

Title

Nut consumption, gut microbiota, and cognitive function: findings from a prospective study in older adults at risk of cognitive decline

Authors: Jiaqi Ni^{1,2,3}, Stephanie K. Nishi^{1,2,3,4,5}, Nancy Babio^{1,2,3}, Clara Belzer⁶, Prokopis Konstati⁶, Jesús Vioque^{7,8}, Dolores Corella^{3,9}, Olga Castañer^{8,10}, Josep Vidal^{11,12}, Isabel Moreno-Indias^{3,13}, Laura Torres-Collado^{7,8}, Patricia Guillem-Saiz^{3,9,14}, Montse Fitó^{8,10}, Miguel Ruiz-Canela^{3,15}, Adrián Hernández-Cacho^{1,2,3}, Francisco J. Tinahones^{3,13} & Jordi Salas-Salvadó^{1,2,3}

Affiliations:

¹Universitat Rovira i Virgili, Departament de Bioquímica i Biotecnologia, Alimentació, Nutrició, Desenvolupament i Salut Mental ANUT-DSM, Reus, Spain

²Alimentació, Nutrició, Desenvolupament i Salut Mental, Institut d'Investigació Sanitària Pere Virgili (IISPV), Reus, Spain

³Centro de Investigación Biomédica en Red Fisiopatología de La Obesidad y La Nutrición (CIBEROBN), Instituto de Salud Carlos III, Madrid, Spain

⁴School of Nutrition, Faculty of Community Services, Toronto Metropolitan University, Toronto, ON, Canada.

⁵Clinical Nutrition and Risk Factor Modification Centre, St. Michael's Hospital, Unity Health Toronto, Toronto, Canada

⁶Laboratory of Microbiology, Wageningen University & Research, Wageningen, the Netherlands

⁷Universidad Miguel Hernández. Instituto de Investigación Sanitaria y Biomédica de Alicante. (UMH. ISABIAL), Alicante, Spain

⁸CIBER de Epidemiología y Salud Pública (CIBERESP), Instituto de Salud Carlos III, Madrid, Spain

⁹Department of Preventive Medicine, University of Valencia, Valencia, Spain

¹⁰Unit of Cardiovascular Risk and Nutrition, Institut Hospital del Mar de Investigaciones Médicas Municipal d'Investigació Mèdica (IMIM), Barcelona, Spain

¹¹CIBER Diabetes y Enfermedades Metabólicas (CIBERDEM), Instituto de Salud Carlos III (ISCIII), Madrid, Spain

¹²Department of Endocrinology, Institut d'Investigacions Biomèdiques August Pi Sunyer (IDIBAPS), Hospital Clinic, University of Barcelona, Barcelona, Spain

¹³Department of Endocrinology and Nutrition, Virgen de la Victoria University Hospital, the Biomedical Research Institute of Malaga and Platform in Nanomedicine (IBIMA-BIONAND Platform), University of Malaga, Malaga, Spain

¹⁴Departamento de Salud, Universidad Europea de Valencia, Valencia, Spain

¹⁵Department of Preventive Medicine and Public Health, University of Navarra, Instituto de Investigación Sanitario de Navarra (IdiSNA), Pamplona, Spain

Fundings:

This work was supported by the official Spanish Institutions for funding scientific biomedical research, CIBER Fisiopatología de la Obesidad y Nutrición (CIBEROBN) and Instituto de Salud Carlos III (ISCIII), through the Fondo de Investigación para la Salud (FIS), which is co-funded by the European Regional Development Fund (six coordinated FIS projects led by JSS and JVi, including the following projects: PI13/00233, PI13/00728, PI13/00462, PI14/01206, PI14/00696, PI16/00533, PI16/00366, PI16/00501, PI17/01441, PI17/00855, PI19/00017, PI19/00781, PI19/00576, PI20/00557, PI21/0046; the Especial Action Project entitled: Implementación y evaluación de una intervención intensiva sobre la actividad física Cohorte PREDIMED-Plus grant to JSS; the Recercaixa (number 2013ACUP00194) grant to JSS; grants from the Consejería de Salud de la Junta de Andalucía (PI0458/2013, PS0358/2016, PI0137/2018); the PROMETEO/2017/017 and PROMETEO/2021/21 grants from the Conselleria de Innovación, Universidades, Ciencia y Sociedad Digital from the Generalitat Valenciana; and by NIH grant R01DK127601. This research was also partially funded by the Eat2beNICE/H2020-SFS-2016-2 EU- H2020 European grant, and the Horizon 2020 PRIME study (Prevention and Remediation of Insulin Multimorbidity in Europe; grant agreement #847879). JN is supported by a predoctoral grant from Ministerio de Ciencia, Innovación y Universidades (FPU 20/00385). SKN is supported by a postdoctoral fellowship from the Canadian Institutes of Health Research (CIHR, MFE-171207). IMI was supported by the "Miguel Servet Type II" program (CPII21/00013) of the ISCIII-Madrid (Spain) and cofunded by the European Union. AHC is supported by a predoctoral grant from Martí Franquès – INVESTIGO research fellowship funded and supported by NextGenerationEU, Servicio Público de Empleo Estatal and Universitat Rovira i Virgili (2022PMF-INV-01). JSS, the

senior author of this paper, was partially supported by ICREA under the ICREA Academia program. None of the funding sources took part in the design, collection, analysis, interpretation of the data, writing the report, or in the decision to submit the manuscript for publication.

Corresponding author:

Prof. Jordi Salas-Salvadó

Universitat Rovira i Virgili, Departament de Bioquímica i Biotecnologia, Food, Nutrition, Development and Mental Health Group (ANUT-DSM), Human Nutrition Unit.

C/ Sant Llorenç, 21, 43201 Reus, Spain.

Email: jordi.salas@urv.cat

Abstract

Background: The diet-microbiota-gut-brain axis emerges as a promising target for preventing neurodegenerative disorders. Nuts are nutrient-dense foods with potential neuroprotective and prebiotic properties, yet their relationship with longitudinal cognitive changes and gut microbiota remains unclear.

Objective: To assess the association of baseline nut consumption with 6-year changes in cognitive function and baseline gut microbiota composition in older adults.

Methods: This prospective study included 747 participants (mean age 65±5 years, 48% women) with overweight/obesity and metabolic syndrome. Baseline nut consumption, assessed via a validated food frequency questionnaire, was categorized as ≤1, 1–3, 3–7, and >7 servings/week. Cognitive function was evaluated at baseline and at 2, 4, and 6 years, using a comprehensive battery of neuropsychological tests. Gut microbiota composition was profiled through 16S rRNA amplicon sequencing. Multivariable linear mixed-effects and linear regression models were utilized.

Results: Participants consuming 3–7 servings of nuts/week showed significantly slower declines in global cognitive function over the follow-up period compared to those consuming ≤1 serving/week (4-year: β [95%CI]=0.170[0.022,0.319], $p=0.024$; 6-year: 0.176[0.020,0.331], $p=0.027$). This nut consumption category was also associated with higher gut microbial diversity (Shannon index: β [95%CI]=0.211[0.008,0.414], $p=0.042$). Thirteen taxa were associated with nut consumption, ten positively, including *Lachnospiraceae UCG-004*, which was further associated with positive changes in global cognitive function (2-year: β [95%CI]=0.020[0.004,0.036], $q=0.050$) and slower decline in attention (6-year: 0.042[0.020,0.064], $q=0.001$).

Conclusions: Moderate nut consumption (3–7 servings/week), was interconnectedly associated with cognitive preservation and favorable gut microbiota composition, underscoring the potential

of dietary modulation of the gut-brain axis for healthy aging.

Keywords: nuts, gut microbiota, cognitive decline, cognitive function, microbiota-gut-brain axis, healthy aging

Key Points:

- Moderate nut consumption (3–7 servings/week) was associated with slower cognitive decline.
- Higher gut microbial diversity was observed in participants with moderate nut consumption.
- Nut-related gut microbiota taxa may play a role in preserving cognitive health.

Abbreviations: AD, Alzheimer’s disease; ASVs, amplicon sequence variants; BDI-II, Beck Depression Inventory; BMI, body mass index; CDT, Clock Drawing Test; CLR, centered log-ratio; DST, Digit Span Test; erMedDiet, energy-reduced Mediterranean diet; FDR, false discovery rate; FFQ, Food Frequency Questionnaire; IQR, interquartile range; LMM, linear mixed-effects models; MEDAS, Mediterranean diet adherence screener; METs, metabolic equivalents; MMSE, Mini-Mental State Examination; PCoA, principal coordinate analysis; PCR, polymerase chain reaction; PERMANOVA, permutational multivariate analysis of variance; PREDIMED-Plus, PREvención con DIeta MEDiterránea; rRNA, ribosomal RNA; SCFAs, short-chain fatty acids; s/wk, serving(s) per week; TMT, Trail Making Tests; VFT, Verbal Fluency Tests; WAIS-III, Wechsler Adult Intelligence Scale-III.

Introduction

Tree nuts and peanuts (hereafter referred to as ‘nuts’) are nutrient-dense foods rich in unsaturated fatty acids, high-quality vegetable protein, dietary fiber, vitamins, non-sodium minerals, phytosterols and polyphenols[1]. These compounds confer anti-inflammatory, antioxidant, and prebiotic properties with potential neuroprotective effects that may support cognitive health[2,3]. Epidemiological studies have demonstrated positive associations between nut consumption and cognitive performance[2]. While cross-sectional studies have more consistently reported beneficial links[4,5,6,7,8,9], prospective findings remain inconsistent, particularly over longer periods[10,11,12]. This gap is concerning given the increasing global burden of mild cognitive impairment and dementia due to aging[13]. Cognitive decline is both a precursor to these conditions and a natural aspect of aging[14,15], with no effective curative treatments available, preventive strategies targeting modifiable risk determinants, such as diet and nutrition, offer a promising approach to mitigate cognitive decline, particularly in older adults with cardiometabolic disorders, conditions linked to higher dementia risk[3,13].

Alterations in gut microbiota have been proposed as a potential mechanism underlying diet-induced changes in cognitive function[16]. Individuals with neurodegenerative diseases have been shown to exhibit aberrant gut microbiota composition, characterized by a reduced microbial diversity and lower abundances of beneficial taxa[17]. Nuts may beneficially shape the gut microbiota composition due to prebiotic properties derived from fermentable fiber, polyphenols and specific lipids[18,19]. Specifically, studies have identified greater abundances of beneficial taxa in nut consumers, such as *Clostridium*, *Lachnospira*, and *Roseburia*, genera predominantly linked to the production of short-chain fatty acids (SCFAs)[19], which are gut microbiota-derived metabolites implicated in glucose and lipid metabolism, blood pressure regulation, inflammation, and also in neuroimmune function[16]. Decreases in the relative abundances of these genera have been observed in individuals with Alzheimer’s disease (AD)[17]. Nut consumption has also been associated with lower levels of *Ruminococcus* and *Parabacteroides*, which have been reported to be increased in AD patients[17,19,20]. In light of these observations and the paucity of evidence, further research is warranted to explore the gut microbiota composition and microbial signatures associated with nut consumption to uncover their potential role in the mechanisms linking nut consumption to cognitive health.

Therefore, in the present study, we aim to explore the intricate relationship between nut consumption, gut microbiota, and changes in cognitive function in a community-dwelling cohort at high risk of cognitive decline. Our objectives are threefold: first, to evaluate the association between nut consumption and changes in cognitive function over time; second, to assess the relationship between nut consumption and gut microbial composition at baseline; and third, to determine whether nut-related microbial signatures are prospectively associated with cognitive function or specific cognitive domains linked to nut consumption.

Methods

Study design and participants

For the present longitudinal cohort study, we used data from the PREDIMED-Plus (PREvención con DIeta MEDiterránea) trial, which is a multicenter, randomized controlled primary prevention trial. A total of 6,874 eligible men and women aged 55-75 years with overweight or obesity and metabolic syndrome[21], free of cardiovascular diseases or dementia at baseline, were randomized in a 1:1 ratio to the lifestyle intervention or control group. Details of the study design are available online (<https://www.predimedplus.com/>), in previous publications[22,23,24] and briefly described in **Appendix_Methods**. In this study, we included participants who provided self-collected stool samples at baseline, baseline dietary intake data, baseline data for the neuropsychological test battery and at least one follow-up cognition assessment. Participants who used an antibiotic within 30 days before stool sample collection or reported implausible baseline energy intakes outside predefined limits (800-4000kcal/day for men and 500-3500kcal/day for women) were excluded[25](**Appendix_Figure_1**).

Assessment of nut consumption

Habitual dietary intake was assessed at baseline using a validated 143-item semi-quantitative food frequency questionnaire (FFQ)[26]. The FFQ included four specific questions on nuts (i.e., almonds, pistachios, walnuts, and other nuts) and one calculation for total nut consumption. Intraclass correlation coefficients of nut consumption for relative reproducibility and validity were 0.80 and 0.55, respectively[26]. Participants reported their consumption frequency with one of nine categories ranging from ‘never to almost never’, to ‘>6 servings/day’ (serving size=30 grams). Total energy intake and nutrients were estimated using Spanish food composition tables[27,28]. To estimate average daily nut consumption, the reported frequencies (using the midpoint for range categories) were multiplied by the serving size.

