Clinical Nutrition 41 (2022) 2565–2576

Contents lists available at ScienceDirect

Clinical Nutrition

journal homepage: http://www.elsevier.com/locate/clnu

Original article

The gut microbiome, mild cognitive impairment, and probiotics: A randomized clinical trial in middle-aged and older adults

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ARTICLE INFO

Article history:
Received 24 May 2022
Accepted 21 September 2022

Keywords:
Gut microbiome
Cogitative aging
Psychobiotics
Lactobacillus rhamnosus GG
Prevotella

SUMMARY

Background: Advancing age coincides with changes in the gut microbiome and a decline in cognitive ability. Psychobiotics are microbiota-targeted interventions that can result in mental health benefits and protect the aging brain. This study investigated the gut microbiome composition and predicted microbial functional pathways of middle-aged and older adults that met criteria for mild cognitive impairment (MCI), compared to neurologically healthy individuals, and investigated the impact of probiotic *Lactobacillus rhamnosus* GG (LGG) in a double-blind, placebo-controlled, randomized clinical trial. A total of 169 community-dwelling middle-aged (52–59 years) and older adults (60–75 years) received a three-month intervention and were randomized to probiotic and placebo groups. Participants were further subdivided based on cognitive status into groups with intact or impaired cognition and samples were collected at baseline and post supplementation.

Results: Microbiome analysis identified *Prevotella ruminicola*, *Bacteroides thetaiotaomicron*, and *Bacteroides xylanisolvens* as taxa correlated with MCI. Differential abundance analysis at baseline identified *Prevotella* as significantly more prevalent in MCI subjects compared to cognitively intact subjects (ALDEx2 $P = 0.0017$, ANCOM-BC $P = 0.0004$). A decrease in the relative abundance of the genus *Prevotella* and *Dehalobacterium* in response to LGG supplementation in the MCI group was correlated with an improved cognitive score.

Conclusions: Our study points to specific members of the gut microbiota correlated with cognitive performance in middle-aged and older adults. Should findings be replicated, these taxa could be used as key early indicators of MCI and manipulated by probiotics, prebiotics, and symbiotics to promote successful cognitive aging.

Registered under ClinicalTrials.gov Identifier no. NCT03080818.

Abbreviations: MCI, mild cognitive impairment; LGG, *Lactobacillus rhamnosus* GG; AD, Alzheimer's disease; PD, Parkinson's disease; IL-8, interleukin-8; OCD, obsessive-compulsive disorder; WGS, whole-genome shotgun; GlnRS, glutaminyl-tRNA synthetase; SIA, species indicator analysis; PCoA, principal coordinate analysis; PICRUSt, phylogenetic investigation of communities by reconstruction of unobserved states; ZIBR, zero-inflated beta regression model with random effects; RCT, randomized clinical trials; MDD, major depressive disorder; BPD, bipolar disorder with current major depressive episodes; MHE, minimal hepatic encephalopathy; HD, Huntington's disease; GABA, gamma-aminobutyric acid; SCFAs, Short-chain fatty acids; POCD, postoperative cognitive dysfunction; ASD, autism spectrum disorders; MIND, mediterranean-DASH intervention for neurodegenerative delay; TSS, total sum scaling; CLR, centered log-ratio; LEfSe, linear discriminant analysis LDA and effect size.

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https://doi.org/10.1016/j.clnu.2022.09.012

1. Introduction

Cognition encompasses long and short-term knowledge acquisition processes and can be divided into different cognitive domains including attention, executive function, memory, visuospatial function, psychomotor speed, and social cognition [1–3]. Advancing age coincides with a decline in cognitive abilities and physiological changes in global and regional brain size [4]. Over the past century, there has been a rapid increase in average life expectancy with the life span of men and women increasing from 48 to 52 years to 76 and 81 years, respectively. Thus the number of individuals with age-associated impairments in cognitive functions is rapidly raising [2,5].

There is growing evidence that the gut microbiome is an important contributor to brain aging. The term gut microbiota refers to the collection of trillions of micro-organisms that colonize the gastrointestinal tract [6]. Pro-inflammatory changes in the gut microbiome have been linked to a range of medical conditions [7–9], and more recently to cognitive impairment and neurological conditions. For example, the composition of the gut microbiome has been associated with the development of Alzheimer's disease (AD), Parkinson's disease (PD), and stroke [10] and was also shown to differ in older adults with and without cognitive dysfunction [11].

Despite these findings, little is known about the association between the composition of the gut microbiome and cognitive function in healthy older adults. A better understanding of this relationship is needed to help clarify the contribution of the gut microbiome to cognitive outcomes. For example, both gut dysbiosis and cognitive decline have been linked to the consumption of a high-fat Western diet [12], a sedentary lifestyle, and cigarette smoking [13–16]. Understanding age-mediated changes and associations impacting the gut microbiome and cognitive function is vital to helping our expanding elderly population maintain functional independence and can also facilitate the development of new therapeutic strategies like probiotics to protect the aging brain.

Psychobiotics are defined as microbiota-targeted interventions including beneficial bacteria (probiotics) or the support for such bacteria (*e.g.*, prebiotics) that result in mental health benefits [17,18]. Probiotics, live microorganisms that can provide health benefits to humans [19], have been shown to enhance the beneficial microbiota and reduce inflammation [20]. Studies have found cognitive benefits of probiotic supplementation in persons with AD [21] and mild cognitive impairment (MCI) [22,23], as well as neurologically healthy older adults [24]. One promising probiotic strain is *Lactobacillus rhamnosus* GG (LGG), which is known for its ability to persist in the gut due to its bile resistance abilities and adhesive properties [25]. LGG has been associated with ample beneficial effects varying from protecting the gut lining and intestinal epithelial cells [26,27], reducing proinflammatory cytokines like interleukin-8 (IL-8) [28,29], and improving levels of neurogenesis in the hippocampus [30]. In mice, administration of LGG has been correlated with decreased anxiety-like [31], obsessive-compulsive disorder (OCD)-like [32], and depressive behaviors [33].

An initial publication from this clinical intervention showed that LGG supplementation was associated with improved cognitive function in persons meeting the criteria for MCI [22]. However, the effects of LGG on the gut microbiome and the contribution of such changes on cognitive function have not been examined. The purpose of the current analysis was to identify differences in the gut microbiota composition of healthy elderly individuals with and without cognitive impairment and investigate the effect of LGG supplementation on microbiome composition associated with amelioration of cognitive decline in middle-aged and older adults with MCI observed in our previous work.

2. Methods

2.1. Participants and study trail

The current study design has been detailed previously and consort flow diagram of the study progress is shown in [22] and can be accessed on the clinicaltrials.gov website (Identifier# NCT03080818). The study was carried out in accordance with the Declaration of Helsinki, and data were obtained in compliance with the regulations by Institutional Review Board at Kent State University (approval no. #16-321).

The clinical trial enrolled a total of two hundred healthy middle-aged and older adults, participants who dropped out of the trial and participants with low compliance (below 80%) were excluded from the analysis. Additionally non-paired subjects were removed from the longitudinal analysis. Briefly, a total of 169 participants, age ranging from 52 to 75 years were included in a randomized, placebo-controlled, double-blinded trial aimed to test the impact of *L. rhamnosus* GG (LGG) supplementation on cognitive functions in healthy middle-aged and older adults. The LGG supplement was in the form of two capsules of the Culturelle Vegetarian Capsules containing a 10 billion CFU blend of *L. rhamnosus* GG and 200 mg prebiotic inulin from chicory root extract (manufactured by iHealth, Inc., Cromwell, CT, USA). The placebo group received two capsules of Culturelle Placebo Veggie capsules containing microcrystalline cellulose, which cannot be fermented by the gut microbiota. The only probiotic supplement that participants had during the trail was the LGG formulation provided. In the event participants received specific medications including antibiotics and proton pump inhibitors, they were excluded. A summary of participants' demographics at baseline is presented in Table 1. Participants had a baseline cognitive function assessment and a three-month

Table 1
Participant's characteristics at baseline (N = 169).[a]

	Placebo group	Probiotic group
Number of participants	83	86
Gender (m/f)	28/55	38/48
Age (years)	64.2 (5.4)	64.4 (5.5)
Body mass (lb.)	172.4 (42.5)	176.1 (50)
Body mass index (kg/m^2)	28.1 (6.8)	27.8 (6.7)
Education (years)	15.3 (2.4)	15.2 (2.6)
Cognitive impairment	23	21

[a] Values are expressed as mean (standard deviation).

