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Abstract: Background: Alzheimer's disease (AD) prevalence is increasing, but its etiology remains elusive. Gut microbes can contribute to AD pathology and may help identifying novel markers and therapies against AD.

Methods: A randomized crossover pilot study of modified Mediterranean-ketogenic diet (MMKD) and American Heart Association Diet (AHAD) intervention is performed on 17 subjects (age: 63.4±3.7yr), of which 11 have mild cognitive impairment (MCI), while 6 are cognitively normal (CN). Subjects undergo MMKD and AHAD intervention for 6-weeks separated by 6-weeks washout periods. Gut microbiome, fecal short-chain fatty acids (SCFAs), and markers of AD in cerebrospinal fluid (CSF) including amyloid β (A β)-40 and A β -42, total tau, and phosphorylated tau-181 (tau-p181) are measured at before and after diet interventions.

Findings: At baseline, MCI and CN subjects show no notable difference in microbiome diversity but several unique microbial signatures are detected in MCI subjects. Proteobacteria correlate positively with A β -42: A β -40 while fecal propionate and butyrate correlates negatively with A β -42 in MCI subjects. Several bacteria are differently affected by the two diets with distinct patterns between CN and MCI subjects. Notably, the abundance of Enterobacteriaceae, Akkermansia, Slackia, Christensenellaceae and Erysipelotriaceae increases while that of Bifidobacterium and Lachnobacterium reduces on MMKD, while AHAD increases Mollicutes. MMKD slightly reduces fecal lactate and acetate while increasing propionate and butyrate. Conversely, AHAD increases acetate and propionate while reducing butyrate.

Interpretation: The data suggest that specific gut microbial signatures may predict the MCI and that the MMKD can modulate the gut microbiome and metabolites in association with improved AD biomarkers.

Research Article

Modified Mediterranean-Ketogenic diet modulates gut microbiome and short-chain fatty acids in association with Alzheimer's disease markers in subjects with mild cognitive impairment

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Short title: Gut microbiome and mild cognitive impairment/ Alzheimer's disease

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Findings: At baseline, MCI and CN subjects show no notable difference in microbiome diversity but several unique microbial signatures are detected in MCI subjects. *Proteobacteria* correlate positively with $A\beta$ -42: $A\beta$ -40 while fecal propionate and butyrate correlates negatively with $A\beta$ -42 in MCI subjects. Several bacteria are differently affected by the two diets with distinct patterns between CN and MCI subjects. Notably, the abundance of *Enterobacteriaceae*, *Akkermansia*, *Slackia*, *Christensenellaceae* and *Erysipelotriaceae* increases while that of *Bifidobacterium* and *Lachnobacterium* reduces on MMKD, while AHAD increases *Mollicutes*. MMKD slightly reduces fecal lactate and acetate while increasing propionate and butyrate. Conversely, AHAD increases acetate and propionate while reducing butyrate.

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Abbreviations:

AD: Alzheimer disease; AHAD: American Heart Association Diet; $A\beta$ 40/42: amyloid β 40/42; ApoE ϵ -4: apolipoprotein-E ϵ -4 allele; CN: cognitively normal; KD: ketogenic diet; MCI: mildly cognitive impaired; MMKD: modified Mediterranean ketogenic diet; SCFAs: short-chain fatty acids; FFAR 2/3: free fatty acid receptor 2/3; LPS: lipopolysaccharide; PD: phylogenetic diversity; OTUs: operational taxonomic units; LDA: linear discrimination analysis; Lefse: LDA effect size.

Research in context

Evidence before this study

Alzheimer's disease (AD) and related dementia is rising worldwide but its etiology and therapies remain unknown. One in every three Alzheimer's cases involves certain modifiable risk elements and hence might be preventable. Dietary profiles are power modulators of brain health. Diet is also one of the major modulators of the gut microbiome. Recent evidences suggest that the gut microbiome is associated with Alzheimer's pathology. Ketogenic diet (KD), a very low-carb and high-fat diet that effectively treats refractory epilepsy, is also known to modulate the gut microbiome as well as the brain function. Several mechanisms of action of KD might also be a modulator of AD pathology such as amyloid aggregation and tau hyperphosphorylation. However, novel gut microbiome and brain biomarkers are needed to pin down the mechanisms by which KD may be protective against AD. Hence, elucidation of gut microbiome during the preclinical stages of AD with no or mild cognitive impairment (MCI) can help identifying novel markers or therapies against AD.

Added value of this study

To our knowledge, this is the first study to demonstrate specific differences in the gut microbiome signature between older-adult MCI patients compared to cognitively normal (CN) counterparts, while also evaluating the impact of a modified Mediterranean-ketogenic diet (MMKD) on gut microbiome composition, short-chain fatty acids (SCFAs) levels, and AD biomarkers. The study identifies specific gut microbiome signatures that are associated with MCI and how these signatures correlate with AD biomarkers including the deposition of β -amyloid ($A\beta$)-40 and $A\beta$ -42 and tau in the cerebrospinal fluid (CSF) of these subjects. In addition, the study demonstrates how MMKD distinctly influences the gut microbiome and SCFAs as well as their association with the Alzheimer's CSF biomarkers in subjects with or without MCI.

Implications of all the available evidence

These findings provide important information to facilitate future intervention and clinical studies to examine the novel diet- and microbiome-related biomarkers and therapeutic targets that may be helpful for precision medicine approaches to AD risk in context to host nutrition and intestinal health.

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia in older adults, with clinical manifestations of progressive cognitive and functional impairment. Nearly 50 million people are living with dementia worldwide and this number is expected to increase to over 65 million by 2030 and 115 million by 2050. AD is a progressive, fatal disease that causes heavy burdens on patients, families, and societies. Despite years of research, the underlying etiology of AD remains unknown, with no available treatments or proven preventative interventions.¹ AD pathology is commonly characterized by extracellular accumulations of amyloid- β -peptides (A β) in senile plaques and intracellular depositions of hyperphosphorylated tau that forms neurofibrillary tangles.² Genetically, Apolipoprotein E (APOE) alleles (ϵ 2, ϵ 3 and ϵ 4) carry different risks for developing AD. Individuals with the ϵ -4 allele have an increased risk compared with individuals with the more common ϵ 3 allele, while the ϵ 2 allele is related to decreased risk.³ Recently, it has become clear that the pathogenesis of AD is far more complex and that amyloid deposition in the brain may precede clinical symptoms by 10-20 years.⁴ There is clear evidence that AD is related to chronic inflammation both in the central nervous system and in the periphery, and that the amyloid plaques are associated with years of inflammatory antimicrobial responses.⁵⁻⁷ Researchers have recently proposed a potential role for gut microbiome in the initiation and exacerbation of AD pathology and revealed that Alzheimer's patients have compositionally distinct and less diverse microbiota from control sex- and age-matched individuals.⁸ In AD, these alterations in the gut microbiome included a greater abundance of pro-inflammatory bacterial taxa along with less anti-inflammatory taxa.⁹

Gut microbiota produce numerous metabolites that directly or indirectly affect brain functions. Among these, short-chain fatty acids (SCFAs) including acetate, propionate and butyrate are known to modulate the peripheral and central nervous systems. Acetate crosses the blood-brain barrier (BBB) and modulates brain signals to regulate food intake.¹⁰ Acetate also impacts microglial cells and reduces blood-brain barrier permeability.¹⁰⁻¹¹ Butyrate is a multifaceted compound that is not only the preferred energy source of intestinal cells but also inhibits histone deacetylases to change the expression of several genes and proteins in intestinal and neuronal cells.¹² Butyrate feeding significantly improves learning and memory by amplifying the expression of learning associated genes in AD mouse models and restoring histone acetylation.¹³ This evidence indicates that restoring the production of SCFAs in the gut may help in preventing or ameliorating AD pathology. Additionally, the gut microbiome is associated with changes in neurotransmitter signaling, synaptic protein expression, long-term potentiation, and myelination.¹⁴ Therefore, examination of gut microbiome could present potential avenues and opportunities to discover and develop specific microbial signatures that are associated with the prognosis of AD progression. In addition, interventions that modulate the gut microbiome and enrich beneficial bacteria and bacterial metabolites may be helpful in ameliorating AD.

Though the precise etiology of AD remains elusive, it is recognized that dietary patterns may play a role in the pathology of AD. Increased risk of AD and dementia is associated with dietary patterns high in saturated fat and simple carbohydrates, also termed 'Western' diets, while diets high in mono- and poly-unsaturated fats, vegetables, fruits, and lean proteins are associated with reduced risk.¹⁵⁻¹⁶ Diet is not only a strong modulator of the gut microbiota but is also a powerful modulator of the brain health. Emerging evidence indicates that specific dietary profiles and nutrients modulate the gut microbiota composition and are associated with the production or aggregation of amyloid proteins.¹⁵⁻¹⁶ Therefore, diets that modulate gut

microbiome may be an effective strategy to impact the development of AD and other forms of dementia.

The very low carbohydrate and high fat ketogenic diet (KD) has been used for the treatment of refractory epilepsy. The KD is remarkably effective for this purpose: 50-70% of patients have >50% seizure reduction and 30% have >90% seizure reduction.¹⁷ In recent years, several adaptations of the KD have been developed that improve compliance and reduce health risks associated with prolonged high intake of saturated fats. One such adaptation, the modified Mediterranean KD (MMKD), has comparable efficacy to the original KD, but allows slightly higher carbohydrate consumption to permit increased intake of vegetables and fruits, while emphasizing fats and proteins derived from healthy sources such as olive oil and fish. Several mechanisms of action of KD, including reduction of neuronal hyperexcitability, enhancement of mitochondrial metabolism, reduced oxidative stress, and inhibited mammalian target of rapamycin, could also be a modulator of AD pathological processes such as amyloid aggregation and tau hyperphosphorylation. In addition, the KD has also been shown to modulate the gut microbiome in mice, while ketone production is also related to the gut microbiome alterations.¹⁸ Propelled by these ever-mounting data connecting the gut microbiome to metabolism, neural activity, and behavior, and also considering that the KD can modulate the gut microbiome composition, we hypothesized that an intervention of MMKD would improve the gut microbiome by maintaining/ restoring the homeostasis of gut microbiota (beneficial and fiber-degrading gut commensal bacteria) and microbial metabolites (e.g., butyrate) along with dementia/AD biomarkers. Herein, we show how the gut microbiome signature differs in participants with mild cognitive impairment (MCI) compared to cognitively normal (CN) counterparts. In addition, the MMKD alters the gut microbiome signature and SCFAs, and these changes are associated with improved cerebrospinal fluid (CSF) AD biomarkers in older adults.

2. Methods

2.1. Study participants

Stool samples used for analyses were collected as part of a pilot study of a 6-week MMKD or American Heart Association Diet (AHAD) intervention in older adults at risk for AD due to baseline cognitive impairment (MCI) or cogni/subjective memory complaints. The study used a randomized cross-over design and included 17 participants (mean age: 63.4 ± 3.7yr) of which 11 participants were diagnosed with MCI (diagnosed using ADNI-2 criteria for early MCI (<http://www.adni-info.org>) and 6 participants were cognitively normal (CN) (Clinical Trials # NCT02984540) (Suppl. Table 1). The study was approved by the Institutional Review Boards of the Wake Forest School of Medicine and was conducted in the Wake Forest Clinical Research Units. Written informed consent was obtained from all participants and/or their legally designated representatives. Diagnoses and eligibility were determined by expert physicians and neuropsychologists based upon the data of cognitive testing, evaluation of medical history, physical examination, and clinical laboratory. Participants were free from alcoholism, head trauma, hypoxia, neurologic disorders except MCI, renal or hepatic disorders, chronic obstructive pulmonary disease, unstable cardiac disease, and psychiatric diseases except mild controlled depression. All study personnel collecting data remained blinded to status or intervention assignment throughout the study. Participants with recent history of use of statins or anti-diabetic medications or agents with prominent CNS effects (except anti-depressants and AD medications) were excluded.

