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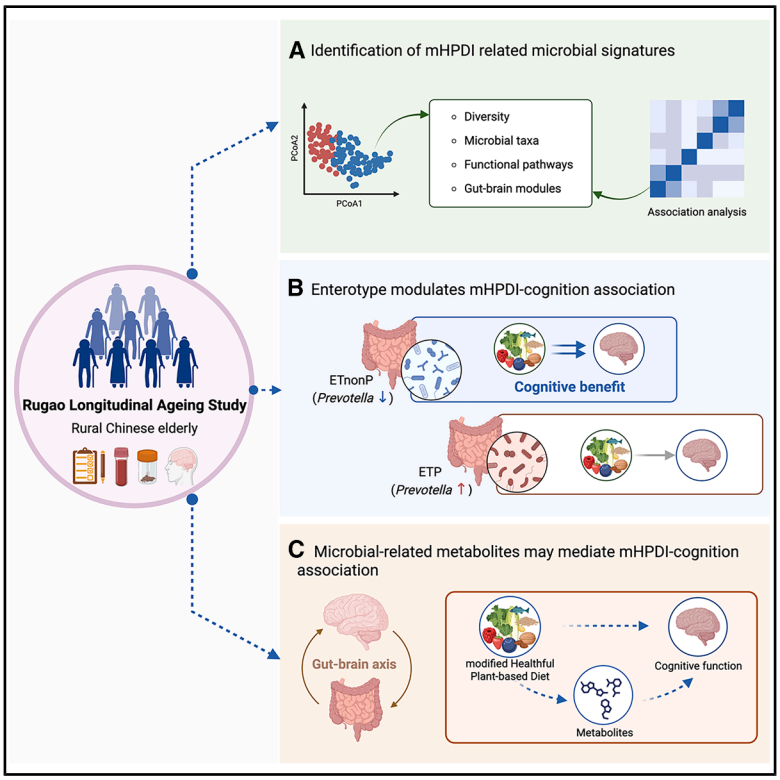
Healthful plant-based diet, gut enterotype, and cognition in a rural Chinese elderly cohort: A longitudinal multi-omics study

Graphical abstract



Highlights

- Healthful plant-based diet linked to distinct gut-microbial structure and taxonomic shifts
- Cognitive benefits of diet vary by enterotype, stronger in non-*Prevotella*-dominant groups
- Circulating microbiota-related metabolites may partly mediate diet-cognition association
- Gut microbial context may shape diet-cognition heterogeneity

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In brief

Shen et al. report that a healthful plant-based diet is associated with distinct gut microbiome features and a 12-metabolite signature. The diet-cognition association varied by gut microbiome enterotype, with stronger benefits in individuals with non-*Prevotella*-dominant enterotypes. These findings suggest that gut microbial context may shape diet-cognition associations in later life.

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Healthful plant-based diet, gut enterotype, and cognition in a rural Chinese elderly cohort: A longitudinal multi-omics study

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SUMMARY

The gut microbiome may shape how diet influences cognitive aging, but population-based evidence remains limited. In 784 older adults living in rural China (70–98 years old) with fecal metagenomics and structured dietary assessment, a modified healthful plant-based diet index (mHPDI) is associated with distinct gut microbial structure and taxonomic shifts (15 species, 17 genera). Among participants with repeated cognitive measurements, higher mHPDI is associated with better global cognition, with stronger benefits in participants with non-*Prevotella*-dominant enterotypes (highest versus lowest tertile β = 0.34, 95% confidence interval [CI], 0.16 to 0.52) than in those with a *Prevotella*-dominant enterotype (0.04, −0.22 to 0.29; *p* interaction = 0.04). Enterotype-associated differences in microbial metabolic pathways, including preQ0 and L-isoleucine biosynthesis, parallel this heterogeneity. Moreover, 12 circulating microbiota-related metabolites (primarily amino acids and short-chain fatty acids) are linked to mHPDI. A composite score comprising these metabolites mediates 11.0% of the mHPDI-cognition association (*p* mediation = 0.02), with branched-chain amino acids as major contributors. These findings suggest that gut microbial context may shape diet-cognition associations.

INTRODUCTION

Neurodegenerative disorders are a major cause of morbidity, disability, and mortality worldwide, with limited effective strategies for prevention.[1,2] Diet is a readily modifiable determinant of cognitive health, and the gut microbiome has emerged as a key mediator linking dietary exposures to brain function.[3–5] Evidence from experimental and observational studies suggests that diet can shape gut microbial communities and their metabolites,[6–9] which in turn influence neuroimmune, neuroinflammatory, and metabolic pathways relevant to cognitive aging.[10–12] However, large-scale, longitudinal, and multi-omics studies that integrate diet, microbiome, and cognition remain scarce, especially in non-Western populations.

A healthful plant-based dietary pattern, characterized by abundant whole grains, vegetables, fruits, legumes, nuts, and healthy fats, has been consistently associated with slower cognitive decline[13–16] and reduced dementia risk.[17,18] To better capture dietary quality in East Asian populations, where fish and seafoods are emphasized for their neuroprotective omega-3 fatty acids, we previously developed a modified healthful plant-based diet index (mHPDI) that incorporates positive scoring for these food groups.[19] Such diets may enhance fiber-degrading and short-chain fatty acid (SCFA)-producing taxa, modulate bile acid and amino acid metabolism, and attenuate systemic inflammation.[11,12] However, most prior studies have been cross-sectional, limited in size, or reliant on 16S rRNA sequencing, constraining both taxonomic and functional resolution.[20–24] Few have addressed whether inter-individual differences in microbiome composition modify the cognitive benefits of plant-forward diets, despite growing evidence that microbial community structure can shape dietary responsiveness in other health domains.[24–26]

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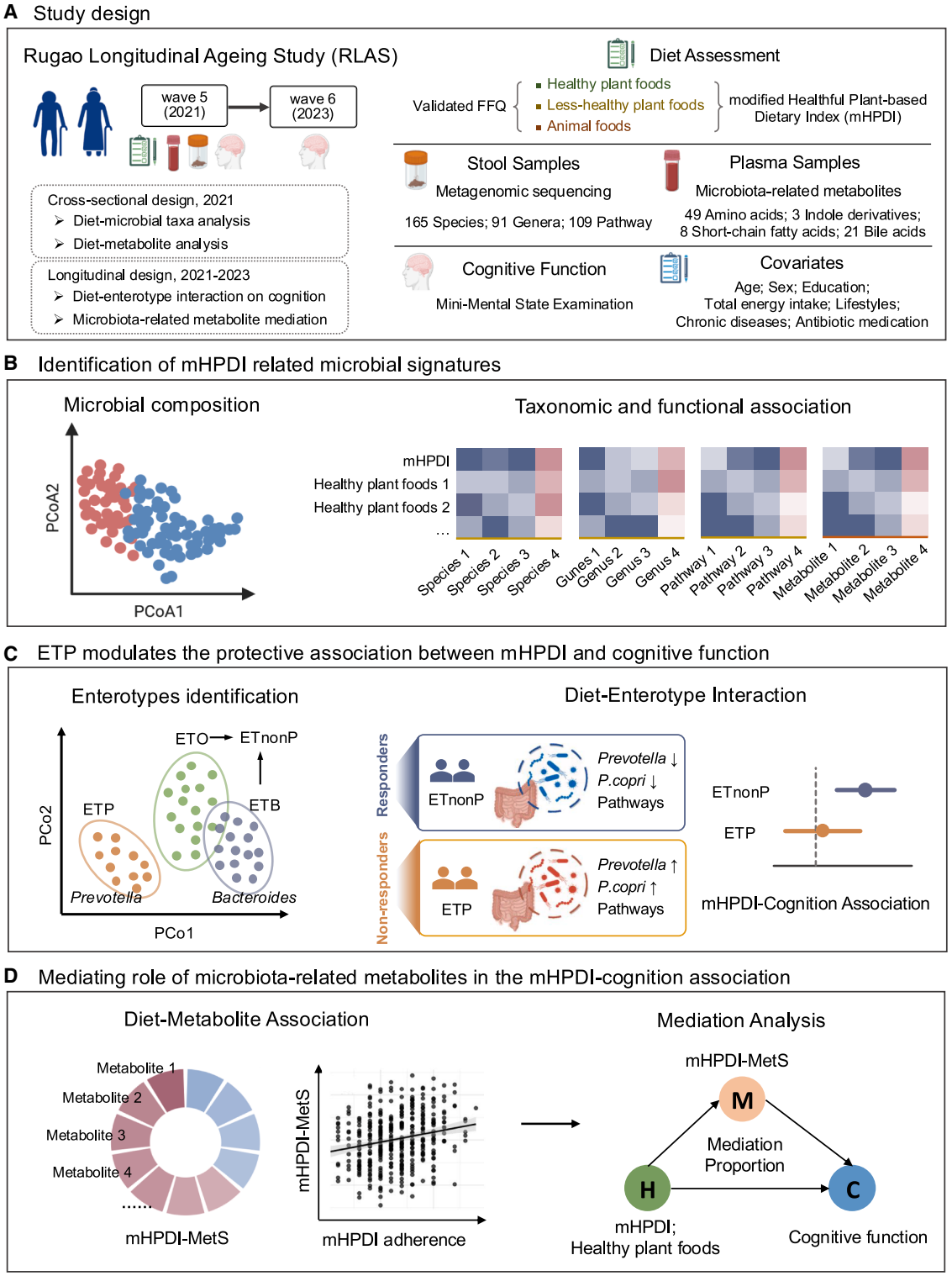

