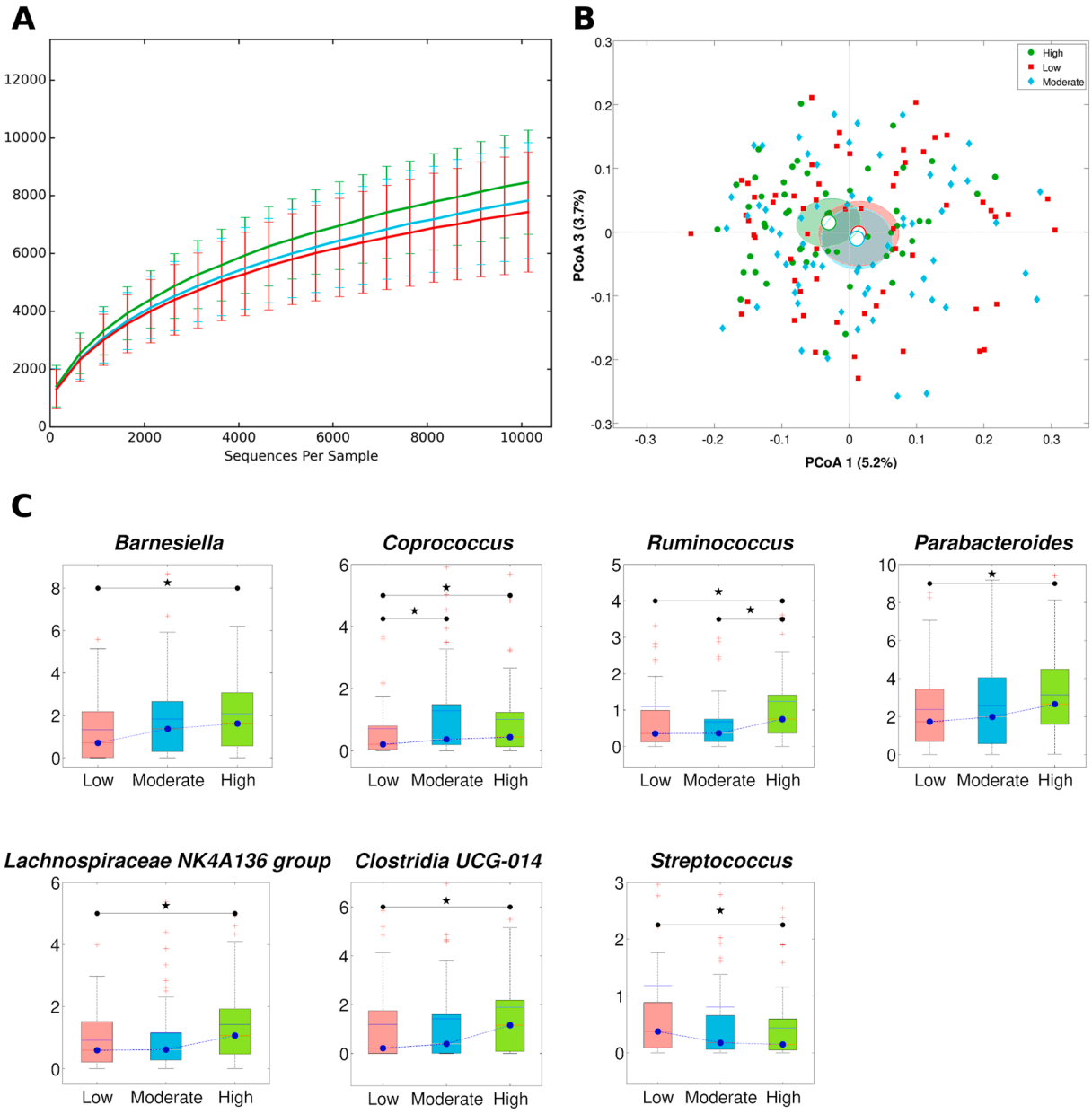

Fig. 1. Differences in gut microbiota among low (n = 61), moderate (n = 65) and high (n = 52) total ORAC. Rarefaction curves compare species richness according to the Chao1 metric among the three ORAC groups. Each line represents the average (with standard deviation error bars) over all samples from the same experimental group (A). Principal Coordinate Plot (PCoA) based on the unweighted UniFrac distance matrix between low, moderate, and high total ORAC. Each point represents a sample with colours representing different groups; centroids represent the average coordinate per group; ellipses are SEM-based confidence intervals. Principal coordinates 1 and 3 are plotted with the percentage of variability explained on the axis (B). Box plots of relative abundances for taxa were found to be differentially abundant between experimental groups (Kruskal–Wallis and Dunn's *post-hoc* tests). Plots represent median, interquartile range, minimum, and maximum values (C).

correlations with IL-6 (r = −0.182). *Coprococcus* showed an inverse correlation with IL-6 (r = −0.195) (Supplementary Fig. S5).

3.4. Associations between total ORAC and overall healthy lifestyle score in relation to gut microbiota diversity, brain and cognitive function

The β-standardized regression coefficients and 95 % confidence intervals (CIs) were derived from multiple linear regression analyses examining the association between total ORAC and Hscore with brain morphometric correlates, global cognitive function, and gut microbiota α-diversity (Chao1 index) (Fig. 4, Supplementary Table S3). The highest tertile of total ORAC was positively and statistically significantly ($p \leq 0.05$) associated with TBV (β-coefficient 34746.04, 95 % CI 14011.93, 55480.15), WMV (19117.10, 95 % CI 7081.26, 31152.93), GMV (15856.51, 95 % CI 2757.62, 28955.39), and the α-diversity index (1111.00, 95 % CI 364.96, 1857.04), independently from other covariates. When ORAC was considered as a continuous variable, it was positively associated with TBV, WMV, and gut α-diversity. A trend towards significance ($p \leq 0.10$) was observed between total ORAC tertiles and MMSE; however, when we considered the score as a continuous variable, the estimates reached significance ($p \leq 0.05$) (Supplementary Table S3). Regarding the role of the Hscore, high adherence (third tertile) was positively and significantly ($p \leq 0.05$) associated with TBV (39360.02, 95 % CI 13014, 65706.04), GMV (26560.95, 95 % CI