2.2. Study Design

Participants were randomly assigned to either a MMKD or an AHAD for 6-weeks, followed by a 6-week washout, and then a 6-week intervention with the second diet (Suppl. Fig. 1). All participants underwent a lumbar puncture (LP) at baseline of diet 1, at the end of diet 1, and again at the end of diet 2. Fasting blood collection and collection of stool samples occurred before and at the end of each diet.

2.3. Intervention.

A registered dietitian developed daily meal plans for each study participant based upon their food preferences and caloric needs as determined by a food diary and resting metabolic rate assessment. Participants had weekly in-person or phone visits for meal planning and adherence assessment. Participants maintained a food record that was reviewed at these visits. Blood ketones were measured weekly to ensure compliance on the MMKD. Participants supplied their own food based upon a daily meal plan, food list, and other provided material. The target macronutrient composition (% of total calories) for the MKMD was <10% carbohydrate, 60-65% fat, and 30-35% protein, and for the AHAD was 55-65% carbohydrate, 15-20% fat, and 20-30% protein. While on the MKMD, participants were supplied with 2L of extra virgin olive oil, and were encouraged to eat fish, lean meats, and nutrient rich foods. While on the AHAD, participants were instructed to limit their fat intake to <40 grams/day, while eating fruits, vegetables, and fiber-containing carbohydrates. Throughout the study, participants received a daily multivitamin and restricted use of supplements with antioxidant or ketone-inducing effects (i.e., resveratrol, coenzyme Q10, fish oil, coconut oil, MCT supplements).

2.4. Lumbar Puncture, CSF biomarkers assays, and ApoE ϵ -4 genotyping

Concentrations of CSF A β -42, A β -40, tau, and phospho-tau (tau-p181) were measured according to the methods described previously.¹⁹ Briefly, CSF (25ml) was collected in the morning after overnight fast as per the standardized best practice guidelines of the National Alzheimer's Coordinating Center using the 20 gauge Sprotte spinal needles and polypropylene tubes (<https://www.alz.washington.edu/BiospecimenTaskForce.html>). CSF was transferred in 0.2 ml aliquots into pre-chilled polypropylene tubes, frozen immediately on dry ice, and stored at -80°C until assay. AD biomarkers (A β -42, A β -40, tau, tau-p181) were measured using the AlzBio3 multiplex assay (FujiRebio Europe). Subjects were stratified based on the carriage of the ApoE ϵ -4 allele, a well-known risk factor for AD that predicts insulin response, as per our previously described method.²⁰

2.5. Fecal microbiome analysis

16S rRNA gene sequencing was performed as previously described.²¹⁻²⁵ Briefly, genomic DNA was extracted from 200mg fecal samples using QiaAmp PowerFecal DNA kit (Qiagen, CA, USA) per manufacturer's instructions. The V4 hypervariable regions of the 16S rDNA gene were amplified using the universal primers 515F (individually barcoded) and 806R; the resulting amplicons were purified with AMPure® magnetic purification beads (Agencourt); the purified products were quantified using the Qubit-3 fluorimeter (Invitrogen); and the amplicon library was generated according to methods of Caporaso et al.²⁶ The purified PCR product was pooled in equal molar concentrations and sequenced on an Illumina MiSeq platform and 2x300bp reagent kit (Miseq reagent kit v3; Illumina Inc.) for paired-end sequencing. Sequences were demultiplexed and quality filtered using QIIME (Quantitative Insights into Microbial Ecology; version 1.9.1) software package per default parameters.²⁷ Operational taxonomic units (OTUs) were chosen by open reference OTU picking based on 97% sequence similarity to the Greengenes database.²⁷ Taxonomy assignment and rarefaction were performed within QIIME. To avoid bias due to different sequencing depth, the OTU

tables were rarefied to the lowest number of sequences per sample (12,151 sequences per sample) for computing alpha-diversity metrics within QIIME using the determination of the number of observed OTUs. Beta diversity of the microbiome was analyzed using principal coordinate analysis (PCoA) of the unweighted and weighted Unifrac distance (using EMPEROR version 0.9.3-dev). LEfSE (Linear discriminatory analysis [LDA] Effect Size) was used to identify bacterial taxa that drive differences between MCI or diet groups.²⁸ To explore the metabolic and other functional activities of the gut bacterial communities in CN vs MCI subjects, an open source bioinformatics tool PICRUST (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) was used.²⁹ The OTUs generated from the 16S amplicon sequencing data were entered into PICRUST software and were analyzed for the prediction of functional genes of the classified members of the gut microbiota resulting from reference-based OTU picking against Greengenes database. Subsequently, the inferred gene families were annotated against KEGG (Kyoto encyclopedia of genes and genomes) orthologs (Kos) and then collapsed into KEGG pathways to generate the functional pathway. The functions were finally categorized and compared at levels 2 and 3.²⁹

2.6. Lactate and SCFAs analyses

Fecal organic acids were measured as per the previously described method.^{21-22, 24} Briefly, an approximately 100 mg aliquot of feces was aseptically mixed with 900 μ L sterile PBS buffer (pH 7.4) in a sterile tube and vortexed for one minute or until uniformly suspended. The suspension was centrifuged at 12,000 $\times g$ for 10 min and the supernatant was filtered through a 0.45 μ m membrane-filter. Final concentrations (μ mol per gram of fecal sample) of lactate, acetate, propionate, and butyrate in fecal specimen were determined using a high-performance liquid chromatography (HPLC; Waters-2695 Alliance HPLC system, Waters Corporation, Milford, MA, United States) with DAD detector at 210 nm, equipped with a Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, United States). Ten μ L sample was injected on the HPLC system; and sulphuric acid (0.005 N) was used to elute the column with a flow rate of 0.6 mL/min at 35°C.

2.7. Statistical analyses

Bacterial diversity, taxon abundance, and SCFAs levels between groups were compared using non-parametric tests in R statistical software package (version 3.4.3; <https://www.r-project.org/>). Alpha-diversity indices, bacterial abundance, and fecal organic acid levels at the baseline between MCI and CN participants was compared using non-parametric unpaired two-tailed t-test. Statistically significant differences between different groups during the dietary interventions were calculated by Kruskal-Wallis test followed by Dunn's post-hoc analysis. Hierarchical clustering and heat-maps depicting the patterns of abundance and log values were constructed within R using the 'heatmap.2' package. Correlation between bacterial abundance and CSF biomarkers was estimated by using Spearman's rank correlation coefficient test (GraphPad Prism software system, version 6.0). Unless otherwise stated, all the values presented herein are means $\pm$ SEM. $P < 0.05$ was considered statistically significant unless specified.

3. RESULTS

3.1. MCI status is associated with gut microbiome signature

The analysis of gut microbiome diversity at baseline demonstrate no notable difference in terms of beta-diversity (weighted unifrac analysis; Fig. 1a) or alpha-diversity (i.e., phylogenetic diversity, observed OTUs, Chao1 index, and Shannon index; Suppl. Fig 2a)

between CN and MCI participants. However, the taxonomical grouping and differential abundance of OTUs at the phylum, family, and genus levels of bacterial taxonomic classification reveal differences in the relative abundance of several taxa between CN and MCI participants (Fig. 1b-n). At phylum level, MCI participants show slightly higher abundance of phylum *Firmicutes* and slightly lower abundance of phylum *Bacteroidetes* (Fig. 1b, d, e) as compared to CN participants. In addition, MCI participants have significantly higher abundance of *Proteobacteria* (Fig. 1f, m), while having slightly higher abundance of *Tenericutes* and slightly lower abundance of *Verrucomicrobia* (Fig. 1b). At family level, MCI participants show significantly higher abundance of families *Enterobacteriaceae* and *Mogibacteriaceae* and insignificantly higher abundance of family *Coriobacteriaceae* (Fig. 1g-i). The genus level comparison and the Linear Discriminatory Analysis (LDA) effect size (Lefse) analysis reveal that the abundance of genera *Phascolarctobacterium* and *Coprococcus* was higher, while that of *Dialister* was lower in MCI participants (Fig. 1j-l). The analysis of fecal concentration of lactate and SCFAs viz. acetate, propionate, and butyrate reveal no significant difference between CN and MCI participants, although MCI participants appear to have slightly lower levels of acetate and propionate (Fig. 2a-d). In addition, the analysis of PICRUSt-inferred functional categorization of the gut microbiome show higher abundance of several disease-related pathways and lower abundance of several metabolism-related pathways in MCI subjects compared with CN subjects (Suppl. Fig. 3).

3.2. Specific gut bacterial taxa correlate with CSF biomarkers of AD at baseline

We further performed correlation analyses to find relationships between any taxa and metabolites with CSF biomarkers including A β 42 and tau-p181 (Fig. 2e). The results show that the phylum *Firmicutes* is positively correlated with tau-p181 (Fig. 2e). Further, *Proteobacteria* are negatively correlated with A β 42 mainly in CN participants; whereas in MCI participants, these correlate positively with A β 42/A β 40 ratio (Fig. 2e). Family *Enterobacteriaceae* is positively associated with tau-p181 and tau-p181/A β 42 ratio, while *Mogibacteriaceae* is positively correlated with A β 42/A β 40 ratio (Fig. 2e). Among fecal organic acids, propionate correlates negatively with A β 42 particularly in MCI participants, while sharing positive relationship with tau-p181 in CN participants (Fig. 2e). In addition, several other bacterial taxa are also found to be associated positively or negatively with these biomarkers (Suppl. Fig. 2b).

3.3. MMKD influences gut microbiome and organic acids distinctly in participants with or without MCI

Six weeks of MMKD and AHAD does not show a strong effect on the overall microbiome in terms of the α - and β -diversity indices, which are not significantly different between the two groups. Similarly, major phyla including *Firmicutes*, *Bacteroidetes* and *Proteobacteria* do not demonstrate any significant change during the two dietary interventions in CN and MCI participants (Fig. 3a-b). However, at family and genus level, several taxa were found to be affected by the two diets (Fig. 3c) with patterns differing between CN and MCI participants (Fig. 3d). In particular, family *Bifidobacteriaceae* and genus *Bifidobacterium* were significantly reduced on MMKD wherein the reduction was more prominent in MCI participants (Fig. 3b; Fig. 4b-c; Suppl. Fig. 4-5). A slight decline in bifidobacteria was seen during AHAD intervention but only in MCI participants (Fig. 3b; 4b-c). On the other hand, the genus *Akkermansia* from phylum *Verrucomicrobia* and family *Verrucomicrobiaceae* was insignificantly increased on MMKD but not on AHAD (Fig. 3b; 4a-c). Both diets lead to a slight increase in the abundance of phylum *Tenericutes* in both of the groups (Fig. 3b; 4a). In addition, MMKD but not AHAD lead to a decline in the abundance of genus *Lachnobacterium* while increasing that of genus *Slackia* (3b; Fig. 4c). Interestingly, the

PICRUSt-inferred predictions of metagenome function reveal that MMKD but not AHAD decreases the abundance of gene families annotated to the Alzheimer's disease (Fig. 5). In addition, the abundance of KEGG pathways associated with type-1 diabetes, type-2 diabetes, and bacterial toxins is also decreased ($p < 0.05$) following MMKD but not AHAD. In terms of metabolism-related pathways, the abundance of gene families associated with carbohydrate digestion and absorption is slightly reduced on MMKD (plausibly due to the ketogenic nature of MMKD) and slightly increased on AHAD, whereas the pathway related to the lipid metabolism i.e., the steroid biosynthesis is slightly increased on MMKD but not on AHAD (Fig. 5).