Figure 1. Conceptual framework of the study

(A) The overall study design, sample collection, and measurements in the Rugao Longitudinal Aging Study (RLAS), an ongoing prospective cohort of community-dwelling older adults aged 70 years and above in China.

(B) This study first incorporated fecal metagenomic data, plasma metabolomic data, and phenotype data to delineate gut microbial signatures (n = 784) and microbiota-related metabolites (n = 636) associated with mHPDI and its components.

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In this study, we integrated fecal shotgun metagenomics, plasma metabolomics, detailed dietary assessments, and longitudinal cognitive assessments in a cohort of community-dwelling older adults in China. We aimed to: (1) identify microbial taxa, functional pathways, and microbiota-related metabolites associated with mHPDI adherence; (2) evaluate whether microbial features modify the longitudinal association between mHPDI and cognitive performance; and (3) quantify the extent to which microbiota-related metabolites mediate these associations.

RESULTS

Study design and participant characteristics

The conceptual framework of this study is shown in Figure 1. We analyzed data from 784 community-dwelling older adults (mean age, 80.3 years; 52.8% women) who were dementia-free at baseline in 2021 and had complete dietary, cognitive, and microbiome profiles (Figure S1). Baseline characteristics by tertiles of the mHPDI are summarized in Table 1. The cohort was predominantly composed of individuals with limited formal education (either illiterate or primary school level) and who reported never smoking or drinking alcohol. Participants with higher mHPDI scores were more likely to have better cognitive performance at baseline, as measured by the Mini-Mental State Examination (MMSE), but not with age, sex, education attainment, physical activity, or the prevalence of diabetes or hypertension. Fasting blood samples were collected in parallel with dietary assessment and fecal sampling, yielding 636 participants for the analysis of plasma metabolites. For longitudinal follow-up, we retained 502 participants with repeated cognitive assessments over a 2-year period.

The mHPDI, adapted from the original healthful plant-based diet index (HPDI)[27,28] to additionally award positive scores for fish and seafood, comprised 16 food groups, with higher scores for healthy plant foods (whole grains, fresh vegetables, fresh fruits, nuts, legumes, tea, and vegetable oils) and fish/seafood, and lower scores for less-healthy plant and animal products. Total scores ranged from 16 to 80, with higher scores indicating greater adherence (STAR Methods). To capture the combined beneficial effects of all healthy plant foods, we also calculated an ''aggregated intake of all healthy plant foods'' score by summing component scores from all healthy plant food groups (Figure S2).

mHPDI associates with distinct gut microbial composition and function

Shotgun metagenomic profiling identified 165 species and 109 clustered functional pathways from baseline metagenome sequencing data (after quality control), and the taxonomic composition at the phylum and genus levels is shown in Figure S3. Adherence to the mHPDI was not associated with α-diversity measures of the gut microbiome (Figure 2A). While mHPDI was not the top driver of overall gut microbiome structure (Figure 2B), permutational multivariate analysis (PERMANOVA) of species-level Bray-Curtis dissimilarity revealed a significant association with respect to taxonomic structure (β-diversity; Figure 2C). The proportion of variation explained by dietary pattern (0.25% for tertile categories) was consistent with prior large-scale diet-microbiome studies.[25,29] Notably, specific healthy plant food components—particularly nuts and whole grains—accounted for a larger share of overall microbiome variation.

Multivariable linear regression identified 15 bacterial species (Figure 2D; Figure S4) and 17 genera (Figure S5) associated with either mHPDI or healthy plant food components, with adjustment for demographic and lifestyle factors, total energy intake, health-related traits, and antibiotic use (all false discovery rate [FDR] $q < 0.25$). An increment in mHPDI was associated with higher relative abundances of *Eubacterium* sp. CAG:38 and *Roseburia* sp. CAG:303 and lower abundances of *Bifidobacterium longum*, *Collinsella aerofaciens*, and *Megamonas hypermegale*, species previously linked to Western-style diets and higher red meat intake.[30,31] Higher aggregated intake of all healthy plant foods was associated with enrichment of *Roseburia* sp. CAG:303, *Adlercreutzia equolifaciens*, and *Asaccharobacter celatus* and depletion of *B. longum*, *Erysipelatoclostridium ramosum*, and *Eubacterium* sp. CAG:274. At the genus level, higher mHPDI or aggregated intake of all healthy plant foods was associated with higher abundances of *Agathobaculum*, *Adlercreutzia*, and *Asaccharobacter* and lower abundances of *Bifidobacterium*, *Lachnospira*, *Megamonas*, *Collinsella*, and *Lawsonibacter*.

Analysis of individual healthy plant foods underscored stable diet-microbiome associations in this aging population. Nut consumption demonstrated the most significant association with overall microbial composition, showing associations with enrichment of *Roseburia inulinivorans*, *Roseburia* sp. CAG:303, and *Veillonella atypica* and depletion of *B. longum*, *Bifidobacterium pseudocatenulatum*, *Catenibacterium mitsuokai*, *Methanobrevibacter smithii*, and *Firmicutes bacterium* CAG:95. Higher tea intake was inversely associated with *Bifidobacterium*, *Ruminococcus*, and *Haemophilus*. While no microbial metabolic pathways were significantly associated with mHPDI, nut consumption was associated with 9 metabolic pathways (all FDR $q < 0.25$; Figure S6). To explore associations between diet and the neuroactive potential of the gut microbiota, we applied a published gut-brain module (GBM) framework that profiles microbial pathways involved in the synthesis or degradation of neuroactive compounds[32] (details in STAR Methods; Table S1). After quality control, 43 GBMs were retained. Greater adherence to the mHPDI or higher healthy plant food intake was associated with 12 GBMs

(C) Second, three enterotypes were identified, dominated by *Prevotella* (ETP), *Bacteroides* (ETB), and other genera (ETO). By integrating longitudinal cognitive assessments (over a 2-year follow-up), we examined interactions between enterotype and mHPDI in relation to cognitive function ($n = 502$), to identify participants showing stronger mHPDI-cognition associations. We conducted interaction analyses focusing on ETP clusters (ETP versus ETnonP), *Prevotella* carriage, *P. copri* carriage, and enterotype-enriched pathways.

(D) We included 81 microbiota-related metabolites contributing to the gut-brain axis based on recent evidence.[10] Among 418 participants with plasma metabolomic data, we further investigated the extent to which microbiota-related metabolites mediate the longitudinal association between mHPDI and cognitive function.

