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RESEARCH PAPER

A red lentils-based synbiotic cookie exerts neuroprotective effects in a mouse model of Alzheimer's disease

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Abstract

Gut microbiota preservation or rational manipulation is a key condition for healthy longevity and a promising strategy to prevent neurodegenerations exploiting the gut-brain axis, with a key role of prebiotics and probiotics. Whether their combination in a functional food can provide a synergistic effect to the host remains controversial. To fill this gap, we supplemented the diet of 3xTg-AD Alzheimer's disease mice with a red lentils (prebiotic)-based cookie enriched with neuroprotective probiotics and we performed behavioural, biochemical and molecular tests. Chronic consumption of this synbiotic preparation (functional cookie) preserved cognition, reduced amyloid load, improved glucose and lipid homeostasis and diminished oxidation and inflammation related damages compared to animals receiving a classic cookie (standard recipe). The synergistic effect was indicated by significantly higher glucose insulinotropic polypeptide concentrations in the functional cookie group compared to probiotic group. Moreover, Ruminoclostridium sp KB18 and Ruminicoccus decreased in the gut of mice supplemented with the functional cookie, partially explaining the improved short-term memory upon treatments and substantiating the combined use over individual components. This synbiotic innovative snack represents a prototype of a simple and affordable dietary approach to promote healthy aging and prevent or delay the onset of neurodegenerations.

Keywords: Functional cookie; Synbiotic; Neurodegeneration; Healthy aging; Gut-brain axis.

1. Introduction

Average human life expectancy is increasing, and the identification of both preventive and therapeutic strategies to counteract age-related neurological disorders and to favor healthy longevity is a global public health priority.

The main cause of dementia among elderly is Alzheimer's disease (AD), which is characterized by brain damages, loss of neurons, shrinkage of the cortex and enlargement of ventricles, resulting in progressive mental decay, memory loss, anxiety and depression [1,2]. AD pathogenesis is complex and multifactorial, including impaired proteolysis and deregulated oxidative and inflammatory patterns [3].

Dyslipidemia can increase the risk of AD onset and can exacerbate neuroinflammation [4]. High-density lipoproteins (HDL), reported to be adversely altered in AD, type 2 diabetes mellitus, and obesity [5], promote cholesterol clearance via reverse cholesterol transport, and have an anti-inflammatory and anti-oxidant ability. In contrast, low-density lipoproteins (LDL) mediate cholesterol transport to the tissues and contribute to the accumulation of

Abbreviations: AD, Alzheimer's disease; AGEs, advanced glycation end products; GIP, glucose insulinotropic polypeptide; GLP-1, glucagon like peptide 1; NOR, novel object recognition.

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amyloid-β (Aβ) in the brain [6]. In addition, elevated plasma levels of triglycerides can influence leptin transport across the blood brain barrier (BBB), with a negative impact on hippocampal neuronal synaptic plasticity, memory, and cognition [7].

Chronic hyperglycemia impaired hippocampal neurogenesis and memory in the 3xTg-AD mouse model [8]. In AD, cerebral glucose absorption and usage are impaired and the downregulated expression of glucose transporters (GLUTs) represents an early event in the pathogenesis of AD. Collaborative functions of glucose transporters are involved to different extents in the maintenance of energy supply to neurons. Glucose uptake into neurons is mainly mediated by GLUT3, due to its high-affinity for glucose [9].

Additionally, AD patients are characterized by altered levels of resistin, a hormone regulating insulin metabolism, glucose uptake and inflammation [10,11]. Aging and impairment of energy metabolism have been associated with changes in the levels of other circulating hormones, such as leptin, ghrelin, glucagon like peptide 1 (GLP-1) and glucose insulinotropic polypeptide (GIP). These neuroprotective hormones are often reduced in AD patients, and this negatively affects glucose homeostasis, lipid profile and cognition [12].

Disturbances in the gut-brain axis, a bidirectional communication network between the central nervous system and the gastrointestinal tract, have been recently identified as key contributors to the onset and progression of AD according to an incompletely characterized mechanism [13,14]. Gut microbial population (the microbiota) can influence brain activity, and alterations in gut microbial diversity, abundance and functionality (dysbiosis) were documented in mild cognitive impaired and AD patients compared to healthy individuals [15], with a demonstrated causal role of dysbiosis in AD [16]. In this context, gut microbiota represents a key target to tackle AD, delay neurodegeneration, and favor healthy longevity, but it is challenging to identify all the pathways involved in the microbiota-gut-AD brain axis [17]. Microbiota composition and metabolism are constantly influenced by lifestyle, social environment, drugs and diet [18]. Health-conscious eating has become a major priority for consumers, and the food industry is interested in the development of functional foods with beneficial activity on gut-brain axis to promote healthy aging. In this context, researchers focused on probiotics, bacterial components of the normal human intestinal flora, for their ability to produce lactate and short chain fatty acids (SCFAs), such as neuroprotective acetate and butyrate [19,20]. Interestingly, oral administration of a multi-strain commercial formulation of lactic acid bacteria and bifidobacteria (SLAB51) reversed gut dysbiosis, counteracted cognitive decline, reduced amyloid aggregates and brain damages, and partially restored the impaired neuronal proteolytic pathways in a mouse model of AD [21]. SLAB51 ameliorated cerebral oxidative status by improving the functionality of antioxidant enzymes and decreasing oxidized proteins, lipids and DNA [22]. It improved also memory function by stimulating the production of SCFAs and neuroprotective gut peptide hormones, such as ghrelin, leptin and GLP-1, and by improving energy homeostasis [23,24].

The composition and function of gut microbiota can be also affected by prebiotics, nondigestible dietary substances that are selectively utilized as energy source by beneficial intestinal microorganisms. Pulse crops such as lentil, common bean, and chickpea are known for their anti-inflammatory and antimicrobial effects. Lentils have been considered beneficial for weight management and blood sugar regulation, due to their low glycemic index [25]. These legumes are rich in protein and low digestible carbohydrates acting as biosynthetic precursors for human microbiota activity, along with prebiotic carbohydrate profiles which are not yet fully characterized [26].

An increasing number of probiotic- and prebiotic-based nutritional interventions in humans have been reported to exert positive effects on gut microbiota [27–29]; these studies primarily involved older adults (including AD patients) with memory deficits, insulin resistance, diabetes, obesity, and cardiovascular diseases that are strongly interconnected [30], but the multi-level mechanism of action of prebiotics and probiotics in functional food requires further preclinical exploration. Considering that lentil seeds contain higher levels of low molecular weight carbohydrates compared to other legumes [31] and based on preliminary experiments [32], we developed a new hypocaloric cookie containing red lentils flour as prebiotic component [33] and coated with chocolate enriched with SLAB51 (probiotic component). This functional cookie (PRE+Pro) was administered to 3xTg-AD triple-transgenic mice to investigate its ability to improve short-term memory and reduce brain amyloid load. Moreover, the effects of the synbiotic combination on lipid profile, glucose homeostasis, inflammation, oxidation, and on the plasma concentration of metabolic hormones (resistin, ghrelin, leptin, GIP, GLP-1), were studied to unravel the multi-level mechanisms of action of the functional cookie components.

2. Experimental section

2.1. Reagents and chemicals

SLAB51 is a commercially available probiotic formulation containing eight different live bacterial strains: *Streptococcus thermophilus* DSM 32245, *Bifidobacterium lactis* DSM 32246, *Bifidobacterium lactis* DSM 32247, *Lactobacillus acidophilus* DSM 32241, *Lactobacillus helveticus* DSM 32242, *Lactobacillus paracasei* DSM 32243, *Lactobacillus plantarum* DSM 32244, *Lactobacillus brevis* DSM 27961. Polyvinylidene difluoride (PVDF) membranes and reagents for western blotting analyses were obtained from Merck KGaA (Darmstadt, Germany). Antibodies for detecting amyloid oligomers, glucose transporters (GLUT 1, 3, 4) advanced glycation end products (AGEs), p27, ionized calcium-binding adapter molecule 1 (Iba-1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), were purchased from AbCam (Milan, Italy). Bcl-2 (sc-7382) was from Santa Cruz Biotechnology. Proteases inhibitors tosyl phenylalanyl chloromethyl ketone (TPCK) and 4- (2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF or Pefabloc) were obtained from Sigma-Aldrich S.r.L. (Milano, Italy). All solvents and reagents used in this experiment were of the highest purity available.

