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Plant Extracts and ω-3 Improve Short-Term Memory and Modulate the Microbiota–Gut–Brain Axis in D-galactose Model Mice

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A B S T R A C T

Background: Aging, characterized by a slow and progressive alteration of cognitive functions, is associated with gut microbiota dysbiosis, low-grade chronic inflammation, as well as increased oxidative stress and neurofunctional alterations. Some nutrients, such as polyphenols, carotenoids, and omega (ω)-3 (n–3), are good candidates to prevent age-related cognitive decline, because of their immunomodulatory, antioxidant, and neuroprotective properties.

Objectives: The objective of this study was to demonstrate the preventive effect of a combination of plant extracts (PE) containing Memophenol™ (grapes and blueberries polyphenols) and a patented saffron extract (saffron carotenoids and safranal) and ω-3 on cognitive function in a mouse model of accelerated aging and to understand the biological mechanisms involved.

Methods: We used an accelerated-aging model by injecting 3-mo-old male C57Bl6/J mice with D-galactose for 8 wk, during which they were fed with a balanced control diet and supplemented or not with PE and/or ω-3 (n = 15–16/group). Short-term memory was evaluated by Y-maze test, following analyses of hippocampal and intestinal RNA expressions, brain fatty acid and oxylipin amounts, and gut microbiota composition (16S rRNA gene sequencing). Statistical analyses were performed (t test, analysis of variance, and Pearson correlation).

Results: Our results showed that oral administration of PE, ω-3, or both (mix) prevented hippocampus-dependent short-term memory deficits induced by D-galactose ($P < 0.05$). This effect was accompanied by the modulation of gut microbiota, altered by the treatment. PE and the mix increased the expression of antioxidative and neurogenesis markers, such as catalase and doublecortin, in hippocampus ($P < 0.05$ for both). Moreover, ω-3 and the mix showed a higher ω-3 amounts ($P < 0.05$) and EPA-derived 18- hydroxyeicosapentaenoic acid ($P < 0.001$) in prefrontal cortex. These changes may contribute to the improvement in memory.

Conclusions: These results suggest that the mix of PE and ω-3 could be more efficient at attenuating age-related cognitive decline than individual supplementations because it targeted, in mice, the different pathways impaired with aging.

Keywords: plant extracts, ω-3, memory, microbiota, D-galactose

Introduction

Brain aging is accompanied by a decline in cognitive functions. These include memory loss and learning deficits [1]. These cognitive disorders occur in 15%–20% of the population aged 65 y and older, and they impair quality of life. Furthermore, the aging process induces alterations in the composition of the intestinal microbiota, involving shifts in the quantity and diversity of microbial bacteria in the gut. More and more studies have reported a link between cognitive function and intestinal microbiota; in fact, the microbiota–gut–brain axis is currently acknowledged as a crucial influencer of behavior during life [2–4]. Furthermore, it has been shown that patients with Alzheimer disease (AD) present altered microbiota composition, metabolites, and bacterial endotoxins [5–9]. Recently, Grabrucker et al. [10] demonstrated that hippocampus-dependent cognitive

Abbreviations: AA, arachidonic acid; DCX, doublecortin; GPx, glutathione peroxidase; PE, plant extracts; SOD, superoxide dismutase; TGF, transforming growth factor; YM, Y-maze.

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deficits are observed in healthy young rats transplanted with gut microbiota of humans diagnosed with AD. The change in the composition of intestinal microbiota with age contributes to the progressive development of chronic inflammation, which is linked to lower cognition, hippocampal neurogenesis, and neuronal plasticity, as well as an increase in oxidative stress [11–14].

During aging, normal age-related cognitive decline can extend to accelerated cognitive decline when associated with risk factors [15]. The occurrence of this cognitive decline can be modified by the regulation of extrinsic factors such as nutrition, which notably influences microbiota [16–18]. Some studies have highlighted the beneficial effect of the Mediterranean diet for the prevention of age-related cognitive disorders [19–21]. Indeed, the Mediterranean diet exhibits positive impacts on the memory of individuals older than 60 y experiencing mild cognitive impairment [22]. Moreover, it increases the microbiota diversity and the number of Bacteroidetes and decreases those of Firmicutes and Proteobacteria [23]. It is notably rich in specific nutrients such as polyphenols and carotenoids, present in fruits and vegetables, as well as omega (ω)-3 derived from marine products. The effects of these nutrients on the prevention of age-related cognitive decline have been individually studied. Indeed, a previous clinical study demonstrated that supplementation with polyphenols from grapes and blueberries can improve episodic memory in healthy elderly with lower levels of memory performance [24]. Moreover, a particular dietary polyphenol profile sourced from specific plant products was linked to a reduced risk of cognitive alteration [25]. Animal studies have reported that the intake of polyphenols can attenuate or delay age-related cognitive decline owing to their neuroprotective properties [26–28]. Moreover, polyphenols may exert a direct impact by modulating the composition of the microbiota and by playing a neuroprotective role through their conversion into bioactive metabolites by the microbiota [29,30]. Saffron carotenoids, picrocrocin and safranal, are also notable bioactive compounds; in a study involving patients with mild-to-moderate AD, saffron exhibited a positive impact on cognitive function [31]. It is able to change gut microbiota diversity and composition in mice, which may impact brain function [32–35]. Carotenoids and safranal also exhibit antioxidant effects by neutralizing free radicals, possess anti-inflammatory properties, and contribute to neuroprotection [36,37]. Moreover, safranal supplementation in aged rats prevents the decrease in antioxidant enzymes [38]. Finally, the beneficial impact of ω-3 on cognitive functions has been widely described in human and animal models [39–45]. Furthermore, in humans, ω-3 could be a way to restore the composition of the microbiota, which is altered during aging [46]. In mice, a diet enriched with EPA and DHA led to a significant rise in the abundance of Firmicutes [47]. Moreover, ω-3 are also well known for their immunomodulatory properties by reducing the concentrations of inflammatory markers and modulating proinflammatory and proresolving oxylipin synthesis [48–51]. In addition, a few studies already investigated the combined effects of polyphenols and lipids, showing that lipid content enhances bioavailability of polyphenols and the accumulation of their metabolites in plasma, describing their protective effects, perhaps by favorizing polyphenols stability during digestion, and their anti-amyloidogenic effects [52–55]. To our knowledge, no study has investigated a combination with saffron carotenoids, but other carotenoids, such as lutein or fucoxanthin, combined with ω-3 have beneficial effects on memory and learning, oxidative stress, and inflammation [56, 57]. Moreover, a combination of EPA, carotenoids, and polyphenols decreases inflammation in BV2 microglia [58].

Hence, the combination of these bioactive food components may have additional or synergistic effects by targeting the different neurobiologic processes involved in aging (neuroinflammation, oxidative stress, and altered neurogenesis) and the microbiota composition to prevent cognitive decline. A recent study showed no effect of combined ω-3 and polyphenols from cocoa on cognition in older adults with memory complaints [59]. However, it focused on a specific class of polyphenols (flavanols). Thus, our study aimed to investigate the impacts of a new formulation combining plant extracts (PE) containing grapes and blueberries polyphenols (Memophenol™) and saffron carotenoids and ω-3 compared with PE or ω-3 alone on the attenuation of age-related cognitive decline in mice. We used a chronic exposure to D-galactose as a model of accelerated aging since its accumulation in the blood induces cognitive decline and leads to the modification of several factors, similar to those altered during aging [60,61]. Indeed, it has been shown that D-galactose induces reactive oxygen species production and increases oxidative stress [62,63]. It also stimulates the expression of proinflammatory factors, reduces hippocampal neurogenesis, and causes microbiota disorders [60,64,65]. In this context, we examined the influence of PE and/or ω-3 supplementations on spatial memory task, classically altered by aging [66]. Additionally, our investigation extended to the evaluation of their impact on gut microbiota composition and on key biologic processes associated with aging, including oxidative stress, neuroinflammation, and altered neurogenesis.