Table 2
PCR primers used in the present study.

Target	Primer set	Sequence (5′ to 3′)	Target Gene	Amplicon size	Reference
Universal 16S rRNA (V4)	F515	GTGCCAGCMGCCGCGGTAA	16S	~300 to 350 bp	[36]
	R806	GGACTACHVHHHTWTCTAAT			
Total *Lactobacillus*	Lactobacillus_F	AGCAGTAGGGAATCTTCCA			[76]
	Lactobacillus_R	CACCGCTACACATGGAG	16S	341 bp	
LGG strain-specific	GG I	CAATCTGAATGAACAGTTGTC	Phage-related gene	470 bp	[77]
	GG II	TATCTTGACCAAACTTGACG			

assessment, in addition to adherence visits every four weeks. Stool samples were collected by all participant using stool kits at baseline and three-month follow-up visits and stored at −80 °C until the time of processing.

2.2. Cognitive performance assessment

For the neuropsychological assessment, participants were asked to complete the computerized NIH Toolbox for the Assessment of Neurological and Behavioral Function — Cognition [34]. Different domains of cognition were evaluated using the Picture Sequence Memory Test, the Auditory Verbal Learning Test, the Dimension Change Card Sort Test, the Flanker Inhibitory Control and Attention Test, the List Sorting Working Memory Test, and the Pattern Comparison Processing Speed Test. Individuals received a total cognitive performance score and were subcategorized into intact and impaired cognitive performance. All tests and assessments have been detailed in our previous study [22].

2.3. DNA isolation from stool samples

Total DNA isolation from stool samples was performed following the Qiagen ClearMag Extraction protocol using the KingFisher Flex Magnetic Bead processing system as described previously [35]. In short, stool samples were transferred to 2 ml screw-cap tubes containing 500 μL Qiagen PM1 buffer (Valencia, CA) and approximately 200 mg (diameter of ≤106 μm) sterile glass beads (Sigma, St. Louis, MO). Followed by mechanical lysis for 5 min using the Qiagen TissueLyser II at 30 Hz. Then samples were centrifuged for 5 min, and the supernatant was transferred to a new tube containing 150 μL of Qiagen IRS solution and incubated overnight at 4 °C. After incubation, samples were briefly centrifuged and the supernatant was aspirated and transferred to KingFisher Deep-well plate containing 450 μLof Qiagen binding buffer with ClearMag magnetic beads (Qiagen). DNA was subsequently purified using KingFisher™ Flex automated System, eluted in DNase-free water, and stored at −20 °C. Total DNA concentration was quantified using the Quant-iTTM PicoGreenR dsDNA reagent (Molecular Probes, Thermo Fisher Scientific, Waltham, MA).

2.4. Amplification and sequencing of the 16S rRNA gene

The microbiota composition was determined by amplification of the hypervariable region V4 of the 16S rRNA gene using universal primers with Illumina compatible adaptors (Table 2) [36]. Briefly, a PCR reaction with a total volume of 25 μL containing 12.5 ng of total DNA, 0.5 μM of forward and reverse primers, and 2× KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, MA) was carried out on sample DNA. Amplicons were purified using the AMPure XP reagent (Beckman Coulter, Indianapolis, IN). Unique barcodes were added to each sample using Illumina dual-index barcodes (Index 1(i7) and Index 2(i5)) (Illumina, San Diego, CA). Libraries were purified and quantified using the same methods mentioned earlier. The final library was prepared by normalizing samples and pooling them in equimolar amounts. Sequencing was performed on the Illumina MiSeq platform using 2 × 250 bp paired-end sequencing.

2.5. Bioinformatic analysis of 16S rRNA amplicon sequencing data

Raw sequencing data were initially converted to fastq format and demultiplexed using Illumina Bcl2Fastq 2.18.0.12. The resulting paired-end reads were processed with the QIIME 2 2018.11 [37] wrapper for DADA2 [38] including merging paired ends, quality filtering, error correction, and chimera detection. Amplicon sequencing units from DADA2 were assigned taxonomic identifiers using the Greengenes [39] and Silva [40] databases. Quantification results were normalized and transformed to relative abundance using Total Sum Scaling (TSS) normalization and Centered log-ratio (CLR) transformation. Diversity analysis and visualization were performed using: QIIME 2, R (version 4.0.3) Phyloseq, Calypso (http://cgenome.net/calypso/), and MicrobiomeAnalyst web tools [41,42]. Species accumulation and rarefaction curves were estimated using QIIME 2 at a rarefaction depth of 5000 sequences per subsample. Observed, Chao1, Shannon, and Simpson alpha diversity indexes were calculated to assess diversity, and the Wilcoxon test was applied to assess the statistical significance of differences between groups. Unpaired and pairwise beta diversity estimates were calculated within Calypso and QIIME 2 using weighted distances as well as Bray–Curtis dissimilarity. Results were visualized through principal coordinate analysis. ANCOM, ANCOM — II, ALDEx2, and ANOVA tests were used to identify significant changes in taxa abundance between groups. Differential abundance analysis was performed using ANCOM, ANCOM — II, and ALDEx2 to the compositional difference of relative abundance. In the case of ANCOM and when required to calculate the compositional difference, a nominal value was applied to ensure non-zero values. Pairwise differential abundance analysis was performed using the zero-inflated beta regression model with random effects (ZIBR) [43]. All *P* values were corrected using Benjamin-Hochberg (FDR) correction. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) 2.3.0-b analysis was performed using KEGG Orthologue functional classifications. PICRUSt results were visualized using Statistical Analysis of Metagenomic Profiles (STAMP) software. Statistical significance of differences between groups was estimated using ANOVA. Linear discriminant analysis LDA and effect size (LEfSe) and species indicator analysis (SIA) were applied to determine the bacterial taxa most likely would be responsible for differences between groups using r package ('lefser') and ('indicspecies ') respectively [44]. An overview of the microbiome analysis is illustrated in Fig. 1.

2.6. Whole genome shotgun (WGS) sequencing

A subset of samples was selected for WGS based on cognitive performance (n = 10 per group). Libraries were prepared using the Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA). Briefly, 5 ng of genomic DNA was fragmented and tagged by adding bridge PCR (bPCR)-compatible adaptors using the Nextera Enzyme Mix. Followed by a limited-cycle PCR program where the Illumina dual-index barcodes and primer sequences for cluster formation were added. Libraries were purified using Agencourt® AMPure® XP Reagent. Clean libraries were quantified, normalized, pooled in an equimolar pool and run on Illumina NextSeq P2/PE/2 × 150 instrument [45].