2.2. Animal model

B6;129-Psen1tm1Mpm Tg (APPSwe, tauP301L)1Lfa/J (named 3xTg-AD) were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). 3xTg-AD are a reliable triple transgenic model of AD containing three mutations (amyloid precursor protein [APP]Swe, tau MAPT P301L, and presenilin-1 M146V) associated with frontotemporal dementia or familial AD. As early as 3–4 months of age Aβ intracellular immunoreactivity can be detected in certain brain regions, and at 10–12 months of age tau hyperphosphorylation occurs. Before the experiment, all mice were housed in plastic (Tecniplast, Buguggiate (VA) Italy) cages (4 animals per cage) in a temperature-controlled room (21±5°C), 60% humidity, 12 h light/dark inverted cycle (light was switched on at 8:00 PM) and were fed laboratory mice diet (Mucedola, Italy) with water ad libitum. 8-week-old mice (weight 15–25 g) were used for the experiments. All experimental procedures were approved and authorized by the Italian Ministry of Health (Approval n° 687/2023-PR), following the national legislation (Legislative Decree 26/2014) and the European Communities Council Directives (2010/63/EU). Appropri-

ate measures minimized pain and discomfort in experimental animals.

2.3. Functional cookies

We developed a hypocaloric cookie with prebiotic-rich ingredients, specifically red lentils, and coated with a probiotic carrier (dark chocolate) containing neuroprotective probiotics (SLAB51). The main (uncoated) cookie is prepared according to a standard recipe modified by substituting wheat flour with red lentils flour and specialty fibers to reduce both sugar (Meltec, Hi-Food SpA [34]) and fat/saturated fat (WF fibre, Hi-Food SpA; [35]) content. Probiotic vitality retention in the chocolate carrier was ensured by the use of mild processing conditions during snack production, and was monitored in the final product. Red lentils were used based on preliminary experiments on the prebiotic activity of different legumes-based cookies on SLAB51, performed according to the internationally standardized INFOGEST *in vitro* digestion protocol [36] (data not shown). A chocolate-coated prebiotic cookie without probiotic (hereinafter referred to as PRE) and a classic cookie (standard recipe with wheat refined flour, sugar, and butter) have also been used.

A preliminary study was performed to assess the probiotics viability in dark chocolate and functional cookies stability. Viable cells count of the probiotic bacteria in chocolate were determined by the standard plate method and values were expressed as colony forming units per gram (CFU/g) of chocolate. Total bacterial counts were determined after 48 h of incubation at 37°C. Probiotics viability was analyzed in triplicate immediately after production and weekly for 68 weeks of storage at room temperature and at 4°C (Supplementary Fig. 1).

2.4. Experimental design

8-week-old 3xTg-AD gender balanced mice were divided into five groups: control group (Water, n=8), with animals receiving normal drinking water and standard laboratory diet, Probiotic group (Pro, n=8), with animals receiving SLAB51 (2×10^{11} bacteria/kg/d) dissolved in drinking water and normal laboratory diet, classic cookie group (n=8), with animals fed with a classic cookie (standard recipe) and normal laboratory diet, prebiotic cookie group (PRE, n=11) with animals fed with the prebiotic cookie and normal laboratory diet, and prebiotic+SLAB51 group (PRE+Pro, n=10), with animals receiving the functional cookie (in order to provide 2×10^{11} bacteria/kg/d of SLAB51) and normal laboratory diet. Animals were treated for a period of 4 months (Supplementary Fig. 6). The drinking solution was replaced every day. Chocolate is not only an optimal vehicle for probiotics, but it is strongly palatable for mice, that typically prefer sweet and fatty foods, and that quickly ate the dietary supplement provided during the entire experimental period. Chocolate coating allowed to deliver the defined daily dose of functional cookie to each mouse. Dietary intake and mice body weights were monitored daily and weekly, respectively, throughout the treatment period to ensure adequate consumption of experimental food with no significant changes among groups during the entire treatment period (Supplementary Fig. 2).

2.5. Behavioral assessments

Three days before the sacrifice, the novel object recognition (NOR) test was performed in a plexiglass arena (45Lx45Wx60H cm) with a white floor and opaque walls. The experiment consisted in three phases: habituation, training and test. All phases were performed during the dark phase under dim light conditions. Before each phase, mice were habituated to the test room for 5 min in individual cages. All phases and all arenas were recorded with a Panasonic USB camera from top view. Experimental data were collected and analyzed with Ethovision XT 7 software (Noldus, Netherlands).

During habituation, mice were placed in the center of the arena and left free to explore it for 10 min. The training phase was performed 24 h after habituation. Mice were placed in the same arena with two identical objects positioned at the opposite angles of the arena and left free to explore for 10 min. The objects were the same for all animals. The time spent exploring each object was measured off-line by two researchers who agreed upon scoring rules beforehand. The animals were considered to be exploring when their nose was within a 2 cm radius from the object and their head was directed towards the object. Time spent sitting on the object or leaning on the object was not counted as exploration unless the head was directed towards the object. Time was measured using the Behavioral Observation Research Interactive Software (BORIS) version 8.22.14 (University of Turin).

The test phase was performed 3 h after the training phase. Mice were placed in the same arena with two objects, one identical to the training phase and the other novel. Objects were placed at the opposite angles of the arena and their position was the same for all animals. Mice were left free to explore for 5 min. The time spent exploring each object was measured offline by two researchers who agreed upon scoring rules beforehand. Exploration definition was the same of the training phase. Time was collected using the BORIS software. Discrimination index was calculated as the ratio between the time spent exploring the novel object and the total time of exploration of the two objects, expressed in percentage.

2.6. Tissue and plasma collection and processing

Upon the 4-month treatments and following the behavioral assessment, mice were euthanized at 24 weeks of age by cervical dislocation.

Blood samples were immediately extracted from the cardiac chambers using a heparinized syringe fitted with a heparinized 26G needle, collected in EDTA-tubes, and centrifuged at 3,500 rpm for 10 min at 4 °C. The plasma supernatant was immediately supplemented with protease inhibitors (1 mM Pefabloc) and stored at -80°C.

Brain tissues were properly collected for subsequent biochemical analyses. In detail, micro-dissected hippocampal and cortical regions of each mouse were flash-frozen in dry ice and stored at -80°C until use. Tissues were homogenized in 50 mM Tris buffer, 150 mM KCl, 2 mM EDTA, and pH 7.5 (1:5 weight/volume of buffer), centrifuged at 13,000×*g* for 20 min at 4 °C, the supernatants were collected and proteinase inhibitors (1 mM TPCK and Pefabloc) were added and stored at -80°C. Protein concentration was determined using the Bradford protein assay [37].

2.7. Cerebral Aβ levels

Murine hippocampus and prefrontal cortex were assayed for Aβ1-40 and Aβ1-42 levels using enzyme-linked immunosorbent assay NOVEX ELISA kits (Invitrogen). Based on preliminary tests, samples were diluted at 1:5 with diluent buffer provided by the kit. Assays were performed according to the manufacturer's directions. Data are expressed as pg/mL ± SD.

2.8. Plasma lipid profile

Total cholesterol, HDL-C, LDL-C, and triglycerides were measured using enzymatic colorimetric kits provided by Chema Diagnostica (Italy). The assays were performed following the manufacturer's instructions. Data are expressed as mean values in mg/dL ± SD.

2.9. Glucose levels

Glycosylated hemoglobin (HbA1c) level is a reliable retrospective glycemic index. Mice HbA1c plasma levels were determined using a mouse HbA1c solid-phase ELISA kit (My BioSource, San Diego, CA, USA) following the manufacturer's instructions. Data are expressed as mean values in ng/mL ±SD.