Methods

Animals and treatments

All experiments were performed with 3-mo-old male C57Bl/6J mice obtained from Janvier Labs (Le Genest-Saint-Isle, France). Mice were maintained under standard housing conditions, on cellulose litter, in a temperature (21 ± 2 °C) and humidity (40%) controlled environment with a 12-h light/dark cycle (7:00–19:00) and with ad libitum access to water and food. Animal husbandry and experimental procedures were done in accordance with the EU Directive 2010/63/EU for animal experiments and were approved by the National Ethical Committee for the Care and Use of Animals (approval ID A27756). A total of 77 animals, separated in 5 groups ($n = 15$–16/group) were used in this study. Mice were fed with an ω-6/ω-3 balanced diet supplemented or not with a formulation containing PE including polyphenols from grape and blueberry extracts (3.69 mg/d, equivalent to 600 mg/d in humans; Memophenol™; Activ'Inside) and carotenoids and safranal from saffron extract (0.1845 mg/d, equivalent to 30 mg/d in humans; patent pending WO2021209455A1; Activ'Inside) and ω-3 in triglyceride form (6.15 mg/d of DHA and 1.51 mg/d of EPA, equivalent to 1000 and 245.5 mg/d and in humans) or with PE or ω-3 alone (composition of diets in Table 1). The amounts of the 3 supplements were chosen on the basis of previous studies and adapted for mice [24,67,68]. During the 8 wk of supplementation, mice received 5 d/wk subcutaneous injections of D-galactose

TABLE 1
Composition of the diets

	Control and D-Gal	D-Gal (PE)	D-Gal (om-3)	D-Gal (PE+om-3)
Ingredients (g/kg of diet)				
Corn starch	460	458.9	460	458.9
Sucrose	230	230	230	230
Cellulose	20	20	20	20
Casein	180	180	180	180
AIN-93M mineral mix	50	50	50	50
AIN-93Vx vitamin mix	10	10	10	10
Fat[1]	50	50	43.5	43.5
Supplements (g/kg of diet)				
Memophenol™[2]	0	1.05	0	1.05
Saffron extract[3]	0	0.527	0	0.527
Fish oil (om-3)	0	0	6.5	6.5

[1] Rapeseed oil, 47%; sunflower oil, 48%; linseed oil, 5%.
[2] Total polyphenol ≥ 75%: flavonoids (flavan-3-ols, flavonols, anthocyanins) ≥ 43%, flavan-3-ols monomers ≥ 20%, flavan-3-ols oligomers (DP ≤ 4) ≥ 22%, flavonols (including quercetin and glycosylated derivatives) ≥ 0.15%, anthocyanins (including malvidin 3-glucoside) ≥ 0.10%, phenolic acids ≥ 0.50%, stilbenes (including resveratrol) ≥ 300 ppm.
[3] Crocins (mainly trans-4-GG, trans-3-Gg; cis-4-GG, trans-2-G) > 3%, safranal > 0.2%, picrocrocin derivatives (mainly picrocrocin; HTCC) > 1%, and kaempferol derivatives (mainly kaempferol-3-sophoroside-7-glucoside and kaempferol-3-sophoroside) > 0.1%.

(Sigma-Aldrich) at a dose of 160 mg/kg, diluted in saline solution (NaCl 0.9%) [69]. Control mice were injected with saline solution. There were no differences in food intake or weight gain between groups. At the end of the experiment, mice were killed. Brain structures of interest—hippocampus, known to play an important role in learning and memory consolidation [70], and prefrontal cortex, implicated in working memory, intestines (ileum and colon), and colon feces were isolated and stored at −80 °C until analysis.

Behavioral testing: Y-maze

Short-term hippocampus-dependent memory was assessed with a Y-maze (YM) test, as described in Chataigner et al. [66], during the last week of supplementation. Briefly, a Y-shaped maze with 3 identical arms was used (34 × 8 × 14 cm) in a testing room with visual cues on the walls and illuminated at 21 lux in the center of the maze. In the first part of the test (acquisition), mice were placed for 5 min in the maze with 1 arm closed by a door and allowed to explore the other 2 arms. After 1 h of intertrial interval, mice were placed again for 5 min in the maze but with all arms opened and were allowed to explore the 3 arms. Closed and start arms were randomly assigned to the mice. To analyze time spent in the arms, animals were video tracked using SMART system (Bioseb). A recognition index (time spent in the new arm/total time spent in all arms × 100) was calculated for the first 3 min to compare the performance of the mice with chance (33%).

Quantitative real-time PCR

Total hippocampal and intestinal RNAs were extracted using the extraction protocol of TRIzol (Invitrogen). Amount and purity of RNA were determined with Nanodrop photospectrometer (Nanodrop One; Life Technologies). Two micrograms of RNA were reverse transcribed to synthesize complementary DNA using Superscript IV (Invitrogen). As previously described, complementary DNAs were amplified by PCR using TaqMan primers specific to target genes studied [49,71]. We focused on the following inflammatory, oxidant, neuroprotective, and neurogenesis markers: IL-6 (Mm00446190_m1), IL-1β (Mm00434228_m1), TNF-α (Mm00443258_m1), transforming growth factor (TGF) β1 (Mm01178820_m1), integrin αM (Mm00434455_m1), fructose transporter type 5 (Mm00600311_m1), cyclooxygenase-2 (Mm00478374_m1), cytokine-inducible nitric oxide synthase (Mm00440502_m1), receptor for advanced glycation end products (Mm00545815_m1), catalase (Mm00437992_m1), glutathione peroxidase (GPx) 1, Mm00656767_g1), superoxide dismutase (SOD) 1 (Mm01344233_g1), SOD2 (Mm01313000_m1), brain-derived neurotrophic factor (Mm04230607_s1), nerve growth factor (Mm00443039_m1), Ca2+/calmodulin-dependent protein kinase II (Mm00437967_m1), doublecortin (DCX; Mm00438400_m1) in the hippocampus and permeability markers zonula occludens 1 (Mm00493699_m1), occludin (Mm00500912_m1), myosin light chain kinase (Mm00653039_m1), and claudin5 (Mm00727012_s1) in ileum and colon. β2-Microglobulin (Mm00437762_m1) and GAPDH expression (Mm99999915_g1) were measured but only β2-microglobulin was used as reference gene considering its higher stability. Fluorescence was measured using a LightCycler 480 instrument II (Roche), and final quantification was analyzed by the comparative threshold cycle method. Results are expressed as relative fold change to control target mRNA expression and are normalized to control or D-galactose groups [49,71].

Lipid analysis

Lipid analysis of the prefrontal cortex was performed by the CSGA (INRAE UMR 1324, Dijon, France) as previously described [45,72,73]. After lipid extraction, fatty acids were methylated following the procedures detailed in previous studies [45,72,73]. The resulting fatty acid methyl esters underwent analysis via gas chromatography using a Hewlett Packard Model 5890 series II gas chromatograph. This system was equipped with a split/-spitless injector, a flame ionization detector, and a CPSIL-88 column (100 m × 0.25 mm internal diameter; film thickness, 0.20 μm; Varian). Hydrogen was used as the carrier gas at an inlet pressure of 210 kPa. The process involved maintaining the

oven temperature at 60 °C for 5 min, then increasing it to 165 °C at a rate of 15 °C/min, holding for 1 min, and subsequently raising it to 225 °C at 2 °C/min. Finally, the temperature was held at 225 °C for 17 min. The injector and detector temperatures were maintained at 250 and 280 °C, respectively. Fatty acid methyl esters were identified by comparison with commercially available standards. The fatty acid composition is expressed as a percentage of the total fatty acids detected.