2.7. Bioinformatic analysis of WGS sequencing data

Raw data processing and demultiplexing of NextSeq data was carried on using Illumina Bcl2Fastq 2.18.0.12. Quality control of the demultiplexed sequencing reads was verified by FastQC (Babraham Institute, Cambridge, UK). Adapters were trimmed using Trim Galore (Babraham Institute, Cambridge, UK). The resulting paired-end reads were classified with Kraken2 [46] and Bracken 2.5 [47] and all reads identified as host were eliminated. Paired-end reads were joined with vsearch 1.10.2 [48]. The resulting single-end reads were again trimmed of any remaining adapters using Trim Galore. Estimates of taxonomic composition, gene family, path abundance, and path coverage were produced from the remaining reads using HUMAnN2 [49]. Alpha diversity was estimated by Shannon index

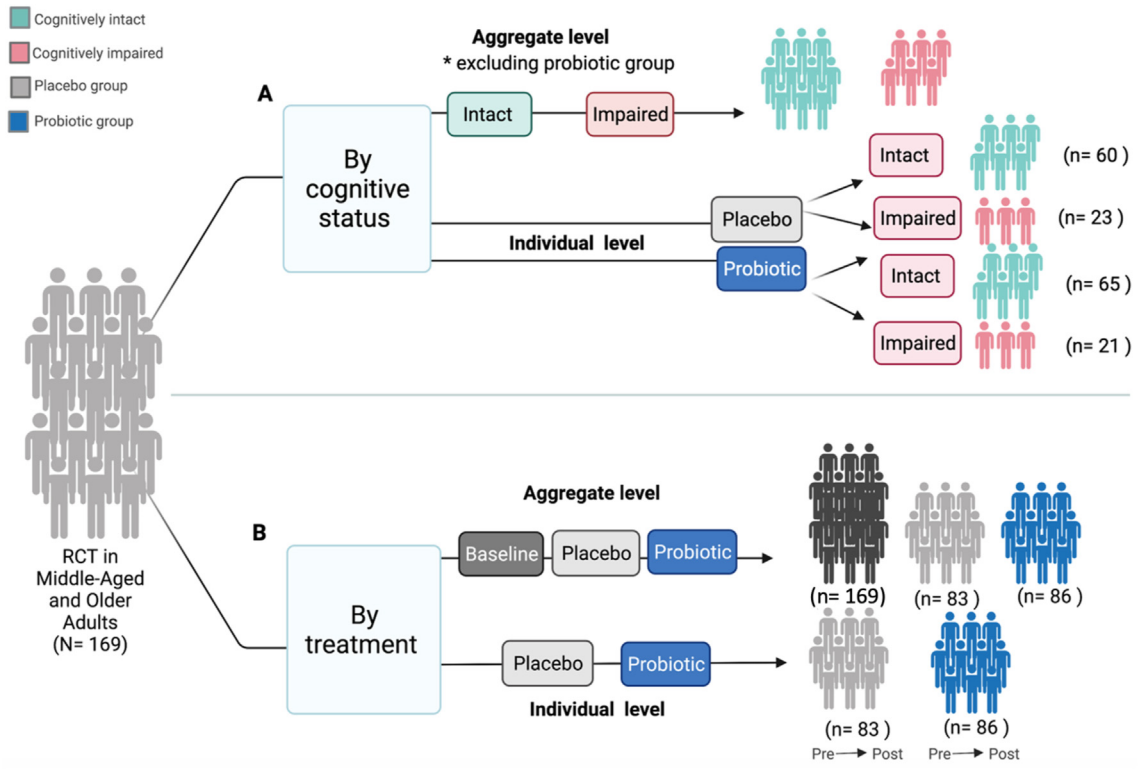

Fig. 1. Overview of data analysis A by cognitive status, and **B** by treatment group.

and observed species count. Beta diversity estimates were measured by Bray–Curtis dissimilarity between samples and plotted using principal coordinate analysis. Visualization of results was performed using QIIME 2 and MicrobiomeAnalyst. 16S rRNA and WGS sequencing data are available upon request from Dr. John Gunstad.

2.8. Quantitative (q)PCR analysis

qPCR was performed on a QuantStudio Q6 (Applied Biosystems). Each reaction had a total volume of 10 μl and included: 1 μl normalized sample DNA, 0.4 uM final primer concentration of each primer set, and 5 μl PowerSybr qPCR master mix (BioRad). For quantitative analysis of the threshold cycle, samples were compared against their standard curve generated earlier under the following cycling conditions: 50 °C for 2 min followed by 95 °C for 10 min, 40 cycles of (95 °C: 15 s, 60 °C: 30 s, and 72 °C: 45 s). Melting curve analysis was carried out to assess off-target amplification and primer dimerization. Data analysis of copy number for each sample in Copies/ng total DNA was carried out with Q6 Software (Applied Biosystems).

For generation of standard curves, DNA was isolated from a microbial community standard (Zymo Research Corporation, Irvine, CA, USA), a consortium of *Lactobacillus* strains (1 ng of *L. rhamnosus* GG AMC0221 (ATCC 53103), *L. rhamnosus* AMC0220 (HN001), *L. paracasei* AMC0143, *L. reuteri* AMC0733, *Lactobacillus plantarum* ATCC BAA 793), and the strain *L. rhamnosus* GG (AMC0221). Using conventional PCR, normalized DNA and target-specific primers were used for standard curve PCR amplification reactions (Table 2). The reaction mix total volume was 50 μl and contained 25 μl KAPA HotStart High Fidelity Master Mix, 2 μl of primer mix (10 nmol), 5 μl DNA template (1 ng), 18 μl H_2O. The thermal cycler was programmed for initial denaturing: 95 °C for 3 min, followed by 30 cycles of (95 °C: 20 s, 60 °C:20 s, 72 °C:30 s) and a final extension at 72 °C for 3 min. Amplicons were run on a 1% agarose gel and single bands were purified using the QiaQuick gel extraction kit (Qiagen, Santa Clarita, CA, USA), and concentrations were quantified using the QuantIT PicoGreen dsDNA reagent kit. Copy numbers were calculated and each sample was subsequently diluted based on copy numbers for standards in qPCR reactions started at 100 copies and increased by 10-fold increments up to 100,000,000 copies. Standard curves were freshly prepared with each qPCR reaction and validated in triplicate.

3. Results

We have reported that the healthy middle-aged and older adults in this cohort that received LGG had improvement on neuropsychological testing over a three-month period in participants with mild cognitive impairment (MCI) at baseline [22]. In this study, using 16S rRNA amplicon and whole-genome shotgun (WGS) sequencing we first investigated the microbiome diversity and composition associated with MCI independent of probiotic treatment, and then determined the impact of *L. rhamnosus* GG on microbiome composition at the aggregate level (baseline, placebo-after, and probiotic-after) and at the individual level (longitudinal analysis) in relation to cognitive status (intact, impaired) Fig. 1.

Overall, the gut microbiota of participants in the trial was dominated by five major phyla, mainly *Bacteroidetes* (48%) and *Firmicutes* (46%), followed by *Proteobacteria* (2.3%), *Actinobacteria* (1.5%), and *Verrucomicrobia* (1.3%) (Supplementary Figs. 1 and 2). The top ten genera in the fecal microbiota were *Bacteroides* (36.8%), *Faecalibacterium* (6.5%), *Alistipes* (4.5%), *Parabacteroides* (3.6%), *Blautia* (2.8%), *Agathobacter* (2.6%), *Prevotella* (2.3%), *Subdoligranulum* (2.2%), *Roseburia* (1.8%), and *Anaerostipes* (1.3%).