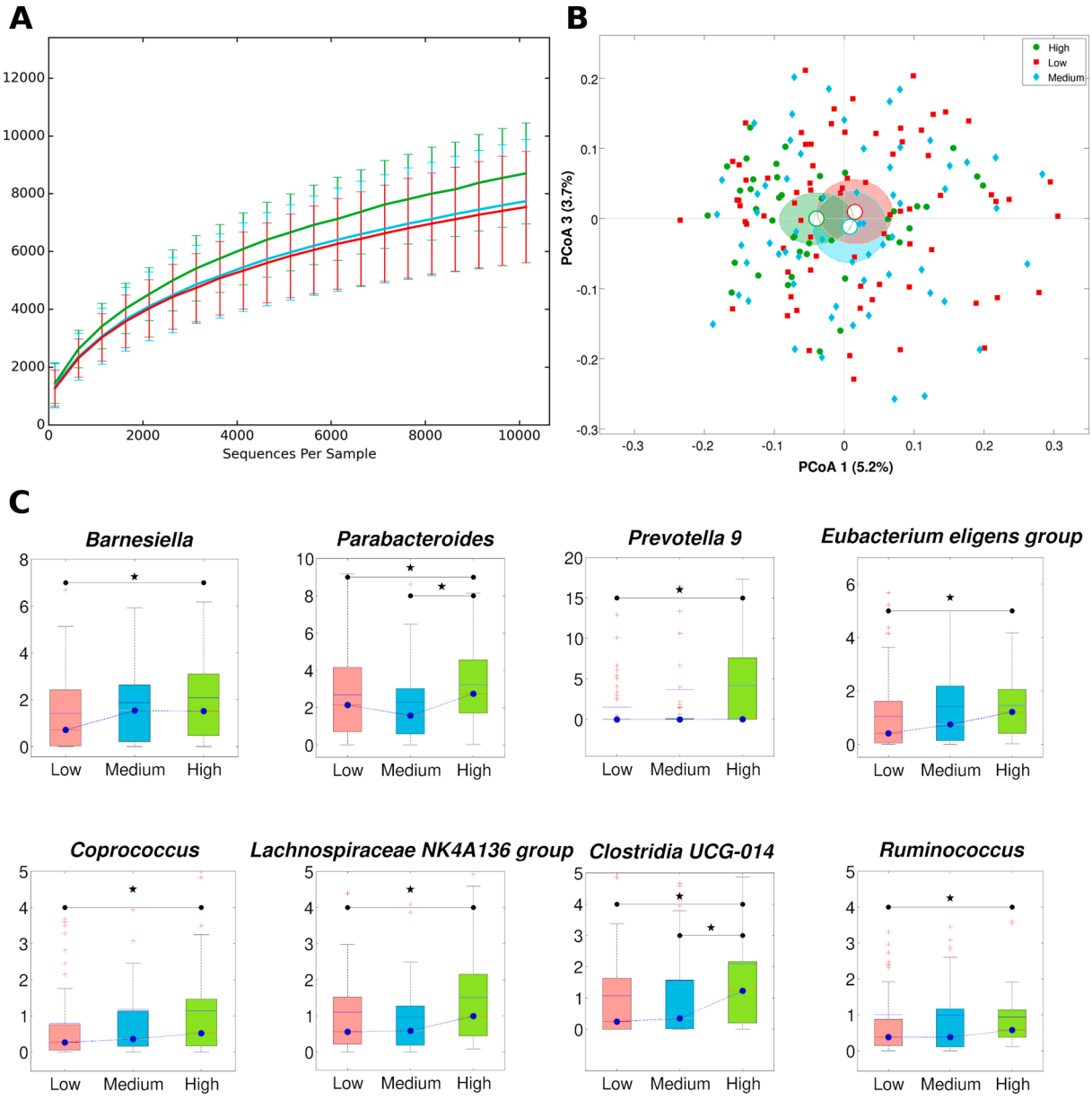

Fig. 2. Differences in gut microbiota between low (n = 75), medium (n = 62), and high (n = 41) healthy lifestyle scores. Rarefaction curves comparing species richness according to the Chao1 metric among the three Hscore groups. Each line represents the average (with standard deviation error bars) over all samples from the same experimental group (A). Principal Coordinate Plot (PCoA) based on the unweighted UniFrac distance matrix between low, medium, and high healthy lifestyle scores. Each point represents one sample with colours representing different groups; centroids represent the mean coordinate per group; ellipses are SEM-based confidence intervals. Principal coordinates 1 and 3 are plotted with the percentage of variability explained indicated on the axis (B). Box plots of relative abundances for taxa were found to be differentially abundant among experimental groups (Kruskal–Wallis and Dunn's *post-hoc* tests). Plots represent median, interquartile range, minimum, and maximum values (C).

6762.94, 46358.96), MMSE (0.55, 95 % CI 0.02, 1.08), and gut α-diversity (1217.58, 95 % CI 491.38, 1943.79). When the Hscore was considered as a continuous variable, it was positively associated with brain measures (negatively with VCSF), MMSE, and gut α-diversity (Supplementary Table S3).

4. Discussion

This study examined the intricate relationships among dietary antioxidant capacity, healthy lifestyle, gut microbiota diversity, cognitive performance, inflammatory markers, and brain morphometry.