Cognitive function assessment

Cognitive function was assessed at baseline and follow-up visits at years 2, 4, and 6 using a comprehensive battery of eight neuropsychological tests including the Mini-Mental State Examination (MMSE)[29,30], Clock Drawing Test (CDT)[31,32,33], Verbal Fluency Tests for Animals and Letter “P”[34,35], Digit Span Test (DST)-Forward and DST-Backward from the Wechsler Adult Intelligence Scale-III[36,37,38], and Trail Making Tests part A and B[38,39,40]. Detailed descriptions of the neuropsychological tests and methods for deriving cohort-specific z-scores have been previously reported[41].

An overall score for Global Cognitive Function (GCF) was obtained by averaging all test scores, adding or subtracting based on whether higher test scores represent better or worse cognitive

performance. To evaluate specific cognitive domains, four composite scores were also calculated by averaging the z-scores of relevant individual tests. These composites included executive function, attention, language, and general cognitive function as a screening-summary measure[42]. Further details regarding the calculation of composite scores are provided in **Appendix_Table_1**. The GCF score and domain-specific cognitive composite scores calculated at each visit were standardized to z-scores using the mean and standard deviation of the respective baseline composite score, with higher values reflecting better cognitive performance.

Taxonomic profiling of gut microbiota

Detailed methods for stool sample collection, microbial DNA extraction, and 16S ribosomal RNA amplicon sequencing have been described previously[43] and are presented in **Appendix_Methods**.

Assessment of covariates

Sociodemographic characteristics, lifestyle information, personal medical history and medication use, and anthropometric data were collected at baseline. Details of the covariate assessment are described in **Appendix_Methods**.

Statistical analysis

Baseline characteristics of the study population were compared across nut consumption categories using one-way ANOVA for continuous variables and chi-square tests for categorical variables. To assess longitudinal associations between baseline nut consumption and changes in cognitive function over the follow-up period, we applied multivariable linear mixed-effects models (LMMs), adjusting for relevant sociodemographic, lifestyle, clinical, and dietary covariates. Gut microbiota diversity and composition were evaluated using multivariable linear regression models, principal coordinate analysis (PCoA), permutational multivariate analysis of variance (PERMANOVA), and MaAsLin2 to identify taxonomic associations with nut consumption. Associations between nut-related microbial taxa and cognitive outcomes were analyzed using LMMs with false discovery rate (FDR) correction for multiple testing. Further details on statistical analyses are provided in **Appendix_Methods**. All analyses were performed using R (version 4.3.1), and statistical significance was determined at $p < 0.05$ (two-tailed), unless specified otherwise.

Results

Study population characteristics

The flow of the study population is shown in **Appendix_Figure_1**, and the final analytical cohort consisted of 747 participants, with a mean age of 65 ± 5 years, of whom 48% were women. Baseline

characteristics of the study population, overall and grouped by baseline nut consumption categories, are presented in **Table_1**. Participants with higher nut consumption tended to have a higher education level, lower BMI and waist circumference, and exhibited greater adherence to the MedDiet, characterized by higher consumption of vegetables, fruits, legumes, and fish. In contrast, participants consuming ≤ 1 serving of nuts/week were more likely to have type 2 diabetes and depressive symptoms, accompanied by greater use of antidiabetic and antidepressant medications.

Nut consumption and cognitive function changes over follow-up

During the follow-up period, participants consuming 3–7 servings of nuts/week at baseline experienced significantly slower GCF decline compared to those consuming ≤ 1 serving/week, particularly at 4 (β :0.170; 95%CI:0.022,0.319; p -value:0.0243) and 6 (β :0.176; 95%CI:0.020,0.331; p -value:0.0267) years of follow-up (**Table_2** and **Appendix_Figure_2**). Participants consuming 1–3 servings/week (β :0.163; 95%CI:0.023,0.303; p -value:0.0229) and >7 servings/week (β :0.183; 95%CI:0.009,0.357; p -value:0.0392) also exhibited reduced cognitive decline after 4 years, but the significant associations were not sustained at 6 years. Furthermore, participants in the 1–3 servings/week category showed significantly slower declines in attention (β :0.218; 95%CI:0.045,0.391; p -value:0.0138) after 6 years of follow-up compared to the reference group. Participants consuming 3–7 servings of nuts/week also showed a non-significant slower decline in attention at 4 and 6 years of follow-up. No significant associations were observed in changes of other cognitive domains. Sensitivity analyses yielded consistent results and showed no significant interactions between nut consumption and education level, physical activity, MedDiet adherence, or the presence of type 2 diabetes, hypertension, hypercholesterolemia, or depressive symptomatology (all p for interaction values >0.05 ; data not shown).

Nut consumption and gut microbiota

The associations between nut consumption and gut microbiota profiles at baseline are presented in **Figure_1**. Participants consuming 3–7 servings of nuts/week had significantly higher gut microbial diversity (Shannon index, β :0.211; 95%CI:0.008,0.414; p -value:0.042) compared to those consuming ≤ 1 serving/week (**Figure_1a-c** and **Appendix_Table_2**). While nut consumption was not a dominant driver of overall gut microbial composition (**Appendix_Figure_3b**), the PERMANOVA result revealed a statistically significant association between nut consumption and overall variation of the gut microbiota composition (p -value:0.047) after adjusting for covariates related to gut microbiota composition (**Figure_1d** and **Appendix_Table_3**). Additionally, nut consumption explained the highest percentages of variation (R^2 :0.46%) in the gut microbiome among a set of variables including age, sex, BMI, physical activity, smoking status, alcohol intake, total energy intake and the prevalence of several diseases (**Appendix_Figure_3b**).

A total of 13 microbial taxa were linked to nut consumption (q -value <0.25 ;**Figure_1e** and

Appendix Table 4). Of these, 10 taxa (i.e., *Lachnospiraceae UCG-004*, *Flavonifractor*, *Oscillibacter*, *Turicibacter*, *Colidextribacter*, *Pseudobutyrvibrio*, *Intestinimonas*, *Hungatella*, *Terrisporobacter*, and *Roseburia*) demonstrated positive associations with higher nut consumption, with the most pronounced associations observed in participants consuming 3–7 servings of nuts/week compared to those consuming ≤ 1 serving/week. Conversely, 3 taxa (i.e., *Phascolarctobacterium*, *Parvimonas*, and *Ruminococcus gauvreauii* group) negatively associated with nut consumption, were observed exclusively in the >7 servings/week category.

Nut consumption, gut microbiota, and changes in cognitive function

Given the significant associations observed between nut consumption and changes in GCF and attention during follow-up, subsequent analyses focused on the relationship between nut-related microbial taxa and these cognitive outcomes. As shown in **Figure 2** and **Appendix Table 5**, *Lachnospiraceae UCG-004*, which was positively associated with higher nut consumption, was significantly associated with a greater GCF after 2 years of follow-up ($\beta:0.020$; 95%CI:0.004,0.036; q -value:0.050). Additionally, higher abundance of *Lachnospiraceae UCG-004* was associated with a slower decline in attention after 6 years of follow-up ($\beta:0.042$; 95%CI:0.020,0.064; q -value:0.001). No other significant associations between nut-related microbial taxa and nut-associated cognitive outcomes were observed after controlling for the FDR.

Discussion

In this longitudinal study of older Mediterranean adults at high risk of cognitive decline, moderate nut consumption was associated with both slower global cognitive decline over a long-term follow-up period and favorable gut microbiota composition profile at baseline. Specifically, participants consuming 3-7 servings of nuts/week at baseline showed the most favorable global cognitive trajectories over time, accompanied by higher baseline microbial diversity and higher relative abundances of key taxa predominant connected to SCFA production. These findings suggest the potential of nut consumption in modulating the gut-brain axis to preserve cognitive health.

Our findings contribute to the growing body of evidence linking nut consumption to cognitive benefits. Prior research in China reported that nut consumers experienced less cognitive decline, as measured by the telephone version of the MMSE, compared to non-consumers after 15-years of follow-up[44]. Similarly, in another large Chinese cohort it has been reported that higher baseline nut consumption was associated with a lower risk of cognitive impairment over a 6-year follow-up period[45]. Our study expands on this by capturing longitudinal cognitive trajectories with comprehensive neuropsychological tests, revealing that moderate and regular nut consumption, rather than very high or low consumption, was associated with more favorable cognitive preservation over time. This may reflect a non-linear dose-response relationship, as

suggested by our non-significant p-trend values, indicating that cognitive benefits may plateau beyond moderate nut consumption. It is also important to consider that the >7 servings/week group comprised a smaller subset of participants, which may have reduced statistical power to detect associations in this category. Additionally, individuals with higher nut consumption may have consumed more salted nuts, potentially increasing sodium intake, which could attenuate the neurocognitive benefits of nuts [46]. These findings underscore the importance of considering both amount and type of nut consumption in relation to cognitive health.

Nuts may support gut health by promoting intestinal homeostasis and microbial diversity due to their unique nutritional composition, including fiber, polyphenols, and unsaturated fatty acids, with potential prebiotic effects[16,19]. In our study, we observed modest but distinct patterns in the gut microbial community across nut consumption categories. Moreover, participants consuming 3-7 servings of nuts/week exhibited the highest value of Shannon index, reflecting greater microbial richness and evenness. This is particularly relevant given that lower microbial diversity has been linked to adverse health outcomes, such as obesity, cardiometabolic conditions, and neurodegenerative disorders[17,47]. Our findings align with a previous systematic review[19], which reported a modest but positive association between nut consumption and gut microbiota diversity. However, results from randomized controlled trials (RCTs) have been inconsistent, likely due to differences in the types of nuts consumed, the duration of the interventions, and other methodological factors[20].

Furthermore, in our study we identified 10 taxa positively associated with nut consumption, including *Lachnospiraceae* UCG-004 and *Roseburia*, bacteria belonging to the *Lachnospiraceae* family, which is known for their predominant capacity to produce SCFAs. The enrichment of these taxa in response to nut consumption has been consistently reported in previous studies[19,20]. A recent 4-week randomized crossover study in healthy, nonelderly adults demonstrated that an unclassified *Lachnospiraceae* ASV was significantly enriched in participants supplemented with 30 g/d of nuts[48]. Similarly, increases in *Roseburia*, *Clostridium*, and *Dialister* have been observed with the consumption of nuts, although these associations have not been consistently replicated across studies[19,20]. Additionally, we observed 3 taxa that were more abundant in individuals who consumed fewer nuts (≤ 1 serving/week). Notably, similar to the *Ruminococcus gausvreauii* group, observed in our study, other taxa within the *Ruminococcaceae* family have also been reported to exhibit lower abundance in nut consumers[19,20]. Importantly, depleted *Lachnospiraceae* and higher *Ruminococcaceae* abundance are among the most consistently reported alternations in the gut microbiome studies in individuals with neurodegenerative diseases such as AD, highlighting their potential role in cognitive health[17].

Previous human studies investigating the relationship between diet, cognitive function, and the gut microbiota are sparse. A 12-month intervention with MedDiet (NU-Age diet) in older adults who were either non-frail or pre-frail demonstrated higher relative abundances of several butyrogenic species, including *Faecalibacterium prausnitzii*, *Anaerostipes*, and *Roseburia*, which were weakly but positively associated with cognitive function[49]. However, a subsequent analysis of the NU-

AGE study assessing the impact of specific food groups, including nuts, revealed a strong association between nut consumption and gut microbiota composition but failed to show a significant association between cognitive outcomes and the gut microbiota[50]. Similarly, the aforementioned 4-week randomized crossover study in adults reported that while nut consumption positively influenced cognition and gut microbiota, these effects appeared to be independent of one another[48]. In our study of older adults with a follow-up of 6 years, we observed that *Lachnospiraceae UCG-004*, a SCFAs-producing genus that was more abundant in individuals with moderate nut consumption, was associated with a slower rate of cognitive decline. Although future research incorporating intervention studies and mediation analyses is needed to clarify the mechanism, our findings highlight the potential of nuts in modulating gut microbiota to preserve cognitive health via the diet–microbiota–gut–brain axis[16].