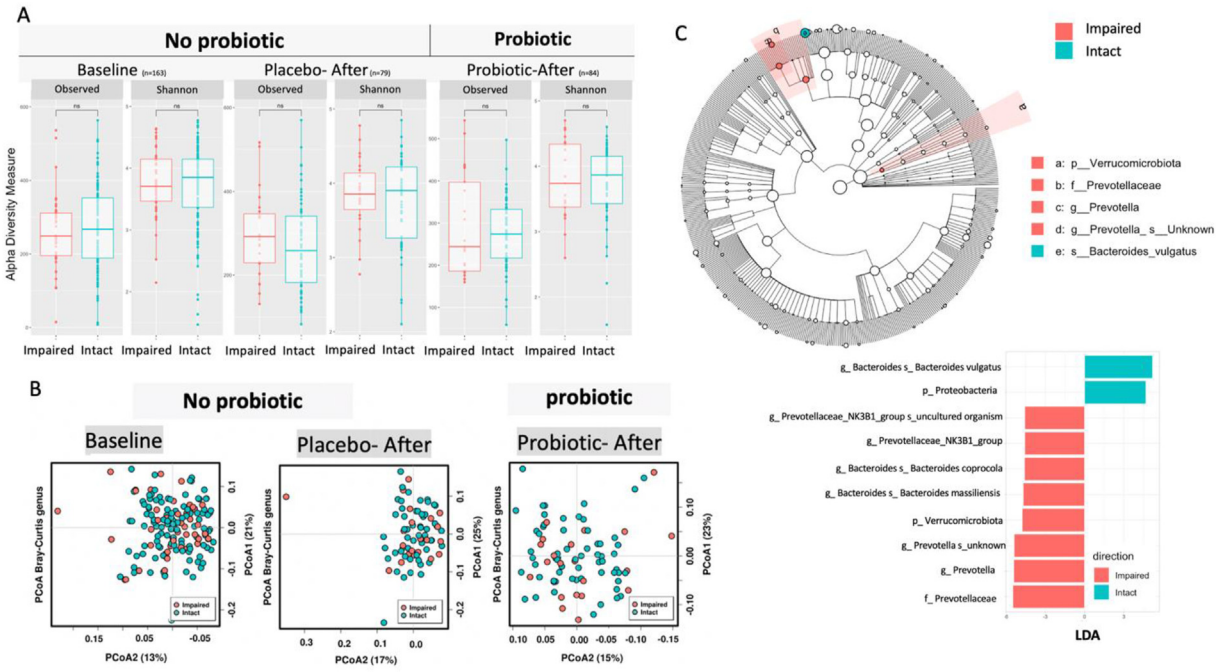

Fig. 2. 16S rRNA-based microbiome composition associated with cognitive performance within treatment groups. (A) Non-paired alpha diversity. (B) Principal Coordinate analysis (PCoA) plot using Bray Curtis distance showing no clustering based on cognitive status. (C) Cladogram and Linear Discriminant Analysis of effect size (Lefse) showing biomarker taxa associated with cognitively intact and impaired individuals. Lefse analysis included non-probiotic groups only. The cladogram was generated using Kruskal–Wallis test = 0.05, Wilcoxon-Rank Sum test = 0.05, and an LDA cutoff score = 4.

3.1. Microbiome diversity and composition associated with cognitive status independent of probiotic effect

With the goal of identifying potential differences in microbiome components associated with intact or impaired cognitive status, participants were grouped by treatment [non-probiotic groups (baseline and placebo), and probiotic group] and then further subdivided based on their cognitive status (intact, impaired). No significant differences were identified in diversity and overall composition associated with cognitive state across non-probiotic and probiotic groups (Fig. 2A and B) by 16S rRNA amplicon sequencing. LEfSe analysis of non-probiotic groups to identify specific taxa most likely to describe each cognitive group identified *Proteobacteria* associated with cognitively intact individuals while *Verrucomicrobia* was associated with the cognitive impaired group. At the genus and species level, *Bacteroides vulgatus* was associated with intact cognition while *Bacteroides coprocola*, *Bacteroides massiliensis*, *Prevotellaceae* NK3B1 group, and an unknown species of the genus *Prevotella* were associated with cognitively impaired individuals (Fig. 2C).

Differential abundance analysis at baseline identified the genus *Prevotella* as more prevalent in cognitively impaired individuals compared to cognitively intact individuals (ALDEx2 $P = 0.0017$, ANCOM-BC $P = 0.0004$). Additionally, univariate comparison analysis showed that the genus *Prevotella* had significantly higher relative abundance in the cognitively impaired group (Adj. $p = 0.0136$) (Fig. 3A). Moreover, using random forest analysis we found that *Prevotella* was ranked as the highest contributor to classification accuracy between cognitive groups (Mean Decrease Accuracy) (Fig. 3B).

PICRUSt analysis identified three features from the EC database and two orthologs from the KO database that were differentially represented between groups. An FMN reductase [NAD(P)H] and glutaminyl-tRNA synthetase (GlnRS) had a higher relative abundance in the impaired group (Adj. $p = 0.045$ and 0.040 for EC, and 0.032 and 0.017 for KO), while an hygromycin B 4-O-kinase had a lower abundance in the intact group (Adj. $p = 0.041$ EC) (Fig. 3C and D). No pathways were significantly differentially represented after correcting for multiple comparisons (Supplementary Fig. 3A).

We next selected the subset of samples of individuals that had the highest *Prevotella* relative abundance in both cognitive groups for WGS sequencing in order to capture most of the species' diversity within the genus. WGS sequencing data showed significant differences in alpha and beta diversity by cognitive performance. Cognitively impaired individuals had a significantly lower evenness at the genus level ($p = 0.0372$), and family level ($p = 0.064$). Furthermore, beta diversity analysis illustrated significant clustering at family and genus levels ($p = 0.03$, and $p = 0.049$, respectively). At the species level, clustering based on the cognitive status continued, but at a lower level of statistical significance ($p = 0.063$) (Fig. 4A). Heat maps showing the relative abundance of top species are presented in (Fig. 4B). SIA and LEfSe analysis identified one species, *Bacteroides thetaiotaomicron* (SIA $p = 0.037$, LEfSe $p < 0.05$) associated with cognitively impaired participants. This observation was consistent with SparCC correlation and random forest analysis, which also identified *Bacteroides xylanisolvens* as correlated with cognitive impairment. On the other hand, cognitively intact subjects were associated with *Bifidobacterium longum*, *Bifidobacterium breve, and Faecalibacterium prausnitzii* (Fig. 4C and D).

Since our initial analysis indicated an increased abundance of *Prevotella* in association with mild cognitive impairment, we next identified *Prevotella* species potentially associated with the phenotype. A total of 12 species of *Prevotella* were identified by Bracken in both cognitive groups and included *Prevotella scopos, Prevotella ruminicola, Prevotella melaninogenica, Prevotella jejuni, Prevotella intermedia, Prevotella fusca, Prevotella enoeca, Prevotella oris, Prevotella denticola, Paraprevotella xylaniphila, Prevotella dentalis*, and *Prevotella* sp. oral taxon 299. *Prevotella ruminicola* was identified by Random Forest analysis as the highest-ranked species by its contributions to classification accuracy (Mean Decrease