Table 1. Characteristics of the study participants by adherence to the modified healthful plant-based diet

		mHPDI			
	Overall	Tertile 1	Tertile 2	Tertile 3	*p* value
	784	303	311	170	
Age, y, mean (SD)	80.3 (4.2)	80.4 (4.3)	80.2 (3.9)	80.0 (4.4)	0.62
Women (%)	414 (52.8)	163 (53.8)	165 (53.1)	86 (50.6)	0.79
Total energy intake, kcal/d, mean (SD)	1685.9 (566.8)	1718.8 (538.1)	1660.6 (572.3)	1673.7 (606.0)	0.42
Educational level (%)					0.18
Illiteracy	343 (43.8)	142 (46.9)	137 (44.1)	64 (37.6)	
Primary school	247 (31.5)	98 (32.3)	94 (30.2)	55 (32.4)	
Middle school and above	194 (24.7)	63 (20.8)	80 (25.7)	51 (30.0)	
BMI, kg/m^2, mean (SD)	23.9 (3.7)	23.7 (3.6)	23.8 (3.9)	24.3 (3.5)	0.31
Smoking status (%)					0.46
Never	588 (75.3)	227 (75.4)	235 (75.8)	126 (74.1)	
Ever	63 (8.1)	30 (10.0)	20 (6.5)	13 (7.6)	
Current	130 (16.6)	44 (14.6)	55 (17.7)	31 (18.2)	
Alcohol consumption (%)					0.53
Never	495 (63.5)	194 (64.7)	199 (64.4)	102 (60.0)	
Ever	69 (8.9)	30 (10.0)	26 (8.4)	13 (7.6)	
Current	215 (27.6)	76 (25.3)	84 (27.2)	55 (32.4)	
Regular exercise (%)	659 (85.6)	251 (84.2)	270 (88.2)	138 (83.1)	0.22
Diabetes (%)	64 (8.2)	24 (7.9)	26 (8.4)	14 (8.2)	0.98
Hypertension (%)	372 (47.4)	140 (46.2)	148 (47.6)	84 (49.4)	0.80
Antibiotic use (%)	50 (6.4)	18 (5.9)	19 (6.1)	13 (7.6)	0.74
Cognitive function score, mean (SD)	20.5 (7.1)	19.9 (7.4)	20.5 (7.0)	21.7 (6.4)	0.04

Baseline cognitive function score of each participant was assessed using the Mini-Mental State Examination. mHPDI, modified healthful plant-based diet index; SD, standard deviation; BMI, body mass index.

(all FDR $q < 0.25$; Figure S7A). For example, higher nut intake was linked to GBMs involved in neurotransmitter, SCFA, and inositol metabolism, histamine degradation, and quinolinic acid degradation, whereas higher tea intake was associated with GBMs related to glutamate synthesis and the degradation of dopamine, tryptophan, or histamine.

Targeted plasma metabolomics analysis focused on 81 microbiota-related metabolites relevant to the gut-brain axis,[10,11] including 49 amino acids, eight SCFAs, three indole derivatives, and 21 bile acids. Principal component analysis revealed significant differences in overall plasma metabolomic profiles by mHPDI adherence (Figure 2E). Specifically, 16 metabolites showed nominal associations with either mHPDI or aggregated intake of all healthy plant foods ($p < 0.05$), including 12 amino acids, two SCFAs, and two bile acids (Figure 2F). For example, compared with the lowest mHPDI tertile, the highest tertile was associated with elevated circulating levels of L-aspartic acid ($\beta = 0.21$, 95% confidence interval [CI], 0.03 to 0.39), pyroglutamic acid (0.21, 0.02 to 0.39), L-glutamic acid (0.21, 0.02 to 0.39), L-lysine (0.19, 0.01 to 0.38), and aminoadipic acid (0.18, 0.00 to 0.37), as well as isocaproic acid (0.25, 0.07 to 0.43).

Prevotella-dominant enterotype modifies the association between mHPDI and cognitive function

Partitioning around medoids (PAM) clustering identified three enterotypes in the study population: *Prevotella*-dominant enterotype (ETP, 35.2% of participants), *Bacteroides*-dominant enterotype (ETB, 26.1%), and other genera-dominant enterotype (ETO, 38.7%) (Figure 3A). Neither mHPDI nor its food components correlated with enterotype distribution (Figure 3B) or with the relative abundance of *Prevotella* or *P. copri* (Figures S4 and S5), consistent with prior findings.[7] Although *Prevotella* was detected in all participants, its relative abundance varied markedly across enterotypes (Figure S8).

In 502 participants with repeated cognitive assessments over 2 years, higher mHPDI adherence was associated with better global cognitive function, independent of demographic and lifestyle factors, total energy intake, and health-related traits (β for highest versus lowest tertile = 0.21, 95% CI, 0.07 to 0.36, p for trend = 0.004; Figure S9). The original HPDI showed a weaker, nominally significant association with better cognitive function (0.12, −0.02 to 0.25, p for trend = 0.09). Higher aggregated intake of all healthy plant foods was also associated with better cognitive function (0.24, 0.06 to 0.41, p for trend = 0.003), with nuts, legumes, tea, and fish/seafood contributing most strongly (Figures S9 and S10). In sensitivity analyses, we used the Hasegawa's Dementia Scale (HDS) as an alternative measure of global cognitive function. Associations between mHPDI and cognitive performance were consistent with those observed using MMSE Z scores (MMSE: $\beta_{T3\ v\ T1} = 0.21$, 95% CI, 0.07 to 0.36; HDS: $\beta_{T3\ v\ T1} = 0.18$, 95% CI, 0.04 to 0.32).

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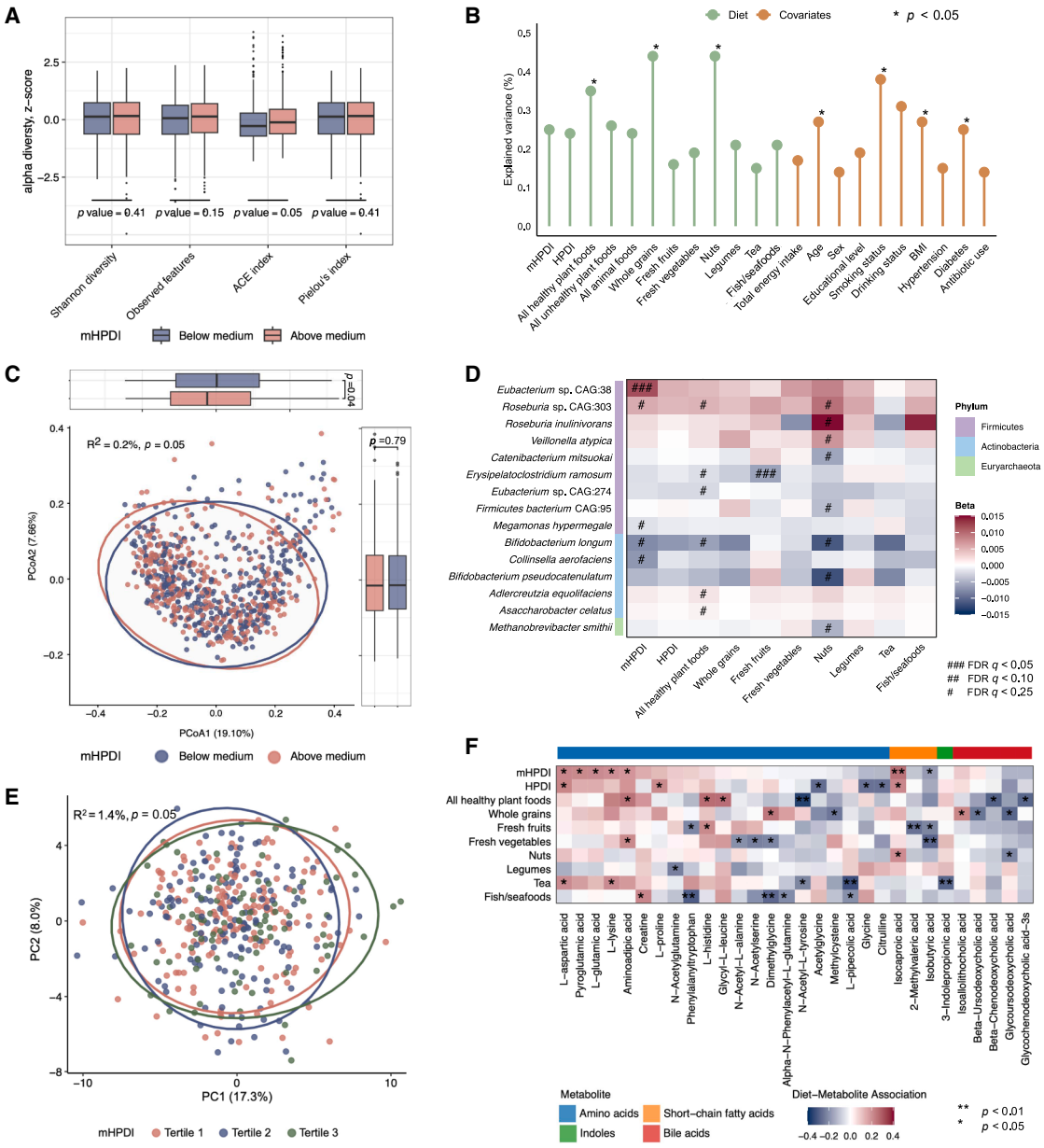

Figure 2. Associations of the modified healthful plant-based diet index with overall gut microbiome configuration, microbial species abundances, and microbiota-related metabolites

(A) Association between mHPDI and α-diversity of the gut microbiome, which was quantified by Shannon diversity, observed features, ACE index (abundance-based coverage estimator), and Pielou's evenness index. Boxplot centers show medians of the α-diversity indices, with boxes indicating inter-quartile ranges (IQRs). Whiskers extend to 1.5 × IQR; dots indicate outliers. *p* values were derived from multivariable-adjusted linear regression models with α-diversity as the dependent variable and mHPDI (stratified by the medium value of the index) as the independent variable.