The benefits of nut consumption on gut microbiota and cognitive function may be attributed to their fiber content. A fiber-deficient diet has been shown to impair cognitive function in mice via gut-microbiota-hippocampal axis disruption, hippocampal synaptic ultrastructure damage, and gut microbiota dysbiosis, distinct from normal brain aging[51]. Fermentation of fiber in the gut leads to the production of SCFAs, predominantly acetate, propionate and butyrate, which regulate various functions including gastrointestinal health, blood pressure, inflammation and neuroimmune function. Altered SCFA levels have been associated with neurological and metabolic disorders, including AD, Parkinson’s disease and obesity[16,52]. Notably, the bacterial genera *Lachnospiraceae UCG-004* and *Roseburia* are prominent producers of butyrate, a microbial metabolite that plays a pivotal role in immune system, reducing neuroinflammation, oxidative stress, and strengthening brain-blood barrier[52]. Moreover, polyphenols in nuts are known to exhibit prebiotic-like properties by promoting the growth of intestinal barrier-protecting bacteria (such as *Lactobacillus* and *Bifidobacteria*), butyrate-producing bacteria (*Faecalibacterium prausnitzii* and *Roseburia*), as well as immune-modulating bacteria (*Bacteroides vulgatus* and *Akkermansia muciniphila*)[47]. Many polyphenols arrive intact in the colon and are extensively metabolized by gut microbiota into bioactive metabolites that may improve cognition and emotional well-being[16]. Unsaturated fatty acids rich in nuts further support brain and cognitive health[2]. A recent randomized crossover trial demonstrated that longer-term mixed nut consumption, without other n-3 polyunsaturated fatty acids, significantly improved insulin action in brain regions involved in the modulation of metabolic and cognitive processes in older adults with overweight/obesity underlying the potential mechanisms of nut consumption on brain insulin signaling[53]. The lipid composition of nuts may also influence the gut microbiota composition and subsequent metabolites[18]. Another trial demonstrated that a walnut-free diet, matched in α -linolenic acid, resulted in a similar enrichment of *Roseburia* as a whole-walnut diet suggesting a potential role for lipids in the prebiotic effect of walnuts[54].

The present study has several limitations. First, because of its observational design, causality cannot be established. Second, while robust adjustments were made for confounders and supplementary sensitivity analyses were conducted, residual confounding cannot be entirely ruled

out. Third, the study focused on Mediterranean older adults with overweight/obesity and metabolic syndrome which limits generalizability to other populations. Fourth, dietary information collected through FFQ does not include details about nut processing and is subject to recall bias and misclassification; however, given the prospective design of the study, baseline nut consumption recall is unlikely to have been influenced by cognitive outcomes over the follow-up period. Fifth, the use of 16S rRNA sequencing provides limited resolution in distinguishing species and identifying functional capabilities. Finally, microbiome data were only collected at baseline, limiting assessment of temporal changes in microbial profiles. Future studies with longitudinal microbiome measures and mediation frameworks are warranted to underly the potential mechanistic pathway. Despite these limitations, our study has several notable strengths. To the best of our knowledge, this is the first study to prospectively investigate the relationship between nut consumption, gut microbiota, and changes in cognitive function in a large Spanish population at high risk of cognitive decline, adding valuable evidence to the promising research on the diet-microbiota-gut-brain axis. The robust assessment of cognitive function by using multiple cognitive tests and composite scores. The prospective design with a long follow-up period of 6 years. Moreover, the rigorous statistical analyses, which accounted for relevant confounders in the associations between nut consumption, gut microbiota, and cognitive function, further enhances the robustness of the study's findings.

In conclusion, moderate nut consumption is associated with cognitive preservation and favorable gut microbiota profiles, highlighting its potential as a dietary strategy to promote healthy aging. Further longitudinal studies and RCTs in humans are required to confirm these findings and elucidate the mechanistic role of the gut microbiome in the diet-cognition relationship.

References

1. Ros E, Singh A, O'keefe JH. Nuts: Natural Pleiotropic Nutraceuticals. *Nutrients* 2021, Vol 13, Page 3269 2021; 13: 3269.
2. Nishi SK, Sala-Vila A, Julvez J, Sabaté J, Ros E. Impact of Nut Consumption on Cognition across the Lifespan. *Nutrients* 2023, Vol 15, Page 1000 2023; 15: 1000.
3. Theodore LE, Kellow NJ, McNeil EA, Close EO, Coad EG, Cardoso BR. Nut Consumption for Cognitive Performance: A Systematic Review. *Advances in Nutrition* 2021; 12: 777–792.
4. Valls-Pedret C, Lamuela-Raventós RM, Medina-Remón A *et al.* Polyphenol-rich foods in the mediterranean diet are associated with better cognitive function in elderly subjects at high cardiovascular risk. *Journal of Alzheimer's Disease* 2012; 29: 773–782.
5. Arab L, Ang A. A cross sectional study of the association between walnut consumption and cognitive function among adult us populations represented in NHANES. *The journal of nutrition, health & aging* 2014 19:3 2014; 19: 284–290.

6. Dong L, Xiao R, Cai C *et al.* Diet, lifestyle and cognitive function in old Chinese adults. *Arch Gerontol Geriatr* 2016; 63: 36–42.
7. De Amicis R, Leone A, Foppiani A *et al.* Mediterranean Diet and Cognitive Status in Free-Living Elderly: A Cross-Sectional Study in Northern Italy. <https://doi.org/101080/0731572420181442263> 2018; 37: 494–500.
8. Tan SY, Georgousopoulou EN, Cardoso BR, Daly RM, George ES. Associations between nut intake, cognitive function and non-alcoholic fatty liver disease (NAFLD) in older adults in the United States: NHANES 2011-14. *BMC Geriatr* 2021; 21.
9. Chen X, Liu Z, Sachdev PS, Kochan NA, O’Leary F, Brodaty H. Dietary Patterns and Cognitive Health in Older Adults: Findings from the Sydney Memory and Ageing Study. *J Nutr Health Aging* 2021; 25: 255–262.
10. Nooyens ACJ, Bueno-De-Mesquita HB, Van Boxtel MPJ, Van Gelder BM, Verhagen H, Verschuren WMM. Fruit and vegetable intake and cognitive decline in middle-aged men and women: the Doetinchem Cohort Study. *British Journal of Nutrition* 2011; 106: 752–761.
11. Samieri C, Grodstein F, Rosner BA *et al.* Mediterranean diet and cognitive function in older age. *Epidemiology* 2013; 24: 490–499.
12. Bishop NJ, Zuniga KE. Investigating walnut consumption and cognitive trajectories in a representative sample of older US adults. *Public Health Nutr* 2021; 24: 1741–1752.
13. Livingston G, Huntley J, Liu KY *et al.* Dementia prevention, intervention, and care: 2024 report of the Lancet standing Commission. *The Lancet* 2024; 404: 572–628.
14. Wilson RS, Leurgans SE, Boyle PA, Bennett DA. Cognitive Decline in Prodromal Alzheimer Disease and Mild Cognitive Impairment. *Arch Neurol* 2011; 68: 351–356.
15. Payton NM, Marseglia A, Grande G *et al.* Trajectories of cognitive decline and dementia development: A 12-year longitudinal study. *Alzheimer’s and Dementia* 2023; 19: 857–867.
16. Schneider E, O’Riordan KJ, Clarke G, Cryan JF. Feeding gut microbes to nourish the brain: unravelling the diet–microbiota–gut–brain axis. *Nature Metabolism* 2024 6:8 2024; 6: 1454–1478.
17. Jemimah S, Chabib CMM, Hadjileontiadis L, AlShehhi A. Gut microbiome dysbiosis in Alzheimer’s disease and mild cognitive impairment: A systematic review and meta-analysis. *PLoS One* 2023; 18: e0285346.
18. Lamuel-Raventos RM, Onge MPS. Prebiotic nut compounds and human microbiota. *Crit Rev Food Sci Nutr* 2017; 57: 3154–3163.
19. Fitzgerald E, Lambert K, Stanford J, Neale EP. The effect of nut consumption (tree nuts and peanuts) on the gut microbiota of humans: A systematic review. *British Journal of Nutrition*

- 2021; 125: 508–520.
20. Creedon AC, Hung ES, Berry SE, Whelan K. Nuts and their effect on gut microbiota, gut function and symptoms in adults: A systematic review and meta-analysis of randomised controlled trials. *Nutrients* 2020; 12: 1–21.
 21. Alberti KGMM, Eckel RH, Grundy SM *et al.* Harmonizing the Metabolic Syndrome. *Circulation* 2009; 120: 1640–1645.
 22. Martínez-González MA, Buil-Cosiales P, Corella D *et al.* Cohort profile: Design and methods of the PREDIMED-Plus randomized trial. *Int J Epidemiol* 2019; 48: 387–388o.
 23. Salas-Salvadó J, Díaz-López A, Ruiz-Canela M *et al.* Effect of a Lifestyle Intervention Program With Energy-Restricted Mediterranean Diet and Exercise on Weight Loss and Cardiovascular Risk Factors: One-Year Results of the PREDIMED-Plus Trial. *Diabetes Care* 2019; 42: 777–788.
 24. Sayón-Orea C, Razquin C, Bulló M *et al.* Effect of a Nutritional and Behavioral Intervention on Energy-Reduced Mediterranean Diet Adherence Among Patients With Metabolic Syndrome: Interim Analysis of the PREDIMED-Plus Randomized Clinical Trial. *JAMA* 2019; 322: 1486–1499.
 25. Willett W. *Nutritional Epidemiology*. Oxford: Oxford University Press 2012.
 26. Fernández-Ballart JD, Piñol JL, Zazpe I *et al.* Relative validity of a semi-quantitative food-frequency questionnaire in an elderly Mediterranean population of Spain. *British Journal of Nutrition* 2010; 103: 1808–1816.
 27. Moreiras O, Carbajal A, Cabrera L, Cuadrado C. *Tablas de composición de los alimentos*. 9th. Ediciones Pirámide. Madrid (España) 2005.
 28. Mataix Verdú J. *Tablas de composición de alimentos*. Instituto de nutrición y tecnología de los alimentos. Granada (España): Editorial Universidad de Granada 2003.
 29. Folstein MF, Folstein SE, Mchugh PR. Mini-mental state" A practical method for grading the cognitive state of patients for the clinician Related papers 'MINI-MENTAL STATE' A PRACTICAL METHOD FOR GRADING THE COGNITIVE STATE OF PATIENTS FOR THE CLINICIAN*. *J gsychiaf Res* 1975; 12: 189–198.
 30. Blesa R, Pujol M, Aguilar M *et al.* Clinical validity of the 'mini-mental state' for Spanish speaking communities. *Neuropsychologia* 2001; 39: 1150–1157.
 31. Shulman KI. Clock-drawing: is it the ideal cognitive screening test? *Int J Geriatr Psychiatry* 2000; 15: 548–561.
 32. Aprahamian I, Martinelli JE, Neri AL, Yassuda MS. The Clock Drawing Test: A review of its accuracy in screening for dementia. *Dement Neuropsychol* 2009; 3: 74–80.

33. Del Ser Quijan T, García De Yébenes MJ, Sánchez Sánchez F *et al.* Evaluación cognitiva del anciano. Datos normativos de una muestra poblacional española de más de 70 años. *Med Clin (Barc)* 2004; 122: 727–740.
34. Benton AL, Hamsher K SA. *Multilingual Aphasia Examination* (3rd ed.). San Antonio, TX: The Psychological Corporation 1994;
35. Peña-Casanova J, Quiñones-Úbeda S, Gramunt-Fombuena N *et al.* Spanish Multicenter Normative Studies (NEURONORMA Project): Norms for Verbal Fluency Tests. *Archives of Clinical Neuropsychology* 2009; 24: 395–411.
36. Wechsler D. *Wechsler Adult Intelligence Scale-III*. San Antonio, Texas: The Psychological Corporation 1997;
37. Wechsler D. *WAIS III. Escala de inteligencia de Wechsler para adultos-III. Manual técnico*. Madrid:TEA Ediciones 1999;
38. Peña-Casanova J, Quiñones-Úbeda S, Quintana-Aparicio M *et al.* Spanish Multicenter Normative Studies (NEURONORMA Project): Norms for Verbal Span, Visuospatial Span, Letter and Number Sequencing, Trail Making Test, and Symbol Digit Modalities Test. *Archives of Clinical Neuropsychology* 2009; 24: 321–341.
39. Reitan R. *Trail Making Test: Manual for administration and scoring*. Tuscon: Reitan Neuropsychology Laboratory 1992;
40. Llinàs-Reglà J, Vilalta-Franch J, López-Pousa S, Calvó-Perxas L, Torrents Rodas D, Garre-Olmo J. The Trail Making Test: Association With Other Neuropsychological Measures and Normative Values for Adults Aged 55 Years and Older From a Spanish-Speaking Population-Based Sample. *Assessment* 2017; 24: 183–196.
41. Ni J, Nishi SK, Babio N *et al.* Higher versus lower nut consumption and changes in cognitive performance over two years in a population at risk of cognitive decline: a cohort study. *American Journal of Clinical Nutrition* 2023; 118: 360–368.
42. Sachdev PS, Blacker D, Blazer DG *et al.* Classifying neurocognitive disorders: the DSM-5 approach. *Nature Reviews Neurology* 2014 10:11 2014; 10: 634–642.
43. Atzeni A, Martínez MÁ, Babio N *et al.* Association between ultra-processed food consumption and gut microbiota in senior subjects with overweight/obesity and metabolic syndrome. *Front Nutr* 2022; 9.
44. Li M, Shi Z. A Prospective Association of Nut Consumption with Cognitive Function in Chinese Adults Aged 55+ _ China Health and Nutrition Survey. *Journal of Nutrition, Health and Aging* 2019; 23: 211–216.
45. Li F, Jiang W, Wang J *et al.* Beneficial Effects of Nut Consumption on Cognitive Function