The two dietary interventions also influence the fecal levels of organic acids including lactate and short-chain fatty acids (acetate, propionate and butyrate) with patterns being different between the diets or between CN and MCI participants (Fig. 6; Suppl. Fig. 5). Overall, both diets reduced lactate (Fig. 6a) and increased propionate (Fig. 6c). Acetate was increased considerably on AHAD but was slightly reduced on MMKD (Fig. 6b). On the other hand, butyrate was increased after MMKD but was reduced after AHAD (Fig. 6d), thereby leading to a significant difference between the effects of the two diets. Further analyses show several differential effects of the two diets on fecal organic acids between CN vs. MCI participants (Fig. 6e-h; Suppl. Fig. 5). For instance, after MMKD, lactate was slightly increased in CN participants, whereas in MCI subjects, it was slightly reduced (Fig. 6e; Suppl. Fig. 5). On the other hand, after AHAD, lactate was significantly reduced in CN participants in contrast to a slight increase in MCI participants (Fig. 6e; Suppl. Fig. 5). Acetate remained largely unaffected by the two diets in CN participants but in MCI participants its levels were increased after AHAD but not after MMKD (Fig. 6f; Suppl. Fig. 5). Propionate shows consistent effect wherein its levels were increased in both CN and MCI participants but only after AHAD (Fig. 6g; Suppl. Fig. 5). Butyrate was slightly increased in both CN and MCI participants after MMKD and slightly reduced after AHAD in both groups (Fig. 6h; Suppl. Fig. 5).

3.4. Diet-induced changes in gut microbiome and organic acids correlate with changes in the CSF biomarkers of AD

Analysis of correlation of changes occurring in the abundance of gut bacterial taxa and fecal levels of SCFAs with changes in the levels of CSF AD biomarkers (A β 42, A β 40, total tau and tau-p181) reveal several distinct patterns of positive and negative association of biomarkers with several gut bacterial OTUs and SCFAs. These patterns are presented in Fig. 7 in the form of a color-based heat-map. For example, phylum *Tenericutes*, which was increased by both diets particularly in MCI participants, was negatively correlated with changes in the CSF levels of A β 42, particularly in MCI participants after MMKD (Fig. 7). In addition, family *Enterobacteriaceae*, which was increased after MMKD in both participant groups, was also negatively correlated with A β 42 but only in MCI participants on MMKD. Family *Lachnospiraceae* was positively associated with A β 42 in both diet groups. Family *Rikenellaceae* and genus *Parabacteroides* were positively associated with A β 42 but only in MCI participants after MMKD, while genus *Oscillospira* was positively correlated with CN participants after MMKD. Alpha-diversity indices and genus *Sutterella* were positively associated, while *Mollicutes* was negatively correlated with tau-p181 in MCI after MMKD. Family *Ruminococcaceae* was positively associated with tau-p181 but only in CN participants after MMKD. Several similar patterns of correlation of gut microbial taxa with CSF biomarkers A β 40 and total tau and the ratio of A β 42/A β 40 and tau-p181/A β 42 were also seen (see Suppl. Fig. 6).

4. DISCUSSION

AD is a progressive age-related neurodegenerative disease with no disease-modifying therapy or validated preventative strategy currently available. Early diagnosis and interventions are prerequisites to prevent or at least slow down the progression of AD. Early diagnosis of AD by molecular markers is not reliably consistent; hence, investigation of novel biomarkers may help strengthen the diagnosis and ultimately the prognosis of AD. Considering their emerging role in brain function and pathology,³⁰⁻³² the gut microbiome and SCFAs may serve as potential candidates for AD biomarkers. Herein, we identify the bacterial taxa that differ between older-adults with MCI and cognitively normal counterparts. Our results suggest that certain bacterial groups show MCI-specific profile and hence may be used as additional and corroborating biomarkers for the detection of MCI.

The overall microbiome diversity in terms of α - and β -diversity indices appears to be comparable between the two groups; however, we note that the MCI subjects have higher abundance of Firmicutes and lower abundance of Bacteroidetes compared to CN counterparts. Notably, increased Firmicutes and decreased Bacteroidetes populations are commonly associated with dysbiotic microbiome signatures and negative health outcome, suggesting that the microbiome in MCI participants is in a dysbiotic state. Although this difference in Firmicutes and Bacteroidetes was not statistically different apparently which might be because of the small sample size; however, our results concur well with those of a recent study that also reported a similar spectrum of gut dysbiosis in AD patients characterized by reduced abundance of bacterial families and genera within the phylum Firmicutes and lower microbiome diversity.⁸ AD patients also had higher abundance of Bacteroidetes.⁸ This disparity from early reports of microbiome composition in AD patients⁸ may be due to differences in the geographical background, dietary habits, and age of subjects. In addition, the participants in our study are likely to be in the earlier stages of AD spectrum (MCI) compared to Vogt et al.⁸ that assessed adults with AD. Hence, considering that MCI is an earlier stage of AD spectrum, the MCI-specific microbiome differences seen in our study cohort suggest that the changes in the gut microbiome composition are linked in neuropathology of AD. Whereas, adults with AD dementia may also have several other health- and lifestyle-related issues that may induce stage-specific microbiome alterations. However, we still observe different patterns of several bacterial groups between MCI and CN subjects, indicating an intricate relationship of host-microbiome interactions and clearly highlights the importance of more comprehensive investigations of the microbiome in AD to establish and validate microbial signatures throughout the spectrum of AD pathology.

The higher abundance of phylum *Proteobacteria*, class *Gammaproteobacteria*, and family *Enterobacteriaceae* (all of which comprise gram-negative bacterial groups) in MCI versus CN participants is intriguing. A wide variety of pathogens belong to the *Proteobacteria* phylum, while *Gammaproteobacteria* is also comprised of several pathobionts including *Enterobacteriaceae*, *Vibrionaceae* and *Pseudomonacaceae* families. Notably, the cell wall of these pathobionts is enriched with highly inflammatory components such as lipopolysaccharides (LPS) that are known to induce low-grade systemic inflammation and aggravate AD pathology.³³ Interestingly, higher abundance of gram-negative bacteria in AD patients compared to normal participants has also been reported previously.³⁴ In addition, we found that MCI participants have higher *Dialister* and lower *Phascolarctobacterium* abundance compared to CN participants, a finding that also concurs with previous report³⁴ and suggests that these genera may be used as a unique biomarker in understanding the progression of MCI to AD dementia. Hence, given the biological applicability of these associations, it would be a worthwhile topic for further studies with larger cohorts to validate and establish these signatures.

Interestingly, the abundance of *Mogibacteriaceae* family, which is also a periodontal environment-linked bacterial group, is significantly higher in MCI vs. CN subjects, hinting that the gastrointestinal tract of MCI subjects may be invaded by microbes living in other bodily niches.³⁵⁻³⁶ Additionally, we find higher abundance of the order *Gamellales* and family *Gamellaceae* in ApoE ϵ -4^{+ve} subjects, suggesting that *Gamella* might somehow be associated with ϵ -4 allele and hence could be used to determine the MCI and/or AD risk in ϵ -4^{+ve} individuals. This finding also concurs with previous study by Vogt et al.³⁴ that also reported higher abundance of *Gamellaceae* and *Gamella* in AD patients. In our study, five out of eleven MCI participants were of APOE ϵ -4^{+ve}. However, no significant difference in gut bacterial diversity, the abundance of specific bacterial groups, or the fecal SCFAs levels was observed between MCI participants with versus without ϵ -4^{+ve} genotype (Suppl. Fig. 7). Further, no consistent or significant pattern was seen in the effect of dietary interventions on bacterial taxa, SCFAs, or their correlation with CSF biomarkers (Suppl. Fig. 8).

MCI is an early stage of AD pathology and accumulating evidence suggests that a substantial proportion of MCI participants remain stable for years,³⁷ indicating that MCI can be targeted preferably by non-pharmacological interventions including dietary and nutritional elements. Propelled by this notion, we herein tested the impact of MMKD and AHAD on CSF biomarkers of AD as well as on the gut microbiome signature and SCFAs levels in MCI and CN participants. While there were no significant differences in the α - and β -diversity of the gut microbiome between participants after MMKD and AHAD interventions, we did notice that several gut bacterial phyla, families, and genera are differentially altered after MMKD and AHAD intervention in MCI versus CN subjects. For example, the phylum *Actinobacteria*, family *Bifidobacteriaceae*, and genus *Bifidobacterium* are significantly decreased in MCI group after MMKD as compared to CN counterparts and AHAD group participants. This pattern coincides with a specific decrease in fecal lactate and acetate levels in MCI-MMKD group, suggesting that the effects of specific diets may differ between MCI versus CN subjects. In addition, this also indicates that the MMKD possibly causes a slight increase in the beneficial SCFAs including butyrate. Because these SCFAs content was measured in the feces, the precise source/origin of these SCFAs remains unknown. However, we speculate that either these might have originated in the liver and were subsequently secreted into the gut (because the participants were on a ketogenic state), or the ketogenic diet might have promoted the intestinal SCFAs production by supplying plant-based fermentable fibers to be fermented by the gut bacteria.

Apart from the observed changes in microbial abundances, diet can also influence the features of host metabolism and immune function by affecting various genes, for e.g., those involved in carbohydrate and lipid metabolism, endocrine functions, inflammatory responses, energy balance, etc. Using PICRUSt-inferred predicted metagenomes of gut microbiome followed by the Clusters of Orthologous Groups (COG) classification, we observe that there are several functional pathways affected differently by the two diets. Most notably, the finding of decreased abundance of KEGG pathways annotated to the Alzheimer's disease as well as the type-1 and -2 diabetes following MMKD but not AHAD is interesting and hints toward the dietary influences beyond the lower gut. While MMKD is already known to be beneficial for metabolic health and related diseases including obesity and diabetes, these data further indicate that MMKD can confer benefits to the neurodegenerative diseases as well. In addition, the reduction in the abundance of pathways associated with bacterial toxins following MMKD further underpins the beneficial influence of MMKD on checking the population of toxigenic pathobionts and/or opportunistic pathogens in the gut thereby helping in maintaining/ restoring the gut microbial homeostasis. No doubt, these functional analyses are helpful in understanding the relationship between the host diet, microbiome and

functional capacities, it must, however, be noted that these predictions are inferred only from the known functions of the gut microbial communities and hence might be over- or underestimated in specific milieus. Future studies employing the whole genome shotgun sequencing and not just the 16S data might provide more insightful data on these functionalities of the gut microbiome.

Recently studies have shown that abnormally higher level of lactate in the gut is associated with brain foginess,³⁸ a transient condition characterized by the combination of symptoms of mental confusion, impaired judgment, poor short-term memory, and difficulty with concentration. Hence, the decreased lactate levels in MCI-MMKD group may be associated with improved memory and cognition in MCI participants. On the other hand, increased butyrate after MMKD may be beneficial in terms of reduced/ homeostatic gut leakiness and restrained LPS diffusion. Butyrate is well known to possess neuroprotective actions and improve brain health³⁹⁻⁴⁰. Butyrate is used as a preferential source of energy by the enterocytes, which results in improved/ homeostatic functions of the gut epithelial and enteroendocrine cells (for the release of gut hormones), goblet cells (for the release of mucus), enterochromaffin cells (for sensory functions), enteric neuronal functions, and mucosal immune cells (to remain in controlled/ homeostatic inflammatory state), as well as enhanced expression of tight junction proteins (required for homeostatic gut permeability). In AD mouse models, butyrate treatment profoundly improves learning and memory ability in unison with improved expression of learning associated genes, that are associated with restoring histone acetylation^{13, 41-44}. Altogether, these studies as well as our data of specific MCI-associated patterns of microbial modulation accompanied by increased butyrate levels and their correlations with AD biomarkers hint that MMKD may be beneficial for participants with MCI.

To our knowledge, this is the first report to demonstrate specific differences in the gut microbiome signature between older adults with MCI versus cognitively normal counterparts, while also evaluating the impact of a modified Mediterranean-ketogenic diet on intestinal microbiome composition, SCFAs levels, and AD biomarkers. Despite our exciting results, our study is not without limitations. For instance, our cohort may be limited in sample size to find statistically robust differences in gut microbiome and metabolite profiling; however, it still represents an ideal model study to investigate the microbiome and its metabolites as biomarkers for MCI versus CN subjects. Although we find interesting differences in the gut microbiome upon dietary interventions that are differentially modulated in MCI versus CN participants, the duration of intervention is relatively short. Further long-term studies of MMKD or similar interventions would help validate these or similar differences in gut microbiome, SCFAs and AD biomarkers in MCI, as well as the progression of MCI to AD dementia. Nevertheless, our results encouragingly suggest that specific gut microbiome signatures and organic acids could be used as biomarkers for the spectrum of AD pathology and hence would facilitate prospective intervention and clinical studies exploring novel nutrition- and microbiome-related biomarkers and therapeutic targets that could be helpful for precision medicine approaches against MCI, dementia and AD risk.