Oxylipin quantification

Targeted lipidomic was performed at the METATOUL platform (MetaboHUB). The distinct metabolites originating from linoleic acid, arachidonic acid (AA), α-linolenic acid, DHA, and EPA were isolated from the prefrontal cortex and underwent analysis via mass spectrometry (LC-MS/MS), following the methods outlined by Le Faouder et al. [74]. Results are expressed as pictograms per milligrams of protein.

Microbiota analysis

These analyses were performed at the UMGC Microbiome Services platform (Genomic Center of the University of Minnesota) on randomly chosen animals ($n = 7$–8). Genomic DNA was extracted from the fecal content using the QIAamp DNA Stool Mini Kit (Qiagen), which involved a bead-beating step following protocol Q as described by Costea et al. [75]. Subsequently, the concentration of double-stranded DNA was measured using the NanoPhotometer GUT MICROBES e2007042-13 Spectrophotometer (Implen). To analyze the gut microbiota composition, Illumina sequencing of the 16S rRNA gene was performed. The V3–V4 region of the 16S rRNA gene was enriched via PCR using primer pairs V3F_Nextera (CCTACGGGAGGCAGCAG) and Meta_V4_806R (GGACTACHVGGTWTCTAAT). The resulting amplicons were purified, quantified, and sequenced using an Illumina Miseq, generating 2 × 300-bp sequencing products at the University of Minnesota Genomics Center. During the sequencing run, each base call was assigned a quality score following Illumina's quality scoring methodology, with a mean quality score per sample exceeding 33.8. The sequence reads were automatically converted to FASTQ, a text file format, using a bcl2fastq converter. The obtained 16S rDNA amplicon sequences underwent analysis using the FROGS (Find Rapidly OTUs with Galaxy Solution) bioinformatics pipeline [76]. Amplicons were filtered based on size and clustered into operational taxonomic units (OTUs) using Swarm (with aggregation parameter d = 1 + d = 3). Chimeras were eliminated using VSEARCH in combination with innovative chimera crossvalidation, and OTUs were retained if they represented 0.005% of the total number of sequences [77]. The OTUs were classified using the reference database Silva138 16S, using a pintail quality of 100 [78]. The relative abundance of each OTU was calculated after data normalization using a threshold of 33133 reads per sample. qPCR targeting the 16S rDNA was used to quantify the abundance of total bacteria and *Bifidobacterium* spp. PCR amplification was conducted under specific conditions, and detection was achieved using the QuantStudio3 instrument and software (Applied Biosystems), using the GoTaq qPCR MasterMix Plus for SYBR Assay (Promega). Bovine serum albumin was added to the samples, and each assay was performed in duplicate within the same run. Standard curves for quantification were constructed using 5-fold dilution series from target species genomic DNA preparations (DSMZ).

Statistical analyses

Statistical analyses were performed using GraphPad Prism software (version 10.1.0). Model efficiency was analyzed by comparing control to D-galactose groups using unpaired *t* test. Then, all groups that received D-galactose injections were compared using 1-way analysis of variance (ANOVA; diet factor). We checked the normality and the equality of variances for each data. All our data are normally distributed except SOD1, SOD2, TGF-β, 18-hydroxyeicosapentaenoic acid (HEPE), and claudin5. However, ANOVA test is fairly robust to deviations from normality, especially with our data whose sample size is >30. We realized Fisher least significant difference post hoc test comparison without correction for multiple comparisons as proposed by Rothman [79]. To analyze the results of the YM test, the groups were compared by a 1-sample *t* test against the chance level (33%) [66]. For microbiota, the analysis of sequenced data involved the utilization of the QIIME (Quantitative Insights Into Microbial Ecology) pipeline (version 1.9.0). The selection of OTUs was conducted against the Greengenes database (version 13.8). Bioinformatic analyses, as well as assessments of gut bacterial diversity, including both α-diversity and β-diversity, were performed using the web interface of MicrobiomeAnalyst (version 2.0, accessible at https://www.microbiomeanalyst.ca/; based in Canada). Finally, Pearson correlation analysis was performed with R Statistical software for the different D-galactose groups (R Core Team). Although the data are not entirely normally distributed, they mostly follow a normal distribution. Pearson correlation is considered robust to minor deviations from normality and can still be validly estimated in skewed distributions [80]. We considered a strong relationship between 2 variables only when the correlation coefficient r exceeded 0.7 or went below −0.7. All data are expressed as means ± SEM unless otherwise stated. Statistical significance was set at $P \leq 0.05$.

Results

PE, ω-3, and the mix of both supplementations prevent short-term memory deficits induced by D-galactose

The short-term memory was evaluated with the YM test. The recognition index of the control group was significantly different from the chance level (33%), indicating that control mice correctly discriminated the new arm and did not present memory deficits ($P = 0.030$) (Figure 1). As expected, D-galactose–treated mice did not discriminate the new arm, but mice supplemented with PE, ω-3, or PE+ω-3 recognized it (PE, $P = 0.028$; ω-3, $P = 0.001$; PE+ω-3, $P = 0.048$). These results suggested that all supplementations prevented the memory deficits induced by D-galactose.

PE and the mix modulate oxidative, neurogenesis, and inflammatory marker expression in hippocampus

In order to understand the mechanisms underlying the improvement of cognitive performance in supplemented groups,

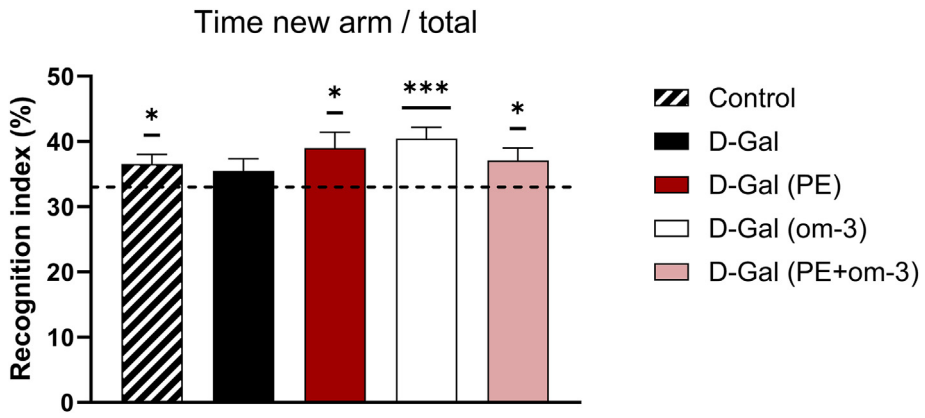

FIGURE 1. Effect of D-galactose treatment and supplementations on short-term memory. Recognition index of the new arm in the Y-maze after 1 h of intertrial interval. The dotted line corresponds to chance level (33%). *$P < 0.05$, ***$P < 0.001$ vs chance level by 1-sample t test, n = 15–16/group).

we analyzed the expression of different genes involved in oxidative stress, inflammation, and neurogenesis in hippocampus (Figure 2). The first column represents the effect of D-galactose treatment compared with control; the other columns represent the effect of the supplementations compared with nonsupplemented D-galactose–treated mice. D-galactose decreased the expression of the antioxidant marker catalase (P = 0.028) and tended to increase the expression of the cytokine TGF-β (P = 0.065) compared with the control group but did not have any significant effect on the other genes explored. However, we observed a supplementation effect on the expression of the antioxidant markers SOD1 [$F_{(3,52)} = 3.743$; $P = 0.017$], GPx1 [$F_{(3,55)} = 3.410$; $P = 0.024$], and catalase [$F_{(3,51)} = 3.360$; P = 0.026] and a slight tendency toward the expression of SOD2 [$F_{(3,47)} = 2.205$; $P = 0.099$]. More precisely, compared with D-galactose control mice, those supplemented with PE exhibited a greater expression of the antioxidant markers GPx1 ($P = 0.008$) and catalase ($P = 0.020$), and those supplemented with PE+ω-3