- Among Elderly: Findings From a 6-Year Cohort Study. *Front Aging Neurosci* 2022; 14: 236.
46. Faraco G, Brea D, Garcia-Bonilla L *et al.* Dietary salt promotes neurovascular and cognitive dysfunction through a gut-initiated TH17 response. *Nature Neuroscience* 2017 21:2 2018; 21: 240–249.
 47. Valdes AM, Walter J, Segal E, Spector TD. Role of the gut microbiota in nutrition and health. *BMJ* 2018; 361: 36–44.
 48. Haskell-Ramsay CF, Dodd FL, Smith D *et al.* Mixed Tree Nuts, Cognition, and Gut Microbiota: A 4-Week, Placebo-Controlled, Randomized Crossover Trial in Healthy Nonelderly Adults. *Journal of Nutrition* 2022; 152: 2778–2788.
 49. Ghosh TS, Rampelli S, Jeffery IB *et al.* Mediterranean diet intervention alters the gut microbiome in older people reducing frailty and improving health status: the NU-AGE 1-year dietary intervention across five European countries. *Gut* 2020; 69: 1218–1228.
 50. van Soest APM, Hermes GDA, Berendsen AAM *et al.* Associations between Pro- and Anti-Inflammatory Gastro-Intestinal Microbiota, Diet, and Cognitive Functioning in Dutch Healthy Older Adults: The NU-AGE Study. *Nutrients* 2020, Vol 12, Page 3471 2020; 12: 3471.
 51. Shi H, Ge X, Ma X *et al.* A fiber-deprived diet causes cognitive impairment and hippocampal microglia-mediated synaptic loss through the gut microbiota and metabolites. *Microbiome* 2021; 9.
 52. Mann ER, Lam YK, Uhlig HH. Short-chain fatty acids: linking diet, the microbiome and immunity. *Nature Reviews Immunology* 2024 24:8 2024; 24: 577–595.
 53. Nijssen KM, Mensink RP, Plat J, Ivanov D, Preissl H, Joris PJ. Mixed nut consumption improves brain insulin sensitivity: a randomized, single-blinded, controlled, crossover trial in older adults with overweight or obesity. *Am J Clin Nutr* 2024; 119: 314–323.
 54. Tindall AM, McLimans CJ, Petersen KS, Kris-Etherton PM, Lamendella R. Walnuts and Vegetable Oils Containing Oleic Acid Differentially Affect the Gut Microbiota and Associations with Cardiovascular Risk Factors: Follow-up of a Randomized, Controlled, Feeding Trial in Adults at Risk for Cardiovascular Disease. *J Nutr* 2020; 150: 806–817.

Acknowledgements

The authors thank all PREDIMED-Plus participants and investigators. CIBEROBN, CIBERESP, and CIBERDEM are initiative of the Instituto de Salud Carlos III (ISCIII), Madrid, Spain. The Hojiblanca (Lucena, Spain) and Patrimonio Comunal Olivarero (Madrid, Spain) food companies donated extra-virgin olive oil. The Almond Board of California (Modesto, CA), American Pistachio Growers (Fresno, CA), and Paramount Farms (Wonderful Company, LLC, Los Angeles, CA) donated nuts for the PREDIMED-Plus pilot study. The authors also thank the PREDIMED-Plus Biobank Network as a part of the National Biobank Platform of the ISCIII for storing and managing the PREDIMED-Plus biological samples.

Authors' contributions

All the principal PREDIMED-Plus investigators contributed to the study concept and design and to data extraction from the PREDIMED-Plus participants. JN, SKN, NB and JSS contributed to the concept and design of the present study. JN wrote the first draft and performed the statistical analyses under the supervision of SKN, NB and JSS. JN and JSS are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. All authors reviewed the manuscript for important intellectual content and approved the final version to be published.

Data availability

The datasets generated and analyzed during the current study are not publicly available due to data

regulations and for ethical reasons, considering that this information might compromise research participants' consent because our participants only gave their consent for the use of their data by the original team of investigators. However, collaboration for data analyses can be requested by sending a letter to the PREDIMED-Plus steering Committee (predimed_plus_scommittee@googlegroups.com). The request will then be passed to all the members of the PREDIMED-Plus Steering Committee for deliberation.

Conflicts of interest

JSS reports serving on the board of and receiving grant support through his institution from the International Nut and Dried Fruit Council, serving on the board of the Instituto Danone Spain and the International Danone institute. None of the other authors declare competing interests.

List of Tables

Table 1 Baseline characteristics of study population overall and according to nut consumption categories 21

Table 2 Mixed-effect linear regression model for the association between baseline total nut consumption and cognitive function changes over follow-up 24

List of Figures

Figure 1. Nut consumption and gut microbiota. **a-c:** associations between nut consumption and alpha diversity metrics (a. Chao1; b. Shannon; c. Simpson). In the boxplots, the central band of the boxplot represents the predicted median of the category, the lower and upper hinges correspond to the first and third quartiles, and the whiskers represent the 1.5× IQR from the hinge, whichever is lower. Alpha diversity metrics are scaled. **d:** PCoA plot illustrating the gut microbial compositions across groups with different nut consumption categories, based on ASV-level Aitchison dissimilarity. The PERMANOVA p-value was calculated with 999 permutations. **e:** Associations between nut consumption and gut microbial features ($q < 0.25$). Values are beta coefficients (95% CI) derived from multivariable-adjusted linear regression models linking categories of nut consumption with genus-level microbial features. Statistical significance was determined using the linear regression model with multiple comparison adjustment via the Benjamini-Hochberg method, with a target false discovery rate of 0.25. ** q-value<0.05, * q-value<0.1. Nut consumption of ≤ 1 serving/week was set as reference. All models were adjusted for age (years), sex (male, female), recruiting center (Alicante, Barcelona, Reus, Valencia), BMI (kg/m^2), physical activity (METs-min/day), smoking status (never, former, current smoker), alcohol consumption in g/day and adding the quadratic term, total energy intake (kcal/day), depressive symptomatology (yes, no), prevalence of diabetes (yes, no), hypertension (yes, no), and hypercholesterolemia (yes, no). Abbreviations: BMI, body mass index; METs, metabolic equivalents; s/wk, serving(s) per week. 25

Figure 2. Associations between nut-related genera and changes in nut-associated cognitive function scores over time. Colors of the heatmap are in correspondence to the beta coefficient for interaction terms between each genus and visit from linear mixed models. All models included participant as random intercept and visit as random slope, and were adjusted for nut consumption categories, age (years), sex (male, female), recruiting center (Alicante, Barcelona, Reus, Valencia), intervention group (control, intervention), educational level (primary, high school, college), civil status (single/divorced/separated, married, widower), BMI (kg/m^2), physical activity (METs-min/day), smoking status (never, former, current smoker), alcohol consumption in g/day and adding the quadratic term, depressive symptomatology (yes, no), total energy intake (kcal/day), prevalence of diabetes (yes, no), hypertension (yes, no), hypercholesterolemia (yes, no), and Mediterranean diet adherence (modified 13-point MEDAS score). Statistical significance was

assessed using the Benjamini-Hochberg method to control the false discovery rate, with q-value (adjusted p-value) reported. * q-value<0.05, + q-value=0.05 (exact values are provided in Appendix Table 5). Abbreviations: BMI, body mass index; MEDAS, Mediterranean Diet Adherence Screener; METs, metabolic equivalents. 26

Table 1 Baseline characteristics of study population overall and according to nut consumption categories

	Total Nut Consumption					P-value ²
	Total	≤1 s/wk ¹	1-3 s/wk	3-7 s/wk	>7 s/wk	
No. of participants	747	185	254	199	109	
Socio-demographic variables						
Age, years	65 ± 5	65 ± 5	65 ± 5	65 ± 5	65 ± 5	0.743
Women, n (%)	361 (48)	98 (53)	127 (50)	89 (45)	47 (43)	0.251
Intervention group, n (%)	360 (48)	84 (45)	119 (47)	94 (47)	63 (58)	0.182
Education level, n (%)						
Primary or less	397 (53)	113 (61)	131 (52)	110 (55)	43 (39)	
Secondary	212 (28)	39 (21)	85 (34)	56 (28)	32 (29)	0.001
College	138 (19)	33 (18)	38 (15)	33 (17)	34 (31)	
Civil status, n (%)						
Single, divorced or separated	99 (13)	25 (14)	33 (13)	30 (15)	11 (10)	
Married	575 (77)	137 (74)	199 (78)	152 (76)	87 (80)	0.715
Widower	73 (10)	23 (12)	22 (9)	17 (9)	11 (10)	
Disease present at recruitment						
Type 2 diabetes, n (%)	204 (27)	61 (33)	58 (23)	52 (26)	33 (30)	0.103
Hypertension, n (%)	607 (81)	140 (76)	204 (80)	177 (89)	86 (79)	0.007
Hypercholesterolemia, n (%)	494 (66)	118 (64)	166 (65)	140 (70)	70 (64)	0.516
Depressive symptomatology, n (%)	145 (19)	56 (30)	40 (16)	33 (17)	16 (15)	0.001
Medication use, n (%)						
Insulin or other antidiabetic drugs	172 (23)	56 (30)	45 (18)	41 (21)	30 (28)	0.01
Antihypertensive agents	579 (78)	138 (75)	187 (74)	167 (84)	87 (80)	0.043
Statins or other hypolipidemic drugs	364 (49)	87 (47)	117 (46)	103 (52)	57 (52)	0.528
Antidepressant	166 (22)	53 (29)	56 (22)	31 (16)	26 (24)	0.022
Anthropometric variables						
BMI, kg/m ²	32.8 ± 3.5	33.4 ± 3.5	32.8 ± 3.4	32.6 ± 3.7	32.2 ± 3.3	0.014

Waist circumference, cm							
	Women	104 ± 10	105 ± 10	104 ± 10	102 ± 9	104 ± 11	0.214
	Men	111 ± 9	113 ± 10	111 ± 9	110 ± 9	109 ± 8	0.029
Lifestyle variables							
Smoking status, n (%)							
	Current smoker	96 (13)	24 (13)	33 (13)	24 (12)	15 (14)	
	Former smoker	292 (39)	62 (34)	109 (43)	72 (36)	49 (45)	0.271
	Never smoked	359 (48)	99 (54)	112 (44)	103 (52)	45 (41)	
Physical activity, METs/min/day		361 ± 344	286 ± 284	348 ± 347	422 ± 374	408 ± 349	0.001
Adherence to modified 13-point Mediterranean diet score*		8.01 ± 1.69	7.68 ± 1.78	8.13 ± 1.65	8.05 ± 1.61	8.23 ± 1.71	0.016
Cognitive performance assessment, raw scores							
Mini-Mental State Examination		28.57 ± 1.63	28.55 ± 1.84	28.58 ± 1.57	28.56 ± 1.56	28.60 ± 1.54	0.993
Clock Drawing Test		5.90 ± 1.23	5.74 ± 1.49	5.94 ± 1.17	5.88 ± 1.16	6.11 ± 0.97	0.077
Verbal Fluency tasks semantical		15.66 ± 4.52	15.63 ± 4.61	15.57 ± 4.55	15.42 ± 4.46	16.36 ± 4.41	0.351
Verbal Fluency tasks phonological		11.97 ± 4.14	11.72 ± 4.35	12.03 ± 4.25	12.01 ± 3.86	12.15 ± 4.06	0.82
Trail Making Test Part A ³		53.50 ± 30.41	56.90 ± 34.14	53.87 ± 31.80	51.84 ± 23.26	49.90 ± 31.69	0.217
Trail Making Test Part B ³		129.00 ± 65.95	136.42 ± 72.41	131.05 ± 66.44	125.34 ± 61.11	118.28 ± 60.68	0.109
Digit Span test forward		9.06 ± 2.55	9.14 ± 2.74	8.92 ± 2.48	8.99 ± 2.41	9.38 ± 2.66	0.42
Digit Span test backward		5.45 ± 2.28	5.39 ± 2.27	5.49 ± 2.24	5.34 ± 2.23	5.68 ± 2.50	0.615
Dietary consumption							
Total energy intake, kcal/day		2,464 ± 497	2,318 ± 524	2,441 ± 469	2,478 ± 464	2,741 ± 462	<0.001
Fiber, g/day		26.8 ± 8.2	24.4 ± 7.3	25.1 ± 7.4	28.1 ± 7.8	32.6 ± 8.6	<0.001
Vegetables, g/day		346.1 ± 140.2	334.1 ± 147.0	325.9 ± 128.5	364.4 ± 134.1	380.0 ± 156.0	0.001
Fruits, g/day		358.8 ± 199.1	335.6 ± 189.4	329.3 ± 176.4	382.3 ± 210.8	423.9 ± 223.5	<0.001
Legumes, g/day		19.8 ± 10.4	19.1 ± 9.1	18.7 ± 9.5	19.8 ± 10.0	23.3 ± 13.7	0.001
Cereals, g/day		159.1 ± 70.2	164.7 ± 79.5	163.5 ± 70.5	149.3 ± 62.8	157.7 ± 64.2	0.108
Total meat, g/day		159.9 ± 55.6	161.7 ± 59.7	162.8 ± 53.3	156.2 ± 56.1	156.4 ± 53.2	0.533
Total fish, g/day		107.8 ± 46.1	93.4 ± 42.5	111.0 ± 50.0	110.1 ± 42.6	120.5 ± 43.2	<0.001

Total dairy, g/day	307.5 ± 177.8	323.9 ± 192.4	275.3 ± 151.5	321.7 ± 181.6	328.4 ± 193.3	0.005
Biscuits, g/day	24.5 ± 26.4	24.8 ± 29.0	23.3 ± 22.6	25.1 ± 29.2	25.9 ± 24.6	0.81
Total olive oil, g/day	45.5 ± 14.6	43.9 ± 14.7	46.9 ± 13.3	46.1 ± 13.9	44.1 ± 17.8	0.107
Coffee, mL/day	41.7 ± 52.8	42.7 ± 54.1	43.7 ± 52.7	39.3 ± 53.5	39.7 ± 49.8	0.801
Tea, mL/day	9.6 ± 26.1	12.1 ± 30.8	8.4 ± 24.9	9.0 ± 25.3	9.1 ± 21.1	0.499
Total alcohol, g/day	10.8 ± 13.8	9.5 ± 13.9	11.7 ± 14.7	10.1 ± 12.0	12.2 ± 14.4	0.209

Data are presented as n (%) and mean ± SD or median [IQR] for categorical and continuous variables, respectively.