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Ethical statement

The study was approved by the Institutional Review Boards of the Wake Forest School of Medicine and was conducted in the Wake Forest Clinical Research Units. All protocols related to the cohorts involved in the study have been reviewed and approved by the Institutional Review Board of the Wake Forest School of Medicine. All experiments and samplings were carried out in accordance with ethical and biosafety protocols approved by the Institutional guidelines.

Conflict of interest

The authors declare no competing interests.

Author contributions

RN: performed microbiome measurement, analyzed data, wrote first draft of the manuscript; BJN, SC: coordinated in collecting human feces from participants enrolled in respective studies; SW: measured fecal organic acids; SC and HY: conceived the idea, supervised the whole study, helped in data interpretations, revised drafts of manuscript. All authors reviewed and approved the final version of the manuscript.

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FIGURE LEGENDS

FIG. 1. Differences in the gut microbiome between subjects clinically diagnosed with mild cognitive impairment (MCI) versus cognitively normal (CN) counterparts. (a) Microbiome beta-diversity in terms of weighted unifracs distance, and (b-c) gut microbiome composition at the level of major phyla (b) and families (c) in CN (n=6) and MCI subjects (n=11). (d-l) Differences in the relative abundance of bacterial taxa in the gut microbiome of CN versus MCI subjects ($*p<0.05$); (m-n): Linear discriminant analysis (LDA) effect size (Lefse) plot and cladogram representing the unique bacterial signatures identified in CN and MCI subjects.

FIG. 2. Gut bacterial taxa and fecal organic acids correlate with cerebral spinal fluid (CSF) biomarkers of Alzheimer's disease (AD) differently in subjects clinically diagnosed with mild cognitive impairment (MCI) versus cognitively normal (CN) counterparts. (a-d) Fecal concentration of organic acids including lactate and short-chain fatty acids in CN (n=6) versus MCI subjects (n=11). (e) Correlation (Spearman R; $*p<0.05$) of gut bacterial taxa and fecal organic acids with CSF biomarkers of AD in CN versus MCI subjects.

FIG. 3. Mediterranean-style ketogenic diet (MMKD) and American Heart Association Diet (AHAD) differently influence gut microbiome in subjects clinically diagnosed with mild cognitive impairment (MCI) versus cognitively normal (CN) counterparts. (a-b) Phyla-level gut microbiome composition at baseline and endpoint of 6-weeks MMKD and AHAD intervention in all participants (n=17) (a) and separately in CN (n=6) versus MCI subjects (n=11) (b). (c) Hierarchical clustering heat-map of gut bacterial taxa that showed >1.0 or <-1.0 Log₂-fold increase or decrease in relative abundance during MMKD or AHAD intervention. (d) Heat-map summarizing the differential patterns of diet-induced alterations (mean Log₂-fold change in relative abundance) in gut bacterial taxa that showed >1.0 or <-1.0 Log₂-fold increase or decrease in relative abundance during MMKD or AHAD intervention in CN and MCI subjects.

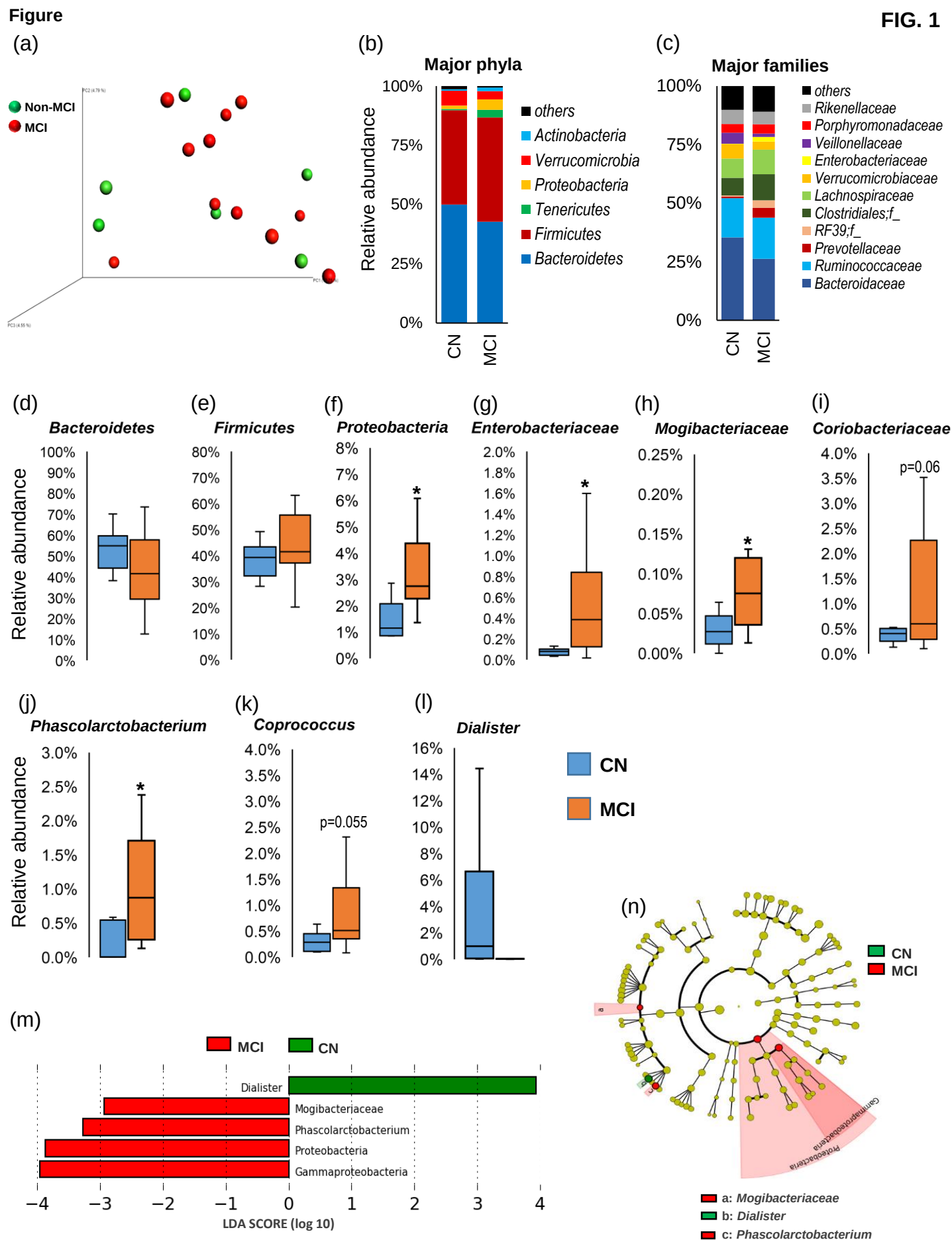
FIG. 4. Mediterranean-style ketogenic diet (MMKD) and American Heart Association Diet (AHAD) induce specific changes in the gut microbiome of subjects clinically diagnosed with mild cognitive impairment (MCI) versus cognitively normal (CN) counterparts. Mean Log₂-fold change in the relative abundance of major phyla (a), families (b) and genera (c) in CN (n=6) versus MCI subjects (n=11) ($*p<0.05$).

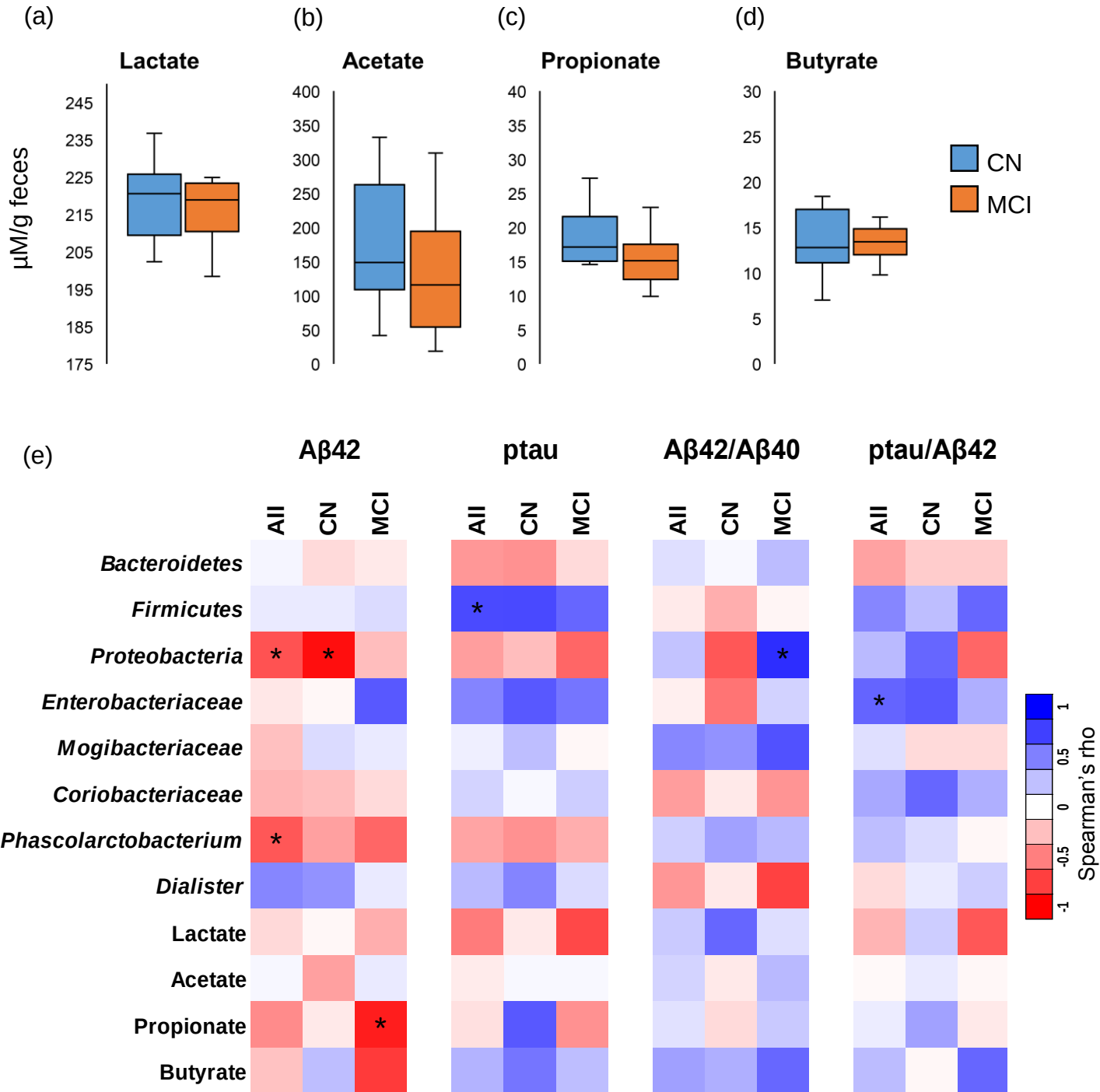
FIG. 5. Differential effects of Mediterranean-style ketogenic diet (MMKD) and American Heart Association Diet (AHAD) on the functional analysis of the gut microbiome. Functional content of the gut microbiota was inferred by using PICRUSt based on the baseline and endpoint 16S amplicon sequencing data. Changes in the functional KEGG pathways during each dietary intervention were calculated by comparing the relative abundance of the KEGG orthologs and pathways (Level 2 and 3) at baseline vs. endpoint data and were interpreted in the form of mean Log₂-fold change.