(B) Explained variance for major dietary components and covariates, estimated using R^2 calculated from PERMANOVA (distance metric: Bray-Curtis dissimilarity). All dietary variables were categorized into tertiles.

(C) Distinct microbial composition by mHPDI adherence (stratified by the medium value), assessed by inter-individual β-diversity (Bray-Curtis dissimilarity). Group differences were tested by PERMANOVA. Side/top boxplots show within-group distance distributions (median and IQR); whiskers = 1.5 × IQR, dots = outliers.

(D) Multivariable-adjusted linear regression analyses for the association of mHPDI (per 10-point increment), aggregated intake of all healthy plant foods (per SD increase), and intake of seven major food components (comparing extreme tertiles) with relative abundance of microbial species. Multiple comparisons were corrected using FDR-adjusted *p* values. All analyses of gut microbial taxa were conducted in 784 participants with metagenomic data, and all models were adjusted for age, sex, educational attainment, total energy intake, smoking status, alcohol consumption, physical activity, body mass index, history of hypertension, history of diabetes, and antibiotic use.

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Prevotella copri has been shown to modulate cardiometabolic benefits of the Mediterranean diet[25] and anti-inflammatory responses to dietary fiber.[26] Based on this prior evidence, we adopted a hypothesis-driven approach and observed that enterotype significantly modified the association between mHPDI and cognitive function (Figures 3C and 3D). Among participants with non-*Prevotella*-dominant enterotypes (ETnonP; comprising ETB and ETO), the highest versus lowest mHPDI tertile was associated with a 0.34-unit higher cognitive *Z* score (95% CI, 0.16 to 0.52), whereas no clear association was observed in ETP (β = 0.04, −0.22 to 0.29; *p* interaction = 0.04). A similar effect modification was observed for HPDI, where ETnonP participants showed stronger HPDI-cognition associations (0.24, 0.08 to 0.41) compared with ETP participants (−0.08, −0.31 to 0.16; *p* interaction = 0.03). Enterotype-specific heterogeneity extended to individual food groups: higher tea intake conferred more pronounced cognitive benefits in ETnonP than in ETP (*p* interaction < 0.05), and fresh vegetables, nuts, tea, and fish/seafood showed directionally consistent trends. Parallel analyses replacing enterotype with *Prevotella* or *P. copri* abundance showed consistent patterns (Figure S11), echoing prior observations of *P. copri*-mediated diet-metabolic health interactions.[25] When the cohort was stratified into three enterotypes (ETP/ETO/ETB), enterotypes consistently modified the association between mHPDI and cognitive function (*p* interaction = 0.01; Figure S12). Specifically, among ETB participants, the highest versus lowest tertile of mHPDI was associated with a 0.41-unit higher cognitive *Z* score (95% CI: 0.17 to 0.65), whereas no clear association was observed in the ETO (β = 0.27, −0.02 to 0.56) or ETP (0.04, −0.22 to 0.29) groups. In addition, ETP membership itself was modestly associated with lower cognitive function over a 2-year follow-up (β = −0.10, 95% CI, −0.19 to −0.01), although *Prevotella* abundance alone was not (Figure S13).

To explore functional correlates of these interactions, we identified 57 of 109 microbial metabolic pathways enriched in ETnonP participants (all FDR *q* < 0.05). Of these pathways, ten showed nominal interactions with mHPDI in relation to longitudinal cognitive function (all *p* interactions < 0.10; Figure 3E). Notably, the protective association between mHPDI and cognition was more pronounced in participants with higher abundances of preQ0 biosynthesis (PWY-6703), inosine-5′-phosphate biosynthesis I (PWY-6123), and L-isoleucine biosynthesis IV (PWY-5104), whereas no association was observed in those with lower pathway abundances (all *p* interactions < 0.05). These pathways have been linked to hippocampal expression of genes involved in neuron generation and migration protection,[33] while L-isoleucine biosynthesis, part of the branched-chain amino acid (BCAA) metabolic pathway, has been implicated in dementia and Alzheimer's disease across multiple cohorts.[34] The descriptions and potential neurobiological relevance of key interactive microbial metabolic pathways are summarized in Table S2.

Taken together, these findings highlight enterotype-specific heterogeneity in the cognitive benefits of a healthful plant-based diet, involving both microbial taxa and metabolic functional potentials.

Microbiota-related metabolites mediate the diet-cognition association

In the longitudinal dataset (*n* = 418), we identified 12 plasma microbiota-related metabolites nominally associated with mHPDI adherence, with adjustment for demographic factors, total energy intake, and lifestyle factors, including nine amino acids and three SCFAs (all *p* < 0.05; Figure 4A; Figure S14). To assess their combined associations, we constructed a composite metabolite score (MetS) by summing these metabolites weighted by their association direction with diet (STAR Methods). The MetS correlated positively with mHPDI adherence (Pearson's r = 0.22, *p* < 0.001; Figure 4B) and partially mediated the association between mHPDI and cognitive function (proportion = 11.0%, *p* mediation = 0.02; Figure 4C). Similarly, this MetS mediated 15.0% of the association between tea intake and cognitive function (*p* mediation = 0.01).

At the individual metabolite level, four amino acids, i.e., three BCAAs (L-isoleucine, L-leucine, L-valine) and alpha-N-phenylacetyl-L-glutamine, significantly mediated the diet-cognition associations (all *p* mediations <0.05; Figure 4D). L-leucine mediated up to 12.9% of the protective association between mHPDI or HPDI and cognitive function, and L-isoleucine accounted for up to 11.3% of these associations. All four metabolites mediated the tea-cognition association, with mediation proportions ranging from 10.6% for L-leucine to 11.6% for alpha-N-phenylacetyl-L-glutamine. Correlation analyses linking microbial taxa with metabolite mediators revealed that *Eubacterium* sp. CAG:38 and *C. aerofaciens* were significantly associated with both mHPDI adherence and higher levels of neuroprotective metabolites (Figure 4E). These metabolite-mediated associations support a coordinated microbial mechanism in which diet-driven modulation of amino acids and SCFAs, metabolites implicated in mediating blood-brain barrier integrity,[10] may contribute to improved cognitive outcomes in older adults.

DISCUSSION

In this cohort of community-dwelling older adults, we integrated dietary assessment, fecal shotgun metagenomics, and plasma metabolomics with repeated cognitive testing to investigate the microbiome's role in diet-cognition relationships. We found that adherence to a modified healthful plant-based diet was associated with distinct gut microbial features and plasma microbiota-related

(E) A total of 81 microbiota-related metabolites contributing to the gut-brain axis were included (49 amino acids, 8 short-chain fatty acids, 3 indole derivatives, and 21 bile acids). Principal-component analysis was performed using microbiota-related metabolites; the first 2 principal components (PCs) were plotted by different adherence to mHPDI.

(F) Associations of mHPDI and its components (highest versus lowest tertile) with microbiota-related metabolites. Linear regression models were conducted with adjustment for age, sex, educational attainment, total energy intake, smoking status, physical activity, and alcohol consumption. "All healthy plant foods" indicates the index of aggregated intake of all healthy plant foods. "All unhealthy plant foods" indicates the index of aggregated intake of all less-healthy plant foods. "All animal foods" indicates the index of aggregated intake of all animal foods.