4.1. Associations between total ORAC and overall healthy lifestyle score with gut microbiota composition

We observed a positive correlation and a significant association between both total ORAC and the healthy lifestyle score with indices of gut α-diversity. Although the impact of diet on the gut microbiota has been widely hypothesized, to our knowledge, this is the first study to use dietary ORAC index and Hscore to specifically investigate differences in both richness (α-diversity) and overall composition (β-diversity). Most findings regarding dietary influence on gut microbiota come from studies focused on plant-based or Mediterranean diet (MD) patterns, which report that a higher α-diversity is positively associated with vegetable intake

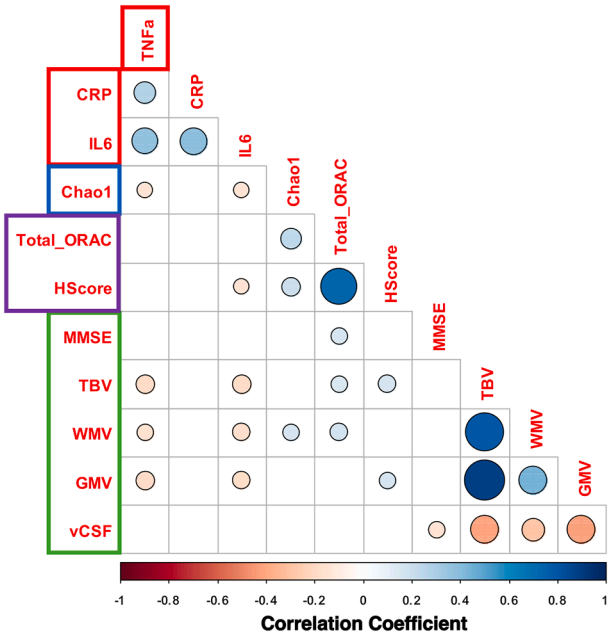

Fig. 3. Heat map of significant Spearman's correlations ($p \leq 0.05$) between gut microbiota α-diversity, brain and inflammatory markers, global cognitive function, total ORAC, and Hscore. The heatmap shows the significant Spearman's correlations ($p \leq 0.05$) between TNFα (Tumour Necrosis Factor-α); CRP (C-reactive protein); IL-6 (Interleukin 6); Chao1 (Chao1 index); Total_ORAC (Total dietary antioxidant capacity); Hscore (Healthy lifestyle score); MMSE (Global cognitive function); WMV (White matter volume); TBV (Total brain volume); GMV (Gray matter volume); VCSF (Ventricular cerebrospinal fluid). The range of correlation coefficients (−1 to +1) is colour coded (red to blue) in the corresponding heat map legend. Method of ordering the correlation matrix: hierarchical clustering.

and adherence to the MD [34]. Indeed, our analysis showed how the dietary ORAC score was related to nutrients such as soluble and insoluble fibers, soluble carbohydrates, vitamins, plant proteins, polyunsaturated fatty acids (PUFAs), and omega-3, whose primary dietary sources were plant-based foods, such as fruits, vegetables, olive and seed oils, cereals, and legumes, all elements typical of the MD. In our study, several bacterial genera (i.e.: *Coprococcus*, *Barnesiella*, *Ruminococcus*, and *Prevotella*) whose abundance increased in individuals with high ORAC or healthy lifestyle scores have been previously described as producers of beneficial short-chain fatty acids (SCFAs) through the saccharolytic fermentation of various plant polysaccharides, including dietary fibre, cellulose, and resistant starch, and potentially improving gut health [35,36]. *Ruminococcus, Barnesiella, Eubacterium eligens* group bacteria, *Lachnospiraceae* NK4A136, *Prevotella* and *Coprococcus* have all been associated to a higher adherence to MD [37,38], vegetarian diet [39], fibre intake [35], vegetables and legumes [40], fruits, nuts [41], salad and green beans/chard/spinach consumption [42]. These genera are considered markers of a more diverse microbiota [43], and have been linked to immune regulation, lower inflammation, inhibition of T-cell proliferation, lower plasmatic trimethylamine–N-oxide (TMAO) levels [39], and improved metabolic health markers, such as lower weight, waist circumference, glucose and glycated hemoglobin (HbA1c) levels [44]. Interestingly, *Coprococcus* has been found to be depleted in individuals with depression [45], Parkinson's disease [46] and children with autism spectrum disorders, suggesting a supportive role in mental health [40]. In our dataset, *Parabacteroides* were found to be increased in individuals with high ORAC/healthy lifestyle. *In vitro* studies have shown that apple consumption increases the abundance of *Parabacteroides*, and research in mouse models suggest

