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Milk fat globule membrane concentrate as a nutritional supplement prevents age-related cognitive decline in old rats: A lipidomic study of synaptosomes

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ABSTRACT

Aging is associated with a decline in cognitive abilities, mainly in memory and executive functioning. A similar but premature deterioration in cognitive capacities is the hallmark of mild cognitive impairment, Alzeimer's disease and dementia. The biochemical mechanisms that cause these neurodegenerative disorders are poorly understood. However, some evidence suggests that insufficient dietary intakes of some phospholipids could impact on brain function and increase the risk of future cognitive impairment and dementia. We evaluated the cognitive and biochemical effects of supplementation with a milk fat globule membrane (MFGM) concentrate in aged rats.

We observed that, compared to control animals, MFGM supplemented rats showed enhanced spatial working memory, but both groups exhibited similar reference spatial learning and emotional memory abilities. No significant differences between BDNF levels in the hippocampus and frontal cortex of treated rats as compared to controls were found. The nootropic effects observed were accompanied by significant changes in the lipid composition of synaptic membranes. MFGM supplementation increased the levels of EPA and DHA acids as well as the plasmalogens content in the synaptosomes isolated from the hippocampus (Synapt-HP) and the frontal cortex (Synapt-FC). In addition enhanced levels of phosphatidyl serine (PS), particularly PS(18:1/18:1), and phosphatidyl inositol (PI) molecular species were observed in Synapt-HP and Synapt-FC of treated animals. Lipidomic analysis also revealed greater concentration of phosphatidyl ethanolamine (PE) molecular species containing very long-chain fatty acids and PE plasmenyls in Synapt-HP as well as an increase of the SM content in Synapt-FC from the MFGM group. Although further studies are needed to confirm the underlying mechanism (individual or synergistic), these results suggest that MFGM supplementation could be employed as a dietary implement to restore the proper cerebral concentration of some bioactive lipids and prevent or slow the progression of age-related cognitive impairment.

1. Introduction

Aging is associated with major declines in different cognitive domains, including memory and executive function (Alexander et al., 2012; Buckner, 2004; Harada, Natelson Love, & Triebel, 2013). Of note, there is high variability in the individual trajectories of cognitive decline between older adults, which can vary from "normal" age-related cognitive decline to mild cognitive impairment, up to dementia (Park, O'Connell & Thomson, 2003; Schmiedek, Lövdén, & Lindenberger, 2013; Dumas, 2017; Petersen et al., 1999).

Animal studies have shown that aging is associated with a decrease in spatial learning and working memory (Foster, Defazio, & Bizon, 2012;

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Hamezah et al., 2017). Acquisition and consolidation of spatial reference memory depend on the correct functioning of the hippocampus (HP), (Morris, Garrud, Rawlins, & O'Keefe, 1982; Wilson, & McNaughton, 1982), while spatial working memory, depends on both the HP (mainly input from dentate gyrus to CA3) (Treves, & Rolls, 1994; Lisman, Talamini, & Raffone, 2005; Jadhav, Kemere, German, & Frank, 2012; Sasaki et al., 2018), and the medial prefrontal cortex (FC) (Baddeley, 2000; Spellman et al., 2015; Miller, 2000). Of interest, these areas of the brain are particularly vulnerable to atrophy during aging (Buckner, 2004; Hamezah et al, 2017; Sowell et al, 2003).