* The nuts consumption item was removed from the original 14-point Mediterranean diet score.

Abbreviations: BMI, body mass index; IQR, interquartile range; METs, metabolic equivalents; s/wk, serving(s) per week.

¹ 1 serving=30 g.

² *p*-value for differences between categories of total nut consumption was calculated by Pearson's Chi-square test or one-way ANOVA, as appropriate.

³ Inverse neuropsychological assessment score, lower scores represent better cognitive performance.

Table 2 Mixed-effect linear regression model for the association between baseline total nut consumption and cognitive function changes over follow-up

Nut consumption		≤1 s/wk		1-3 s/wk		3-7 s/wk		>7 s/wk		p-trend
		β, [95% CI]	p-value	β, [95% CI]	p-value	β, [95% CI]	p-value			
Global cognitive function (n=744)										
Year 2 vs Baseline	Ref.	0.098, [-0.024, 0.221]	0.117	0.077, [-0.053, 0.207]	0.245	0.010, [-0.143, 0.164]	0.895	0.901		
Year 4 vs Baseline	Ref.	0.163, [0.023, 0.303]	0.023	0.170, [0.022, 0.319]	0.024	0.183, [0.009, 0.357]	0.039	0.077		
Year 6 vs Baseline	Ref.	0.073, [-0.073, 0.220]	0.326	0.176, [0.020, 0.331]	0.027	0.041, [-0.138, 0.221]	0.652	0.370		
General cognitive function (n=746)										
Year 2 vs Baseline	Ref.	0.085, [-0.108, 0.278]	0.387	0.183, [-0.022, 0.388]	0.080	0.076, [-0.165, 0.316]	0.538	0.360		
Year 4 vs Baseline	Ref.	0.156, [-0.092, 0.405]	0.217	0.243, [-0.020, 0.505]	0.071	0.288, [-0.020, 0.596]	0.067	0.060		
Year 6 vs Baseline	Ref.	-0.10, [-0.378, 0.176]	0.476	0.157, [-0.137, 0.452]	0.295	0.041, [-0.299, 0.381]	0.813	0.284		
Executive function (n=745)										
Year 2 vs Baseline	Ref.	0.032, [-0.097, 0.160]	0.630	0.036, [-0.100, 0.172]	0.605	0.011, [-0.150, 0.171]	0.896	0.905		
Year 4 vs Baseline	Ref.	0.084, [-0.052, 0.220]	0.228	0.086, [-0.057, 0.230]	0.238	0.106, [-0.062, 0.274]	0.217	0.291		
Year 6 vs Baseline	Ref.	0.036, [-0.103, 0.176]	0.609	0.091, [-0.057, 0.239]	0.230	-0.020, [-0.192, 0.152]	0.822	0.964		
Attention (n=745)										
Year 2 vs Baseline	Ref.	0.123, [-0.025, 0.272]	0.104	-0.026, [-0.184, 0.131]	0.742	-0.103, [-0.289, 0.083]	0.276	0.051		
Year 4 vs Baseline	Ref.	0.148, [-0.011, 0.306]	0.068	0.091, [-0.077, 0.258]	0.287	0.034, [-0.163, 0.231]	0.736	0.891		
Year 6 vs Baseline	Ref.	0.218, [0.045, 0.391]	0.014	0.154, [-0.030, 0.338]	0.100	0.053, [-0.160, 0.267]	0.625	0.931		
Language (n=747)										
Year 2 vs Baseline	Ref.	0.049, [-0.103, 0.201]	0.526	0.123, [-0.037, 0.284]	0.131	0.113, [-0.075, 0.301]	0.240	0.143		
Year 4 vs Baseline	Ref.	0.040, [-0.113, 0.193]	0.606	0.127, [-0.035, 0.288]	0.124	0.024, [-0.165, 0.213]	0.805	0.512		
Year 6 vs Baseline	Ref.	0.059, [-0.106, 0.225]	0.482	0.157, [-0.018, 0.333]	0.079	-0.042, [-0.245, 0.161]	0.687	0.985		

The multivariable linear mixed-effects model was adjusted for age (years), sex (male, female), recruiting center (Alicante, Barcelona, Reus, Valencia), intervention group (control, intervention), educational level (primary, high school, college), civil status (single/divorced/separated, married, widower), BMI (kg/m²), physical activity (METs-min/day), smoking status (never, former, current smoker), alcohol consumption in g/day and adding the quadratic term, depressive symptomatology (yes, no), total energy intake (kcal/day), prevalence of diabetes (yes, no), hypertension (yes, no), hypercholesterolemia (yes, no), and Mediterranean diet adherence (modified 13-point MEDAS score). Abbreviations: BMI, body mass index; METs, metabolic equivalents; s/wk, serving(s) per week.

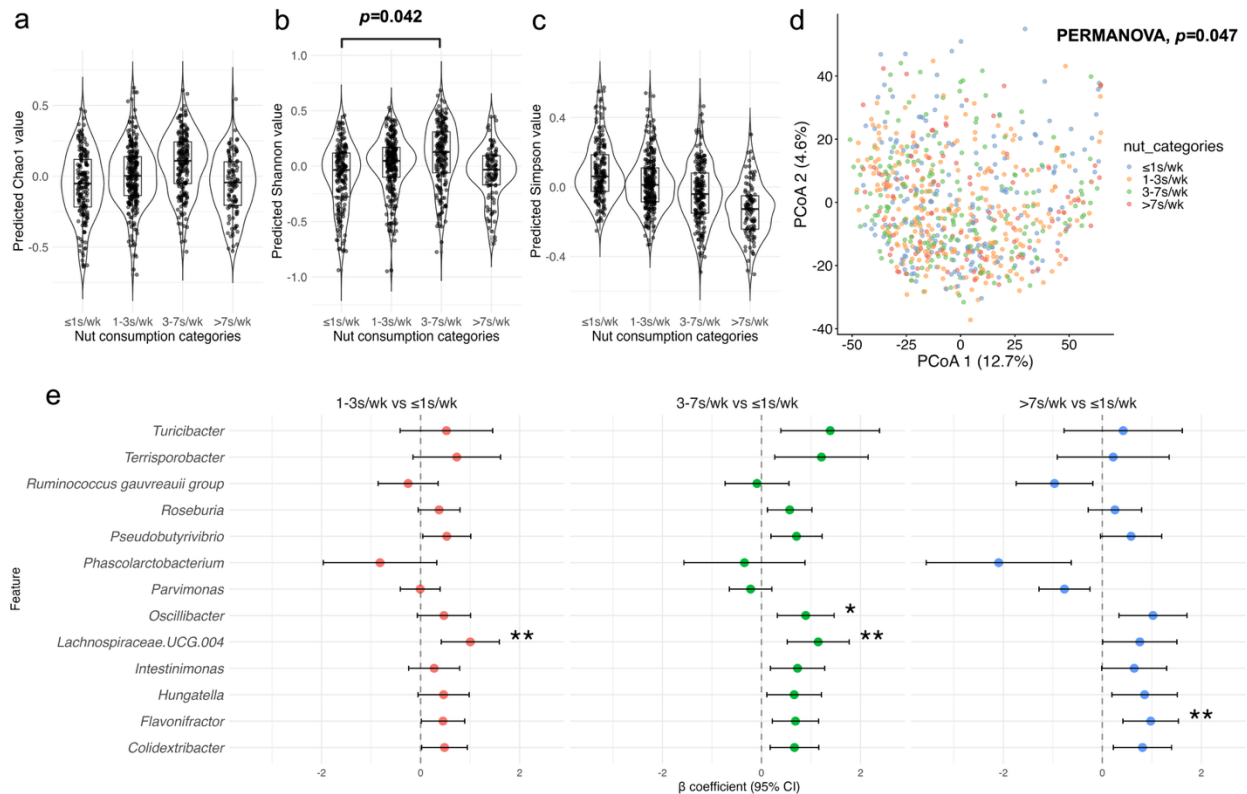


Figure 1. Nut consumption and gut microbiota. **a-c:** associations between nut consumption and alpha diversity metrics (a. Chao1; b. Shannon; c. Simpson). In the boxplots, the central band of the boxplot represents the predicted median of the category, the lower and upper hinges correspond to the first and third quartiles, and the whiskers represent the $1.5 \times$ IQR from the hinge, whichever is lower. Alpha diversity metrics are scaled. **d:** PCoA plot illustrating the gut microbial compositions across groups with different nut consumption categories, based on ASV-level Aitchison dissimilarity. The PERMANOVA p-value was calculated with 999 permutations. **e:** Associations between nut consumption and gut microbial features ($q < 0.25$). Values are beta coefficients (95% CI) derived from multivariable-adjusted linear regression models linking categories of nut consumption with genus-level microbial features. Statistical significance was determined using the linear regression model with multiple comparison adjustment via the Benjamini-Hochberg method, with a target false discovery rate of 0.25. ** q-value < 0.05, * q-value < 0.1. Nut consumption of ≤ 1 serving/week was set as reference. All models were adjusted for age (years), sex (male, female), recruiting center (Alicante, Barcelona, Reus, Valencia), BMI (kg/m^2), physical activity (METs-min/day), smoking status (never, former, current smoker), alcohol consumption in g/day and adding the quadratic term, total energy intake (kcal/day), depressive symptomatology (yes, no), prevalence of diabetes (yes, no), hypertension (yes, no), and hypercholesterolemia (yes, no). Abbreviations: BMI, body mass index; METs, metabolic equivalents; s/wk, serving(s) per week.