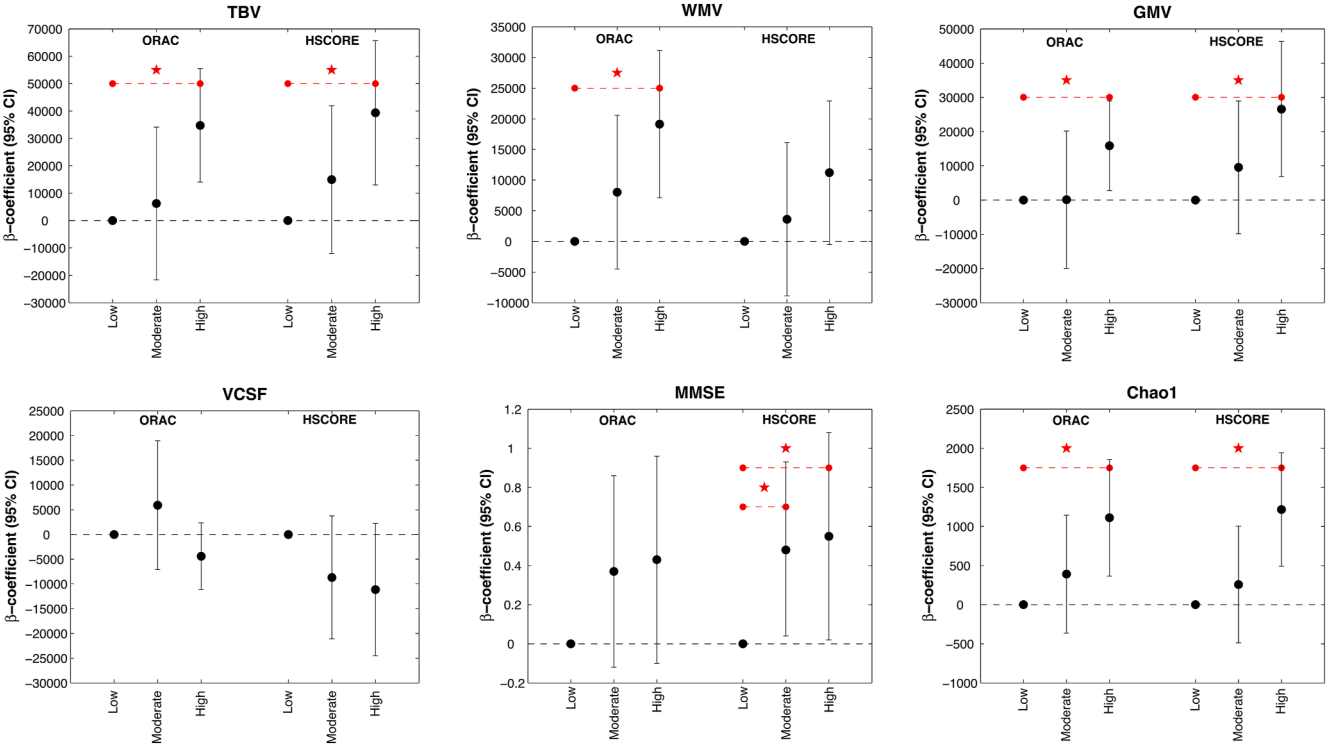

Fig. 4. β (standardized regression coefficient) and 95 % CI (confidence intervals) derived from the multiple linear regression of the association between total ORAC and healthy lifestyle score with brain morphometric correlates, global cognitive function and gut microbiota α-diversity index. ORAC (Oxygen Radical Absorbance Capacity), Hscore (Healthy lifestyle score), TBV (Total brain volume), WMV (White matter volume), GMV (Gray matter volume), VCSF (Ventricular cerebrospinal fluid), MMSE (Global cognitive function), Chao1 (Chao1 index).

that cranberries can boost the relative abundance of *Parabacteroides* by up to 15 % [47].