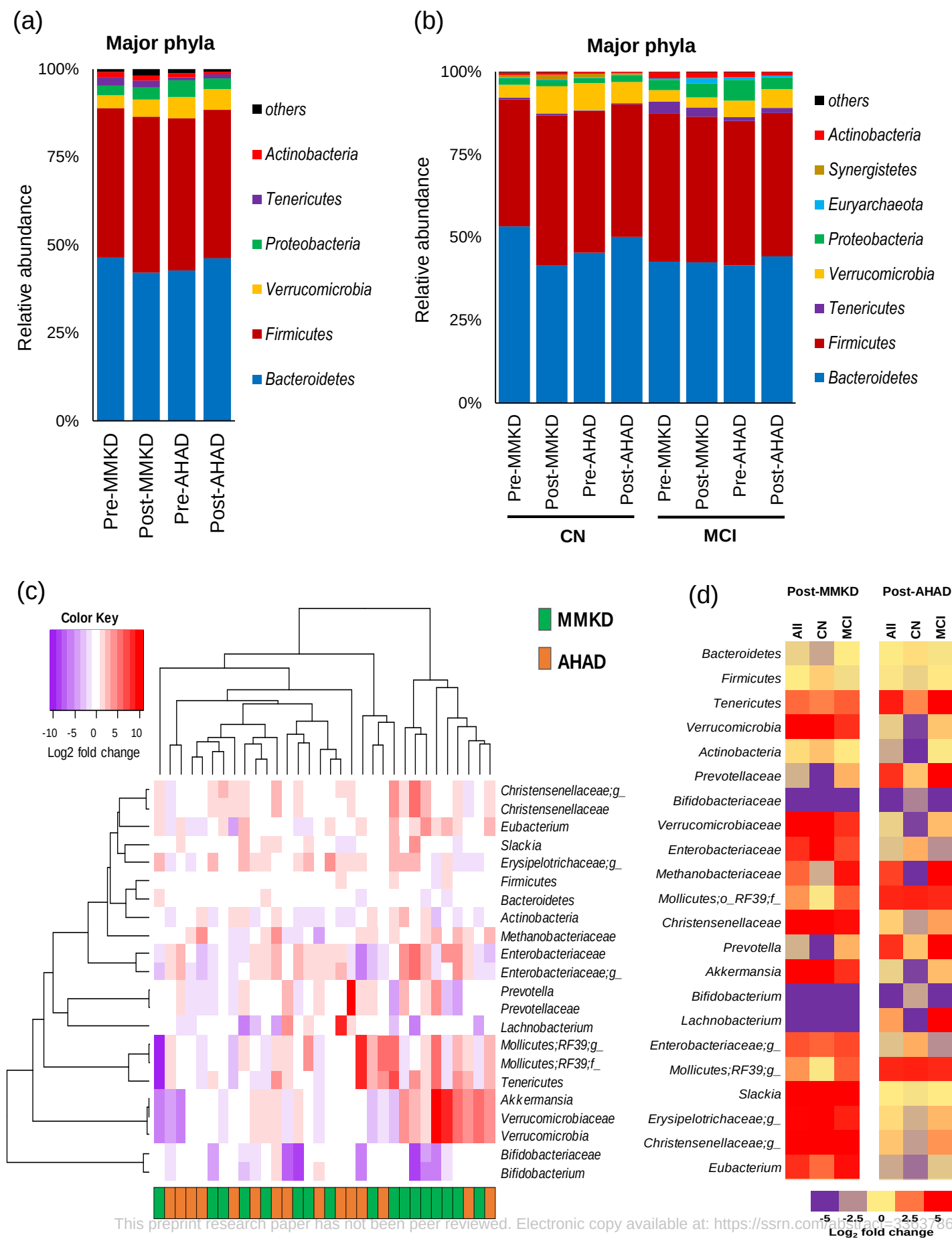
FIG. 6. Mediterranean-style ketogenic diet (MMKD) and American Heart Association Diet (AHAD) differently influence gut microbial metabolites in subjects clinically diagnosed with mild cognitive impairment (MCI) versus cognitively normal (CN) counterparts. Mean percent change in the fecal concentration of intestinal organic acids in all participants (n=17) (a-d) and separately in CN (n=6) versus MCI subjects (n=11) (e-h) ($*p<0.05$).

FIG. 7. Diet-induced changes in gut microbiome and fecal organic acids are associated with changes in cerebral spinal fluid (CSF) biomarkers of Alzheimer's disease (AD) in

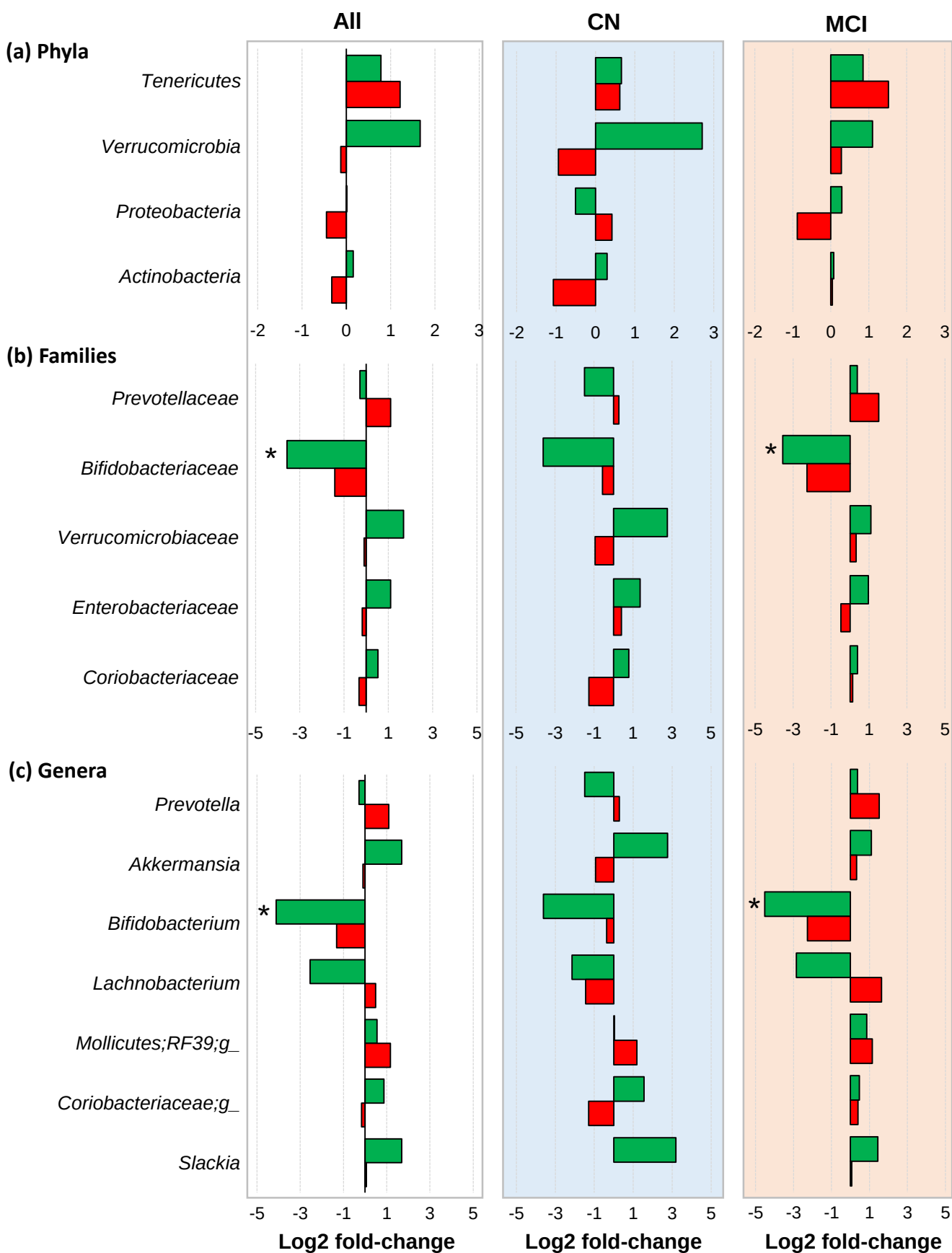
subjects clinically diagnosed with mild cognitive impairment (MCI) versus cognitively normal (CN) counterparts. Heat-map depicting the correlation patterns (Spearman R; $*p < 0.05$) of changes (percent-fold) in gut bacterial taxa and fecal organic acids with changes (percent-fold) in CSF biomarkers of AD in CN (n=6) versus MCI subjects (n=11) (e-h).