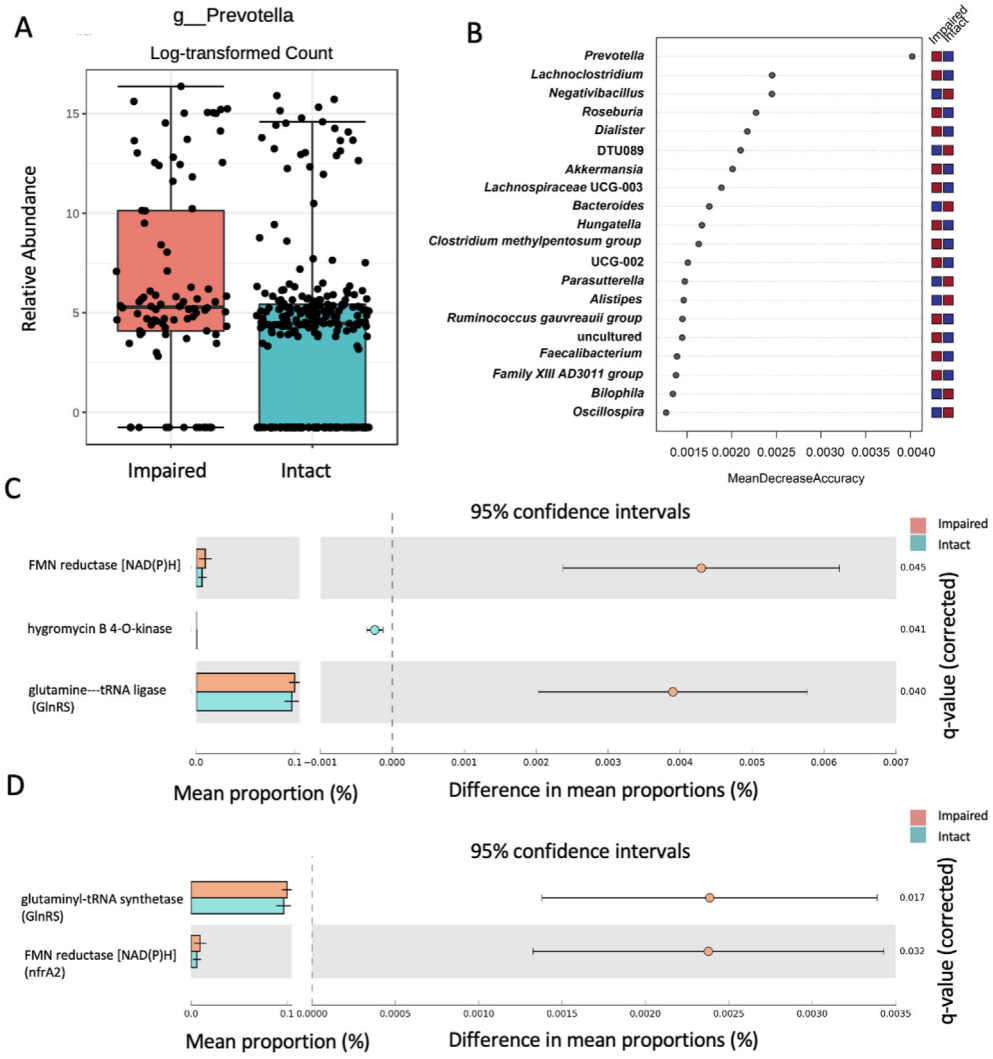

Fig. 3. Relative abundance and predicted functional pathways by cognitive status (A) Univariate comparison analysis showing a significant difference in the relative abundance of *Prevotella* between cognitively intact and impaired groups (Kruskal–Wallis Adj. $p = 0.0136$). (B) Random Forest analysis between cognitive groups at the genus level (tree n = 5000). Genera were ranked by their contributions to classification accuracy (Mean Decrease Accuracy). Extended error bar plot of PICRUSt2 predictions using Welch's test of C ECs and D KOs. All *p* values were corrected using Benjamin-Hochberg correction.

Accuracy) and was associated with the cognitively impaired group. Similar results were obtained with SparCC correlation analysis (Fig. 4C and D). This analysis suggested an association between species of *Prevotella* and *Bacteroides* and mild cognitive impairment in healthy middle-aged and older adults. The relative abundance of species belonging to both genera is presented in Supplementary Fig. 4.

3.2. Absolute abundance of Lactobacillus species and LGG strain determined by quantitative (q) PCR

To assess LGG persistence in the gut, 88 randomly selected samples from 44 subjects receiving either placebo or probiotic treatments were analyzed by high-throughput qPCR targeting the 16S rRNA gene, total *Lactobacillus*, and LGG strain-specific primers (Table 2). Participants from the probiotic group had significantly more copies of LGG DNA/ng compared to baseline and the placebo group (Adj $P < 0.0001$) (Fig. 5A). Furthermore, *Lactobacillus* was 11.5 times more abundant in the probiotic compared to the placebo group (3.8-fold). Finally, using universal *Eubacteria* primers targeting the 16S rRNA gene we observed no differences in total bacterial cell counts after probiotic supplementation suggesting that LGG did not increase the total microbial load, but rather displaced other taxa (Supplementary Fig. 5).

3.3. LGG had a marginal impact on diversity and predicted functional pathways of the gut microbiome at the aggregate level

Sequencing of 16S rRNA amplicons targeting the hypervariable region 4 of the ribosomal gene was carried on fecal samples at baseline and post supplementation corresponding to aggregate treatment groups: (1) Baseline (n = 169), (2) Placebo (n = 83), (3) Probiotic (n = 86) (N = 338). Overall, no significant differences in diversity were observed between placebo and probiotic treatment groups (Fig. 5B). Similarly, no clustering was observed in a Bray Curtis distance Principal Coordinate Analysis (PCoA) plot based on treatment type (Fig. 5C). LEfSe analysis to identify taxa specifically associated with treatment showed unknown/uncultured species of the genus *Lactobacillus* and *Mogibacterium* associated with the probiotic treatment, while *Lachnospira was* associated with placebo (Fig. 5D). Finally, no significant differences were observed in the predicted functionality of species between the placebo and

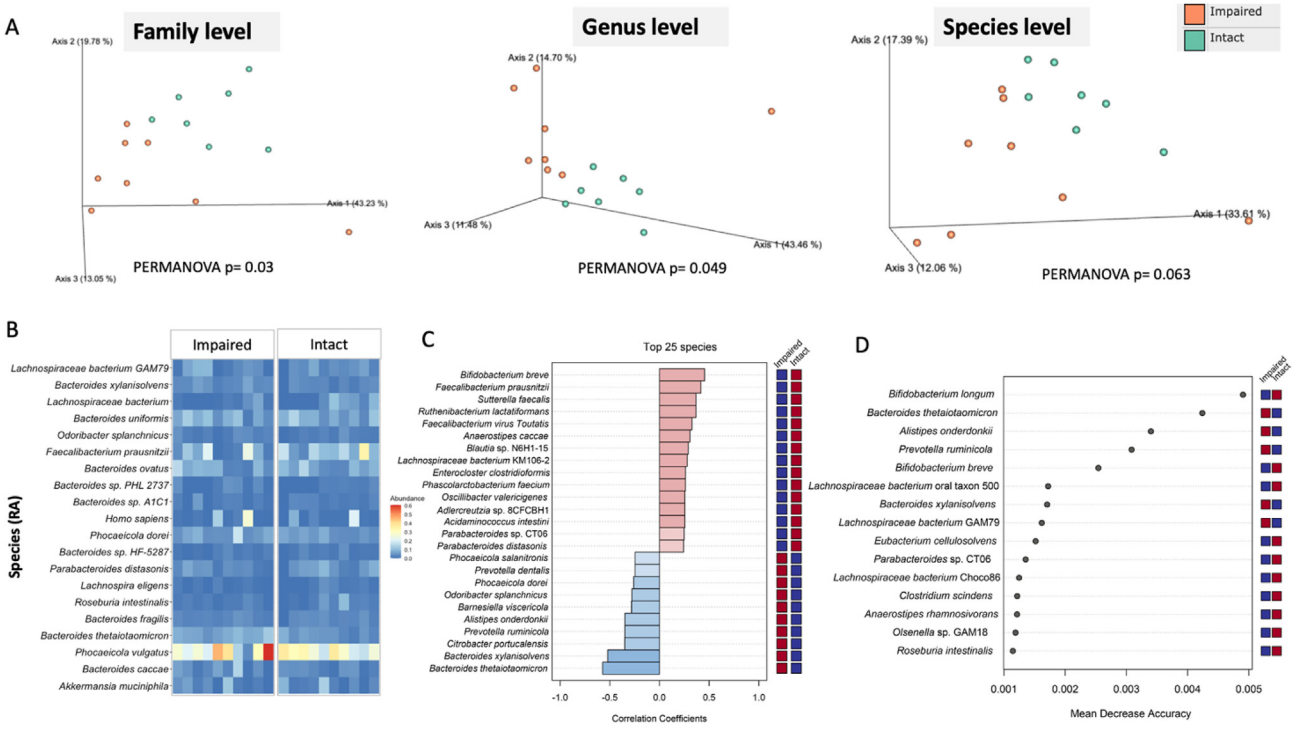

Fig. 4. Composition of the gut microbiome of individuals with high abundance of *Prevotella* by cognitive status at baseline (n = 10/group) determined by WGS analysis. (A) Bray Curtis Principal Coordinate Analysis (PCoA) plots at family, genus, and species levels showing clustering based on cognitive status (PERMANOVA p = 0.03, 0.049, and 0.063) respectively. (B) Heat map of relative abundance at genus and species level (top 20), (C) SparCC correlation analysis showing the top 25 species ranked by their correlation with cognitive status. The deeper color indicates a stronger correlation (dark blue or red). (D) Random Forest analysis of cognitive groups at the species level (tree n = 5000). Species were ranked by their contributions to classification accuracy (Mean Decrease Accuracy). The mini heat map on the right indicates whether the abundance was correlated with MCI or non-MCI groups (red = high, blue = low).

probiotic groups using predictive functional profiling (PICRUSt) (Supplementary Fig. 3B).