4.2. Associations between total ORAC and overall healthy lifestyle score with brain volume measures

In our study, we observed a positive and significant independent association between higher ORAC and TBV, GMV, and WMV. Only one study has examined the role of total dietary antioxidant capacity, as measured by the ferric-reducing antioxidant power (FRAP) assay, on key structural brain volumes in older adults. This study found that higher dietary FRAP scores were associated with greater TBV, but did not show associations with GMV, WMV, or white matter lesions in fully adjusted models [12]. Looking at nutritional patterns, a 'Fiber & Antioxidants' pattern was positively associated with larger TBV, less white matter damage [48,49], and lower brain atrophy [50]. More broadly, several prospective observational and cross-sectional studies in healthy older individuals have reported that greater adherence to diets rich in plant-based and polyphenol-rich foods—such as vegetables, fruits, nuts, whole grains, fish, and Mediterranean and DASH diets are associated with larger TBV, GMV, WMV, cortical thickness, and hippocampal volumes [51,52]. These diets also help preserve temporal lobe health and slow brain atrophy in aging populations [53]. In line with these findings, our study also found that an overall healthy lifestyle score was positively associated with brain volumes. While only a few studies have investigated the combined effects of various lifestyle factors, they generally report similar outcomes: adherence to a healthier lifestyle is associated with indices of preserved brain properties in older adults [54,55].

4.3. Associations between total ORAC and overall healthy lifestyle score with global cognitive function

We found a trend towards significance between global cognitive function and total ORAC tertiles; however, when we considered the score as a continuous variable, the estimates reached significance, which is consistent with some previous studies. For instance, a study in a Chinese population [8] reported that higher total antioxidant capacity (TAC) in a midlife diet was associated with lower odds of cognitive impairment in later life. Similarly, TAC and the Composite Dietary Antioxidant Index (CDAI) [9] were linked to a decreased risk of cognitive dysfunction in the NANHES cohort [10]. However, Devore et al. did not find a significant correlation between FRAP and cognitive function in older women from the Nurses' Health Study [11]. Interestingly, the effect of diet on cognitive function was stronger and statistically significant when combined with other lifestyle factors, suggesting that adherence to multiple lifestyle recommendations has a significantly greater impact on cognitive function than following a single behaviour. Additionally, we observed a negative correlation between MMSE scores and VCSF, reinforcing the established relationship between cognitive performance and brain atrophy [30].

4.4. Effect of gut microbiota α-diversity on neurocognitive outcomes

Finally, our study revealed that higher gut microbiota α-diversity was positively correlated with WMV and negatively correlated with VCSF, suggesting that a richer gut microbiota diversity may be linked to healthier brain outcomes. The data aligned with previous research shows that changes in gut microbial composition, diversity, and activity influence brain structure, function, and cognition. For instance, significant associations were found between gut microbial diversity and multiple neuroimaging measures across key brain networks and these neuroimaging biomarkers may mediate the relationship between gut microbiota diversity and cognitive function [56]. Gut microbiota diversity and specific bacterial enterotypes influence functional connectivity within and between large brain networks. These connectivity differences mediate the links between gut microbiota composition and behaviours such as sleep quality and executive function [57]. Altered gut microbiota composition in patients with arteriosclerotic cerebral small vessel disease (aCSVD) is linked to poorer disease outcomes and increased inflammaging [58]. A higher gut microbiota diversity was associated with larger GMV in both hippocampus and nucleus accumbens and to higher global cognitive performance [59] whereas reduced microbial diversity was associated with reduced GMV in patients with schizophrenia [60]. Notably, among bacterial genera, *Barnesiella* was found positively correlated to MMSE, TBV, GMV, and negatively to VCSF. This genus has previously been reported to be negatively correlated with all cerebral small vessel disease (cSVD) markers, having a decreased abundance in individuals with a high burden of markers for cSVD [61], and showed a positive association with cognitive functions (as estimated by tests such as Digit Symbol Substitution Test and category fluency) in middle-aged adults [62]. Moreover, higher *Barnesiella* and *Ruminococcus* abundances were found as directly correlated to better cardiovascular and cognitive health and to be mediators in the relationship between cardiovascular health and cognitive performance, through their anti-inflammatory properties and the production of short-chain fatty acids, critical for maintaining gut-brain axis integrity [59].