2.10. Hormones determination

Plasmatic concentration of resistin was measured using the RayBio Mouse Resistin ELISA Kit (Ray biotech Inc.). Ghrelin, leptin, GIP, GLP-1 were determined by ELISA kits from Merk-Millipore (Milan, Italy) respectively, following the manufacturer's instructions. Data are expressed as mean values in pg/mL ±SD.

2.11. Circulating lipopolysaccharide

A double antibody sandwich ELISA was used to quantify the plasma concentration of lipopolysaccharides (LPS) in the plasma of control and treated animals (LPS ELISA Kit, Mybiosource, Cat No. MBS261904), following the kit manufacturer protocol Data are expressed as mean values in ng/mL ±SD.

2.12. Western blotting

Amyloid oligomers, glucose transporters, AGEs, Iba-1, Bcl-2, and p27 were analyzed through western blot. In detail, 30 μg total protein of brain homogenates were loaded on 10-12% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto PVDF membranes. After incubation with the specific antibodies, the immunoblot detection was carried out with an ECL western blotting ChemiDocTM System (Biorad, Milan, Italy). Each gel contained molecular weight markers ranging from 6.5 to 205 kDa. GAPDH was utilized to guarantee equal protein loading and to normalize western blot data. ImageJ software [38] was used for the densitometric analysis of the western blot results. For amyloid oligomers 120-30 kDa-molecular weight smear and low-molecular weight tetramers (∼17.2 kDa) and trimers (∼ 13.5 kDa) were selected in the Image J analysis. Monomers were not detected.

2.13. Microbiota analysis

Feces were collected 3 d before the sacrifice. An aliquot of 100 mg (wet weight) of each fecal sample DNA was extracted with a DNA isolation kit (MoBio Power soil, MoBio Laboratoroies, USA) following the manufacturer's instructions.

A single-step 30-cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen) was performed under the following conditions: 94°C for 3 minutes, followed by 28 cycles (5 cycles used on PCR products) of 94°C for 30 s, 53°C for 40 s, and 72°C for 1 min, after which a final elongation step at 72°C for 5 min was performed. Illumina sequencing of V4 region of the bacterial 16S rRNA genes was performed using primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) to 806R (5′- GGACTACVSGGGTATCTAAT-3′′) at the MR DNA laboratory (www.mrdnalab.com, Shallowater, TX, USA).

The demultiplexed sequences were imported into QIIME2 (v. 2024.5) for analysis. The DADA2 denoising procedure was used to remove chimeric sequences and identify amplicon sequence variants (ASVs) [39]. To assign taxonomy SILVA release 138 was used with the VSEARCH algorithm. To determine phylogenetic relationships sequences were aligned using MAFFT and a phylogenetic tree was constructed with FastTree. The Samples were rarefied at the lowest per sample depth, 139,800 reads, for even depth of analysis. Alpha diversity was calculated using the Chao1, observed features, and Shannon metrics to estimate community richness and evenness. A Kruskal-Wallis test was used to test differences in alpha diversity between groups. Beta diversity was calculated using Bray-Curtis, unweighted Unifrac, and weighted Unifrac dissimilarity matrices to estimate differences in overall community structure. Pairwise permutational multivariate analysis of variance (PERMANOVA) was calculated for all beta diversity measures using 999 permutations. *P*-values were corrected for multiple comparisons using the Benjamini-Hochberg procedure.

To assess differences in the abundance of identified bacteria at each taxonomic level analysis of compositions of microbiomes with bias correction (ANCOMBC) was applied [40]. The Holm method was used globally to adjust for multiple comparisons, and a q-value of <0.05 was defined as being statistically significant. PICRUSt2 was then used to predict abundances of KEGG orthologs based on the amplicon sequence variants [41]. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) was applied to predict functional potential of the bacterial community defined by the amplicon sequence variants. The PICRUSt2 analysis utilized the Kyoto Encyclopedia of Genes and Genomes and MetaCyc databases to define functional profiles.

2.14. Statistical analysis

Biochemical data are presented as mean values ± SD. Data were analyzed using one-way ANOVA with group as a categorical variable. Where ANOVA revealed significant differences ($p<.05$), a post-hoc Bonferroni or Tukey HSD test was performed. Statistical analyses were performed using Sigma-stat 3.1 software (SPSS, Chicago, IL, USA). *P*-values $P<.05$ were considered to be significant.

3. Results

3.1. The functional cookie preserved cognitive function in AD mice

The deterioration of short-term memory function is a hallmark of AD. We performed the novel object recognition (NOR) test to evaluate the effect of treatments on memory, by comparing the discrimination index of mice between groups. The discrimination index is a common parameter to measure memory retrieval and is defined as the ratio between the time of exploration of the new object and the total exploration time. Results showed a significant difference between the groups (One way ANOVA for group: $F\ [4,40] = 6.54$, $P=.0004$). Post hoc analysis revealed that mice treated either with SLAB51 in water (Pro), prebiotic (PRE) and the combination of prebiotic and probiotic (PRE+Pro) all showed a significantly higher discrimination index when compared to water group (post hoc Tukey HSD test: Pro vs water $P=.007$; PRE vs water $P=.01$; PRE+Pro vs water $P=.0004$). Mice receiving the functional cookie showed a significant higher discrimination score compared to mice consuming a classical cookie (PRE+Pro vs classic cookie $P=.04$). These data suggest that all treatments are effective in ameliorating memory in AD mice, although to a different extent, with the functional cookie providing a cognitive improvement compared to a classic cookie (Fig. 1, Panel A). Furthermore,