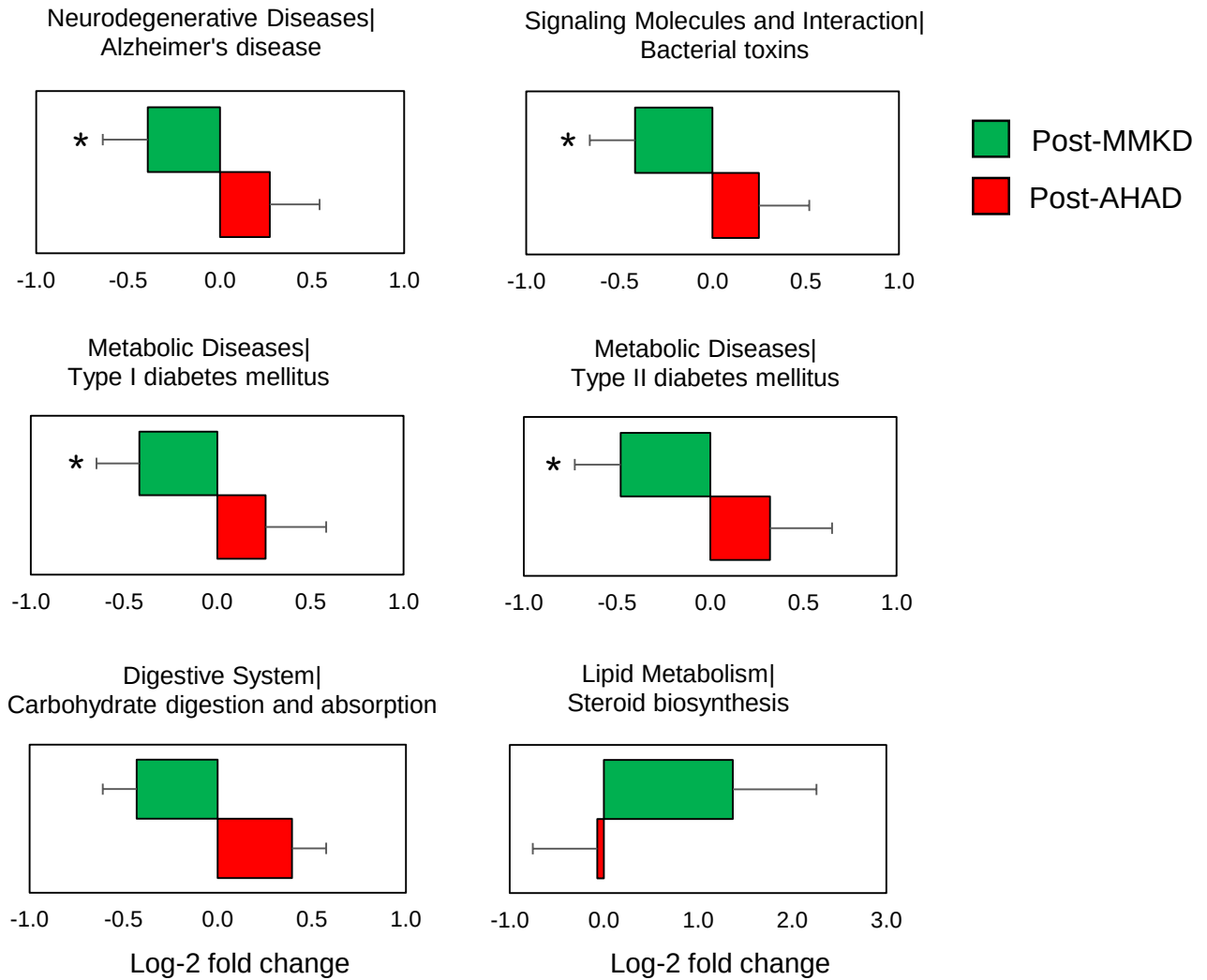


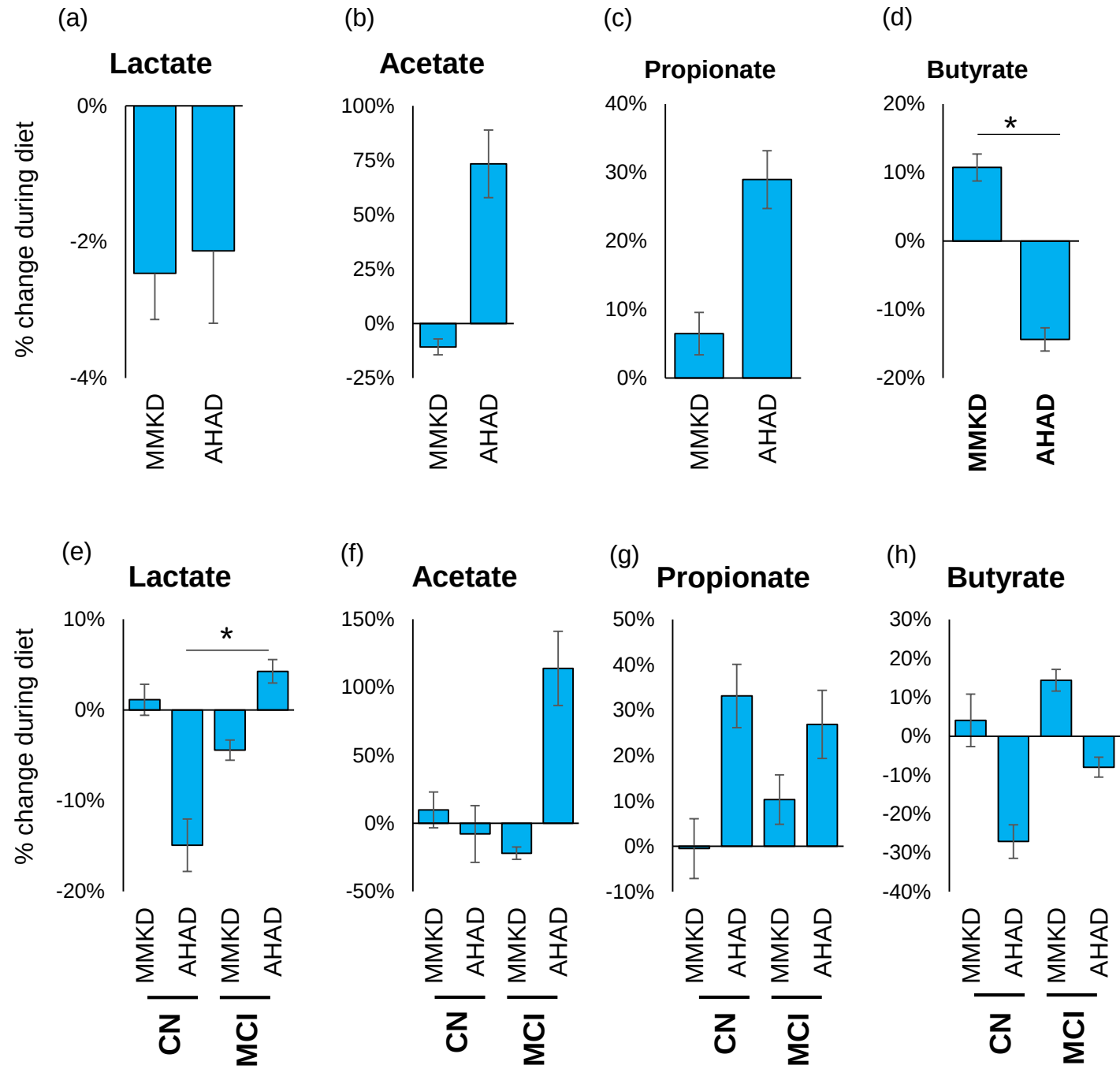


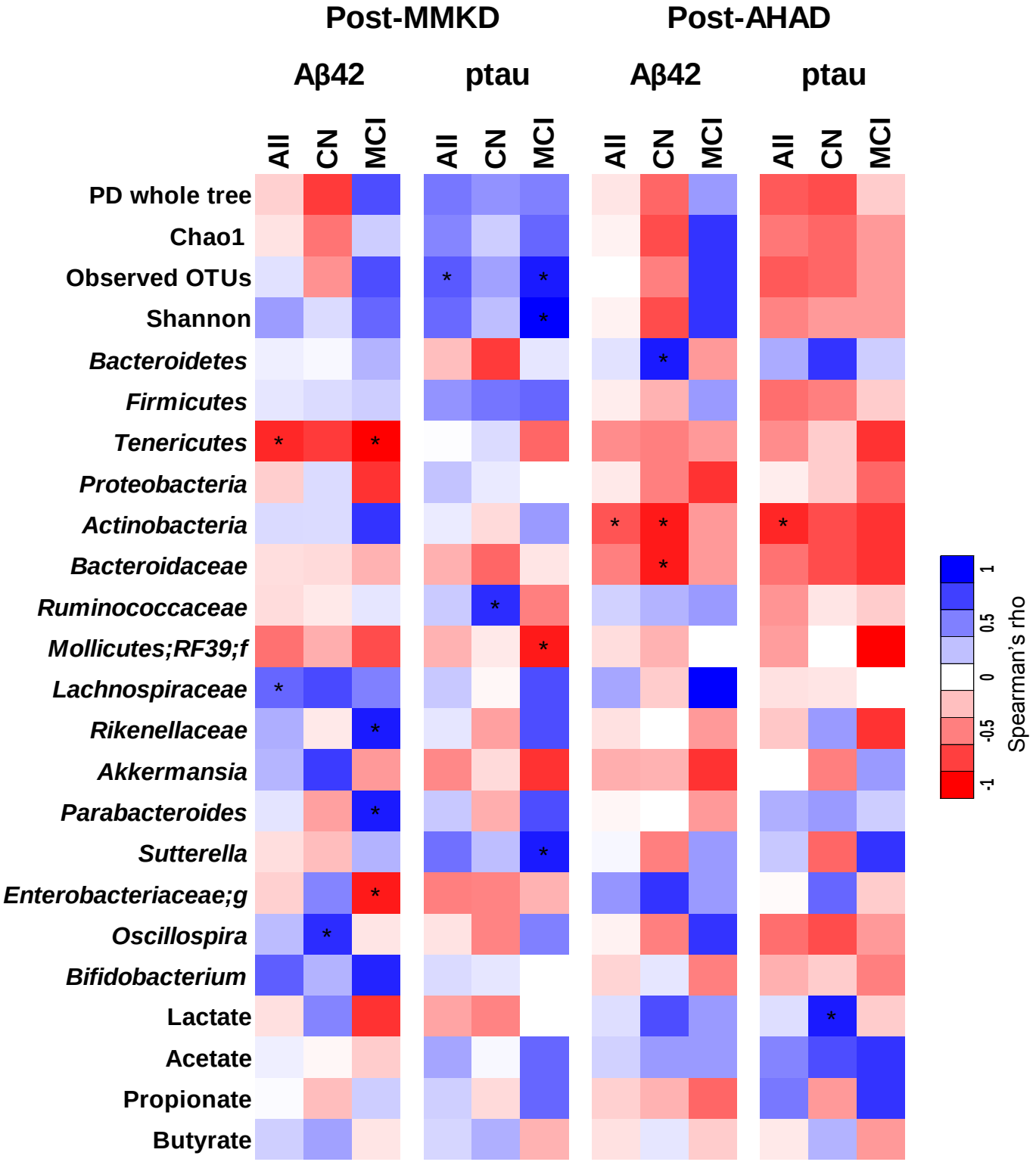


Post-MMKD Post-AHAD









Supplementary Figures

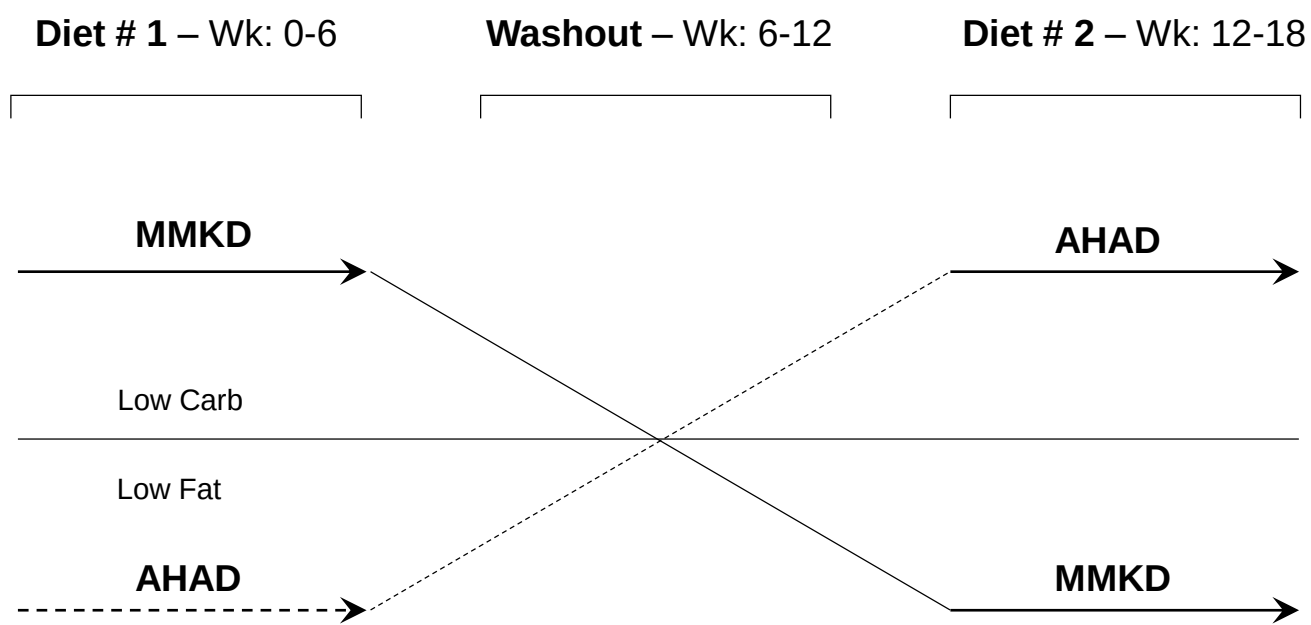
Nagpal et al.