4.5. Possible underlying mechanisms

Our study revealed negative correlations between inflammatory markers and ORAC, healthy lifestyle scores, gut microbiota diversity, and certain brain morphometry parameters - underscoring the potential role of inflammation in linking an antioxidant-rich plant-based diet and a healthy lifestyle to gut-brain health. Our findings are consistent with a review by Hyzy and colleagues, which showed that antioxidant supplements (such as vitamins C, B2 and D, and polyphenols) can enhance cognitive and gut health by boosting microbiota diversity, reducing inflammation and oxidative stress, and strengthening the integrity of the gut barrier, a factor associated with cognitive decline and neurodegenerative disease [13]. The negative correlations observed between inflammatory markers and brain measures highlight the detrimental effects of chronic low-grade inflammation on brain health. Elevated cytokines, through their role in neuroinflammation and blood–brain barrier disruption, play an important role in the pathophysiology of brain atrophy in neurodegenerative diseases [63]. Research consistently shows a strong association between inflammatory markers, gut microbiota, and brain volumes, particularly in ageing and neurodegeneration. Elevated levels of inflammatory markers such as IL-6, CRP and TNFα were associated with reductions in TBV, GM and WM integrity [64,65]. Studies also suggested an inverse association between the diversity of the gut microbiome and the expression of inflammatory markers. For example, a study on a severe trauma rat model showed that α -diversity decreased and levels of inflammatory cytokines (IL-1α, IL-6, IL-8 and TNFα) increased [66]. Conversely, a study on DDS-induced colitis showed that levels of IL-1β, IL-6, PGE2, and TNFα were reduced and bacterial diversity was significantly improved after probiotics supplementation [67].

Plant-based foods are abundant in antioxidants due to the thousands of bioactive phytochemicals – such as flavonoids, tannins, phenolic acids, lignans, selenium, omega-3 fatty acids, vitamin C, vitamin E and carotenoids - found in fruits, vegetables,

nuts, seeds, olive oil, and legumes. These compounds, neutralise free radicals, prevent their formation, and help maintain a balance between pro- and anti-oxidants, as well as pro- and anti-inflammatory markers, thereby preserving cell and mitochondrial integrity, modulating transcription factors, and suppressing pro-inflammatory cytokines (e.g. TNFα, IL-1B, IL-6, IL-8), all of which are crucial for a healthy immune response [68].