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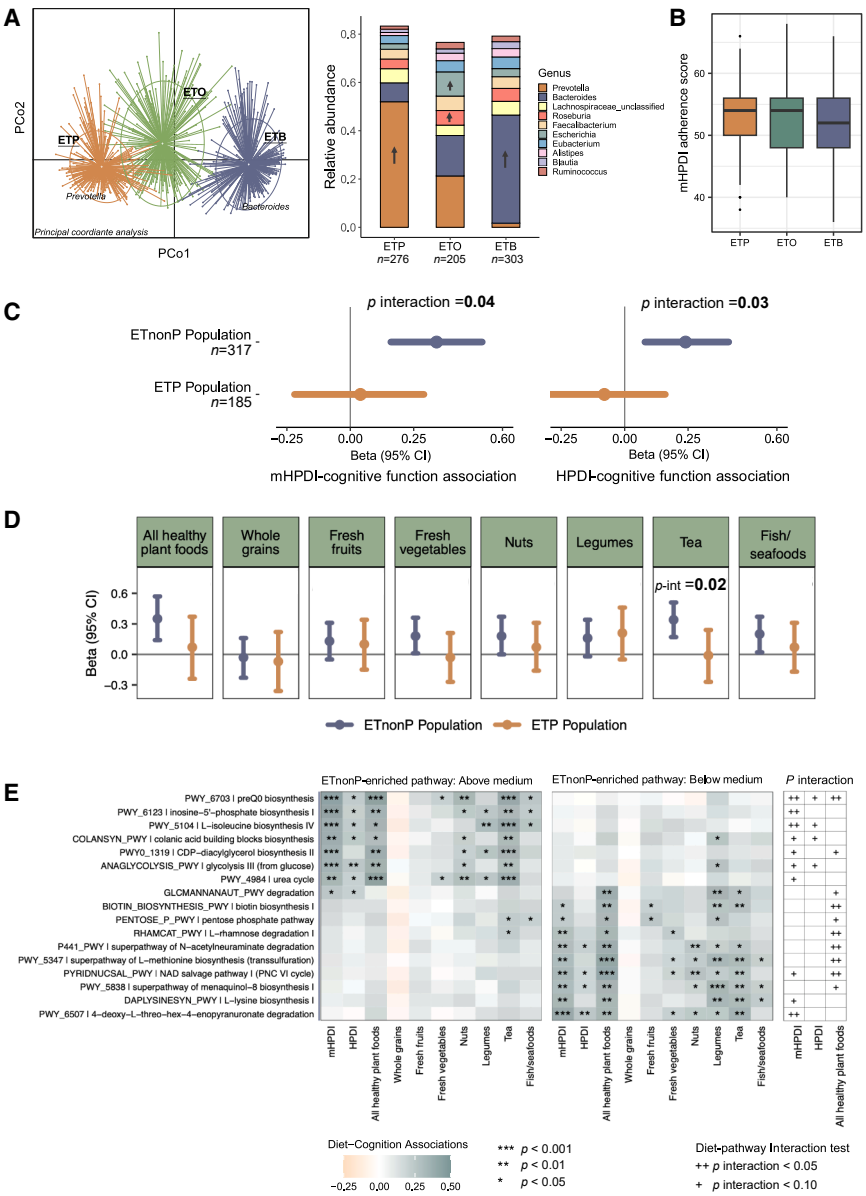

Figure 3. *Prevotella*-dominant enterotype modulates the protective association between the modified healthful plant-based diet and longitudinal cognitive function

(A) Left: Enterotype clustering analysis was performed using Jensen-Shannon divergence (JSD) and partitioning around medoids (PAM) among the overall population (n = 784), identifying *Prevotella* (ETP), *Bacteroides* (ETB), and other genera (ETO) enterotypes. Right: Changes in the distribution of genera across the three enterotypes, as indicated by arrows.

(B) Enterotypes were not associated with mHPDI adherence after adjustment for major covariates (multinomial logistic regression; $p > 0.05$). Boxplot centers show medians of mHPDI, with boxes indicating inter-quartile ranges. Whiskers extend to 1.5 × IQR; dots indicate outliers.

(C) Longitudinal associations between mHPDI and cognitive function over a 2-year follow-up across ETnonP (n = 317) and ETP (n = 185) subgroups. The protective association of mHPDI and HPDI with cognitive function was stronger in ETnonP participants but diminished in those with ETP (p interaction < 0.05). β coefficients and 95% CIs were estimated using linear mixed-effects regression models for each diet index.

(D) Longitudinal associations between major food component intakes and cognitive function over time across ETnonP (n = 317) and ETP (n = 185) subgroups. β coefficients and 95% CIs were estimated using linear mixed-effects regression models for each food group.

(E) We first identified 57 pathways positively associated with ETnonP (derived from logistic regression after controlling for major covariates). The heatmap presents 17 ETnonP-enriched pathways that showed significant interactions with either mHPDI or aggregated intake of all healthy plant foods in relation to cognitive function over time, among which 10 pathways showed significant mHPDI-pathway interactions. All dietary variables were modeled comparing the highest versus lowest tertile. Longitudinal analyses were conducted using linear mixed-effects regression models, with adjustment for age, sex, educational attainment, total energy intake, smoking status, alcohol consumption, physical activity, body mass index, history of hypertension, history of diabetes, antibiotic use, and follow-up duration. "All healthy plant foods" indicates the index of aggregated intake of all healthy plant foods.

metabolites and that these associations varied by enterotype. Specifically, participants with ETnonP exhibited stronger protective associations between mHPDI and cognitive health, whereas benefits were attenuated among those with ETP. A composite score of 12 mHPDI-associated microbiota-related metabolites partially mediated the mHPDI-cognition association, with BCAAs and SCFAs primarily contributing to this mediation. Collectively, these findings highlight the potential value of personalizing dietary advice based on gut microbiome features to promote healthy cognitive aging. We note that these insights are drawn from an East Asian elderly population, and extension of these findings to other populations will require validation in cohorts with different cultural, socioeconomic, and age characteristics.

Our taxonomic results are directionally consistent with prior large, diet-focused metagenomic studies that link plant-forward dietary quality to enrichment of saccharolytic taxa (e.g., *Roseburia* spp., *Eubacterium* spp.) and depletion of taxa (e.g., *Bifidobacterium* spp., *Collinsella* spp.) associated with animal-product intake.[7–9,20–24] In this older Chinese cohort, higher mHPDI, particularly from aggregated healthy plant foods intake, was associated with higher abundances of *Roseburia* sp. CAG:303 and *Eubacterium* sp. CAG:38 and lower abundances of *B. longum* and *C. aerofaciens*, a pattern also observed in Western cohorts,[7–9,20,24] albeit with cohort-specific differences likely reflecting dietary context,[24] host genetics,[7,8] and microbial strain structure.[9,20] Notably, nuts, a key healthy plant food component,