Modified Mediterranean-Ketogenic diet modulates gut microbiome
and short-chain fatty acids in association with Alzheimer's disease
markers in subjects with mild cognitive impairment

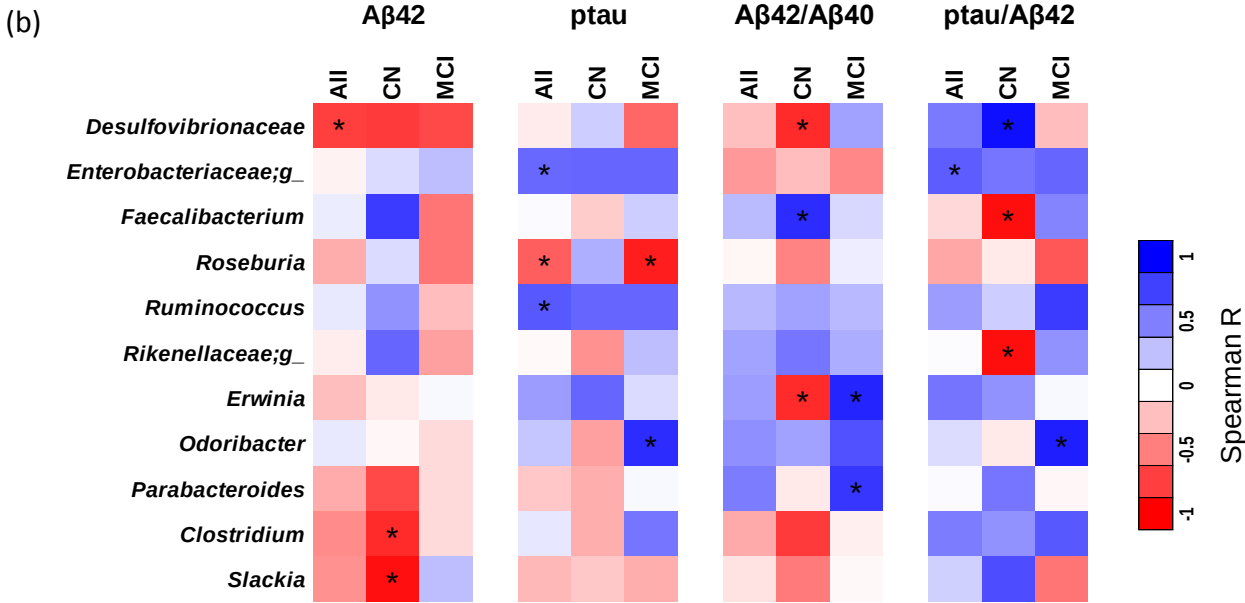
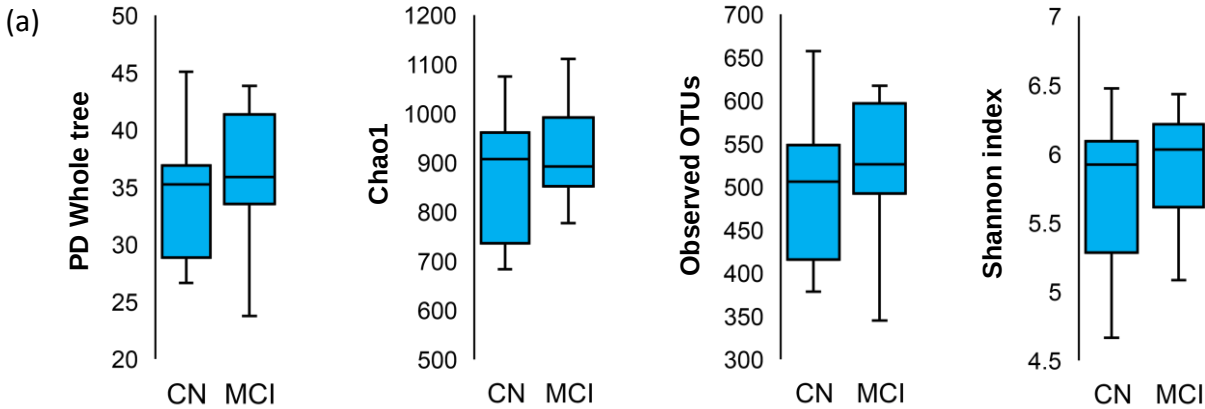
Suppl. Table 1. General characteristics of the participants enrolled in the study.

Total number of subjects	17	
Age, years (mean±SD)	63.6 ± 3.7	
Study groups	Cognitively normal (CN)	Mild cognitive impairment (MCI)
Number of subjects	6	11
N, APOE ε4 (E4) genotype	1	5

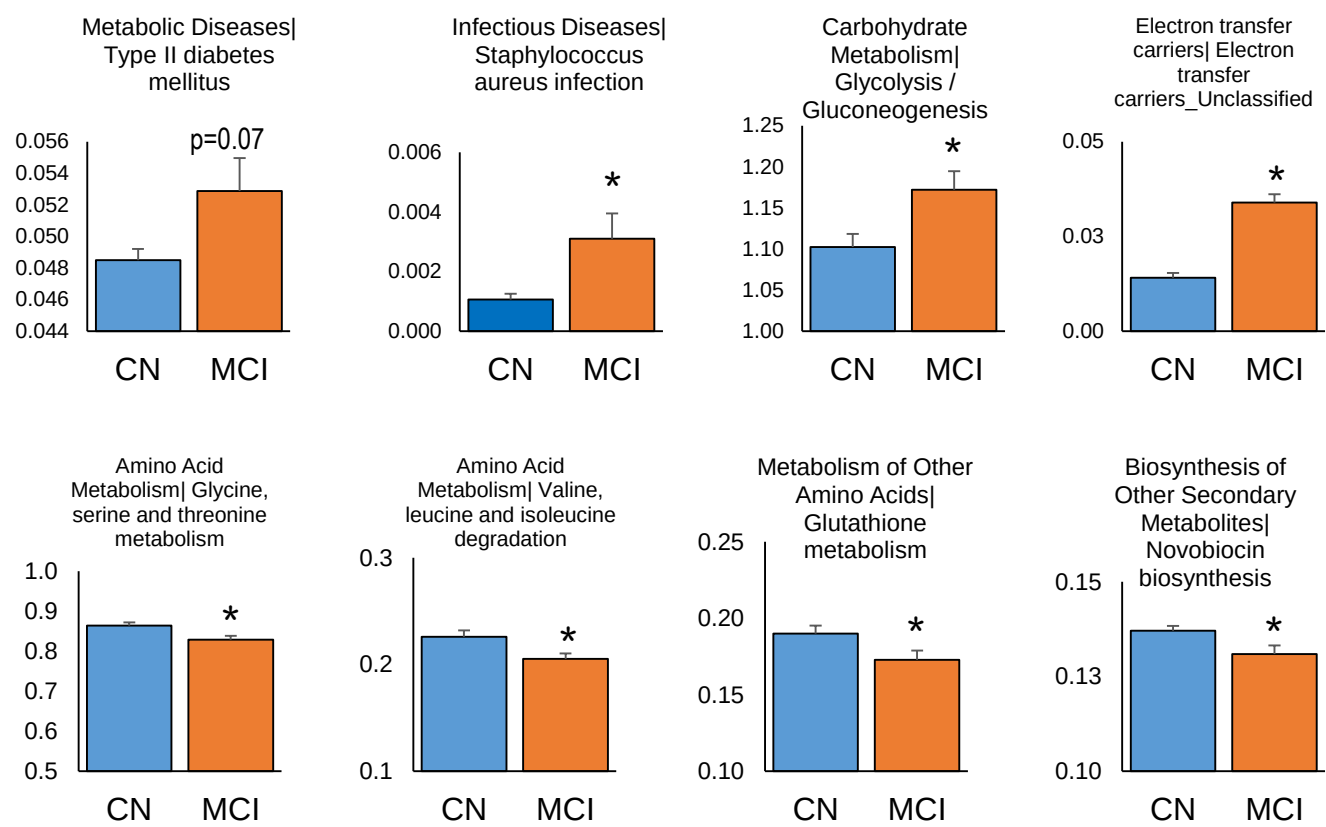
Suppl. Fig. 1. Study design.



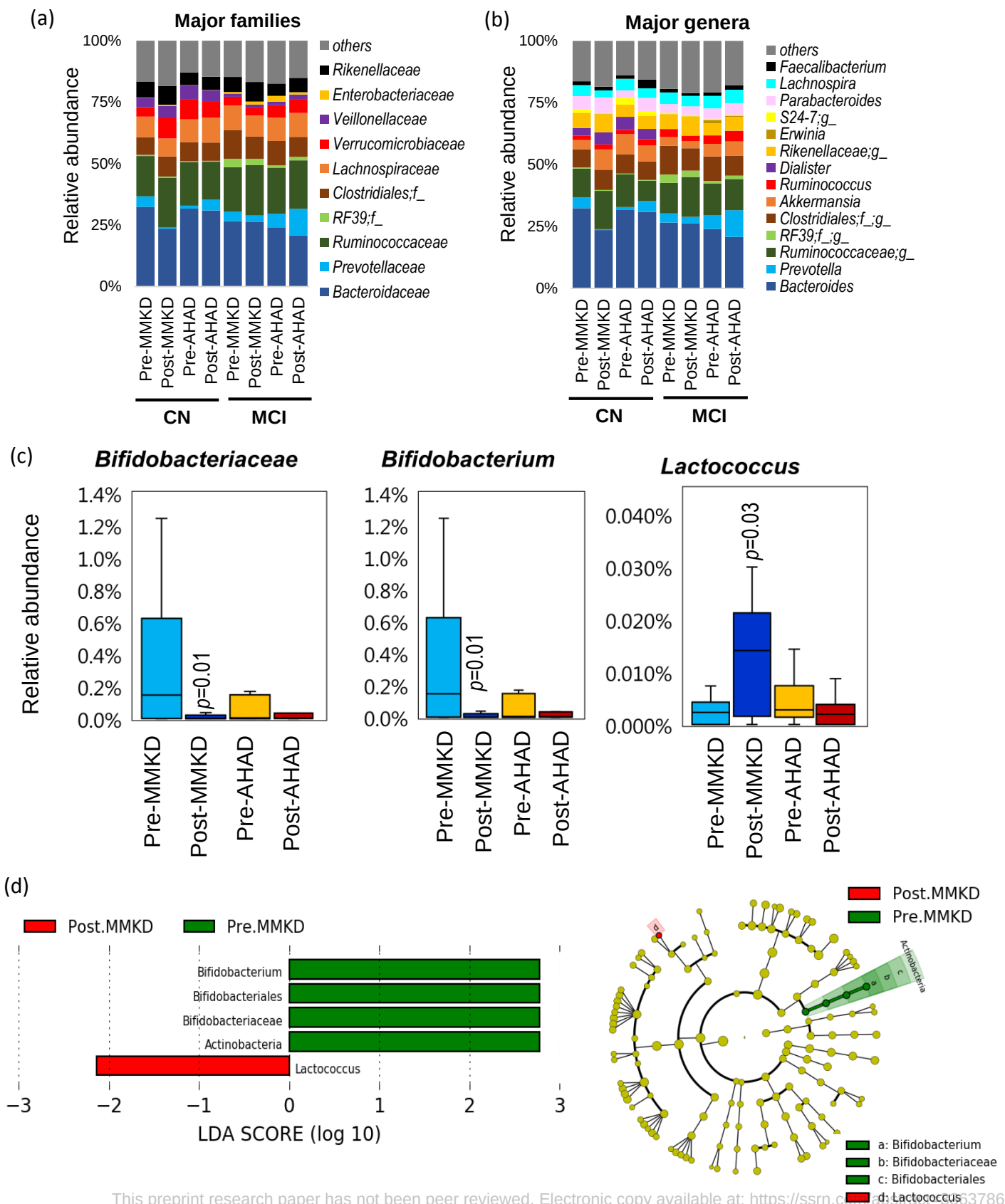
Suppl. Fig. 2. (a) Indices of the gut microbiome alpha-diversity in cognitively normal (CN) subjects versus subjects with mild cognitive impairment (MCI). (b) Association of gut bacterial taxa with cerebrospinal fluid biomarkers of Alzheimer's disease in CN and MCI subjects (*p<0.05).