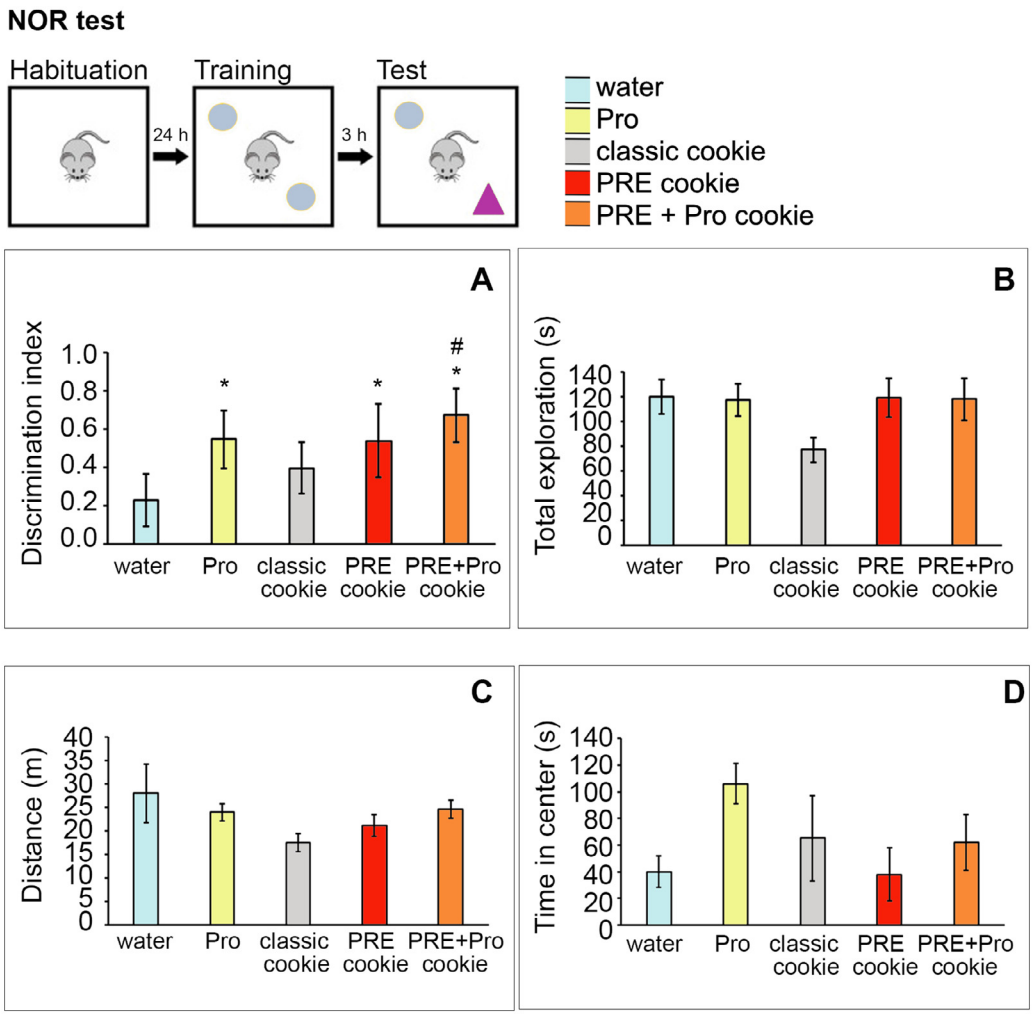

Fig. 1. Cookies supplementation improved memory function. Short term memory changes in 3xTg-AD mice supplemented with SLAB51 in water (Pro) or with a classic cookie or with a hypocaloric prebiotic cookie (PRE) or with a prebiotic cookie enriched with probiotics (PRE+Pro). Panel A: NOR test results expressed as discrimination index. Panel B: total exploration time (s) during NOR. Panel C: Distance travelled in the center of the open field arena. Panel D: Total time spent in the center of the open field arena. Data points marked with an asterisk are significantly different compared to water group (*P<.05). Data points marked with a hashtag are significantly different compared to mice supplemented with the classic cookie (#P<.05).

we evaluated the total exploration time for the two objects during the training session, to check whether differences in exploring could account for the differences in the discrimination index. Results show that the total exploration time did not differ among groups (One-way ANOVA for group: F [4,40] = 1.88, P=.133). This suggests that the differences in the test phase are specific to memory and do not reflect a different propensity to explore (Fig. 1, Panel B).

During the habituation phase, which was carried out in an open field, we measured the total distance travelled by mice, to evaluate locomotory activity, and time spent in the center of the arena to assess anxious behavior. The groups showed no difference in distance travelled (one-way ANOVA for group: F [4,40] = 1.83, P=.14). This suggests that the observed increase in the discrimination index does not relate with locomotory activity, and further supports the hypothesis that the effect of treatments was specifically correlated to memory (Fig. 1, Panel C). No difference was detected also in time spent in center (one-way ANOVA for group: F [4,40] = 2.14, P=.09), which suggests that the treatments had no significant effect on anxiety (Fig. 1, Panel D).

3.2. Aβ1-42 is reduced in both the hippocampus and the cortex of AD mice supplemented with the functional cookie

Early cognitive decline in AD is associated with the deposition of amyloid aggregates in specific brain regions. Treatments affected amyloid aggregates levels (F [4,40] = 3.97, P=.0023). Specifically, mice treatment with probiotic (in water or in the functional cookie) reduced brain amyloid oligomers compared to untreated 3xTg-AD mice (P=.0025) (Fig. 2A). Interestingly, a significant reduction of amyloid oligomers in the PRE or PRE+Pro groups was observed compared to the classical cookie group (Fig. 2, Panel A). Aβ1-40 and Aβ1-42 levels were separately detected through ELISA in the hippocampus and the prefrontal cortex of control and treated 3xTg-AD mice. No quantitative differences were observed for Aβ1-40 either in the hippocampus (F [4,40] = 1.46, P=.21) or in the prefrontal cortex (F [4,40] = 2.65, P=.09) (Supplementary Fig. 3). Conversely, brain levels of Aβ1-42 increased significantly with age, from 1.9±0.45 pg/mL at 2 months to 5.8±0.68 pg/mL at 6 months, more evidently in the prefrontal cortex compared to the hippocampus (Supplementary Fig. 3). Upon 4-month

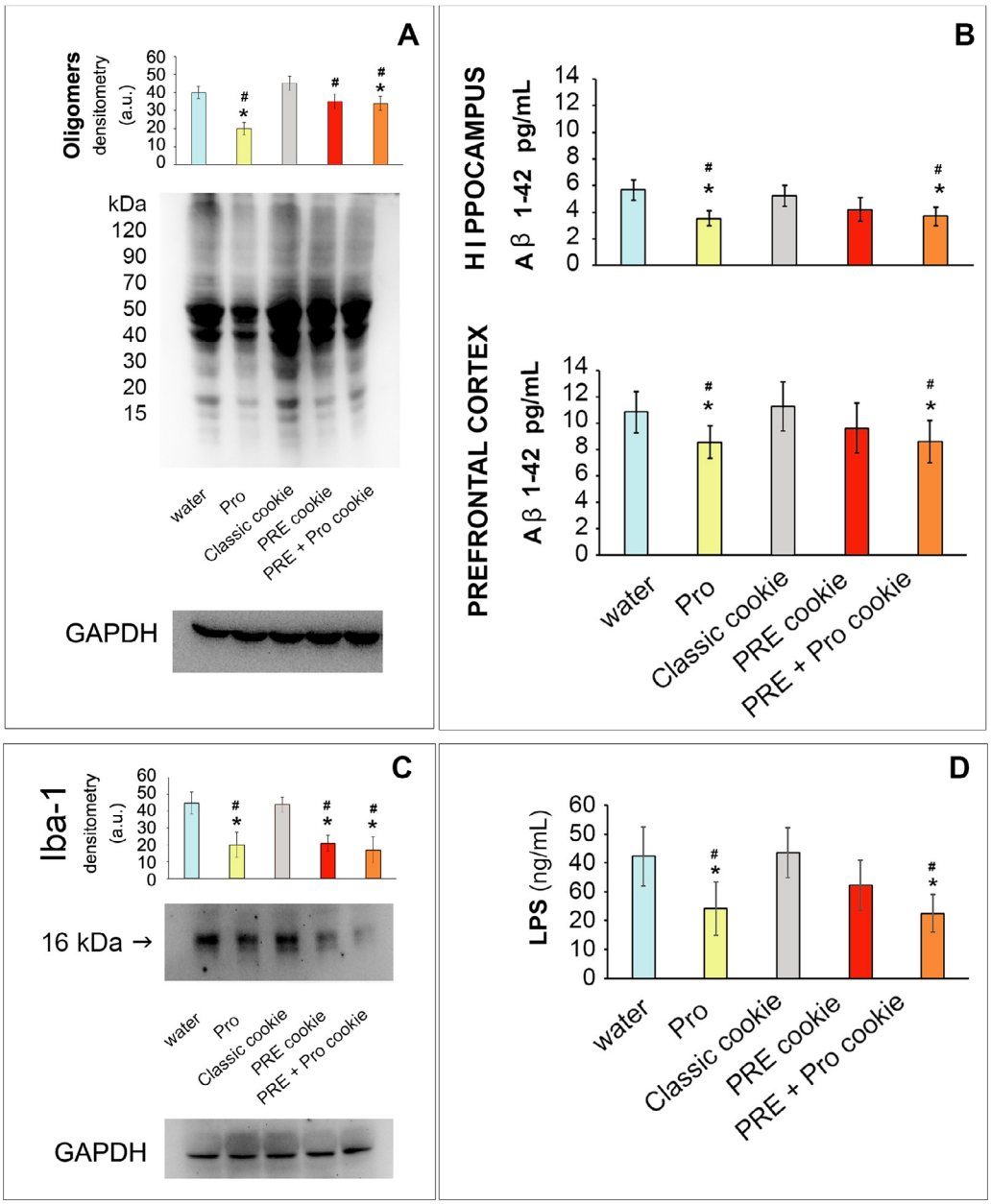

Fig. 2. Decreased amyloid load and inflammation in the brain of treated mice. Panel A: Western blotting of brain amyloid oligomers. The densitometry from three separate blots and a representative immunoblot are reported. Equal protein loading was verified by using an anti-GAPDH antibody. The detection was performed by ECL. Panel B: ELISA for Aβ1-42 in both the hippocampus and the prefrontal cortex of 3xTg-AD mice upon treatments. Aβ1-42 levels are expressed as pg/mL. Panel C: Iba-1 brain levels (western blotting); Panel D: LPS plasma levels (ng/mL) measured through ELISA. Data points marked with an asterisk are statistically significant (ANOVA) compared to water group mice ($^*P<.05$). Data points marked with a hashtag are significantly different compared to mice supplemented with the classic cookie (#$P<.05$).

of probiotic supplementation, Aβ1-42 decreased in the hippocampus (F [4,40] = 7.33 P=.0002), this event being critical to short-term memory for associative information. In fact, the hippocampus plays a key role in the encoding and retrieval of novel associative information in short-term memory. Interestingly, the functional cookie exerted a comparable effect (P=.0006), meaning that probiotic ability to reduce brain amyloid levels is maintained in the functional snack.