3.4. LGG preparation effect on the diversity of the gut microbiome at the individual level

Smoking, alcohol consumption, drug use, and physical activity of participants were recorded and explored in our analysis. We conducted paired and longitudinal analysis that minimized inter-individual variations during the supplementation period. The analysis showed that the observed changes were not attributed to age and BMI, two significant factors that have been associated with changes in the gut microbiota composition and cognitive functions. Non-phylogenetic and phylogenetic pairwise alpha and beta diversity analysis by BMI showed that both matrices were not significant (alpha diversity p-values = 0.19, and 0.96 respectively) (beta diversity p-values = 0.87, and 0.61 respectively). Additionally non-phylogenetic and phylogenetic pairwise alpha and beta diversity analysis by age were also non-significant (alpha diversity p-values = 0.12, and 0.31 respectively) (beta diversity p-values = 0.34, and 0.15 respectively).

Examining the probiotic effect, pairwise diversity analysis showed a significant difference in paired beta diversity primarily found in non-phylogenetic diversity metrics (Bray Curtis $P = 0.018$). The difference was less significant when using phylogenetic beta diversity metrics (Weighted UniFrac $P = 0.09$) suggesting a change in relative abundance but not necessarily taxa introduction or depletion of existing taxa (Fig. 5E). Paired differential abundance of the compositional difference between time points by treatment group indicated one taxon, *Cyanobacteria* chloroplast, increased differentially in the probiotic group (ANCOM-II; W = 192 $P < 0.05$, ALDEx2; $P = 0.007$ BH = 0.348, ANCOM; W = 6 $P < 0.05$) (Supplementary Fig. 6). This taxon is unlikely to indicate the presence of *Cyanobacteria* chloroplast but rather related to the chloroplast in the inulin found in the prebiotic formulation. However, *Cyanobacteria* chloroplast was not found to be significant in the longitudinal differential abundance analysis (ZIBR) indicating that the chloroplast pairwise differential abundance is due to the treatment of zeros required for compositional difference (Supplementary Fig. 7).

3.5. Cognitively impaired individuals had a different response to probiotic treatment

Two-part ZIBR analysis [43] to estimate differential abundance in longitudinal compositional data within the cognitively intact cohort identified four genera differentially abundant with respect to treatment in either the number of subjects with non-zero relative abundance or the relative abundance, when present: *Lactobacillus*, *Oscillospira*, *Clostridium* (*Erysipelotrichaceae* family), and an unnamed *Clostridiales* genus of the family EtOH8 (Supplementary Fig. 8).

Among the cognitively impaired cohort, two genera were identified as differentially abundant with respect to LGG treatment: *Dehalobacterium* and *Prevotella* (Fig. 6). A significant treatment effect was observed in *Dehalobacterium* for the non-zero relative abundance, with a downward trend when present ($P = 2.07e{-}09$, Adj. = 2.032e−07). There was no statistically significant treatment effect for *Prevotella* in the number of subjects containing the genus; however, examining the non-zero relative abundance was found to significantly decrease in cognitively impaired individuals in response to LGG treatment ($P = 2.33e{-}05$, Adj. = 0.001). Notably,

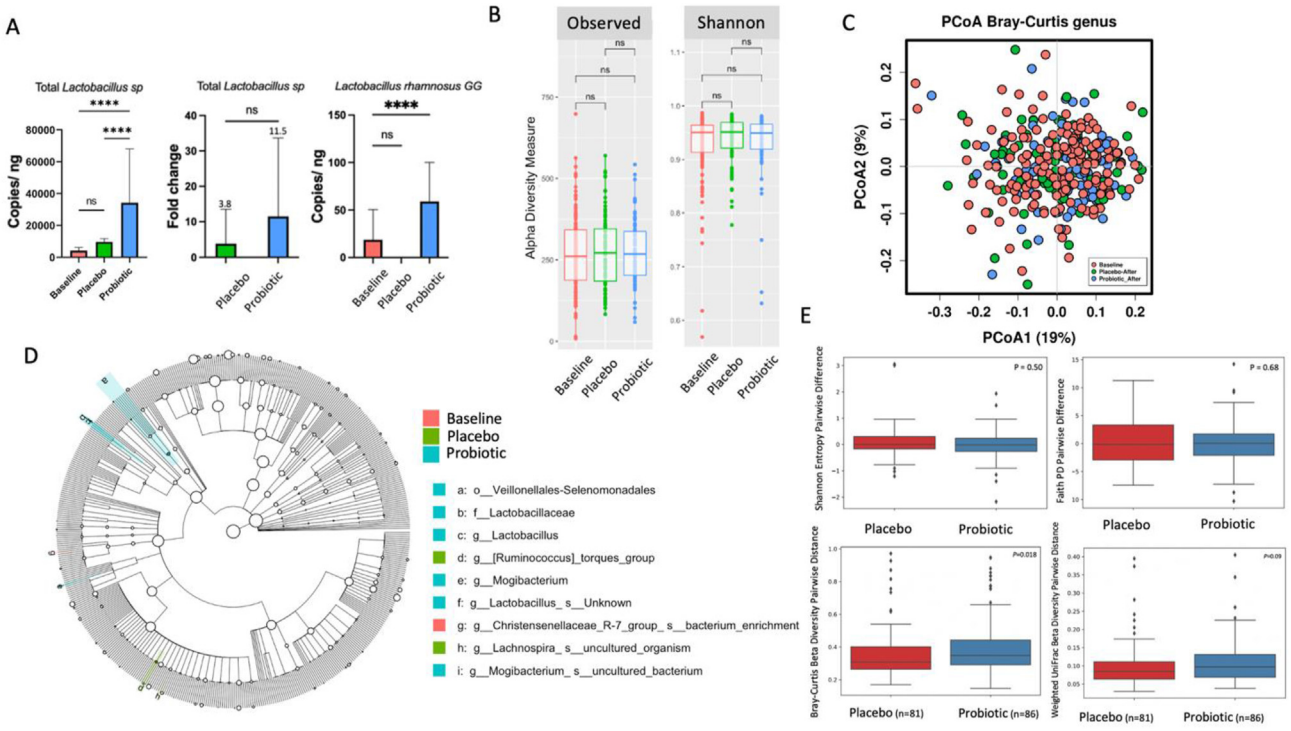

Fig. 5. LGG impact on the gut microbiome. (A) Absolute quantification of LGG strain using qPCR with LGG strain-specific primers and the fold change of the genus *Lactobacillus*. (B) Non-paired alpha diversity analysis by treatment group. (C) Bray Curtis distance Principal Coordinate Analysis (PCoA) plot showing no clustering by treatment. (D) Cladogram and Linear Discriminant Analysis of effect size (Lefse) showing biomarker taxa for each group. The cladogram was generated using Kruskal–Wallis test = 0.05, Wilcoxon-Rank Sum test = 0.05, and LDA cutoff score = 4. (E) Pairwise alpha (top) and beta (bottom) diversity analysis by treatment using phylogenetic and non-phylogenetic diversity matrices.