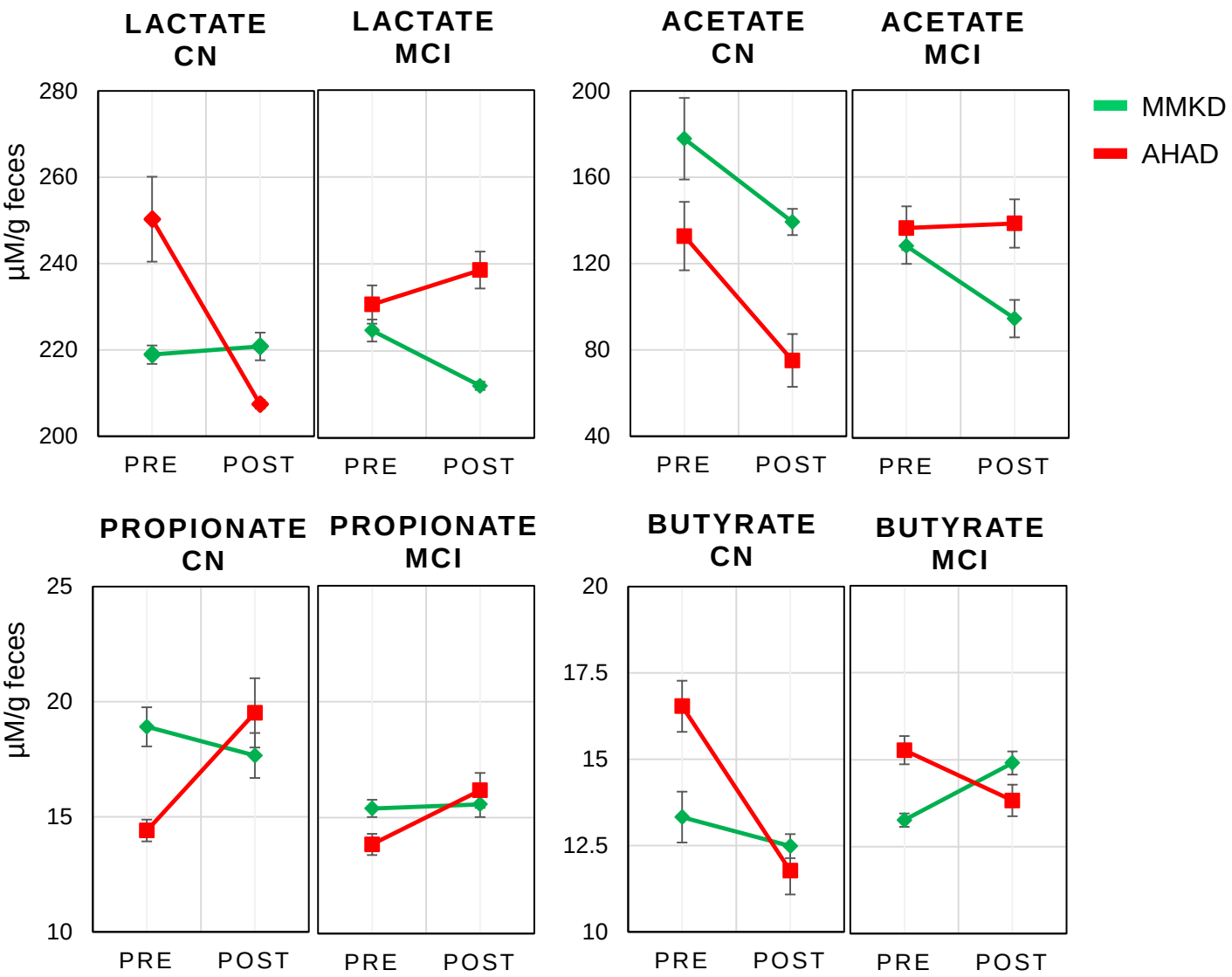
Suppl. Fig. 3. Differences in the functional analysis of the gut microbiome in subjects with MCI versus cognitively normal (CN) subjects. The relative abundance of the functional content (Level 2 and Level 3 KEGG pathway) of the gut microbiome was inferred by using PICRUST based on the 16S amplicon sequencing data. Only the pathways showing considerable difference between the two groups are shown here (* $p < 0.05$).



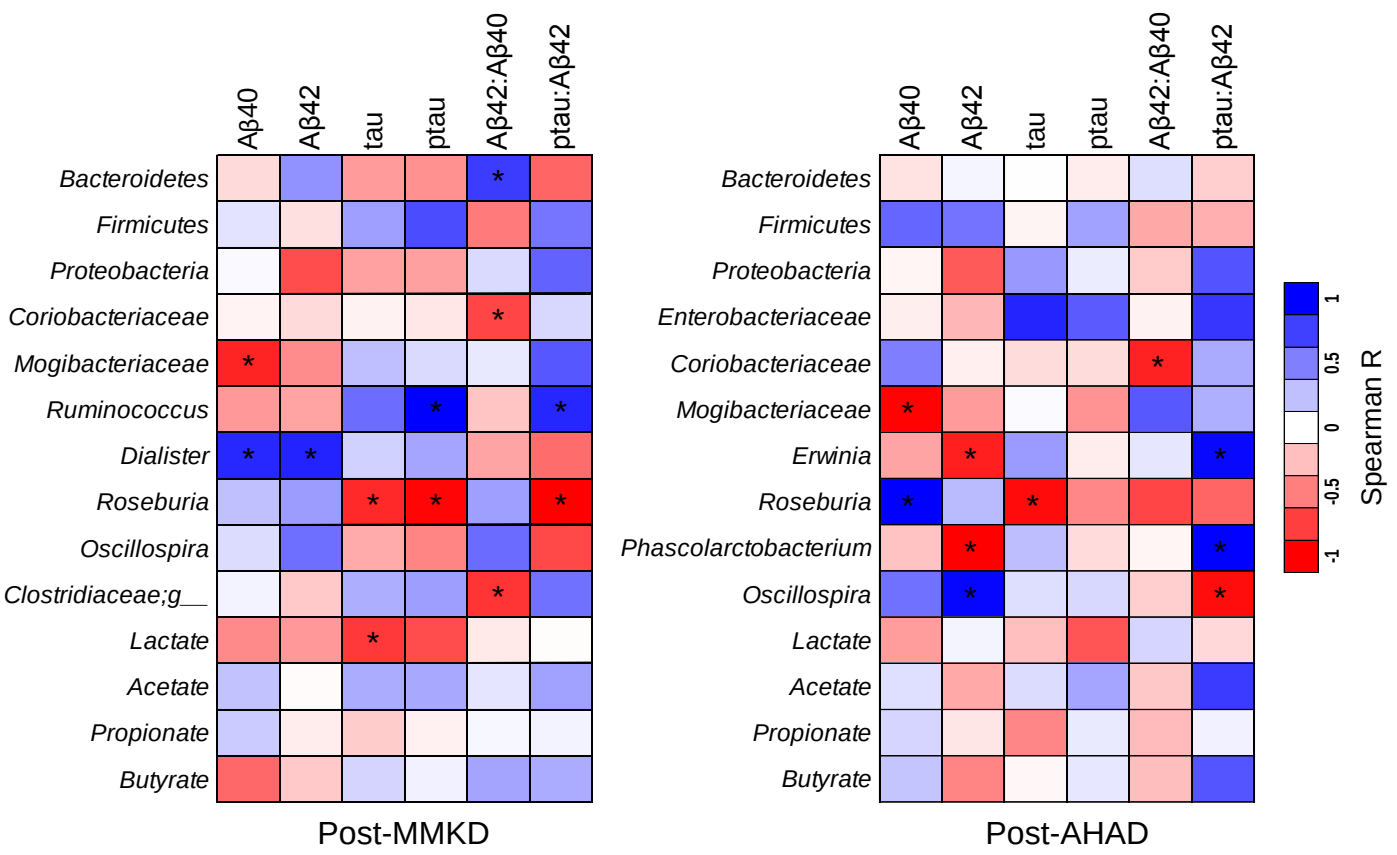
Suppl. Fig.4. Comparison of family- and genus-level gut microbiome composition (a-b) and the relative abundance of selected bacterial taxa (c) at baseline and endpoint of MMKD and AHAD intervention in subjects with mild cognitive impairment (MCI; n=11) versus cognitively normal (CN) counterparts (n=6). (d) Lefse analysis showing bacterial taxa contributing to the microbiome difference between the two diet groups.



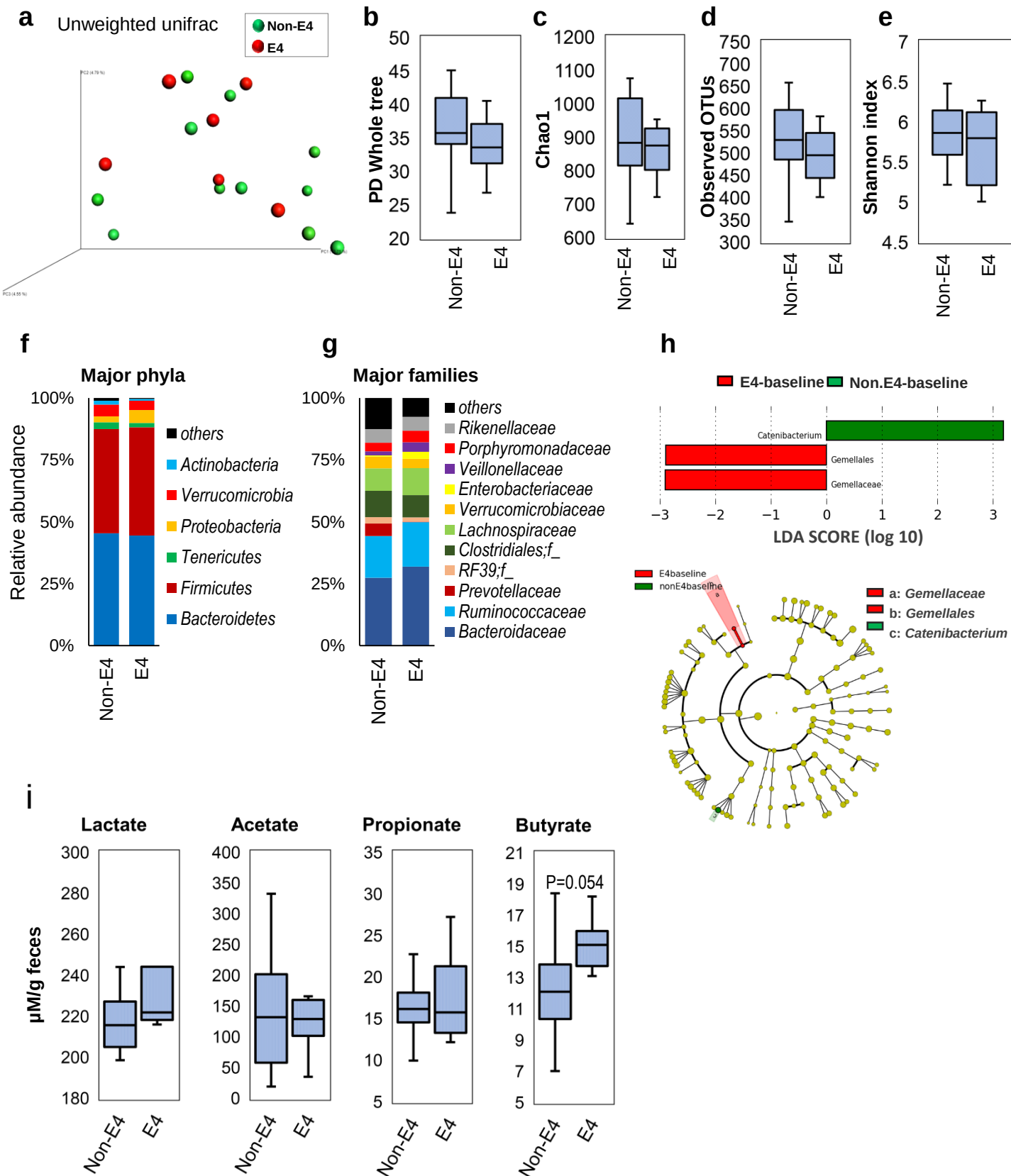
Suppl. Fig. 5. Fecal concentration of lactate and short-chain fatty acids at baseline and endpoint of MMKD and AHAD intervention in subjects with mild cognitive impairment (MCI; n=11) versus cognitively normal (CN) counterparts (n=6).



Suppl. Fig. 6. Association of gut bacterial taxa and fecal organic acids with cerebral spinal fluid biomarkers of Alzheimer's disease in all subjects combined (n=17) (*p<0.05).



Suppl. Fig. 7. Beta-diversity, (a) alpha-diversity indices (b-e), genus- and family-level gut microbiome composition (f-g), and fecal concentration of organic acids (i) in MCI subjects with or without EpoE-4 (E4) genotype. (h) Lefse analysis showing bacterial taxa contributing to the microbiome difference between the two groups.



Suppl. Fig. 8. Gut microbiome composition at phylum-, family- and genus level (a-c) at baseline and endpoint of MMKD and AHAD intervention in subjects with or without EpoE-4 (E4) genotype. (d) Mean Log₂-fold change in major bacterial taxa and fecal organic acids during the two diets.