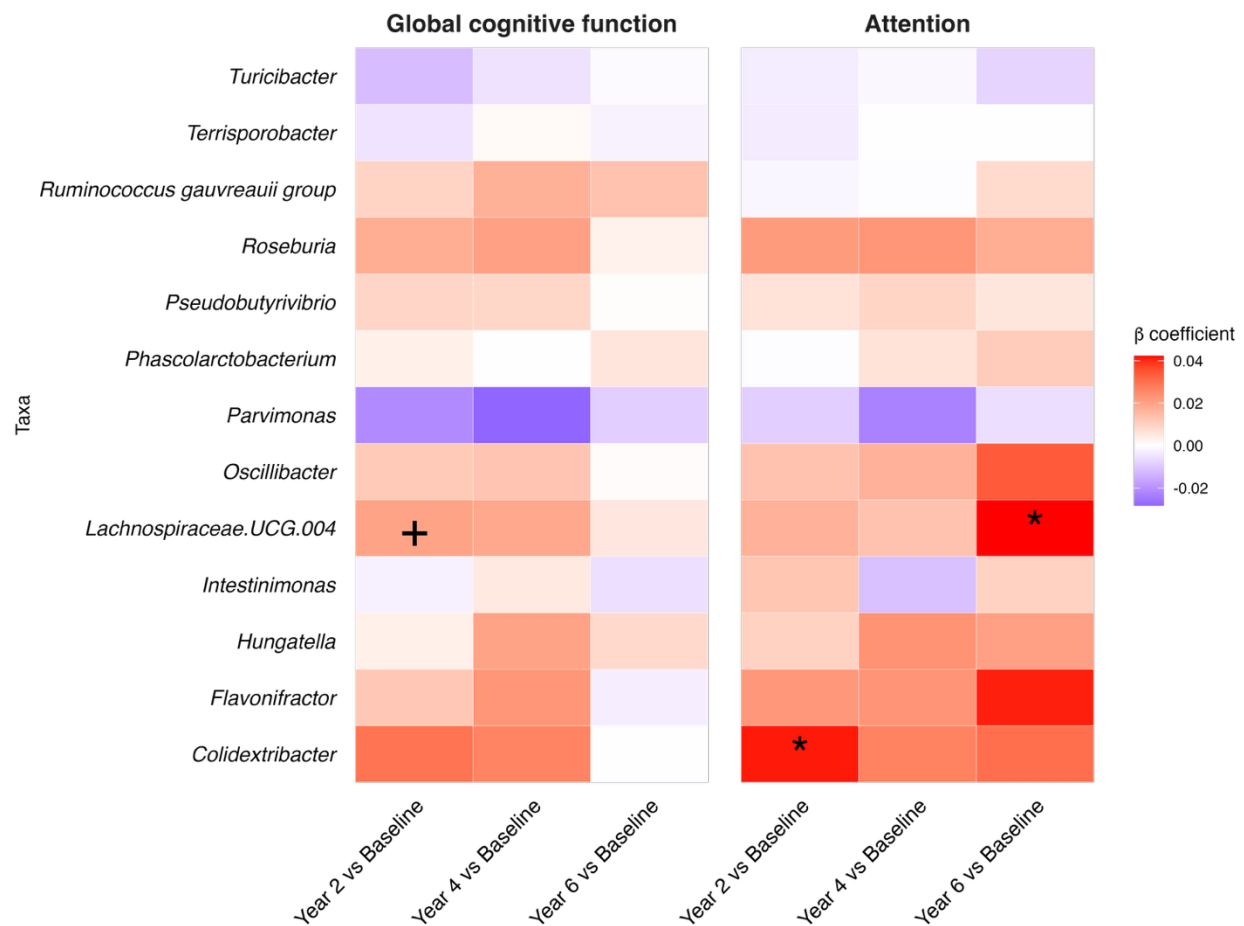


Figure 2. Associations between nut-related genera and changes in nut-associated cognitive function scores over time. Colors of the heatmap are in correspondence to the beta coefficient for interaction terms between each genus and visit from linear mixed models. All models included participant as random intercept and visit as random slope, and were adjusted for nut consumption categories, age (years), sex (male, female), recruiting center (Alicante, Barcelona, Reus, Valencia), intervention group (control, intervention), educational level (primary, high school, college), civil status (single/divorced/separated, married, widower), BMI (kg/m²), physical activity (METs-min/day), smoking status (never, former, current smoker), alcohol consumption in g/day and adding the quadratic term, depressive symptomatology (yes, no), total energy intake (kcal/day), prevalence of diabetes (yes, no), hypertension (yes, no), hypercholesterolemia (yes, no), and Mediterranean diet adherence (modified 13-point MEDAS score). Statistical significance was assessed using the Benjamini-Hochberg method to control the false discovery rate, with q-value (adjusted p-value) reported. * q-value<0.05, + q-value=0.05 (exact values are provided in Appendix Table 5). Abbreviations: BMI, body mass index; MEDAS, Mediterranean Diet Adherence Screener; METs, metabolic equivalents.

Title: Nut consumption, gut microbiota, and cognitive function: findings from a prospective study in older adults at risk of cognitive decline

Supplementary Data:

Appendix Methods.

Appendix Figure 1. Flow chart of the study population.

Appendix Figure 2. Estimated marginal means of cognitive function through follow-up by nut consumption categories.

Appendix Figure 3. Nut consumption and overall gut microbiota composition.

Appendix Table 1. Composite cognitive function assessment equations.

Appendix Table 2. Associations between baseline nut consumption and gut microbial alpha diversity.

Appendix Table 3. PERMANOVA test results based on Aitchison distance across baseline nut consumption categories.

Appendix Table 4. Results of MaAsLin2 analysis showing taxa significantly associated with baseline nut consumption ($q < 0.25$).

Appendix Table 5. Mixed-effect linear regression model for the association between nut-related genera and changes in nut-associated cognitive function scores.

Appendix Methods.

PREDIMED-Plus study design

The PREDIMED-Plus (PREvención con DIeta MEDiterránea) is a multicenter, randomized controlled primary prevention trial designed to evaluate the effects of an intensive lifestyle intervention, including an energy-reduced Mediterranean diet (erMedDiet), physical activity promotion, and behavioral support (intervention group), compared to usual care with ad libitum MedDiet recommendations (control group) on a composite cardiovascular endpoint as the primary outcome. Neurodegenerative diseases and changes in cognitive function are pre-defined secondary and intermediate outcomes in this trial. All participants provided written informed consent, and the trial protocol was approved by the research ethics committees of all participating institutions. The trial was registered at the International Standard Randomized Controlled Trial (Number: ISRCTN89898870) in 2014.

Taxonomic profiling of gut microbiota

At baseline, participants collected stool samples at home following standardized instructions provided with detailed illustrations. Samples were immediately frozen after collection and transported to the laboratory under cold conditions to ensure preservation. Upon receipt, stool samples were stored at -80°C until microbial DNA extraction was performed. DNA quality and concentration were evaluated prior to sequencing. The V4 region of the 16S rRNA gene was amplified in triplicate polymerase chain reaction (PCR), and the resulting amplicons were sequenced on the Illumina NovaSeq platform. Quality control measures included the use of artificial mock communities with known composition as positive controls and negative control samples to detect potential contaminant sequences. Raw sequencing data were processed using the DADA2 pipeline with default parameters[1], which involved paired-end reads demultiplexing, quality filtering, and the generation of amplicon sequence variants (ASVs). Taxonomic assignment of ASVs was performed using the Silva reference database (v138.1)[2].

Assessment of covariates

Sociodemographic characteristics (e.g., age, sex, education level, and civil status), lifestyle information (e.g., smoking status, physical activity, and adherence to the Mediterranean diet (MedDiet)) were collected by trained personnel via interviewer-administered questionnaires. Physical activity was estimated using a validated Spanish short version of the Minnesota Leisure Time Physical Activity Questionnaire (the REGICOR questionnaire)[3]. Adherence to the traditional MedDiet was evaluated using a validated 14-item MedDiet adherence screener (MEDAS)[4]. The original MEDAS score ranges from 0 to 14, with higher values indicating greater adherence to the MedDiet. However, to avoid over-adjustment in statistical analyses, the item specifically related to nut consumption, defined as consuming ≥ 3 servings of 30 g per week for 1 point, was removed. Therefore, a modified 13-item MedDiet score was derived and used as a covariate in the statistical models.

Personal medical history (e.g., prevalence of type 2 diabetes, hypertension, and hypercholesterolemia), and medication use, was obtained from participant self-report or collected from medical records. Depressive symptomatology was evaluated using the Beck Depression Inventory (BDI-II), with a score of ≥ 14 established as the threshold for identifying individuals at risk for depression[5,6].

Anthropometric measurements, including weight and height, were taken by trained personnel using calibrated scales and wall-mounted stadiometers, with participants wearing light clothing and no footwear or accessories. BMI was calculated as weight in kilograms divided by height in meters squared. Waist circumference was measured at the midpoint between the lowest rib and the iliac crest using a flexible anthropometric tape.

Detailed statistical analysis

The study population was categorized into four groups of baseline nut consumption: ≤ 1 serving/week, 1–3 servings/week, 3–7 servings/week, and > 7 servings/week. These categories

were determined based on the distribution of nut intake within the study population, recommendations for nut consumption[7,8], and prior research findings[9]. Baseline characteristics of the study population were compared across nut consumption categories and are presented as means \pm SD using one-way ANOVA for continuous variables, and numbers (percentages) for categorical variables using the chi-square test. Baseline nut consumption, classified into these categories, was used as the primary exposure variable for the study, with ≤ 1 serving/week functioning as the reference group for all analyses. Missing data in covariates were minimal, with one missing value each for civil and smoking status, which were imputed using the most frequent category. No imputations were performed for cognitive function outcomes.

To evaluate the association between baseline nut consumption and cognitive function changes over the follow-up period, multivariable linear mixed-effects models (LMMs) were applied. Given the nonlinear trajectory of cognitive function over time, the follow-up visit was modeled as a categorical variable. Fixed effects included nut consumption categories, visit, and their interaction term, alongside baseline covariates: age (years), sex (male or female), recruiting center (Alicante, Barcelona, Reus, or Valencia), intervention group (control or intervention), educational level (primary, high school, or college), civil status (single/divorced/separated, married, or widower), BMI (kg/m²), physical activity (METs-min/day), smoking status (never, former, or current smoker), alcohol intake (g/day and adding the quadratic term), total energy intake (kcal/day), type 2 diabetes status (yes or no), hypertension status (yes or no), hypercholesterolemia status (yes or no), depressive symptomatology (yes or no), and Mediterranean diet (MedDiet) adherence score as a measure of diet quality. Random effects included participants as intercepts and visit as slopes. Additionally, linear trends in nut consumption categories were assessed by assigning the median nut intake value for each category of participants. Several sensitivity analyses were conducted to assess the robustness

of our findings. Potential effect modification by education level, physical activity, diet quality (13-point MEDAS score), presence of type 2 diabetes, hypertension, and hypercholesterolemia, and depressive symptomatology was evaluated with the likelihood ratio test by comparing models with and without the multiplicative interaction term between these variables and nut consumption categories in the fully adjusted models. Additionally, we conducted analyses excluding participants with a baseline MMSE score <24[10] to further test the robustness of the results.

In the microbiome analysis, we first applied multivariable linear regression models to assess associations between nut consumption and gut microbial alpha diversity indices with adjustment for age (years), sex (male or female), recruiting center (Alicante, Barcelona, Reus, or Valencia), BMI (kg/m²), physical activity (METs-min/day), smoking status (never, former, or current smoker), alcohol consumption in g/day and adding the quadratic term, total energy intake (kcal/day), prevalence of diabetes (yes or no), hypertension (yes or no), and hypercholesterolemia (yes or no), and depressive symptomatology (yes or no). Alpha diversity metrics, including Chao1, Shannon, and Simpson indices[11,12,13], were calculated based on 16,256 ASV-level absolute abundance counts and standardized to z-scores for comparability. Beta diversity was calculated in terms of Aitchison distance metric (Euclidean distance over centered log-ratio (CLR)-transformed ASV-level abundance counts), incorporating all 16,256 ASVs [14]. To assess the overall gut microbial community structure, dimensionality reduction was performed using principal coordinate analysis (PCoA) based on the Aitchison distance metric. The first two PCoA axes (PCoA1 and PCoA2) were extracted to summarize the primary variation in microbial community composition. Beta diversity differences across nut consumption categories were tested using permutational multivariate analysis of variance (PERMANOVA)[15] with 999 permutations, adjusting for the same covariates as in the alpha diversity analysis models.

To identify specific microbial genera associated with nut consumption, we used the MaAsLin2[16] with adjustments for the same covariates. Before the analysis, we applied quality control filtering for taxonomic features at genus level by excluding features with a relative abundance of <0.1% in more than 10% of samples. A total of 221 microbial genera met these criteria and were included in the per-feature analysis. All high-dimensional tests were corrected for multiple comparisons using the Benjamini–Hochberg method with a false discovery rate (FDR) threshold of 0.25 for q values, as commonly applied[17].

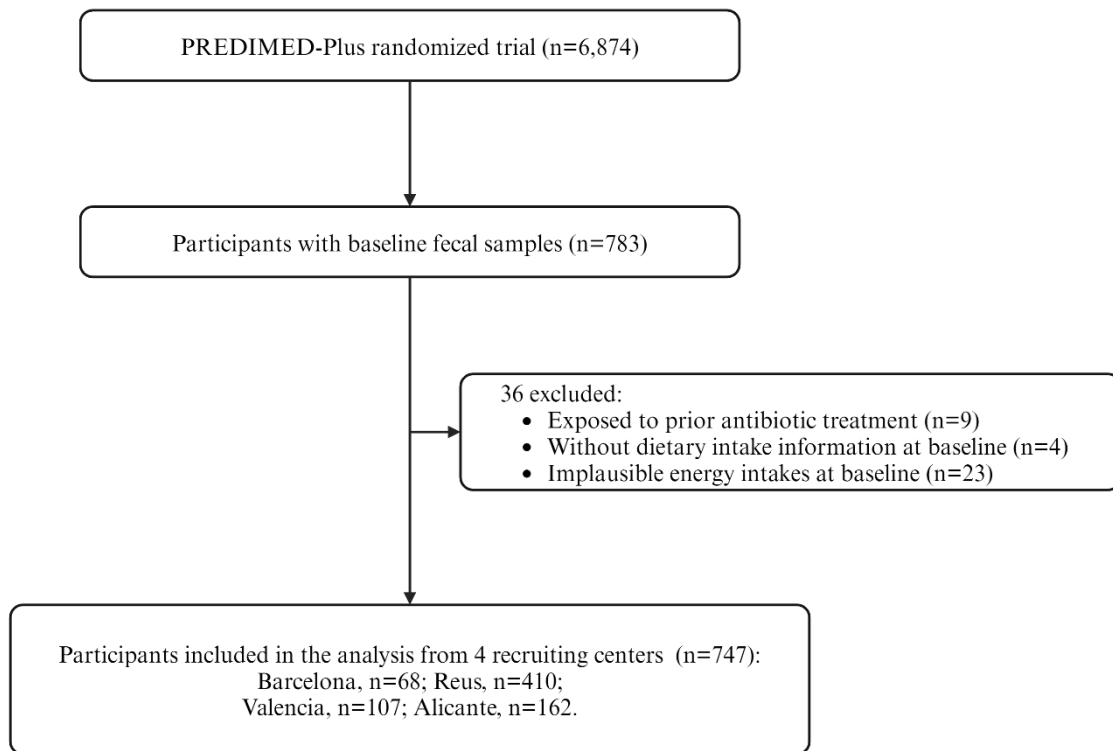
Linear mixed-effects models were subsequently employed to evaluate the associations between nut-related microbial genera and nut-associated cognitive function changes. These models included similar adjustments as previous LMMs, with the addition of the nut consumption variable as a covariate. The Benjamini–Hochberg method was used to account for multiple testing, and an adjusted p -value threshold of <0.05 was considered statistically significant.