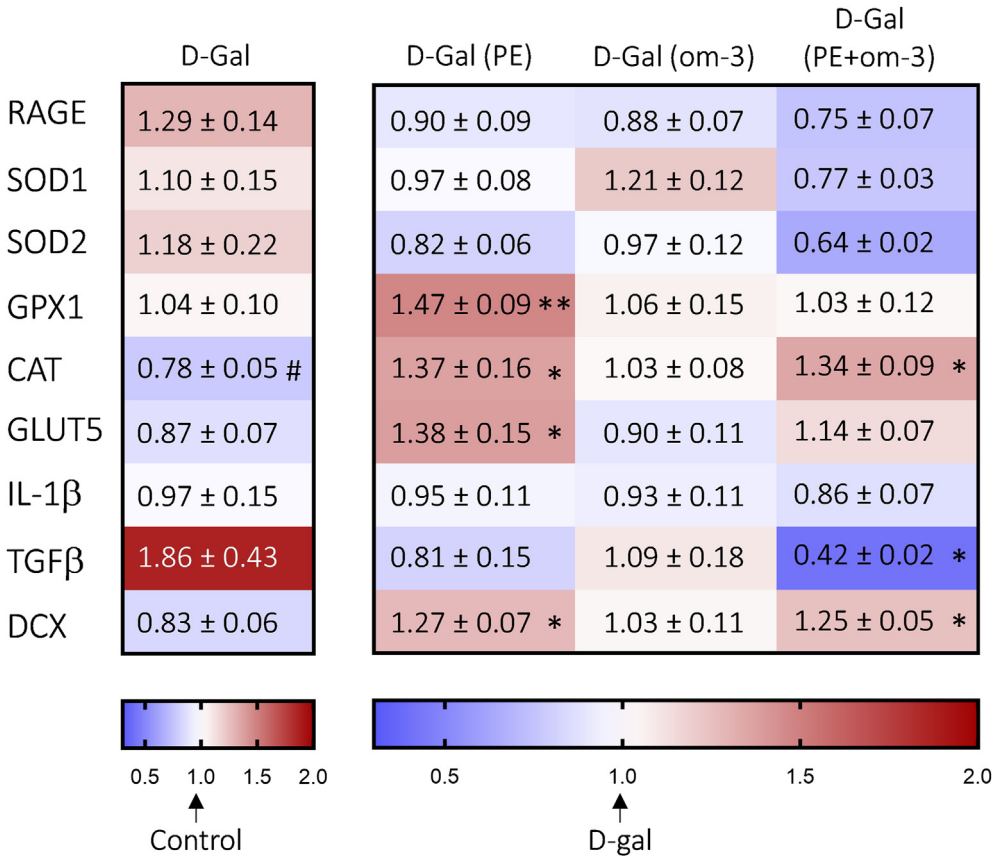

FIGURE 2. Effect of D-galactose treatment and supplementations on oxidative, inflammatory, and neurogenesis markers expressions in hippocampus. #$P < 0.05$ vs control by t test, *$P < 0.05$, **$P < 0.01$ vs D-galactose by 1-way ANOVA and Fisher least significant difference post hoc test; n = 15–16/group. Relative expressions are presented as mean ± SEM.

had a higher expression of catalase ($P = 0.025$). Supplementations also exhibited an effect on inflammatory markers: Glut5 [$F_{(3,51)} = 3.772$; $P = 0.016$], which is a marker of microglial homeostasis, and TGF-β [$F_{(3,52)} = 3.650$; $P = 0.018$]) [81]. Indeed, PE supplementation increased the expression of Glut5 ($P = 0.015$), and PE+ω-3 supplementation had a 2 times lower expression of TGF-β ($P = 0.014$) than D-galactose control. Finally, PE and PE+ω-3 supplementations increased the expression of the neurogenesis marker DCX as compared with D-galactose control [ANOVA: $F_{(3,50)} = 3.333$; $P = 0.027$; PE compared with D-galactose control: $P = 0.019$; PE+ω-3 compared with D-galactose control: $P = 0.026$]. In addition, ω-3 supplementation did not have any effect on these markers. Supplementations had no impact on receptor for advanced glycation end products and IL-1β. D-galactose treatment and supplementations did not have any effect on the other inflammatory and neuroprotective markers tested (data not shown).

ω-3 and PE+ω-3 supplementations impact PUFA and oxylipin amounts in prefrontal cortex

The impact of the supplementations on fatty acid and oxylipin compositions was investigated in prefrontal cortex, as it seems to be comparable with the hippocampus in terms of fatty acid composition and susceptibility to changes caused by dietary supplementation [82]. D-galactose treatment did not have any effect on these compositions (data not shown), but the supplementations significantly affected the ω-6/ω-3 ratio and AA, DHA, and EPA and its derivate 18-HEPE amounts [ω-6/ω-3 ratio: $F_{(3,57)} = 23.87$; $P < 0.001$; AA: $F_{(3,57)} = 23.14$; $P < 0.001$; DHA: $F_{(3,57)} = 2.936$; $P = 0.041$; EPA: $F_{(3,57)} = 5.009$; $P = 0.004$; 18-HEPE: $F_{(3,55)} = 14.30$; $P < 0.001$] (Figure 3A–E). Indeed, compared with D-galactose control, ω-3, and PE+ω-3 supplementations significantly decreased the ω-6/ω-3 ratio ($P < 0.001$ for both) and AA amounts ($P < 0.001$ for both) and increased DHA (D-galactose compared with ω-3 ($P = 0.044$), D-galactose compared with PE+ω-3 ($P = 0.028$), and EPA amounts (D-galactose compared with ω-3: $P = 0.002$; D-galactose compared with PE+ω-3: $P = 0.016$) as well as 18-HEPE amounts ($P < 0.001$ for both). PE did not have any effect on fatty acid and oxylipin concentrations.

PE and PE+ω-3 supplementations attenuate alterations in gut permeability

To evaluate gut permeability, we analyzed the expression of different genes in ileum and colon (Figure 4). The first column represents the effect of D-galactose treatment compared with control, the other columns represent the effect of the supplementations compared with nonsupplemented D-galactose–-treated mice. D-galactose control mice had a lower expression of zonula occludens 1 in colon than control mice ($P = 0.023$), indicating an increase in gut permeability. D-galactose treatment did not affect the expression of the other markers in colon or ileum. Supplementations significantly impacted the expression of claudine5 [$F_{(3,52)} = 3.507$; $P = 0.022$], occludin [$F_{(3,55)} = 6.859$; $P = 0.001$], and myosin light chain kinase [$F_{(3,57)} = 2.985$; $P = 0.039$] in ileum. More precisely, when compared with D-galactose treatment, PE supplementation decreased occludin expression ($P = 0.027$), and PE+ω-3 supplementation increased claudine5 and occludin expression ($P = 0.009$ and $P = 0.026$, respectively). Moreover, ω-3 supplementation had no effect on these markers. These results suggested that D-galactose treatment altered gut permeability and that PE+ω-3 supplementation attenuated the alteration of permeability.