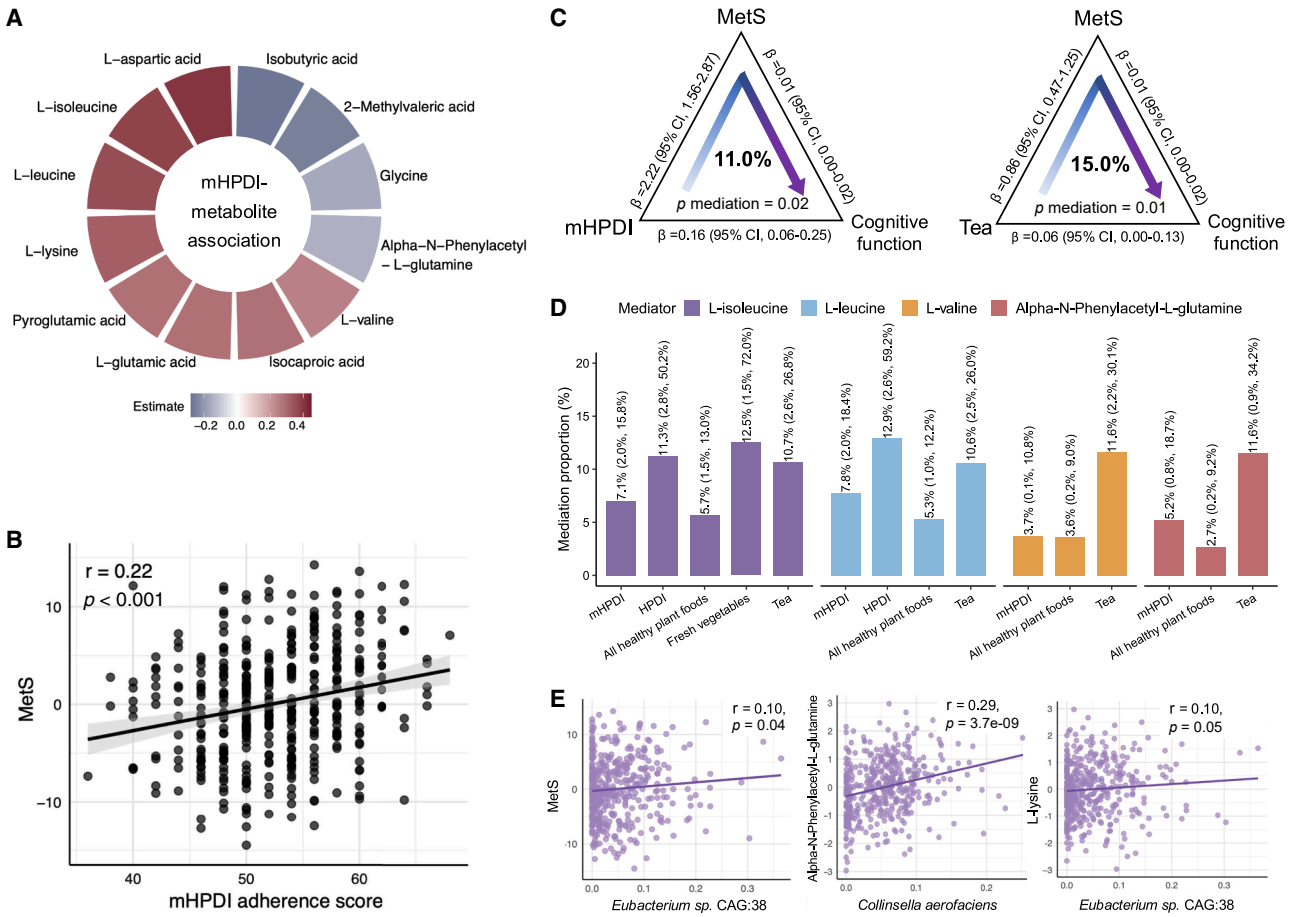

Figure 4. Plasma microbiota-related metabolites mediated protective associations between the modified healthful plant-based diet and longitudinal cognitive function

(A) Based on 418 participants with repeated cognitive assessments, 12 microbiota-related metabolites were identified that were significantly associated with mHPDI (highest versus lowest tertile; all *p* values < 0.05), using multivariable-adjusted linear regression models.

(B) We developed a composite metabolite score (MetS) based on the 12 metabolites associated with mHPDI and examined its correlation with mHPDI adherence. Regression line with 95% CI shown.

(C) The MetS mediated 11.0% of the longitudinal association between mHPDI (per 10-point increment) and cognitive function (*Z* score) over a 2-year follow-up.

(D) Mediation analyses for individual selected metabolites in the mHPDI-cognition association over time (all *p* mediations < 0.05). Dietary indices were treated as per 10-point increment and food groups as *Z* scores.

(E) Partial Spearman correlation between microbial species and MetS. All above models were adjusted for age, sex, educational attainment, total energy intake, smoking status, physical activity, and alcohol consumption.

showed the strongest and most coherent associations with both community composition and *Roseburia* abundance, in line with intervention evidence that nut intake can remodel microbial communities.[35] These convergent observations suggest that multiple healthy plant foods, and nuts in particular, may act through overlapping microbial pathways that favor saccharolytic fermentation and production of neuromodulatory metabolites.

We observed that enterotype modified the diet-cognition association; i.e., older adults with ETnonP derived a clear cognitive benefit from higher mHPDI adherence, whereas participants with ETP did not. Parallel analyses substituting enterotype with *Prevotella*/*P. copri* abundance supported this interaction. Mechanistically, *Prevotella*-sparse communities may favor metabolic routes that more efficiently convert plant substrates (e.g., fiber and polyphenols) into metabolites linked to neuroimmune homeostasis, such as butyrate or indole derivatives.[25] While Western studies associate *Prevotella* with improved glucose metabolism under high-fiber conditions,[36,37] its enrichment has also been linked to risk of neurodegenerative conditions.[38–40] In our Chinese cohort, *Prevotella* abundance did not show association with cognition over time ($p > 0.05$), potentially reflecting the dominance of non-Western *P. copri* clades with distinct genetic and functional profiles.[41] These findings may suggest that clade- or strain-level heterogeneity within *Prevotella*/*P. copri*, which differs between non-Western and Western populations, may yield functional outputs that blunt diet benefits in some settings.[41,42] Utilizing the metagenomic data obtained in this study, future work could perform strain-level analysis of *P. copri* to investigate whether specific *P. copri* strains or genotypes are associated with a "non-responder" status to plant-based diets. Such analyses

would provide finer-grained insights into how intraspecies heterogeneity contributes to inter-individual differences in diet responsiveness and cognitive outcomes. The enrichment of ETnonP-linked pathways (for example, preQ0 and inosine-5′-phosphate biosynthesis) that intensified the mHPDI-cognition association further supports pathway-level modulation of dietary responsiveness, while underscoring that "enterotype" likely proxies a broader, functionally meaningful community context. However, the appropriateness and stability of discrete enterotype classifications remain actively debated.[43] Some argue that partitioning the gut microbiota into enterotypes oversimplifies a more nuanced, gradient-like ecological reality. Conversely, others maintain that discrete clustering retains utility, particularly if it predicts differential responses to interventions, thereby reflecting the functional role of the microbiota as a metabolic modulator. From this perspective, the "ETP" identified in this study likely represents not merely a taxonomic cluster but a specific microbial functional state. Our study suggests that this state may collectively reduce metabolic responsiveness to plant-based diets, making it a more predictive marker than *Prevotella* abundance alone. Key taxa such as *Prevotella* may therefore be more precisely interpreted as biomarkers of long-term diet and lifestyle, rather than as stable, recurrent microbial communities.[44]

Plasma metabolomics provided complementary evidence for a diet-microbiome-brain axis.[10,11] A composite MetS, comprising 12 mHPDI-associated metabolites, accounted for approximately 11% of the observed diet-cognition association. Additional parallel mechanistic pathways may include systemic inflammation, vascular and metabolic health, gut barrier integrity, and the production of other neuroactive microbial or diet-related metabolites (e.g., polyphenols) that were not captured in the current analysis.[11,45] Among the individual mediators, BCAAs were notable, consistent with prior population studies linking circulating BCAAs to cognitive outcomes.[34,46] SCFAs, commonly considered neuroprotective,[47] displayed heterogeneous associations in plasma: higher isocaproic acid levels were associated with better cognitive performance, whereas isobutyric acid and 2-methylvaleric acid were associated with poorer cognition. Such divergence mirrors reports in other cohorts and may reflect biological compartmentalization (fecal versus plasma pools),[48] differences in host metabolic handling,[48] and variation in gut barrier integrity.[49] The longitudinal design and temporally aligned metabolite profiling strengthen the plausibility of a directional pathway from diet to microbiota-related metabolites to cognition. However, as metabolite concentrations were measured only at baseline, the observed mediation is correlational. Future intervention studies or repeated metabolite measurements at multiple time points are needed to establish the dynamic causal role of their changes in the process of diet influencing cognition.

In summary, our study links a modified healthful plant-based diet to distinct gut microbiome features, metabolic pathways, and a 12-metabolite plasma signature associated with better cognition in older adults. Benefits were strongest in those lacking a *Prevotella*-dominant enterotype, underscoring the potential of microbiome-tailored nutrition to preserve brain health and lower neurodegenerative risk.