References:

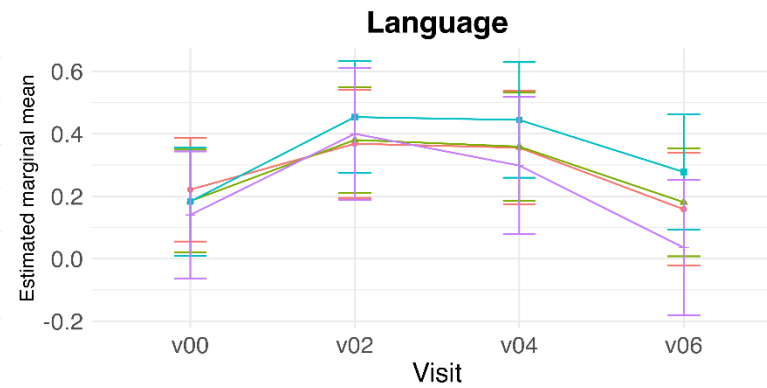
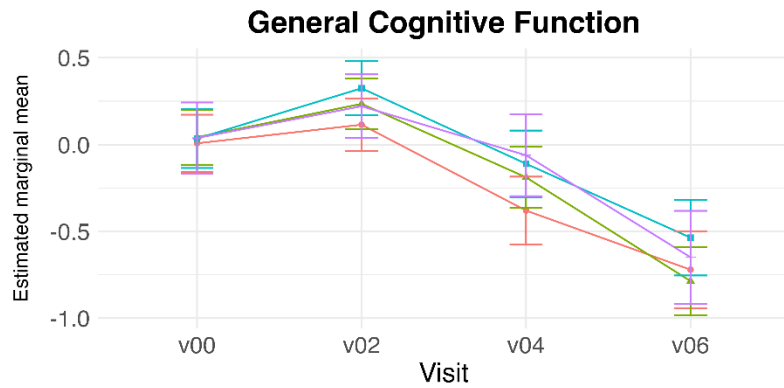
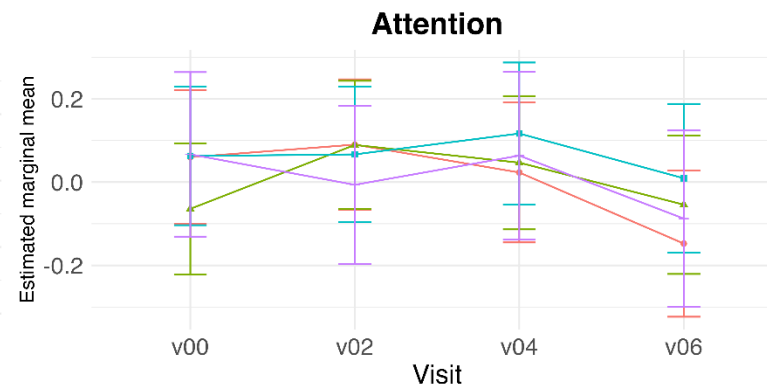
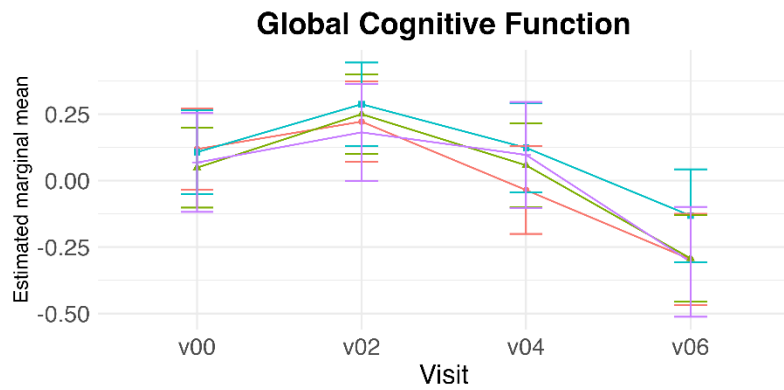
1. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods* 2016; 13:7 2016; 13: 581–583.
2. Quast C, Pruesse E, Yilmaz P *et al.* The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* 2013; 41.
3. Molina L, Sarmiento M, Peñafiel J *et al.* Validation of the Regicor Short Physical Activity Questionnaire for the Adult Population. *PLoS One* 2017; 12: 168148.
4. Schröder H, Fitó M, Estruch R *et al.* A Short Screener Is Valid for Assessing Mediterranean Diet Adherence among Older Spanish Men and Women. *J Nutr* 2011; 141: 1140–1145.

5. Beck A, Steer R, Brown G. BDI-II. Beck depression inventory–II–Second Edition Manual. San Antonio, TX: The Psychological Corporation 1996;
6. Sanz J, Luis A, Carmelo P, Resumen V. CLÍNICA Y SALUD 249 ARTÍCULOS Adaptación española del Inventario para la Depresión de Beck-II (BDI-II): 2. Propiedades psicométricas en población general The spanish adaptation of Beck’s Depression Inventory-II (BDI-II): 2. Psychometric properties in the general population. 2003; 14: 249–280.
7. Food-Based Dietary Guidelines recommendations for nuts and seeds | Knowledge for policy. https://knowledge4policy.ec.europa.eu/health-promotion-knowledge-gateway/food-based-dietary-guidelines-europe-table-12_en (14 January 2025, date last accessed).
8. Neale EP, Tran G, Brown RC. Barriers and Facilitators to Nut Consumption: A Narrative Review. International Journal of Environmental Research and Public Health 2020, Vol 17, Page 9127 2020; 17: 9127.
9. Ni J, Nishi SK, Babio N *et al.* Higher versus lower nut consumption and changes in cognitive performance over two years in a population at risk of cognitive decline: a cohort study. American Journal of Clinical Nutrition 2023; 118: 360–368.
10. Mini-Mental State Exam (MMSE) Test for Alzheimer’s / Dementia. <https://www.dementiacarecentral.com/mini-mental-state-exam/> (7 October 2022, date last accessed).
11. A C. Estimating the population size for capture-recapture data with unequal catchability. Biometrics 1987; 43: 783–91.
12. Shannon CE. A Mathematical Theory of Communication. Bell System Technical Journal 1948; 27: 379–423.
13. Simpson EH. Measurement of Diversity. Nature 1949 163:4148 1949; 163: 688–688.

14. Aitchison J, Barceló-Vidal C, Martín-Fernández JA, Pawłowsky-Glahn V. Logratio analysis and compositional distance. *Math Geol* 2000; 32: 271–275.
15. Anderson MJ. A new method for non-parametric multivariate analysis of variance. *Austral Ecol* 2001; 26: 32–46.
16. Mallick H, Rahnavard A, McIver LJ *et al.* Multivariable association discovery in population-scale meta-omics studies. *PLoS Comput Biol* 2021; 17: e1009442.
17. Wang DD, Nguyen LH, Li Y *et al.* The gut microbiome modulates the protective association between a Mediterranean diet and cardiometabolic disease risk. *Nat Med* 2021; 27: 333–343.

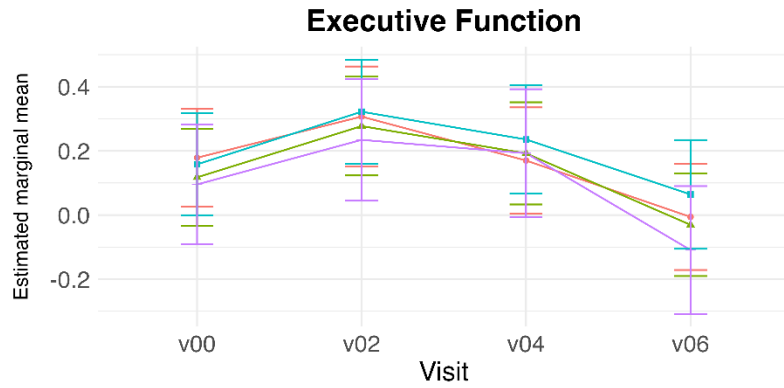


Appendix Figure 1. Flow chart of the study population.

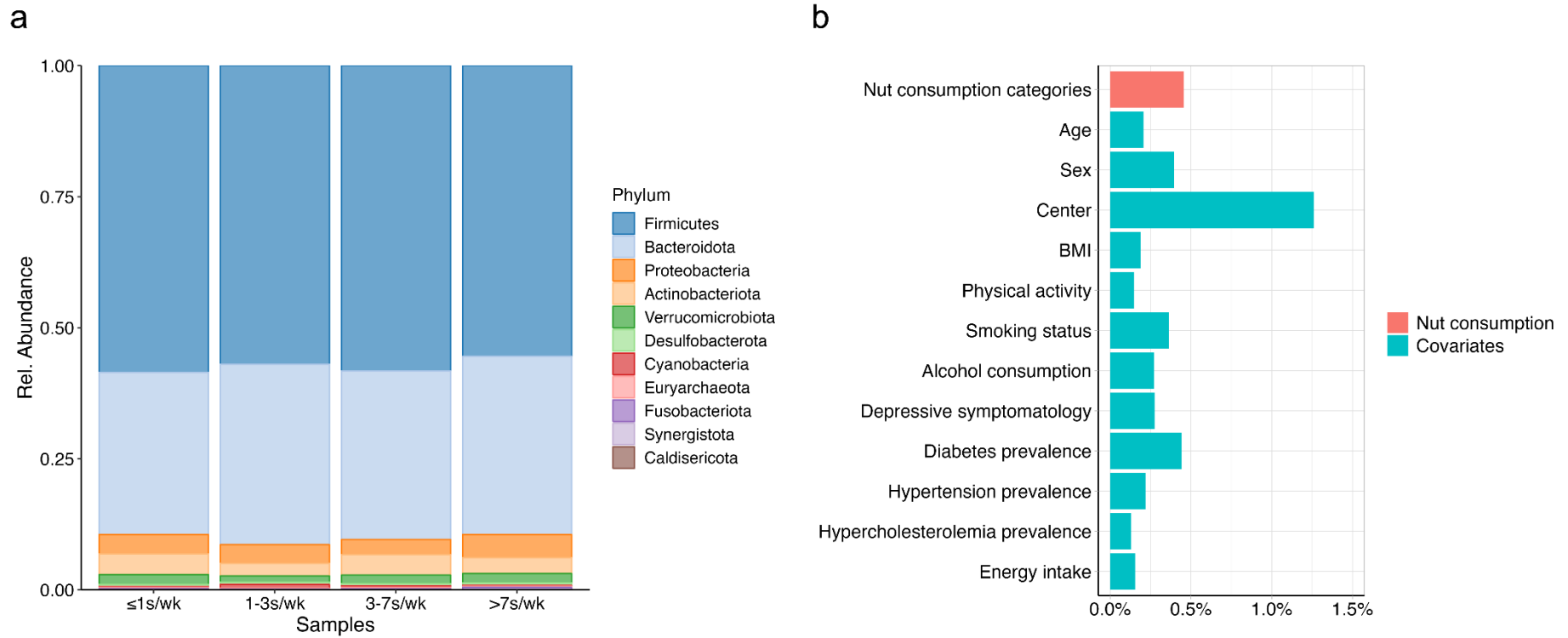


nut_categories

- ≤1s/wk
- 1-3s/wk
- 3-7s/wk
- >7s/wk



Appendix Figure 2. Estimated marginal means of cognitive function through follow-up by nut consumption categories. Abbreviations: s/wk, serving(s) per week and 1 serving of nuts = 30g.



Appendix Figure 3. Nut consumption and overall gut microbiota composition. **a.** Composition bar plot of relative abundance of phylum rank by nut consumption categories. **b.** Proportion of variation in gut microbial taxonomic composition explained by the nut consumption and covariates, as quantified by two-sided permutational multivariate analysis of variance based on ASV-level Aitchison distance metric. Abbreviations: s/wk, serving(s) per week.

Appendix Table 1. Composite cognitive function assessment equations¹.