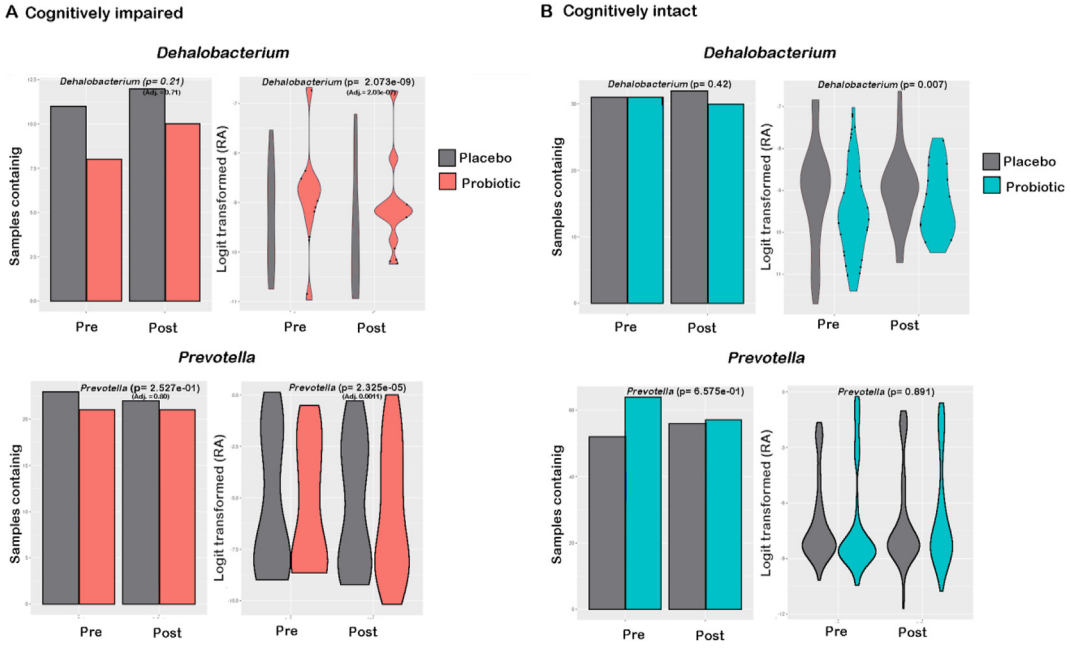

Fig. 6. Differential abundance analysis by zero-inflated beta regression model with random effects (ZIBR) identified three genera associated with **A** the cognitively impaired group, and **B** the cognitively intact group. Left panels show the number of samples containing each genus within their respective treatment group (placebo or probiotic). The right panels show the logit-transformed non-zero abundance for each group.

this decrease was not observed in cognitively intact individuals also receiving the probiotic intervention.

Consistent with the ZIBR analysis, the log-ratio relative abundance of the family *Prevotellaceae* before and after treatment by the cognitive group showed a decrease at follow-up regardless of treatment (Adj. $p = 0.037$). Finally, heat tree plots were generated to depict differences between microbial communities by cognitive group and time (pre vs. post probiotic treatment) for cognitively

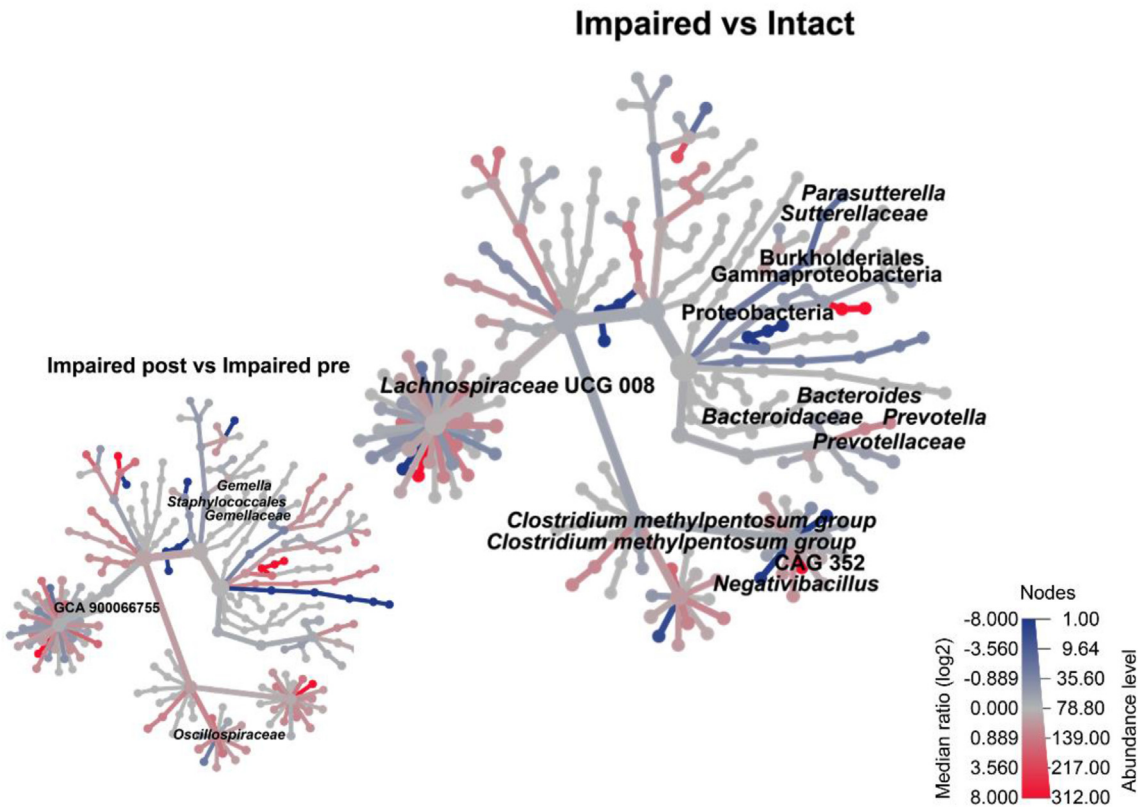

Fig. 7. Heat trees depicting differences between microbial communities by cognitive grouping (left) and by time for cognitively impaired individuals (right) using a non-parametric Wilcoxon rank-sum test at ($p < 0.05$). The size and color of nodes and edges are correlated with the abundance of taxa, only significant taxa are labeled.

impaired individuals. Heat trees comparing impaired to intact individuals showed a higher abundance of *Proteobacteria, Gammaproteobacteria, Burkholderiales, Sutterellaceae, Parasutterella, Bacteroidaceae, Bacteroides, Prevotellacea, Prevotella, Lachnospiraceae* UCG 008, *Clostridium methylpentosum group, and Negativbacillus* in cognitively impaired individuals ($p < 0.05$). Further, when we compared impaired individuals pre and post LGG intervention, we observed an increased abundance of *Oscillospiraceae, Gemellaceae, Staphylococcales, Gemella,* and GCA 900066755 in the post probiotic group ($p < 0.05$) (Fig. 7).

4. Discussion

There is a growing interest in the association between gut microbiota and cognitive performance aimed to achieve successful aging and maintain functionality and independence. Our study examined differences in the gut microbiome composition in a cohort of individuals with and without mild cognitive impairment. Additionally, we investigated the impact of *L. rhamnosus* GG supplementation on aggregate and individual gut microbiome composition [22]. We identified indicator taxa associated with the MCI group in an elderly population, namely members of the genera *Prevotella* and *Bacteroides*. Furthermore, we found that LGG treatment had a marginal impact on overall microbiome diversity at both aggregate and individual levels, nonetheless, we observed differences in taxa responses to LGG treatment by subjects' cognitive status.