Limitations of the study

The observational design of this study limits causal inference between diet, microbiome, and cognitive outcomes. However, we mitigated this by leveraging a well-characterized longitudinal cohort with repeated cognitive assessments, enabling temporal alignment of exposures and outcomes. Potential measurement error from self-reported dietary intake was addressed through the use of a validated food frequency questionnaire (FFQ) specifically adapted to Chinese dietary patterns, improving cultural and dietary relevance. Moreover, cognitive function was primarily assessed using the MMSE, which evaluates a limited range of cognitive domains and lacks the resolution to capture domain-specific cognitive changes. This limitation restricts detailed inference regarding specific cognitive processes. Furthermore, mediation analyses were conducted in the overall population and therefore reflect average indirect effects across heterogeneous microbiome enterotype subgroups. Enterotype-stratified mediation analyses were limited by sample size, underscoring the need for larger, adequately powered studies to confirm potential enterotype-specific metabolic pathways linking diet to cognitive function. Finally, the near-universal prevalence of *Prevotella* in this Chinese cohort constrains generalizability to populations with lower abundance and different clade distributions. However, this homogeneity provided a rare opportunity to investigate diet-microbiome interactions in an aging East Asian population, where *Prevotella* is common but underrepresented in global microbiome research.[41,50] Notably, several *Prevotella*-associated metabolites identified in this cohort, including BCAAs and certain SCFAs, were linked to cognitive performance, suggesting that differences in *Prevotella* prevalence and clade composition across populations may shape the interconnected pathway from diet and gut microbiome to circulating metabolites and cognition. Future multi-ethnic and interventional studies are warranted to confirm these associations and clarify causal mechanisms.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yan Zheng (yan_zheng@fudan.edu.cn).

Materials availability

This study did not generate new, unique reagents.

Data and code availability

The datasets generated and analyzed during the current study from the Rugao Longitudinal Aging Study cohort, including metadata, metagenomic sequencing data, and metabolite data, have been deposited in the National Center for National Omics Data Encyclopedia (NODE): OEP001391 (https://www.biosino.org/node/home). This study did not generate original code. All custom scripts used for data analysis were written using existing software packages, as described in the STAR Methods section, and are available from the lead contact upon request. Any additional information required to reanalyze the data reported in this work is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

J.S. and Z.S. contributed equally to this work. Y.Z., C.Y., and X.W. contributed to the design and interpretation of the study. J.S. and Z.S. conducted the data analyses and drafted the manuscript. J.S., Z.S., H.S, Y.P., and Y.Z. contributed to the generation of metagenomics data. H.S., Y.P., P.W., G.H., Y.H., Z.M., H.C., L.H., X.W., C.Y., and Y.Z. critically revised and edited the manuscript. Y.Z., C.Y., and Z.S. obtained funding. All authors read and approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no conflicts of interest.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Fecal and blood samples	This paper	N/A
Critical commercial assays		
DNeasy PowerSoil Pro Kit	Qiagen	Cat #47021
Q300 Metabolite Assay Kit	Human Metabolomics Institute	HY5023R
Deposited data		
Metadata, metagenomic sequencing data and metabolite data	This study	National Center for National Omics Data Encyclopedia (NODE): OEP001391 (https://www.biosino.org/node/home)
Gut-brain module (GBM) database	Valles-Colomer et al.[32]	https://raeslab.org/software/gbms.html
Software and algorithms		
R software (version 4.4.1)	R project	https://www.r-project.org
vegan R package	R project	https://cran.r-project.org/web/packages/vegan/index.html
cluster R package	R project	https://cran.r-project.org/web/packages/cluster/index.html
stats R package	R project	https://cran.r-project.org/web/packages/STAT/index.html
mediation R package	Baron and Kenny[51]	https://cran.r-project.org/web/packages/mediation/index.html
MetaPhlAn (version 3)	Beghini et al.[52]	https://github.com/biobakery/MetaPhlAn
HUMAnN 3.0	Beghini et al.[52]	https://github.com/biobakery/humann
MassLynx v4.1	Xie et al.[53]	Waters, Milford, MA

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

The present study was conducted within the Rugao Longitudinal Aging Study (RLAS), an ongoing community-based prospective cohort study in rural areas of Rugao City, Jiangsu Province, China. Briefly, the RLAS was initiated in 2014 and recruited residents aged 70 years and above.[54] Participants have been followed up approximately every 1.5 years since baseline. At each study visit, each participant completed structured face-to-face questionnaires, underwent standardized physical examinations, and provided multiple biological specimens for laboratory analyses. The study protocol was approved by the Human Ethics Committee of the School of Life Sciences, Fudan University, and all participants provided written informed consent.

A total of 784 dementia-free participants from the RLAS, all of whom completed dietary assessment and provided stool samples at wave 5 (2021) were included for diet-gut microbiome association analysis. Of these, 502 participants with repeated cognitive assessments at both wave 5 and wave 6 (2023) were retained for evaluating diet-microbiome interactions in relation to longitudinal cognitive function over a 2-year follow-up. Finally, 418 of these participants who had available plasma samples underwent targeted metabolomic profiling at wave 5 were included to investigate the mediating role of microbiota-related metabolites in the diet-cognition association (Figure S1).

METHOD DETAILS

Measurement of adherence to modified healthful plant-based dietary pattern

Dietary information was assessed using a validated quantitative FFQ.[55] The original FFQ was adapted with minor modifications, through the addition of several food items to enhance the assessment of total energy and nutrient intake and mitigate the risk of underestimation, resulting in a 32-item version for the present study (wave 5). Participants reported the frequency and portion size of their usual food intake over the past year. Nutrient intakes, including total energy intakes were estimated by multiplying the intake amount of each item by its corresponding nutrient content, based on China Food Composition Table (Standard Edition 6), and then summing across all food items.

In the current study, we incorporated seven healthy plant foods (whole grains, fresh vegetables, fresh fruits, nuts, legumes, tea, and vegetable oils), four less-healthy plant foods (refined grains, potatoes, sugar sweetened beverages, and sweets and desserts), and five animal foods (fish/seafoods, animal fat, dairy, eggs, and meat). Most food groups were scored on a scale from 1 to 5 according to cohort-specific tertile distributions, with 1 representing the lowest and 5 the highest consumption levels. For vegetable oils and animal fat, binary scores were assigned (5 for 'as main cooking grease' and 1 for not). As originally conceptualized by Satija et al.,[27,28] the HPDI is not a vegetarian diet score but rather emphasizes the quality of plant foods while jointly accounting for animal foods intake. The HPDI assigns positive scores to higher consumption of healthy plant foods, and reverse scores to less-healthy plant foods and animal foods. In light of consistent evidence supporting the neuroprotective benefits of regular fish consumption,[56] we developed a modified HPDI (mHPDI) by additionally assigning a positive score to fish/seafood intake. This modification improves relevance in populations with habitual fish consumption and has been applied in large-scale cohort studies linking to lower risks of mortality, cardiometabolic disease, and dementia.[18,19,28,57] The adherence indices of each participant were calculated by summing across all food groups, yielding a theoretical range from 16 (low adherence) to 80 (perfect adherence). In the current study, the mHPDI was prioritized as the primary dietary exposure to better reflect dietary quality in East Asian populations, while the original HPDI was also calculated and analyzed as a reference. Additionally, to evaluate the aggregated effects of different food categories, we generated three composite indices— "aggregated intake of all healthy plant foods", "aggregated intake of all less-healthy plant foods", and "aggregated intake of all animal foods"—by summing the total scores based on the corresponding food groupings.

Stool sample collection and metagenomic sequencing

Stool samples of each participant were self-collected at local community clinics under the guidance of trained technicians according to a standard protocol.[58] Immediately after collection, stool samples were stored at −20°C and transferred to the biobank at Fudan University within 24 h, where they were stored at −80°C until processing. Fecal DNA was extracted using the advanced version of DNeasy 96 PowerSoil Pro QIAcube HT Kit (QIAGEN, Germany). Subsequent library preparation and whole-genome sequencing adhered to previously established protocols,[59] yielding an average depth of 10 gigabytes per sample. Quality control was performed as previously described.[60] In this study, an average of 69.6 million high-quality reads per sample were obtained after quality control.

Taxonomic and functional profiling

Taxonomic profiling was conducted using MetaPhlAn 3.0, and functional profiling was performed using HUMAnN3 (version 3.0.0).[52] Taxonomic features (species-level) with relative abundance $\geq$0.01% in at least 10% of all samples were retained for feature-wise tests. Similarly, microbial functional pathways with relative abundance <0.001% in greater than 90% of all samples were excluded. To reduce redundant testing, pathways were clustered using Ward's hierarchical clustering (R function hclust in the stats package with a cutree height of 0.1) with a dissimilarity measure of 1 minus the Spearman correlation coefficient. Eventually, a total of 165 species, 91 genera, and 109 pathways clusters were retained for downstream analysis. The relative abundances of taxonomic features were arcsine square root transformed before the regression analyses. Four α-diversity indices were calculated at the species level, including Shannon diversity, observed features (a measure of richness), Pielou's evenness index, and ACE index (Abundance-based Coverage Estimator, a measure of richness).