Reduced amyloid load is in agreement with ameliorated inflammatory status demonstrated by lower cerebral levels of Iba-1 inflammatory marker in treated mice (Fig. 2C) an the reduced plasma concentrations of LPS in the same animals (Fig. 2D) after treatment.

Additionally, upon treatments, lower cerebral levels of p27 and increased levels of Bcl-2 were detected compared to both water and classic cookie groups (Fig. 3A), indicating a decreased apoptotic activity in treated AD mice that display an altered apoptotic index especially in the hippocampus [21].

3.3. Probiotics and the functional cookie ameliorated blood lipid and glucose profile of AD mice

Plasma lipid and glucose profiles were assessed upon chronic consumption of the functional cookie. There was a statistically significant difference for cholesterol between groups as determined by one-way ANOVA (F [4,40] = 5.26, P=.002), HDL-C (F

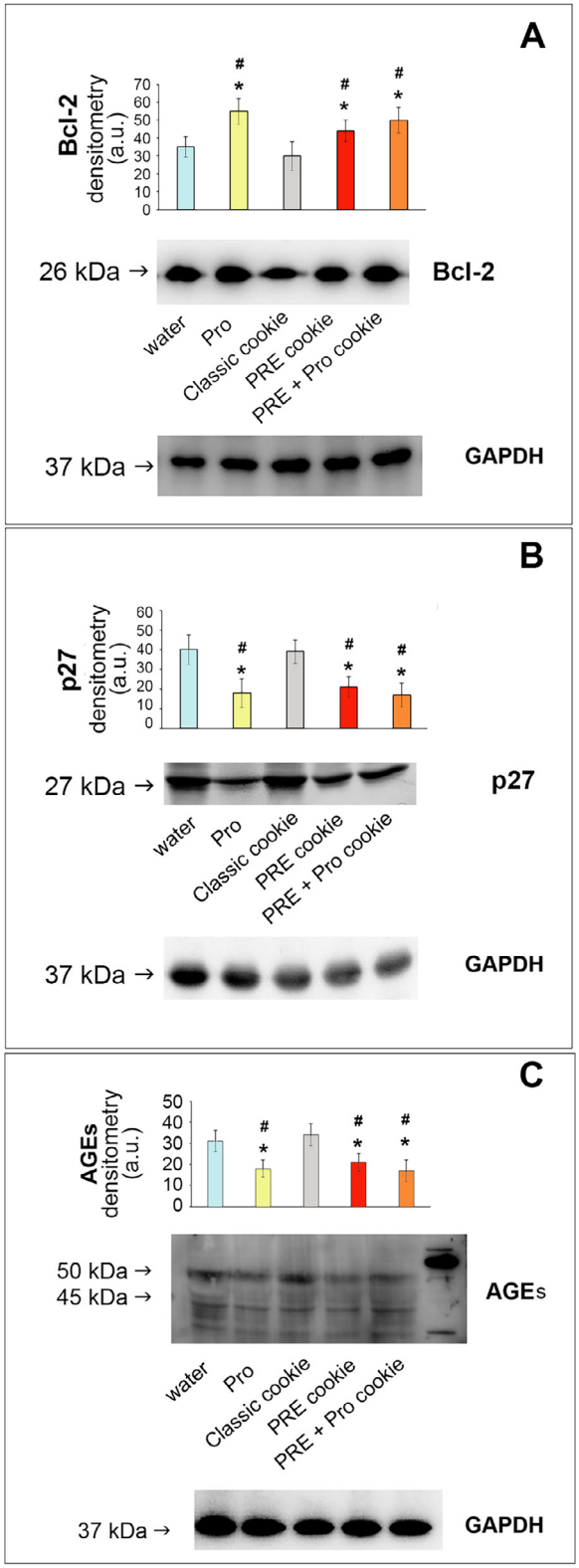

Fig. 3. Decreased apoptotic activity and AGEs in the brain. Bcl-2 (panel A), p27 (panel B) and AGEs (panel C) brain levels upon treatments. Representative immunoblots and corresponding densitometric analysis obtained from 3 separate blots are shown. Equal protein loading was verified by using an anti-GAPDH antibody. The detection was executed by an ECL western blotting analysis system. Data points marked with an asterisk are statistically significant (ANOVA) compared to water group mice (*P<.05). Data points marked with a hashtag are significantly different compared to mice supplemented with the classic cookie (# P<.05).

[4,40] = 4.06, P=.037), LDL-C (F [4,40] = 3.22, P=.024), and triglycerides (F [4,40] = 5.19, P=.002). Post-hoc tests revealed that the supplementation with SLAB51 in water or in the functional food significantly reduced triglycerides and total cholesterol concentration, and increased HDL-C compared to both water group and to the group receiving the classic cookie. SLAB51 (Pro) treatment decreased LDL-C compared to both water and classic cookie treatment. Moreover, the functional cookie treatment decreased LDL-C compared to classic cookie treatment. Interestingly, the supplementation with the prebiotic cookie reduced triglycerides, total cholesterol and LDL-C and increased HDL-C compared to the classic cookie group. The HDL-C increase in the prebiotic cookie group was significant compared to the water group (Table 1).

Chronic hyperglycaemic states have been associated with poor cognitive function (such as learning and memory), increased risk of dementia and morphological alterations in key brain structures, such as the hippocampus [42]. Being HbA1c an indicator of long-term glycemic control, the plasma concentration of this parameter was measured upon 4-month treatments. As expected, HbA1c was significantly reduced in mice supplemented with the probiotics compared with the water group (F [4,40] = 3.26, P=0.021), whereas plasma HbA1c concentrations were significantly higher in AD mice supplemented with all the tested cookies (classic (P=0.019), PRE (P=.021), and PRE+Pro (P=.037) groups) compared to water group (Table 1).

Nevertheless, compared to the classic cookie group, both PRE and PRE+Pro groups showed significantly lower concentrations of HbA1c (P=.033 and .003 respectively). Interestingly, the functional cookie treatment significantly reduced HbA1c levels compared to prebiotic cookie treatment (P=.046) (Table 1).

The preservation of normal glucose levels in the blood resulted in reduced formation of AGEs in the brain of mice supplemented with probiotics or with the prebiotic cookie or the PRE + Pro cookie (Fig. 3, Panel B), in agreement with decreased plasma level of resistin (Table 2), a hormone involved in insulin resistance. Specifically, Pro, PRE and PRE+Pro treatments reduced the plasma concentration of resistin compared to both water or classic cookie supplemented groups (P=.03, P=.049, P=.047 respectively). Moreover, resistin concentration was significantly higher in female mice compared to male mice (data not shown), in agreement with previous evidences [43].

As shown in Figure 4, the supplementation with Pro, PRE and PRE+Pro increased the expression of cerebral glucose transporter GLUT1. SLAB51-based treatments particularly affected GLUT3 (Fig. 4), the major protein responsible for brain glucose delivery and utilization. This is an interesting result, considering that GLUT3 has a higher affinity for glucose than GLUT1, -2, or -4 and it has at least a fivefold greater transport capacity than GLUT1 and -4. No significant variation in GLUT4 expression was observed upon treatments.

3.4. Probiotics and the functional cookie restored plasma levels of neuroprotective hormones

The plasma concentration of ghrelin, leptin, GLP-1 and GIP were measured considering their neuroprotective effects and potential as therapeutic targets. No changes in hormone plasma levels were observed in mice supplemented with the classic cookie compared to the water group, except for GIP, which significantly decreased (Table 2). As expected, compared to water group mice, treatment with SLAB51 significantly increased the plasma concentration of ghrelin, leptin and GIP, and functional cookie treatment significantly increased concentration of ghrelin, GIP and GLP-1, confirming the positive effect of probiotics and of the functional cookie on neuroprotective hormones (Table 2). The neuroprotective hor-

Table 1
Effect on blood lipid and glucose profile.