4.6. Limits and strengths

Several limitations should be acknowledged. First, the cross-sectional design of NutBrain limits the ability to infer causality between exposures and outcomes, raising the potential issue of reverse causation. For example, individuals with better brain function and decision-making capacity are more capable of making and maintaining healthier dietary choices [69]. Second, data on diet and other lifestyle factors were self-reported, which may introduce recall and potential misclassification bias. Third, we only had a single sampling of the gut microbiota, although its composition is generally considered stable over time [70]. Moreover, the 16S-based sequencing allowed us to characterize the microbiota profile in a larger number of individuals, but it has not a direct functional consequence; thus, we speculated about the possible positive effects and the production of SCFAs based on the existing literature. Furthermore, microbial taxa reported as significantly altered with different ORAC or Hscores must be taken with caution due to the FDR threshold (i.e.: 0.2) employed. This choice was made on account of the relatively limited sample size of our study and the exploratory nature of the analysis, given all the variables involved. Fourth, due to the nature of observational studies, residual confounding effects cannot be fully excluded, even though we made extensive adjustments for potential confounders. Fifth, this study assesses dietary antioxidant intake using the standardized dietary ORAC index, which measures antioxidant capacity *in vitro* but does not reflect antioxidant bioavailability and metabolism in individuals and should be used with caution as a proxy for dietary antioxidant capacity in a biological context. Sixth, the size of the analytical sample varied across outcomes and some key subgroups (e.g.: high Hscore individuals) were relatively small, possibly limiting the statistical power and generalisability of the findings. However, given that our data were obtained from a well-defined, broadly representative population, we are confident that our findings are robust and that the significant associations observed are not due to chance alone. Finally, participants in phase II were slightly older and non-smoker than non-participants, suggesting the possibility of selection bias, as the sample may not accurately represent younger people and current smokers, which could influence the observed associations. Therefore, caution is warranted when extrapolating these results to the general population.

Nevertheless, the study boasts several notable strengths including the individual-level microbiota and imaging data from a well-established population-based cohort, providing robust, evidence-based multi-data integration. Participants were also thoroughly characterized, with blood specimens collected to measure APOE genotyping and inflammatory markers, enabling us to control for several potential confounding factors. All subjects in the NutBrain cohort were free-living individuals with no *a priori* selection bias, providing a good representation of the general population over the age of 65. Furthermore, the total dietary antioxidant capacity index reflects the antioxidant effect of a wide range of dietary nutrients, including lesser-known micronutrients like flavonoids, and captures interactions among these components within foods, offering advantages over studying single antioxidant components. The ORAC score was derived from a 3-day food diary, which provides reliable and accurate estimates of portions consumed based on weighted food records, thus reducing the issue of food omissions due to memory failure.

5. Conclusions

Our study provides evidence that an antioxidant-rich diet is significantly and independently associated with higher gut microbiota diversity, cognitive function and brain morphometry outcomes in older adults. Remarkably, these benefits are particularly pronounced when combined with a favourable lifestyle. A reliable hypothesis could be that such a healthy diet may reduce neurodegenerative processes by altering brain structure, modulating gut composition, and lowering pro-inflammatory markers. These findings emphasize the potential of the *diet–gut microbiota–brain axis*, offering a promising frontier for the development of precision nutrition strategies aimed at supporting cognitive health throughout the lifespan.

Author contributions

FP, AM, MS: Conceived and designed the study; SB, SC, AC, MCR, NJ, EP, AP, Acquired data; CC, TC: Performed microbiota sample processing; AP, AM, GR: Acquired or performed processing and analysis of MRI data; OP, ES: Performed blood samples processing and biomarker assessment.; FP, MS: Conducted the statistical and bioinformatic analysis; FP, AM, MS: Wrote the first draft of the manuscript; All: Revised the manuscript critically for intellectual content; FP, MS: Had primary responsibility for the final content. All authors read and approved the final version of the manuscript. No contributor eligible for the authorship has been excluded from the list of authors.

Data sharing statement

Data described in the manuscript, code book, and analytic code will be made available upon request of pending application and approval.

Ethics approval, study registration and permissions

All participants provided formal written informed consent to participate in the study. For those unable to provide full written informed consent, a caregiver or guardian was identified to provide guidance regarding participation. The study protocol and amendments were reviewed and approved by the Medical Ethics Committee of Pavia, Italy (approval ref. prot. 20180036036, 20/04/2018; amendment ref. prot. 20190045757, 21/05/2019).

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used Microsoft Copilot and DeepL.com in order to enhance the clarity and quality of their writing. After using these tools, the authors reviewed and edited the content as needed and took full responsibility for the content of the published article.

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Conflict of interest

The authors declare that they have no competing interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2026.106585.

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