All supplementations modulate gut microbiota composition

α-Diversity was measured with Chao1 index to measure the richness and Shannon index to measure the richness and evenness. D-galactose treatment significantly increased both indexes compared with control (Chao1: $P = 0.001$; Shannon: $P = 0.006$)

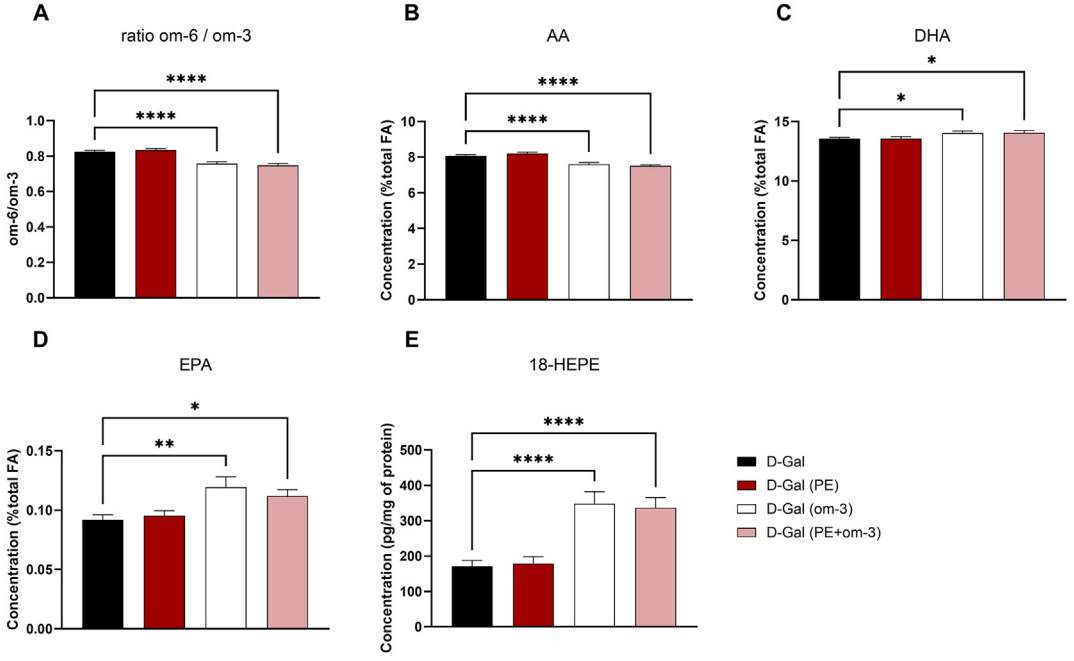

FIGURE 3. Effect of supplementations on fatty acid and oxylipin concentrations in prefrontal cortex (A–E). *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$ vs D-galactose by 1-way ANOVA and Fisher least significant difference post hoc test; $n = 15$–16/group. Data are presented as mean ± SEM.

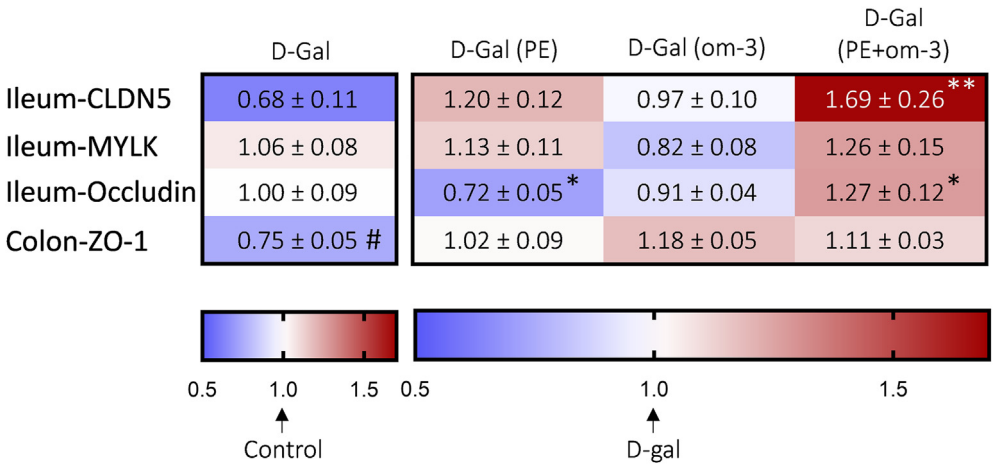

FIGURE 4. Effect of D-galactose treatment and supplementations on gut permeability marker expression in ileum and colon. #$P < 0.05$ vs control by t test, *$P < 0.05$, **$P < 0.01$ vs D-galactose by 1-way ANOVA and Fisher least significant difference post hoc test; $n = 15$–16/group. Relative expressions are presented as mean ± SEM.

(Figure 5A,B). Supplementations did not have any effect on Chao1 index (Figure 6A) but have an effect on Shannon index [$F_{(3,25)} = 3.391$; $P = 0.034$]. Indeed, PE+ω-3 induced a greater diversity than PE ($P = 0.025$) and ω-3 ($P = 0.008$) alone (Figure 6B). To go further in the diversity analyses, β-diversity was also evaluated using principal coordinate analysis with Bray–Curtis dissimilarity. It revealed differences in microbiota composition between control and D-galactose–treated mice ($P = 0.04$) (Figure 5C) and between all D-galactose–treated groups ($P = 0.001$) (Figure 6C).

Relative abundance of gut microbiota at phylum, family, and genus levels was analyzed (Figure 7). The first 2 columns represent the effects of D-galactose treatment compared with control; the other columns represent the effects of the supplementations compared with nonsupplemented D-galactose–-treated mice. Two phyla were affected by D-galactose treatment and 3 by supplementations. Indeed, D-galactose significantly decreased the abundance of Actinobacteria ($P = 0.041$) and increased that of Deferribacteres ($P = 0.019$). Further, ω-3 and PE+ω-3 supplementations had an effect on the abundance of Actinobacteria [$F_{(3,25)} = 4.044$; $P = 0.018$], Proteobacteria [$F_{(3,25)} = 3.491$; $P = 0.031$], and Patescibacteria [$F_{(3,25)} = 6.337$; $P = 0.002$]. More precisely, ω-3 increased the abundance of Actinobacteria ($P = 0.042$) and decreased the abundance of Proteobacteria when compared with D-galactose control ($P = 0.011$). PE+ω-3 increased the abundance of Patescibacteria compared with D-galactose control ($P = 0.003$). PE supplementation did not have any significant effect at the phylum level.

Five families were modulated by D-galactose treatment compared with the control group: 4 were increased (Lachnospiraceae: $P = 0.010$, Desulfovibrionaceae: $P = 0.037$, Ruminococcaceae: $P = 0.009$, and Helicobacteraceae: $P = 0.019$), and 1 was decreased (Bifidobacteriaceae: $P = 0.022$). Three families' abundances were changed by supplementations: Bifidobacteriaceae [$F_{(3,25)} = 3.901$; $P = 0.021$], Desulfovibrionaceae [$F_{(3,25)} = 4.089$; $P = 0.017$] and Saccharimonadaceae [$F_{(3,25)} = 6.336$; $P = 0.002$]. More specifically, PE group showed a lower abundance of Desulfovibrionaceae ($P = 0.045$) than D-galactose group. Further, ω-3 reversed the effect of D-galactose treatment on Bifidobacteriaceae ($P = 0.031$) and Desulfovibrionaceae ($P = 0.031$). PE+ω-3 supplementation increased the abundance of Saccharimonadaceae compared with D-galactose control ($P = 0.003$).

D-galactose treatment affected the abundance of 6 genera. Indeed, *Ruminiclostridium* ($P = 0.003$), *Tyzzerella* ($P = 0.022$), *Lachnoclostridium* ($P = 0.041$), *Oscillibacter* ($P = 0.018$), and *Helicobacter* ($P = 0.019$) genera had a higher abundance in D-galactose–treated mice than those in control mice, whereas *Bifidobacterium* genus was reduced by the treatment ($P = 0.022$). Supplementations affected the abundance of 6 genera compared with D-galactose treatment: the concentrations of *Bifidobacterium* [$F_{(3,25)} = 3.901$; $P = 0.021$], *Faecalibaculum* [$F_{(3,25)} = 6.052$; $P = 0.003$], *Candidatus Saccharimonas* [$F_{(3,25)} = 6.336$; $P = 0.002$], *Lachnospiraceae_NK4A136* [$F_{(3,25)} = 3.240$; $P = 0.039$], *Turicibacter* [$F_{(3,25)} = 4.164$; $P = 0.016$], and *Lachnoclostridium* [$F_{(3,25)} = 4.020$; $P = 0.018$]. Indeed, PE supplementation increased the concentration of *Faecalibaculum* ($P = 0.047$) and decreased that of *Lachnoclostridium* ($P = 0.012$) compared with D-galactose control. Similarly, mice supplemented with ω-3 revealed greater abundances of *Bifidobacterium* ($P = 0.031$) and *Faecalibaculum* ($P = 0.001$) and a lower abundance of *Lachnoclostridium* ($P = 0.016$) than D-galactose control mice. Finally, *Candidatus Saccharimonas*, *Lachnospiraceae_NK4A136*, and *Turicibacter* were increased by PE+ω-3 supplementation ($P = 0.003$; $P = 0.034$; and $P = 0.012$, respectively).