	Water	Pro	Classic cookie	PRE cookie	PRE+Pro cookie
Total cholesterol (mg/dL)	129.65 ± 10.00	112.79 ±7.84*#	156.81 ±13.94	128.61 ±14.69*#	115.70 ±13.17*#
LDL cholesterol (mg/dL)	45.01 ±6.24	25.21 ±4.00*#	48.05 ±5.97	33.04 ±3.29*#	29.11 ±3.19*#
HDL cholesterol (mg/dL)	50.83 ±5.81	83.54 ±566*#	69.46 ±7.98	79.87 ±7.30*#	82.32 ±9.55*#
Triglycerides (mg/dL)	162.189 ±48.61	129.61 ±51.18*#	191.29 ±39.74	127.49 ±48.58*#	131.16 ±48.89*#
HbA1c (ng/mL)	31.01 ±5.10	18.523.91*#	85.11 ±6.21*	65.21 ±7.05*#	43.05 ±6.12*# °

Plasma concentrations (mg/dL) of total cholesterol, HDL-C, LDL-C, triglycerides, and HbA1c in 3xTg-AD mice after 4-month supplementation with water, SLAB51 (Pro), classic cookie, prebiotic cookie (PRE), probiotics enriched prebiotic cookie (PRE+Pro). (*P<.05 vs water; # P<.05 vs classic cookie; P<.05 vs PRE).

Table 2
Plasma concentrations of key hormones.

	Water	Pro	Classic cookie	PRE cookie	PRE+Pro cookie
GIP (pg/mL)	423.25 ±109.53	778.5 ±180.17**#	227.51 ±47.88*	432.37 ±132.02# °	970.62 ±132.02*#°+
GLP-1 (pg/mL)	106.87 ±17.88	120.62 ±28.42	99.01 ±22.95	132.00 ±36.61	146.02 ±37.89*#
ghrelin (pg/mL)	398.37 ±72.95	542.87 ±66.35*	448.62 ±67.86	497.87 ±84.12	668.62 ±142.81#°
leptin (pg/mL)	3.94 ±2.13	6.07 ±1.25*#	2.18 ±0.49	2.40 ±1.78	4.67 ±2.74#
resistin (pg/mL)	49.56 ±14.29	27.25 ±14.78*#	44.34 ±20.21	32.45 ±12.23*#	34.94 ±16.02*#

GIP, GLP-1, ghrelin, leptin, and resistin were determined in the plasma of 3xTgAD mice supplemented with Probiotics (Pro) or classic cookies or prebiotic cookies (PRE) or PRE+Pro cookies. Data points marked with an asterisk are statistically significant compared to water group (*P<.05). Data points marked with hashtag are statistically significant compared to the group supplemented with the classic cookie (# P<.05). PRE+Pro cookie vs prebiotic cookie is indicated with (P<.05); PRE+Pro vs to Pro is indicated with + (P<.05) (one way ANOVA, followed by the Bonferroni test).

mones increased in mice supplemented with the functional cookie compared to the classic cookie group (Table 2). Mice receiving the functional cookie had considerably higher ghrelin concentration compared to the group supplemented with the prebiotic cookie, and higher levels of GIP compared to both PRE and Pro groups (Table 2), suggesting a synergistic effect of the synbiotic association and substantiating the combined use over individual components. Notably, GLP-1 concentrations were significantly increased only after supplementation with functional cookie compared to classic cookie treatment (Table 2).

3.5. Effects of the functional cookie on gut microbiota composition

The ability of SLAB51 to strategically shift gut microbiota composition on 3xTg-AD mice was previously published [21]. Upon chronic supplementation with the same probiotic formulation (Pro), or with the prebiotic cookie (PRE) or with the prebiotic cookie enriched with the probiotics (PRE+Pro), 16s rRNA sequencing of fecal microbiota showed no significant differences in alpha diversity indices, revealing no difference in species richness (Fig. 5, Panels A and B), in line with previously published data [21]. PCoA plots based on unweighted Unifrac distances indicated a different microbiota structure in treated mice. Moreover, the classic cookie didn't significantly affect the composition of gut microbiota (Fig. 5, panel C; Supplementary Fig. 4).

Pairwise permutational multivariate analysis of variance (PERMANOVA) indicated statistically significant alterations ($q < 0.05$) in the prebiotic cookie (PRE), probiotic in water (Pro), and the prebiotic cookie enriched with probiotics (PRE+Pro) compared to the water group. Statistically significant differences were observed in PRE and Pre+Pro compared to Pro (Table 3). Additionally, PERMANOVA results show that the classic cookie did not have a statistically significant effect on the overall gut microbiota composition (P=.054, q=0.067).

Instead, few changes were observed in PRE and PRE+Pro groups compared to water group. As expected, *Lactobacillus plantarum* increased upon supplementation with the functional cookie, indicating that this probiotic strain effectively colonized the gut of the animals. Interestingly, both Ruminoclostridium sp KB18 (or Hungateioclostridiacee) and Ruminoccoccus species from Oscillospiracee decreased in AD mice chronically supplemented with the prebiotic (PRE) cookies and the PRE +Pro cookies. Limited information exists regarding Ruminoclostridium sp KB18, but some authors observed its decrease in C57BL6/J mice upon treatment with cannabidiol rich cannabis extract with a consequent positive effect on inflammatory status [44]. Additionally, decreased Ruminiccoccus in AD mice upon PRE and PRE+Pro treatment could be associated to changes in lipid metabolism. Ruminiclostridium has been found to modulate lipid metabolism, produce short-chain fatty acids, stimulate the development of beige adipocytes in white adipose tissue, reduce inflammation, and improve intestinal barrier function [45,46] . Additionally, the observed shifts in gut ecology have could be associated with anti-inflammatory effects of the

Table 3
Pairwise PERMANOVA summary table for unweighted UniFrac PCoa.

	R^2	q-value
Water vs. Pro	0.13	0.018*
Water vs. Classic Cookie	0.09	0.067
Water vs. PRE Cookie	0.12	0.018*
Water vs. PRE+Pro Cookie	0.12	0.013*
Pro vs. Classic Cookie	0.12	0.018*
Pro vs. PRE Cookie	0.16	0.005*
Pro vs. PRE+Pro Cookie	0.19	0.005*
Classic Cookie vs. PRE Cookie	0.06	0.242
Classic Cookie vs. PRE+Pro Cookie	0.07	0.068
PRE Cookie vs. PRE+Pro Cookie	0.06	0.192

Pairwise permutational multivariate analysis of variance (PERMANOVA) indicates statistically significant alterations (* q<0.05) in the prebiotic cookie (PRE), probiotic in water (Pro), and the prebiotic cookie enriched with probiotics (PRE+Pro) compared to the water group.