Gut–brain module analysis was conducted using a published metabolic reconstruction framework that translates shotgun metagenomic data into microbial neuroactive metabolic potential.[32] GBM coverage was calculated as the proportion of pathway steps with at least one detected orthologous group, and modules with coverage >66% were considered present. After filtering for abundance (relative abundance >0.0001%), 43 GBMs were retained for downstream analyses. Details regarding GBM functions, pathways, and structures are provided in Table S1. Associations between these GBMs and cognitive function are shown in Figure S7B.

Blood sample collection and plasma metabolites profiling

Fasting plasma samples were collected by trained nurses for metabolite profiling. Targeted metabolomic measurement of plasma samples were performed using an ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) system. The Q300 Kit (Metabo-Profile Biotechnology Co. Ltd, Shanghai, China) was utilized, covering a broad range of established gut microbiota-related metabolites, as well as key host-derived metabolites. Raw data were processed using MassLynx software (v4.1, Waters, Milford, MA, USA), including peak integration, calibration, and quantitation for each metabolite, as described previously.[53]

Accumulating evidence indicates that the microbiota-related metabolites like SCFAs, amino acids, indole derivatives, and bile acids play important roles in gut–brain interactions and the regulation of brain function.[10,11] Accordingly, we focused on investigating the potential role of 81 plasma microbiota-related metabolites (including 49 amino acids, eight SCFAs, three indole derivatives, and 21 bile acids) in the associations between diet, gut microbiota, and cognitive health. Metabolite concentrations were normalized using inverse rank-based transformation followed by quantile normalization.

Cognitive assessment and covariate measurement

The cognitive function of each participant was assessed using two objective cognitive tests by trained interviewers, including MMSE and HDS.[61,62] The MMSE was employed as the primary global cognitive instrument in main analyses. The MMSE instrument evaluated five cognitive domains: orientation, memory, and attention, and language and visuospatial ability. The global cognitive score

was then calculated as the sum of all domain scores (ranges 0–30 points), with a higher score indicating better cognitive performance. This global cognitive score was subsequently z-standardized for all analyses. Apart from the shared cognitive domains of orientation, memory, and attention, HDS focused on the domains of abstract and naming while MMSE focused on the domains of language and visuospatial ability.[63] The HDS was employed in sensitivity analyses to verify the robustness of the findings.

Information on potential confounders was collected using a standardized questionnaire, including age, sex, educational attainment, smoking status, alcohol consumption, physical activity, body mass index, history of hypertension or diabetes, and antibiotic use. Total energy intake was calculated from the FFQ concurrent with the diet indices.

QUANTIFICATION AND STATISTICAL ANALYSIS

Baseline characteristics of the study population were summarized across tertiles of mHPDI adherence and group comparisons were performed using chi-square test for categorical variables and the Wilcoxon rank-sum test for continuous variables. Multivariable-adjusted linear regression models were employed to examine the associations of mHPDI and its components with α-diversity, taxonomic features (species, genus, and pathways), and selected microbiota-related metabolites. All models were adjusted for age, sex, total energy intake, body mass index, educational attainment, smoking status, alcohol consumption, physical activity, history of diabetes, history of hypertension, and antibiotic use. In employing the GBM framework, multivariable-adjusted linear regression models were used to assess associations between mHPDI (and its components) and GBM abundances, adjusted for the full set of covariates as described above. For per-feature analyses of microbial taxa, pathways, and GBMs, multiple hypothesis testing was controlled using the FDR, with a target rate of 0.25 for *q* values considered statistically significant, consistent with prior exploratory microbiome studies.[25,64] To determine inter-individual variability in microbial composition, we calculated the Bray-Curtis dissimilarity matrix between samples with relative abundance of taxonomic data at species level. PERMANOVA was applied to quantify the proportion of variance in microbial composition explained by dietary variables and covariates using the 'adonis' function in the R package 'vegan'. The distance matrix was visualized through principal coordinates analysis. For metabolomic data, principal component analysis was performed for descriptive visualization of the data composition structure. Vegetable oils and animal fat were not included in the microbial and metabolic analyses because the intake was recorded only as binary responses rather than continuous quantification. For analyses of global microbial community composition (β-diversity), mHPDI was dichotomized at the median to enable a balanced two-group comparison for ordination and PERMANOVA-based testing, whereas other analyses modeled mHPDI in tertiles.

Among the overall population, we assessed longitudinal associations of mHPDI and its food components with cognitive function (*Z* score) using linear mixed-effects regression models, which accounted for repeated cognitive assessments. All models were adjusted for age, sex, total energy intake, body mass index, educational attainment, smoking status, alcohol consumption, physical activity, history of diabetes, history of hypertention, and follow-up duration, with additional mutual adjustment for other food components in analyses of indicidual components. To assess the robustness of cognitive measurement, we conducted sensitivity analyses using the HDS as an alternative global cognitive instrument. Enterotype clustering was performed using Jensen–Shannon divergence (JSD) and the PAM algorithm based on genus-level abundance.[65] The optimal number of clusters was determined using the Calinski–Harabasz index, which peaked at k = 3 (average silhouette width = 0.16; Figure S15A). PAM clustering identified three enterotypes, dominated by *Prevotella* (ETP), *Bacteroides* (ETB) or other genera (ETO). Clustering stability was confirmed using random sample-removal analyses and alternative distance metrics, which showed high concordance with the primary JSD-based results (Figures S15B and S15C). To investigate how diet interacts with microbial features in relation to cognitive health, we conducted interaction analyses focusing on enterotype clusters (ETP versus ETnonP), *Prevotella* carriage, *P. copri* carriage, and enterotype-enriched pathways. Effect modification by microbial features was evaluated by including an interaction term between mHPDI and the microbial features, along with their main effects, confounding variables (fixed effects), and per-subject random effects to account for within-subject variation. Statistical significance of the interaction was evaluated using a two-sided likelihood ratio test comparing models with and without the interaction term, with p interaction <0.05 considered significant. We then performed stratified analysis to quantify the associations between the mHPDI and cognitive function in subgroups defined by different levels of microbial features separately. The interactions between healthy plant food components and enterotype features in relation to cognitive outcomes were subsequently tested using the same analytical approach. In addition, to assess robustness, we conducted sensitivity analyses by stratifying participants into three enterotype clusters (ETP/ETO/ETB) and tested their interactions with mHPDI in relation to cognitive outcomes.

Mediation analysis was conducted among participants with both plasma metabolomic data and repeated cognitive assessments to examine whether microbiota-related metabolites contributing to diet mediated the protective association between mHPDI and longitudinal cognitive health. To characterize the collective metabolic signature associated with mHPDI, we constructed a composite MetS by integrating 12 metabolites identified in the longitudinal dataset. Following established approaches in metabolomics studies,[66] the MetS was calculated as the sum of standardized metabolite concentrations (*Z* scores), each weighted by the direction of its association with mHPDI (+1/-1). This unweighted directional scoring scheme was used to capture the overall dietary metabolic pattern at the population level, rather than to optimize individual-level prediction. Mediation analysis was conducted to quantify the proportion of the mHPDI-cognition association mediated by the MetS. Briefly, our approach followed and fulfilled the logical steps outlined by Baron and Kenny's,[51] while employing modern statistical techniques for estimation and inference. We first confirmed a significant total effect of the exposure variable (mHPDI) on cognitive function (*Z* score) using a linear mixed-effects model, with

adjustment for demographic factors, total energy intake, and lifestyle factors. We then estimated whether mHPDI was associated with the mediator (MetS). Only when this association was statistically significant ($p < 0.05$) did we proceed to add the MetS into the total-effect model to estimate its effect on cognition while retaining mHPDI and all other covariates. The indirect (mediated) effect and its proportion were then assessed using the Preacher & Hayes bootstrapping method (1,000 simulations), with p mediation <0.05 considered statistically significant. Exposure–mediator interaction was tested and found to be non-significant. Individual metabolites associated with mHPDI and cognitive function were considered as potential mediators using the same analytical framework. To test potential microbial linkages underlying the diet-metabolite-cognition axis, partial Spearman correlations were calculated between mHPDI-related microbial species and key microbiota-related metabolites, adjusting for major covariates. All statistical analyses were performed using R version 4.4.1.