The current study is one of few randomized clinical trials (RCT) in the USA that investigated the microbiome composition associated with MCI compared to their cognitively normal counterparts in healthy individuals using 16S rRNA amplicon and WGS sequencing. Additionally, a limited number of studies assessed the effect of probiotic interventions among healthy elderly and those with MCI. Those studies have been conducted in Japan [50], South Korea [23], and China [51].

We found no significant differences in microbiome diversity associated with cognitive status independent of treatment effect. In alignment with our findings, a previous study [52] found no significant differences in diversity indexes between cognitively impaired and cognitively intact groups in a pilot study of 17 patients. In our study, we identified species of *Bacteroides* and the closely related genus *Prevotella* associated with the cognitive impairment group at baseline. In accordance with our findings, a study comparing the microbiome of 82 elder Japanese individuals in association with their cognitive performance identified a correlation between MCI and increased abundance of *Bacteroides* [50]. Likewise, the relative abundance of *Prevotella* has been associated with worse cognitive scores in obese and nonobese subjects [53]. Conversely, the link between species of *Prevotella* and *Bacteroides* and cognitive status identified in our study is in partial agreement with other published reports. Guo, Peng [54] assessed the microbiome composition of newly diagnosed unmedicated AD and MCI patients and found a decline in the relative abundance of *Bacteroides* and an increase in *Prevotella,* which could promote inflammation with disease progression.

There is an overall discordance on the role of *Prevotella* in brain function with both eubiotic and dysbiotic proposed roles [55–57]. A higher abundance of *Prevotellaceae* including *P. ruminicola* was reported in major depressive disorder (MDD) patients compared to bipolar disorder with current major depressive episode (BPD) patients [58]. Conversely a negative association of *P. ruminicola* with cognitive impairment due to minimal hepatic encephalopathy (MHE) has been reported [59]. Additionally, a lower abundance of *Prevotella oralis*, *P. ruminicola,* and *Prevotella tannerae* was reported

in autistic compared to neurotypical children [60]. Notably, most studies have reported genus level observations when data from our study and others suggest different roles at the species and strain levels. Our study presents some insight into *P. ruminicola* (formerly known as *Bacteroides ruminicola*) and its potential involvement in cognitive decline. *P. ruminicola* is one of the most predominant species in the rumen gut and has known functions in the generation of short-chain fatty acids (SCFAs) where it consumes butyrate and produces propionic, acetic, formic, and succinic acids [61]. Using shotgun sequencing, Kong, Ellul [62] analyzed the gut microbiome composition and metabolomics of a Huntington's disease (HD) mouse model and found that *P. ruminicola* was positively correlated with butyrate levels and negatively correlated with ATP and pipecolic acid levels. *P. ruminicola* was reduced in HD fecal samples suggesting that it is one of the species that could have a mechanistic role in regulating plasma metabolites in HD preceding significant cognitive and motor dysfunction, highlighting the potential role of the microbiome-gut-brain axis signaling in the pathogenesis of cognitive decline disorders [62].

We observed a correlation between higher abundance of *B. thetaiotaomicron* and *B. xylanisolvens* and MCI. *B. thetaiotaomicron* can ferment glutamate, the most abundant excitatory neurotransmitter that transports signals to the brain via the vague nerve [63]. Glutamate is also the precursor of gamma-aminobutyric acid (GABA), another well-known neurotransmitter. Both glutamate and GABA have been reported to decrease with aging and have been suggested as markers for early diagnosis of AD [64]. Moreover, predicted gene function using PICRUSt identified a higher abundance of glutaminyl-tRNA synthetases (GlnRS) in individuals with cognitive impairment. *B. xylanisolvens* is a xylan-degrading, acetate producer species. SCFAs have been speculated to play a pivotal role in neuro-immunoendocrine regulation, yet the underlying mechanisms of their role have not been fully elucidated [65,66]. The species has also been reported to secrete taurine which has been proposed as a potential biomarker for autistic individuals, where taurine is known to play a role in supporting nerve growth [67]. Additionally, a high abundance of *B. xylanisolvens* has been reported in non-IBDD depression patients, and in gout patients [58,68]. Our findings suggest the involvement of several taxa as contributors to MCI, implying that the impact of the gut microbiome is not dominated by one species.

Overall, our data showed a minimal probiotic impact on the aggregate microbiome composition and predicted functional pathways. In our cohort, improvement in cognition scores was correlated with a marginal probiotic impact on overall competition and diversity of the gut microbiota. This is consistent with previous reports of probiotic clinical trials, where improvements in cognitive function coincided with subtle changes in the gut microbiome profiles [23,69,70]. Conversely, other studies [71,72] reported no beneficial impacts in individuals fed probiotic milk containing *Lactobacillus casei* or *L. rhamnosus* JB-1, respectively. Differences in outcome might be due to shorter intervention periods, a gap between intervention and testing, interindividual variability, and/or the selected probiotic strain. A recently published meta-analyses by Lv, Ye [73], which included 7 controlled clinical trials and 11 animals studies, as well as a systematic review by Baldi et al. [69] which included 23 papers showed that a single strain probiotic and a duration of 12 weeks were effective in human studies.

In our study, each cognitive group had a significantly different response to the probiotic intervention. Specifically, individuals in the MCI group had significantly lower relative abundances of *Dehalobacterium* and *Prevotella* in response to the probiotic treatment. Reports in animal studies identified *Dehalobacterium* as an indicator of postoperative cognitive dysfunction (POCD) in aged mice [74] and a driver of autism spectrum disorders (ASD) pathological traits like altered behavior and TNF-alpha expression [75].

Our work has a number of limitations, the most importantis that our observations are limited to DNA level findings, while further studies will be needed to assess gene expression and metabolic activities linked to the organisms identified in this study. Additionally, specific conditions often associated with aging like low-grade inflammation, increased intestinal permeability, and obesity can add to the complexity of host–microbe interactions in this context. Finally, linking healthy dietary patterns (for example, the Mediterranean and MIND diets) with gut microbiome components in relation to cognition will be essential to ensure successful aging.

This study contributes to the understanding of the gut microbiota role on brain aging in the elderly population. Taken together, our results indicate that LGG had a marginal impact on overall microbiota composition and points to a specific response based on the hosts initial cognitive status. Contrarily to most studies, which reported on the microbiome composition of MCI patients compared to other forms of cognitive dysfunction like AD, we compared MCI to healthy individuals aiming to provide information that could help in the early detection of cognitive aging. Finally, our analysis identified *Prevotella ruminicola* and *Bacteroides thetaiotaomicron* as taxa that could be modulated with pre, pro, or synbiotics to prevent or slow the progression of cognitive impairment.

5. Data share

Data and materials used in the analysis are available upon request from the corresponding authors for the purposes of reproducing or extending the analysis.

Author's contributions

MAA-P, JG were responsible for the conception and/or design of the study. JG, UB were responsible for the acquisition and/or analysis of clinical data. MA, MAA-P, JR were responsible for the acquisition and/or analysis of microbiome data. JR, MA, JG conducted the statistical analyses. MA, MAA-P, UB were responsible for writing the original draft. All authors participated in revising and editing the manuscript and have read and approved the final manuscript.

Conflicts of interest

Dr. John Gunstad reports grants from DSM Royal, during the conduct of the study. The authors declare no other potential conflict of interest to disclose.

Acknowledgments

The Microbiome Core is funded in part by the Center for Gastrointestinal Biology and Disease (CGIBD P30 DK034987) and the UNC Nutrition Obesity Research Center (N ORC P30DK056350). This work was supported by i-Health, Inc., a division of Royal DSM.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2022.09.012.

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