Correlation analysis

To understand the link between memory and the biological markers, we performed a correlation analysis in the different D-galactose groups (Supplemental Figure 1). We only describe the correlations between variables that were significantly modulated within each group. Interestingly in D-galactose group, although microbiota was not correlated with cognitive, antioxidative, inflammatory, or intestinal permeability parameters, the relative abundance of certain genera of adverse bacteria were correlated with each other. Indeed, the abundance of *Lachnoclostridium* genus was positively correlated with that of *Oscillibacter* ($r = 0.84$; $P = 0.009$), *Ruminiclostridium* ($r = 0.89$; $P = 0.003$), and *Tyzzerella* ($r = 0.86$; $P = 0.006$) genera. Moreover, the abundance of *Oscillibacter*

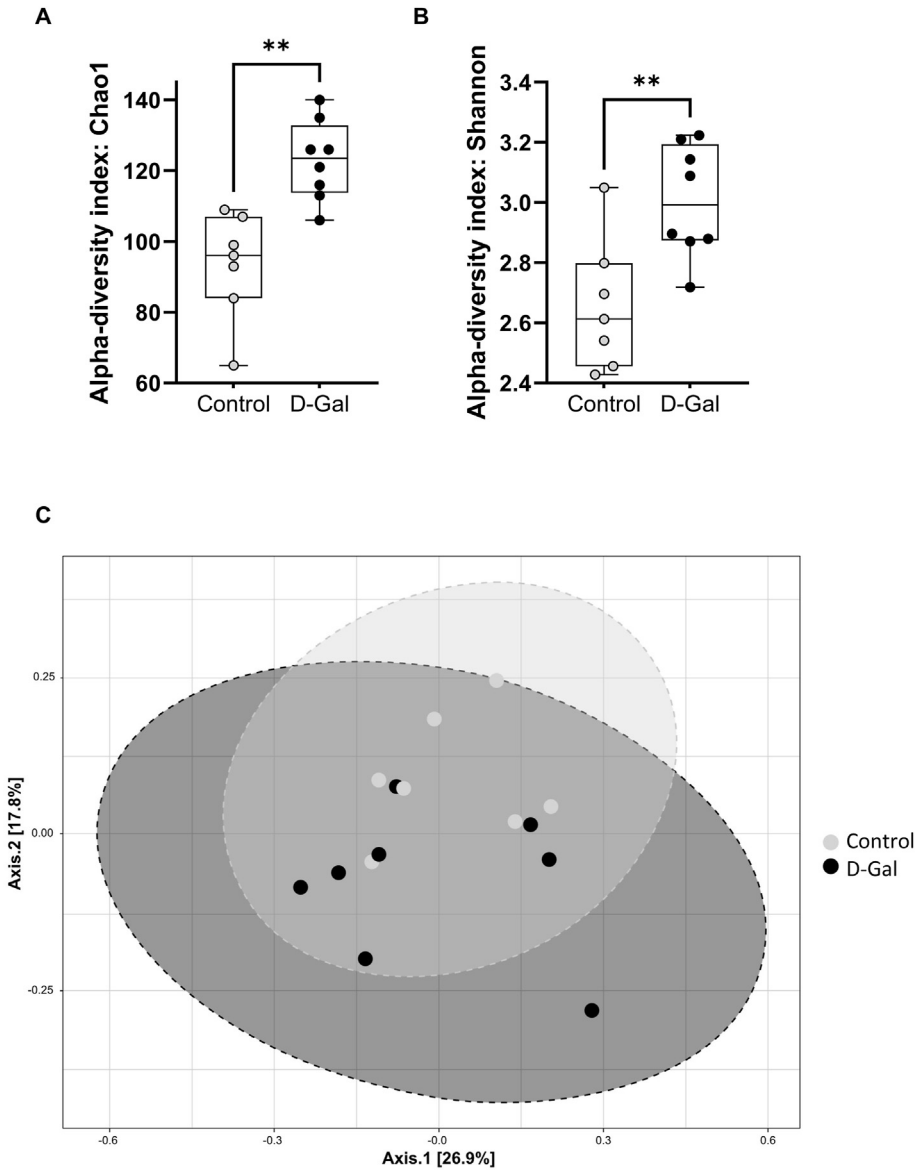

FIGURE 5. Gut microbial diversity analysis of control and control D-galactose groups. (A) Chao1 index. (B) Shannon index. $^{**}P < 0.01$ by *t* test). Data are presented in boxes and whiskers where the whiskers are minimum to maximum. (C) Principal coordinate analysis with Bray–Curtis dissimilarity ($n = 7$–8/group).

was also positively correlated with that of *Ruminiclostridium* ($r = 0.78$; $P = 0.023$) and *Tyzzerella* ($r = 0.77$; $P = 0.027$). In PE and ω-3–supplemented groups, cognitive parameter was significantly correlated with microbiota suggesting that microbiota played a role in preservation of cognitive function in these groups. Indeed, the YM recognition index was positively correlated with *Faecalibaculum* in the PE group ($r = 0.83$; $P = 0.021$) and positively correlated with *Bifidobacterium* in the ω-3 group ($r = 0.80$; $P = 0.053$). In the PE group, the relative abundance of *Faecalibaculum* was positively correlated with antioxidant markers GPx1 ($r = 0.76$; $P = 0.049$) and catalase ($r = 0.80$; $P = 0.032$), indicating a function of microbiota in their regulation. Moreover, *Faecalibaculum* was negatively correlated with occludin expression in ileum ($r = -0.81$; $P = 0.049$). In the mix-supplemented group, none of the bacteria significantly modulated by the diet correlated with the YM recognition index. However, microbiota was associated with the modulation of oxidative and neurogenesis parameters and brain fatty acid concentration. Indeed, the abundance of *Candidatus Saccharimonas* was positively correlated with catalase expression ($r = 0.76$; $P = 0.030$) and the abundance of *Lachnospiraceae_NK4A136* was negatively correlated with DCX expression ($r = -0.71$; $P = 0.048$) and with the DHA concentration ($r = -0.81$; $P = 0.014$). Moreover, DHA concentration was also negatively correlated with ω-6/ω-3 ratio ($r = -0.81$; $P < 0.001$).