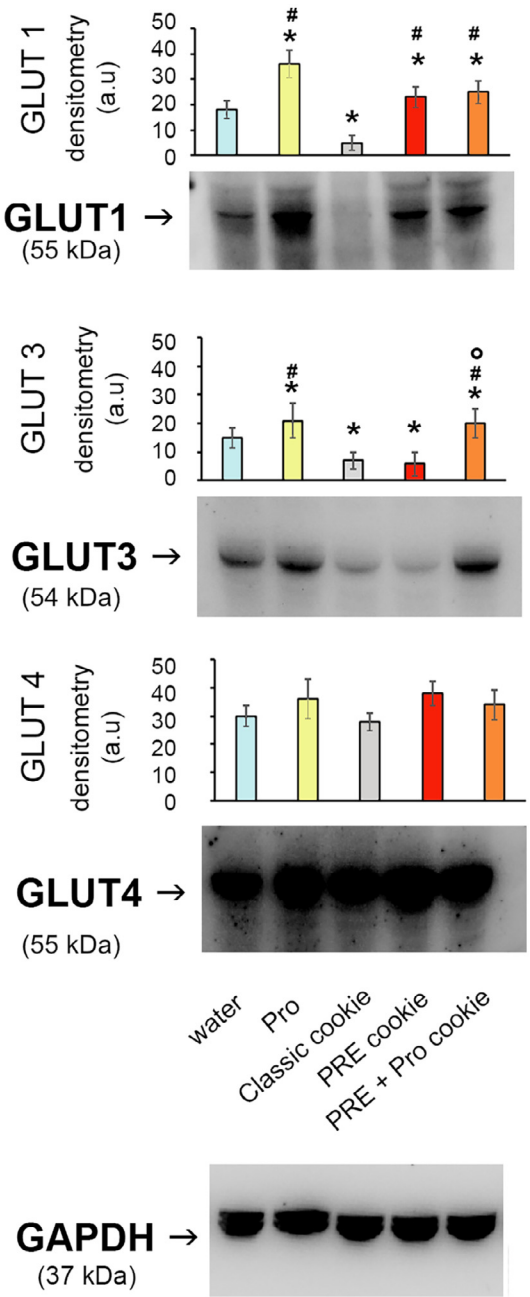

Fig. 4. Glucose transporters brain expression in 3xTg-AD mice supplemented with the functional cookies. Representative immunoblots and corresponding densitometric analysis obtained from 3 separate blots are shown. Equal protein loading was verified by using an anti-GAPDH antibody. The detection was executed by an ECL western blotting analysis system. Data points marked with an asterisk are statistically significant compared to water group mice (*P<.05). Data points marked with a hashtag are significantly different compared to mice treated with the classic cookie (#P<.05). ° indicate a significant difference (P<.05) between PRE and PRE+Pro group.

functional cookies, demonstrated by decreased cerebral Iba-1, reduced plasmatic LPS, increased blood levels of HDL-cholesterol, improved memory function upon 4-month dietary intervention with the functional cookie.

4. Discussion

Global population aging and higher incidence of metabolic diseases contribute to increase the prevalence of individuals with dementia. Public health measures will play an important role in addressing reversible factors in varied cultural and socioeconomic environments with the final aim to protect world populations from cognitive impairment. It is recognized that both gut microbiota dysbiosis and metabolic deficits are involved in the pathogenesis of non-communicable diseases like AD and precede cognitive symptoms, with incompletely defined mechanisms [47]. In this context, dietary interventions using prebiotics and/or probiotics to strategically manipulate the microbiota–gut–brain axis have been experimented as preventative and therapeutic approaches in neurodegenerations. In this work a hypocaloric prebiotic-based cookie enriched with neuroprotective SLAB51 [21,47] has been developed and supplemented for 4-months in 3xTg-AD mice. The aim of the study is to provide a functional food containing live neuroprotective bacteria and to explore their potential synergistic effects on AD signs derived from the combination of probiotics with prebiotic lentils flour. As explained by Kleerebezem et al., "the synergy underlying the synbiotic concept intrinsically implies that these functional foods elicit a superior effect compared to those elicited by the separate administration of their constituents" and it is very complicated to scientifically establishing a synergistic and/or complementary effect on health [48]. In this study, chronic supplementation with the functional cookie improved memory function in 3xTg-AD mice, as indicated by NOR test. In fact, after 3 h from the presentation of the familiar object, the recognition of novelty was significantly improved in animals that received SLAB51 freshly prepared in water as well as SLAB51 contained in the cookie, indicating that the probiotic component ameliorates cognitive skills. This is in agreement with a previous study showing an increased discrimination index in AD mice treated with probiotics compared to AD untreaded mice and comparable with wildtype age-matched untreated mice [21], that at this specific age exhibit a similar performance to the AD counterpart in tasks assessing recognition memory [49]. Additionally, improved cognition was also observed in mice receiving the prebiotic cookie, suggesting that the prebiotic component has a role not only in in maintaining bacteria viability, in line with preliminary prebiotic assays on different legumes (data not shown), but it can also contribute to neuroprotection most likely through the stimulation of the growth and activity of beneficial bacteria. Analysis of microbiota showed that *Ruminiclostridium sp KB18* and *Ruminococcus* decreased upon supplementation with the functional cookies. These data can partially explain the improved short-term memory upon treatments, mostly evident in animals supplemented with PRE + pro cookies (Pro vs water P=.007; PRE vs water P=.01; PRE+Pro vs water P=.0004) substantiating the combined use over individual components, although further studies are needed to unravel the vast complexity of host-microbiome interactions.

High levels of total cholesterol and LDL-C and low levels of HDL-C are associated with vascular dementia and, indirectly, with AD [50]. The prebiotic cookie (in the absence of SLAB51) significantly increased HDL-C and decreased LDL-C in the plasma of 3xTg-AD mice compared to control (water) group and to mice supplemented with the classic cookie, respectively, indicating that red lentils have a significant role in ameliorating lipid profile, in agreement with animal studies on lentils hypocholesterolemic and prebiotic properties [51] and with prospective epidemiological studies reporting that the consumption of phenolic-rich lentils was inversely associated with the incidence of both obesity and diabetes [52]. Treatment with SLAB51 in water (Pro) or with the prebiotic cookie (PRE) or with the functional cookie (PRE+Pro) produced comparable effects on blood lipid profile, without showing a synergistic effect. Other studies showed a synergistic effect of jackfruit seed sourced resistant starch (JSRS) and *Bifidobacterium pseudolongum subsp. globosum* on ameliorated hyperlipi-

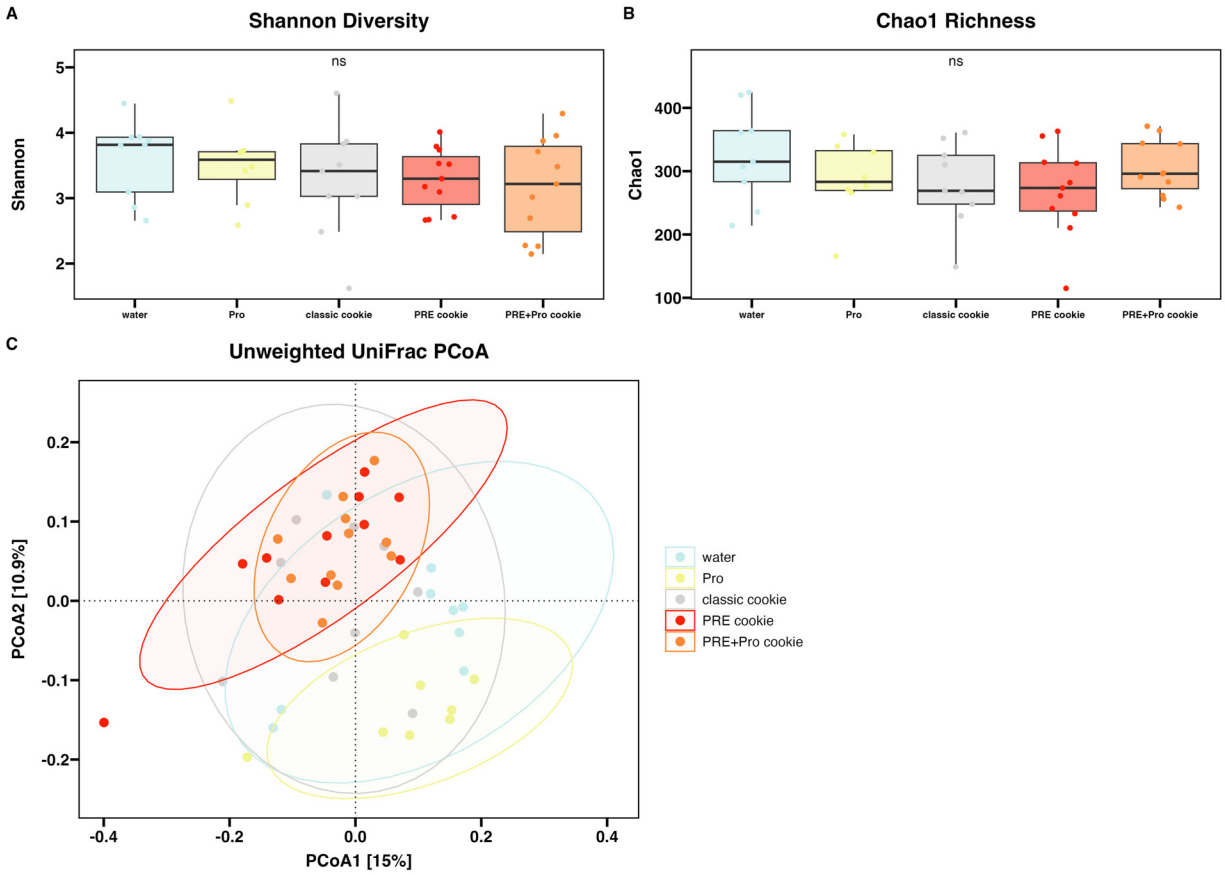

Fig. 5. Microbiota analysis. Panel A: Diversity (Shannon H index) and (Panel B) richness (Chao1 index) of the microbiota in mice upon 4-month diet supplementation (feces were collected three days before the sacrifice). Panel C: Principal coordinate analysis (PCoA) based on unweighted UniFrac distances, representing differences in microbial community structure between samples.