Composite cognitive domain	Composite component score
<i>Attention</i>	$= \frac{(-zTMT-A) + zDST-f}{2}$
<i>Executive Function</i>	$= \frac{zVFT-a + zVFT-p + (-zTMT-B) + zDST-b}{4}$
<i>Language</i>	$= \frac{zVFT-a + zVFT-p}{2}$
<i>General Cognitive Function</i>	$= \frac{zMMSE + zCDT}{2}$
<i>Global Cognitive Function</i>	$= \frac{zMMSE + zCDT + zVFT-a + zVFT-p + (-zTMT-A) + (-zTMT-B) + zDST-f + zDST-b}{8}$

¹z-scores of the TMT-A and TMT-B for generating composite scores were inverted, so that higher scores would represent better cognitive performance. Abbreviations: CDT, Clock Drawing Test; DST-b, Digit Span test - backward; DST-f, Digit Span test - forward; MMSE, Mini-Mental State Examination; TMT-A, Trail Making Test Part A; TMT-B, Trail Making Test Part B; VFT-a, Verbal Fluency tasks semantical; VFT-p, Verbal Fluency tasks phonological.

Appendix Table 2. Associations between baseline nut consumption and gut microbial alpha diversity.

		≤ 1 s/wk			1-3 s/wk			3-7 s/wk			> 7 s/wk		
		β	CI (95%)		p-value	β	CI (95%)		p-value	β	CI (95%)		p-value
Chao 1	Ref.	0.040	-0.153	0.233	0.681	0.151	-0.055	0.356	0.152	-0.030	-0.276	0.217	0.814
Shannon	Ref.	0.048	-0.143	0.238	0.622	0.211	0.008	0.414	0.042	0.010	-0.234	0.253	0.937
Simpson	Ref.	-0.053	-0.249	0.142	0.591	-0.149	-0.357	0.059	0.161	-0.240	-0.490	0.011	0.061

The linear regression models was adjusted for age (years), sex (male, female), recruiting center (Alicante, Barcelona, Reus, Valencia), BMI (kg/m²), physical activity (METs-min/day), smoking status (never, former, current smoker), alcohol consumption in g/day and adding the quadratic term, total energy intake (kcal/day), depressive symptomatology (yes, no), prevalence of diabetes (yes, no), hypertension (yes, no), and hypercholesterolemia (yes, no). Alpha diversity metrics are scaled. Abbreviations: s/wk, serving(s) per week.

Appendix Table 3. PERMANOVA test results based on Aitchison distance across baseline nut consumption categories.

	Df	SumOfSqs	R²	F	Pr(>F)
Total nut consumption					
Model 1	3	48235	0.00455	1.1361	0.079
Model 2	3	48235	0.00433	1.1477	0.066
Model 3	3	48235	0.00455	1.1522	0.047

PERMANOVA with 999 permutations was performed to test for significant beta diversity clustering across nut consumption categories, using the `adonis2` function with the argument “by” set to “term” in the R package `vegan` (version 2.6-4).

Model 1 adjusted for age (years), sex (male, female), and energy intake (kcal/d).

Model 2 further adjusted for recruiting center (Alicante, Barcelona, Reus, Valencia), BMI (kg/m²), physical activity (METs-min/day), smoking status (never, former, current smoker), alcohol consumption in g/day and adding the quadratic term.

Model 3 further adjusted for depressive symptomatology (yes, no), prevalence of diabetes (yes, no), hypertension (yes, no), and hypercholesterolemia (yes, no).

Pr(>F) < 0.05 deemed as significant.

Abbreviations: BMI, body mass index; METs, metabolic equivalents; PERMANOVA, permutational multivariate analysis of variance; SumOfSqs, sum of squares.

Appendix Table 4. Results of MaAsLin2 analysis showing taxa significantly associated with baseline nut consumption (q<0.25).

feature	metadata	value	coef	stderr	pval	qval
Lachnospiraceae.UCG.004	nut_categories	3-7s/wk	1.14612573	0.31905875	0.00034997	0.0236476
Flavonifractor	nut_categories	>7s/wk	0.97678081	0.28589347	0.00066937	0.03747606
Lachnospiraceae.UCG.004	nut_categories	1-3s/wk	1.00409507	0.29922088	0.00083278	0.0421305
Oscillibacter	nut_categories	3-7s/wk	0.89412133	0.2923452	0.00230656	0.08591808
Oscillibacter	nut_categories	>7s/wk	1.02355531	0.35021023	0.00357796	0.10886837
Parvimonas	nut_categories	>7s/wk	-0.7642889	0.26255147	0.00371287	0.10979104
Flavonifractor	nut_categories	3-7s/wk	0.68613118	0.23865546	0.00415852	0.12032252
Phascolarctobacterium	nut_categories	>7s/wk	-2.0923445	0.7469619	0.00522757	0.14253613
Turicibacter	nut_categories	3-7s/wk	1.38869865	0.50856247	0.00647436	0.15558153
Colidextribacter	nut_categories	>7s/wk	0.81156189	0.30037482	0.00705665	0.16461591
Pseudobutyrvibrio	nut_categories	3-7s/wk	0.70775757	0.26350917	0.00739868	0.1675065
Colidextribacter	nut_categories	3-7s/wk	0.66711987	0.25074407	0.0079731	0.17285404
Intestinimonas	nut_categories	3-7s/wk	0.72887248	0.27952319	0.00930619	0.19155232
Hungatella	nut_categories	>7s/wk	0.85378418	0.33678716	0.01145053	0.2162106
Terrisporobacter	nut_categories	3-7s/wk	1.21123784	0.48116487	0.01203964	0.22427121
Roseburia	nut_categories	3-7s/wk	0.57056465	0.22964958	0.01319732	0.23783498
X.Ruminococcus..gavreaultii.group	nut_categories	>7s/wk	-0.9660781	0.39459148	0.01458852	0.24601289

The linear regression models were adjusted for age (years), sex (male, female), recruiting center (Alicante, Barcelona, Reus, Valencia), BMI (kg/m²), physical activity (METs-min/day), smoking status (never, former, current smoker), alcohol consumption in g/day and adding the quadratic term, total energy intake (kcal/day), depressive symptomatology (yes, no), prevalence of diabetes (yes, no), hypertension (yes, no), and hypercholesterolemia (yes, no). Abbreviations: coef, coefficient; pval, p-value; q-val, q value; stderr, standard error; s/wk, serving(s) per week.

Appendix Table 5. Mixed-effect linear regression model for the association between nut-related genera and changes in nut-associated cognitive function scores.

Nut-related taxa	Global cognitive function					Attention				
	β	CI (95%)	p -value	q-value	β	CI (95%)	p -value	q-value		
Lachnospiraceae.UCG-004										
Year 2 vs Baseline	0.020	0.004	0.036	0.012	0.050	0.017	-0.002	0.036	0.082	0.181
Year 4 vs Baseline	0.019	0.001	0.037	0.037	0.096	0.013	-0.007	0.034	0.196	0.340
Year 6 vs Baseline	0.005	-0.014	0.024	0.575	0.702	0.042	0.020	0.064	0.000	0.001
Flavonifractor										
Year 2 vs Baseline	0.012	-0.013	0.037	0.340	0.534	0.022	-0.008	0.052	0.147	0.262
Year 4 vs Baseline	0.023	-0.005	0.051	0.111	0.262	0.023	-0.008	0.055	0.150	0.262
Year 6 vs Baseline	-0.003	-0.033	0.027	0.843	0.960	0.041	0.006	0.076	0.023	0.075
Oscillibacter										
Year 2 vs Baseline	0.012	-0.012	0.035	0.332	0.560	0.013	-0.015	0.042	0.361	0.595
Year 4 vs Baseline	0.013	-0.014	0.040	0.356	0.560	0.017	-0.013	0.047	0.272	0.472
Year 6 vs Baseline	0.001	-0.028	0.030	0.956	0.956	0.034	0.000	0.068	0.053	0.159
Parvimonas										
Year 2 vs Baseline	-0.021	-0.051	0.008	0.158	0.302	-0.009	-0.045	0.028	0.636	0.837
Year 4 vs Baseline	-0.028	-0.062	0.006	0.104	0.244	-0.023	-0.061	0.015	0.238	0.447
Year 6 vs Baseline	-0.009	-0.044	0.026	0.626	0.795	-0.006	-0.048	0.036	0.779	0.845
Phascolarctobacterium										
Year 2 vs Baseline	0.003	-0.008	0.015	0.566	0.778	-0.001	-0.014	0.013	0.938	0.938
Year 4 vs Baseline	0.000	-0.013	0.013	0.957	0.957	0.006	-0.008	0.021	0.395	0.652
Year 6 vs Baseline	0.006	-0.008	0.019	0.403	0.738	0.011	-0.005	0.027	0.161	0.380
Turcibacter										
Year 2 vs Baseline	-0.012	-0.025	0.001	0.070	0.166	-0.003	-0.019	0.012	0.665	0.783
Year 4 vs Baseline	-0.005	-0.020	0.009	0.482	0.758	-0.001	-0.018	0.015	0.867	0.894

	Year 6 vs Baseline	-0.001	-0.016	0.015	0.902	0.950	-0.008	-0.026	0.011	0.413	0.649
Colidextribacter											
	Year 2 vs Baseline	0.029	0.006	0.053	0.014	0.057	0.041	0.013	0.070	0.004	0.025
	Year 4 vs Baseline	0.026	0.000	0.053	0.053	0.125	0.027	-0.003	0.057	0.082	0.159
	Year 6 vs Baseline	0.000	-0.029	0.028	0.976	0.976	0.030	-0.003	0.064	0.076	0.156
Pseudobutyrvibrio											
	Year 2 vs Baseline	0.009	-0.010	0.029	0.354	0.584	0.006	-0.017	0.030	0.595	0.840
	Year 4 vs Baseline	0.009	-0.014	0.031	0.444	0.684	0.009	-0.016	0.035	0.469	0.774
	Year 6 vs Baseline	0.000	-0.023	0.024	0.973	0.973	0.005	-0.022	0.033	0.700	0.840
Intestinimonas											
	Year 2 vs Baseline	-0.002	-0.023	0.018	0.817	0.917	0.013	-0.012	0.038	0.323	0.592
	Year 4 vs Baseline	0.005	-0.019	0.028	0.686	0.906	-0.011	-0.038	0.015	0.403	0.666
	Year 6 vs Baseline	-0.006	-0.030	0.019	0.642	0.883	0.010	-0.019	0.039	0.502	0.753
Hungatella											
	Year 2 vs Baseline	0.003	-0.016	0.023	0.729	0.860	0.010	-0.013	0.033	0.405	0.607
	Year 4 vs Baseline	0.020	-0.002	0.042	0.074	0.175	0.023	-0.001	0.048	0.064	0.164
	Year 6 vs Baseline	0.008	-0.015	0.031	0.483	0.705	0.021	-0.007	0.048	0.136	0.314
Terrisporobacter											
	Year 2 vs Baseline	-0.005	-0.017	0.007	0.429	0.708	-0.004	-0.018	0.011	0.643	0.826
	Year 4 vs Baseline	0.001	-0.013	0.015	0.878	0.934	0.000	-0.016	0.015	0.972	0.984
	Year 6 vs Baseline	-0.002	-0.017	0.012	0.750	0.872	0.000	-0.017	0.017	0.984	0.984
Roseburia											
	Year 2 vs Baseline	0.018	-0.006	0.042	0.149	0.307	0.022	-0.007	0.051	0.141	0.328
	Year 4 vs Baseline	0.021	-0.006	0.048	0.136	0.302	0.023	-0.008	0.053	0.144	0.328
	Year 6 vs Baseline	0.003	-0.025	0.031	0.830	0.913	0.018	-0.016	0.052	0.296	0.477
Ruminococcus.gauvreauii.group											
	Year 2 vs Baseline	0.009	-0.008	0.027	0.288	0.453	-0.002	-0.023	0.020	0.884	0.941
	Year 4 vs Baseline	0.017	-0.003	0.037	0.093	0.219	-0.001	-0.023	0.022	0.965	0.965

Year 6 vs Baseline	0.013	-0.008	0.034	0.212	0.349	0.008	-0.017	0.033	0.521	0.819
--------------------	-------	--------	-------	-------	-------	-------	--------	-------	-------	-------

The multivariable linear mixed-effects model was adjusted for nut consumption categories, age (years), sex (male, female), recruiting center (Alicante, Barcelona, Reus, Valencia), intervention group (control, intervention), educational level (primary, high school, college), civil status (single/divorced/separated, married, widower), BMI (kg/m²), physical activity (METs-min/day), smoking status (never, former, current smoker), alcohol consumption in g/day and adding the quadratic term, depressive symptomatology (yes, no), total energy intake (kcal/day), prevalence of diabetes (yes, no), hypertension (yes, no), hypercholesterolemia (yes, no), and Mediterranean diet adherence (modified 13-point MEDAS score). Abbreviations: BMI, body mass index; MEDAS, Mediterranean Diet Adherence Screener; METs, metabolic equivalents.