Discussion

Aging induces memory impairment associated with biochemical changes and dysbiosis, which can lead to a significant decrease in life quality. This study demonstrated that cognitive deficits can be prevented by nutritional intervention in a model of accelerated aging. More particularly, we found complementary effects between phytonutrients from PE (Memophenol™ and a patented saffron extract) and ω-3 from fish on

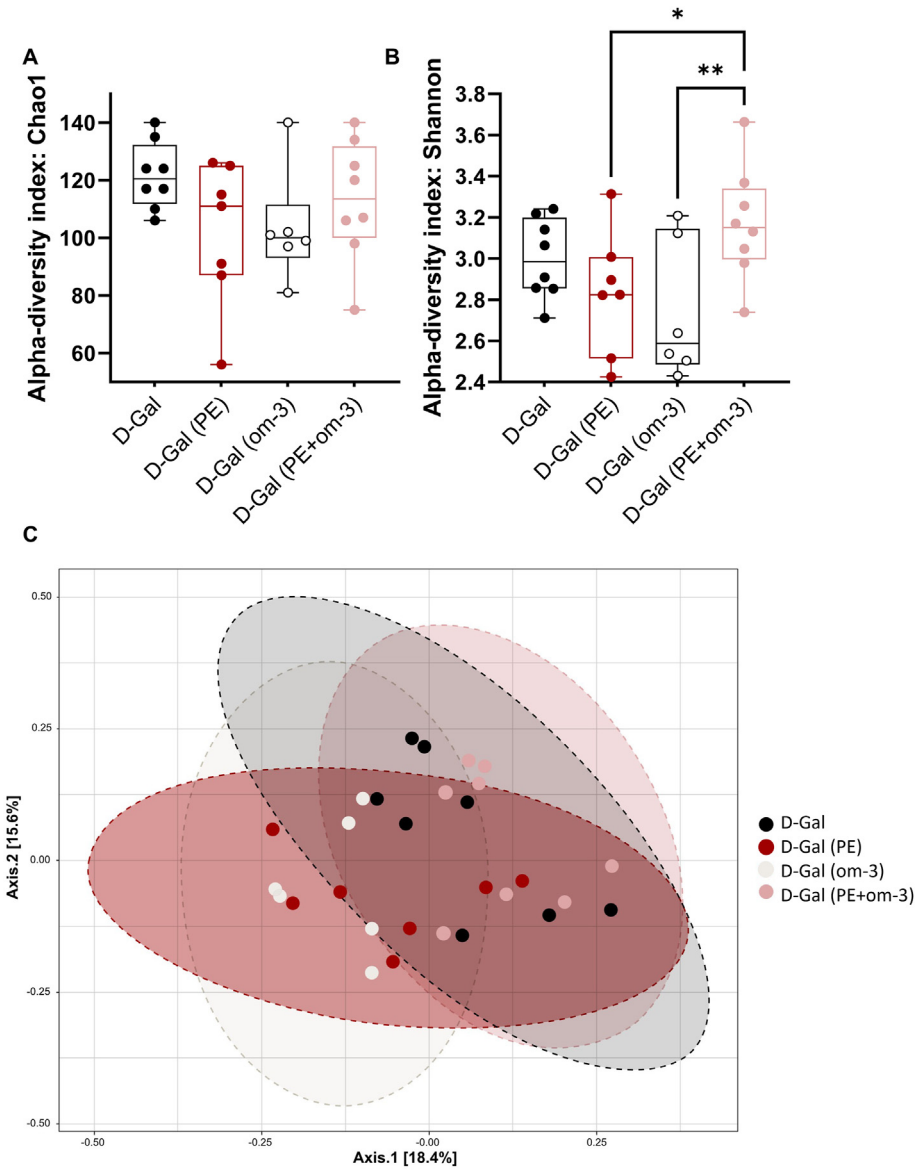

FIGURE 6. Gut microbial diversity analysis of D-galactose groups, supplemented or not. (A) Chao1 index. (B) Shannon index. *$P < 0.05$, **$P < 0.01$ by 1-way ANOVA and Fisher least significant difference post hoc test. Data are presented in boxes and whiskers, where the whiskers are minimum to maximum. (C) Principal coordinate analysis with Bray–Curtis dissimilarity ($n = 7$–8/group).

the attenuation of age-related alterations. This effect was associated with a modulation of gut microbiota diversity and composition and gut permeability as well as an increase in cerebral ω-3 and antioxidant and neuroprotective effects.

We used a D-galactoseactose accelerated-aging model widely described that induces cognitive deficits, oxidative stress, inflammation, and dysbiosis [60,62,63,65]. Our results are in accordance with those of previous studies showing short-term spatial memory impairments in D-galactose–treated mice [83, 84]. These deficits were not found in supplemented mice, indicating that PE (Memophenol™ and the patented saffron extract) and ω-3, alone or in combination, can prevent age-related cognitive decline. It is crucial to understand the molecular mechanisms contributing to the prevention of memory deficits in order to assess the effectiveness of our supplementations.

Aging is accompanied by changes in the gut microbiota composition, which can affect memory via the gut–brain–axis [2, 11,85]. In our study, we confirmed that D-galactose treatment increased α-diversity, suggesting that it impacted the integrity of gut microbiota [86]. We also demonstrated that D-galactose treatment induced a change in microbiota profile by inducing higher abundance of adverse bacteria correlated with each other, all belonging to Firmicutes phyla, known to be increased by a Western diet [23]. It increased the abundance of *Tyzzerella*, which was correlated with cognitive deficits in humans [87]. D-galactose–treated mice also showed greater abundances of the inflammation-associated genera *Lachnoclostridium*, *Oscillibacter*, and *Ruminiclostridium*, the last also being more abundant in model mice with AD [88–90]. Even if we did not detect inflammation in hippocampus, inflammation is generally related to a decrease in cognitive performances, and the modulation of inflammation-associated genera may influence global inflammation [91,92]. Moreover, D-galactose decreased the abundances of Actinobacteria and, more specifically, *Bifidobacterium*

		Control	D-Gal	D-Gal (PE)	D-Gal (om-3)	D-Gal (PE+om-3)
PHYLUM	Actinobacteria	19.670	13.130 #	15.350	21.140 *	8.321
	Proteobacteria	2.137	3.539	1.875	0.992 *	3.342
	Patescibacteria	0.483	0.315	0.164	0.202	1.480 **
	Deferribacteres	0.009	0.055 #	0.021	0.017	0.109
FAMILIES	Bifidobacteriaceae	17.550	10.810 #	13.800	19.360 *	6.940
	Lachnospiraceae	2.262	5.423 ##	2.945	2.588	6.373
	Desulfovibrionaceae	1.009	2.628 #	1.025 *	0.817 *	3.017
	Ruminococcaceae	0.654	1.629 ##	0.891	0.912	1.676
	Saccharimonadaceae	0.483	0.315	0.164	0.202	1.479 **
	Helicobacteraceae	0.009	0.055 #	0.021	0.017	0.109
GENUS	*Bifidobacterium*	17.550	10.810 #	13.800	19.360 *	6.940
	Faecalibaculum	2.166	1.810	4.959 *	7.820 ***	2.389
	Candidatus Saccharimonas	0.483	0.315	0.164	0.202	1.479 **
	Lachnospiraceae_NK4A136	0.368	0.497	0.358	0.282	1.307 *
	Turicibacter	0.196	0.533	0.516	0.191	2.075 *
	Ruminoclostridium	0.076	0.305 ##	0.090	0.144	0.270
	Tyzzerella	0.073	0.198 #	0.134	0.079	0.143
	Lachnoclostridium	0.041	0.100 #	0.033 *	0.033 *	0.089
	Oscillibacter	0.012	0.110 #	0.082	0.073	0.130
	Helicobacter	0.009	0.055 #	0.021	0.017	0.109

FIGURE 7. Effect of D-galactose treatment and supplementations on gut microbiota composition. #$P < 0.05$, ##$P < 0.01$ vs control by *t* test; *$P < 0.05$, **$P < 0.01$ vs D-galactose by 1-way ANOVA and Fisher least significant difference post hoc test; $n = 7$–8/group. Relative abundances are presented as mean.

(Bifidobacteriaceae), which are reported to be lower in humans with poor cognitive scores and in those with AD [93,94].