demia in mice; JSRS as a prebiotic showed limited preventive effects on body weight and blood lipid profiles, but the synergistic effect of JSRS and probiotic *B. pseudolongum* counteracted hyperlipidemia [53]. This point highlights the importance of preclinical testing of synbiotic combinations prior to their administration in patients.

Interestingly, decreased cholesterol is consistent with reduced amyloid load, in agreement with studies showing that inhibition of cholesterol biosynthesis decreased γ-secretase activity and amyloid formation [54], and with the ability of SLAB51 to modulate activity and hepatic expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate-limiting enzyme of hepatic cholesterol biosynthesis [24].

As anticipated in the results section and as expected, Aβ1-42 concentrations were significantly higher in the prefrontal cortex compared to the hippocampus. Interestingly, chronic consumption of the probiotic containing functional cookie decreased Aβ1-42 particularly in the hippocampus, which is critical to short-term memory for associative information and has an essential role in memory for the order of events in everyday experiences [55]. SLAB51 is the neuroprotective component, but NOR test suggested that the prebiotic ingredient exerted an independent contribute on preserving cognitive function, that could be due to the ability of prebiotics to improve lipid profile, exert antioxidant and anti-inflammatory effects (demonstrated by reduced plasmatic LPS, decreased AGEs and Iba-1 in the brain and inhibited neuronal apoptotic activity (p27 and Bcl-2 expression levels), but the exact mechanism needs to be further elucidated.

Improvement on cognitive function is also supported by increased plasma concentration of ghrelin, leptin, GLP-1 and GIP. Previous studies have shown that AD patients have a time-dependent plasmatic reduction of these peptide hormones that play a role in regulating neural functions such as learning and memory. In particular, ghrelin has been shown to counteract memory deficits and synaptic degeneration in animal models of AD [56], while leptin was described as a neurotrophic factor and exerted neuroprotective effects against Aβ oligomer-induced toxicity *in vitro* [57]. In neurodegenerations energy status is unbalanced also because of reduced levels of neuroprotective and the antidiabetic incretins GIP and GLP-1 [58]. Oral administration of SLAB51 and the functional cookie favored higher plasma levels of such hormones, with the combination of lentils flour ingredient and SLAB51 potentiating the effect particularly for GIP. In general, prebiotics are known to promote the SCFAs production by probiotics [59], and this activity can explain the increased secretion of the incretin upon supplementation with the complete functional cookie, in agreement with previous studies [60]. Regarding GIP, the partial synergistic effect upon combining lentils flour and SLAB51 in the functional cookie was consistent with the reduced levels of glycosylated hemoglobin and resistin, suggesting that the regular consumption of this synbiotic food can effectively counteract impaired glucose homeostasis and insulin resistance that are commonly associated with AD and unhealthy aging. Chronic hyperinsulinemia is a condition that links AD and diabetes, being involved in regulating cerebral Aβ accumulation and clearance. [61]. Moreover, during diabetes pathogenesis, receptors for AGEs are also altered and AGEs

are increased in AD brain. Here, we observed that chronic supplementation with the functional cookie caused a reduced brain concentration of these harmful products contributing to reduce oxidation and inflammation related damages, as also confirmed by brain levels of Iba-1 and plasma concentrations of LPS. As expected, the probiotic treatment mitigated the glycaemic index HbA1c, whereas daily consumption of all the cookies exhibited a propensity to elevate blood HbA1c levels and this is due to differences between cookie ingredients (including chocolate and sugar) and normal laboratory diet. It is interesting to note that the regular consumption of hypocaloric prebiotic cookies enriched with these probiotics successfully counteracted glucose metabolism dyshomeostasis compared to the regular consumption of most commercially available cookies, representing a healthy snacking habit for consumers. In fact, flavonoids in lentils can inhibit both α-glucosidase and lipase, consequently modulating post-meal blood glucose concentration and body weight [52], consistently with our results. Moreover, studies have shown that the combination of prebiotic (oligofructose) and probiotic (*Bifidobacterium animalis*) possesses a synergistic effect in lowering glycemia [62]. Here, the combination of lentils flour with the probiotics reduced blood glucose levels to a higher extent compared to both classic cookie and prebiotic cookie. This enhanced effect may be associated with enhanced brain expression of GLUTs. Evidence suggests that chronic hyperglycemia reduces glucose absorption and utilization in the brain by downregulating glucose transporters (GLUTs) expression, ultimately leading to impaired energy supply and neuronal activity, contributing to cognitive decrease [63]. Treatments containing SLAB51 increased the expression of GLUT1 and GLUT3 compared to control. It is noteworthy that functional cookies significantly increased GLUT3 compared to classic and prebiotic cookie; in fact, GLUT3 has a higher affinity for glucose than other GLUTs and has at least fivefold greater transport capacity than GLUT1 and -4 [64], largely contributing to correct glucose utilization and proper cognitive ability. Consistently, GLUT4 was observed to be unaltered in several AD rodent models compared to the other GLUTs [65].

Concluding, partial synergistic and complementary effects can be observed using this specific prebiotic and probiotic combination, mainly demonstrated by glucose insulinotropic polypeptide being significantly higher in the functional cookie group compared to probiotic treated mice. Moreover, few changes in the gut microbiota composition can partially explain the improved short-term memory upon treatments, which was mostly evident in animals supplemented with the functional cookie, substantiating the combined use of lentils flour and SLAB51 over individual components in a functional hypocaloric snack. Moreover, the use of a multi-strain formulation instead of a single probiotic is a strength because it provides favourable environmental conditions for the growth of desired beneficial strains, representing a unique aspect of these bacteria.

Moreover, this PRE+Pro cookie represents an optimal vehicle that is able to ameliorate both lipid and glucose profile compared to daily consumption of classic consumed cookies and with 1 year of shelf life at room temperature (Supplementary Fig. 1). This tailored functional food possesses improved properties with respect to commercially available healthy cookies, contributing to maintain an innovative, sustainable and globally competitive health-related industry, similarly to SLAB51 freshly dissolved in water.

Beyond providing a cognitive improvement, this synbiotic innovative snack, exerted anti-inflammatory and antioxidant effects on the host, representing a prototype of a simple and affordable dietary approach to promote healthy aging and prevent or delay the onset of age-related neurodegenerations.

CRediT authorship contribution statement

Laura Bonfili: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Francesco Maria Grasselli:** Investigation. **Massimiliano Cuccioloni:** Writing – review & editing, Validation, Formal analysis, Data curation. **Valentina Cecarini:** Writing – review & editing, Validation, Formal analysis, Data curation. **Daniela Lufrano:** Writing – review & editing, Validation. **Elena Vittadini:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Livio Galosi:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Gregorio Sonsini:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Massimo Ubaldi:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Jonathan Louis Turck:** Software, Investigation, Data curation. **Luis Fernando da Costa Medina:** Validation, Software, Data curation. **Jan Suchodolski:** Validation, Software, Investigation. **Anna Maria Eleuteri:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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