In this study, the mix of PE (Memophenol™ and the patented saffron extract) and ω-3, which prevented cognitive alterations, was also associated with gut microbiota changes. The mix supplementation increased the abundance of some beneficial bacteria (*Lachnospiraceae_NK4A136*, *Candidatus Saccharimonas*, and *Turicibacter*). Moreover, it had an effect on gut permeability. Age-induced intestinal dysbiosis weakens the integrity and function of the intestinal barrier, leading to the release of LPS into the circulation, accelerating inflammation and the onset of cognitive disorders [11]. Mice supplemented with the mix showed an increased expression of gut permeability markers, indicating greater integrity of the gut barrier, which could therefore play a role in the prevention of cognitive decline. This effect could be explained by a higher abundance of *Lachnospiraceae_NK4A136,* which is known to be associated with an improvement of intestinal barrier function in aged rats and is negatively correlated with inflammation [95,96]. Moreover, the mix supplementation increased *Candidatus Saccharimonas*, which has an anti-inflammatory effect by secreting metabolites (such as lactate and acetate) and maintaining intestinal homeostasis [97,98]. In addition, *Candidatus Saccharimonas* was positively correlated with the antioxidant gene catalase. The mix supplementation also increased the abundance of *Turicibacter*, known to be reduced in an AD mouse model and in patients with AD, in whom it is negatively correlated with YKL-40, a biomarker of inflammation and glial activation, in cerebrospinal fluid [9,99]. These effects of the mix supplementation could be due to the combined effects of PE and ω-3. Indeed, both PE and ω-3 supplementations prevented cognitive alterations. The mitigating effect of PE (Memophenol™ and the patented saffron extract) supplementation alone could be attributed to its ability to modulate the gut microbiota composition: it increased the abundance of *Faecalibaculum* and decreased that of *Lachnoclostridium*. These 2 genera have been linked to oxidative stress: antioxidant effects are positively correlated with *Faecalibaculum* and negatively correlated with *Lachnoclostridium* [100,101]. This was confirmed in our study by the positive correlation of *Faecalibaculum* with antioxidant genes and cognitive performance. The preventive effect of ω-3 supplementation alone on memory could also be attributed to its ability to modulate the gut microbiota composition: it has already been described that ω-3 can restore gut microbiota impaired during aging [46]. In fact, a prolonged administration of high doses of EPA/DHA decreased the abundance of the proinflammatory phylum Proteobacteria, as reported by Pusceddu et al. [102] in female rats and observed in this study in male mice. Moreover, we showed an increase of the abundance of *Bifidobacterium* and *Faecalibaculum*, which are linked to better cognitive function [93,103]. In fact, we observed a positive correlation between *Bifidobacterium* and cognition in ω-3–supplemented mice. Thus, by modulating gut microbiota, the mix diet can act on central nervous system and induce oxidative stress and neuroinflammation inhibition, contributing to the prevention of cognitive decline, directly by blood circulation or indirectly by vagal pathway, via metabolite production, not evaluated in our study.

Moreover, the bioactive food components involved in the supplementations can act independently of the microbiota pathway on central nervous system because both PE supplementation and ω-3 supplementation affected different pathways as demonstrated in several in vitro and in vivo studies [26–28,36–38,48–50]. Indeed, PE (Memophenol™ and the patented saffron extract) supplementation demonstrated antioxidant and

neuroprotective properties that were not highlighted by ω-3 supplementation. In fact, PE supplementation modulated the expression of catalase and DCX in hippocampus. Interestingly, these effects were maintained in the mix supplementation group. This result confirmed those of previous studies. Indeed, a mix of curcumin and hesperetin, 2 polyphenols, can recover catalase expression, altered in rat brains after D-galactose treatment [104]. Polyphenols from cacao increase the expression of antioxidant markers catalase and GPx1 in the hippocampus of D-galactose–treated rats [105]. Saffron also shows antioxidant capacities in aged rat hippocampus by increasing the antioxidant status and decreasing oxidative markers [106]. Moreover, supplementation with DHA or EPA in D-galactose–induced aging mice was found to increase oxidative stress, suggesting that cosupplementation with antioxidants might be necessary [107]. It has been shown that cognitive dysfunction is linked to oxidative stress during the aging process. Reviews of populations with mild cognitive impairment and dementia reveal evidence of positive associations between oxidative stress and cognitive decline [108,109]. In mice, vascular oxidative stress is linked to neurovascular damage and cognitive impairment [110]. Moreover, Memophenol™, a polyphenol-rich extract from grapes and blueberries induces an increase in the rate of DCX-IR cells with dendrites in the hippocampus of aged mice, associated with better cognitive performance [111]. Crocin-rich saffron extract promotes neurogenesis in hippocampus [112]. Some studies in humans and animals show that aging is associated with an important decrease of neurogenesis, which contributes to the development of cognitive deficits [113–115]. Furthermore, ω-3 supplementation exhibited an anti-inflammatory effect by modulating the brain fatty acid composition and oxylipin concentration. Specifically, it increased ω-3 which exert their anti-inflammatory effect via oxylipin synthesis, particularly via 18-HEPE derived from EPA [49,50]. 18-HEPE has been shown to be negatively correlated with inflammation and depressive symptoms in patients with major depressive disorder supplemented with EPA, and it is also known to be reduced in APOE4 mice, which have an increased risk for AD [116,117]. PE supplementation did not affect oxylipin concentration but interestingly, the mix increased 18-HEPE synthesis and decreased TGF-β expression.

However, future studies should aim to elucidate other mechanisms more clearly. Other potential mechanisms, such as the mitochondrial damage pathway, could contribute to the observed positive effects. Administering D-galactose to animals can induce brain aging similar to human brain aging, including increased mitochondrial DNA mutations and impaired mitochondrial structure [118–121]. Mitochondrial damage accumulates over time and progressively contributes to neuronal decline, akin to neurodegenerative conditions [122]. Notably, ω-3 long-chain PUFAs in the inner mitochondrial membrane have been shown to affect oxidative stress by suppressing and scavenging reactive oxygen species, providing neuroprotective benefits. Consequently, our bioactive supplementation could also act by improving mitochondrial function that will contribute to attenuate oxidative stress and to limit cognitive alteration. To highlight this mechanism, further studies should assess by RT-qPCR the integrity of mitochondrial genes such as *Rac1* and *NOX* genes, which are involved in the communication between the endoplasmic reticulum and mitochondria and in the electron transfer of oxygen, respectively. These different pathways have to be confirmed in a model of aged mice. In addition, this study has another limitation as it was conducted solely on male mice, despite known differences between the sexes [123]. Hence, our study demonstrated that a mix between PE and ω-3 supplementation attenuates D-galactose–induced cognitive decline and associated alterations.

In conclusion, this study displayed evidence of the beneficial cognitive effect of a mix containing PE (Memophenol™ and a patented saffron extract) and ω-3 on D-galactose–induced memory impairment. The mix supplementation prevented cognitive alterations, induced oxidative stress and neuroinflammation inhibition, and enhanced neurogenesis by acting at the same time on indirect pathways through the modulation of gut microbiota composition and on direct pathways. Our results confirmed the interest to target gut microbiota composition to treat age-related behavioral alterations. This study confirmed the interest in the multitargeting action of nutrients to mitigate cognitive alterations induced by age.

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Author contributions

The authors' responsibilities were as follows – MM, LP, DG, SL, VP, CJ, A-LD: designed research; MM, MB, CL: conducted research; MM: analyzed data; MM, AP: performed statistical analysis; MM: wrote the article with support of LP, CJ, and A-LD; and all authors: read and approved the final manuscript.

Conflict of interest

DG, coordinator of Silver Brain Food project, reports financial support was provided by Public Investment Bank France. MM, LP, and DG are funded by Activ'Inside. Activ'Inside employees were mainly involved in the design of the study and in the choice of extract doses to be used, based on previous research and their expertise on grape and blueberry polyphenols extract and saffron extract. The other authors report no conflicts of interest.

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Data availability

Data described in the manuscript, code book, and analytic code will be made available upon request pending application and approval.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tjnut.2024.09.015.

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