An interplay between genetic and environmental factors that contribute to age-related cognitive decline and brain atrophy has been reported (Fotuhi, Hachinski, & Whitehouse, 2009). Among the latter, proper diets and lifestyles appear to play preventive roles. In addition, the use of nutraceuticals/functional foods as pharma-nutritional tools is proposed to lessen the burden of cognitive decline before it worsens (Dumas, 2017; Visioli, & Burgos-Ramos, 2016). In fact, there is growing interest in evaluating the effects of interventions that use bioactive ingredients from functional foods to prevent age-related cognitive impairment and promote healthy lifespan (de Cabo, Carmona-Gutierrez, Bernier, Hall, & Madeo, 2014; Le Couteur, et al., 2016; Solon-Biet, et al., 2019; Visioli, Ingram, Beckman, Magnusson, & Hagen, 2022). Among such bioactive ingredients, polar lipids are particularly valuable because they are highly abundant in the brain for example, phospholipids (PL) such as phosphatidylcholine (PC) (Castro-Gómez, Garcia-Serrano, Visioli, & Fontecha, 2015; Pérez-Gálvez, et al., 2018; Fontecha, et al., 2020).The rationale behind the use of polar lipids to prevent cognitive decline is that, during aging, the central nervous system becomes depleted of polyunsaturated fatty acids (PUFA), such as docosahexaenoic acid (DHA) (Thomas, Thomas, Radcliffe, & Itsiopoulos, (2015). In this respect, the LipiDiDiet study (Soininen et al., 2017) tested the effects of a multinutrient combination containing, among others docosahexaenoic (DHA) and eicosapentaenoic (EPA) acids, which did not improve a neuropsychological test battery in Alzheimer's disease patients. Another trial, i.e. the PLICAR (Scholey et al., 2013) is testing the effects of a milk protein concentrate rich in natural, non-synthetic milk phospholipids (Lacprodan® PL-20) in memory-impaired patients.

An interesting source of polar lipids is the milk fat globule membrane (MFGM) (Bourlieu et al., 2018; Dewettinck et al., 2008; Fontecha et al., 2020; Gassi et al., 2016; Verardo, Gomez-Caravaca, Arraez-Roman, & Hettinga, 2017). Buttermilk (BM) is a by-product of butter manufacturing with a high content of MFGM; It is particularly rich, i.e. up to 20 % of total fat, in polar lipids (Castro-Gómez, et al., 2015; Castro-Gómez, et al, 2014; Calvo, et al., 2020), namely phosphatidylserine (PS) and sphingomyelin (SM).

In summary, MFGM concentrate from BM (BMC) appears to be a suitable candidate for the preparation of nutraceuticals to be employed in the cognitive decline arena (Pérez-Gálvez, et al., 2018). We had previously tested a concentrate rich in MFGM, obtained from BM fat by pressurized liquid extraction (PLE), in a rat model of aging. This concentrate, given as daily doses in the form of a jelly lollipop, alone or in combination with a krill oil concentrate, was able to modulate miRNA expression (Crespo, et al.,2018), to improve hippocampal insulin resistance and synaptic signaling (Tomé-Carneiro, et al., 2018) and to lower the emotional memory (contextual fear conditioning) in aged rats (García-Serrano, et al., 2020).

The aim of this study was to assess whether dietary supplementation with a BMC, enriched in polar lipids, by membrane filtration process, was able to improve the cognitive abilities of aged rats.

2. Materials and methods

2.1. Preparation of MFGM concentrate from buttermilk (BMC)

The liquid pasteurized buttermilk (BM), used as a starting material, was purchased from a local dairy manufacturer. BM was a classical food-grade by-product from the aqueous phase released during churning of cream into butter. BMC was obtained by means of an ultrafiltration-diafiltration (UF-DF) process (Fig. 1) carried out at the facilities of the INNOLACT S.L. company (Lugo, Spain), using a pilot-scale filtration system. The BM was subjected to the first UF step, and after the removal of permeate, a concentrated BM was obtained. Then, water was recirculated through the system (DF step), to overcome the viscosity increase and reduce the lactose concentration in the retentate.

Subsequently, the mixture was ultrafiltered again and once the permeate was removed, a BM eightfold more concentrated (BMC) than the starting material was obtained. BMC was spread evenly, as a 1 cm thick layer, on a metal plate, frozen at −80 °C, and then lyophilized in a Lyobeta-15 Freeze-Dryer (Telstar Technologies, Terrassa, Spain), to remove the remaining moisture. The final dry product was cut into square-shaped "cookie" pieces (4 g/unit). Finally, the global composition of the MFGM concentrate (BMC) was analyzed and its lipid fractions were studied in detail (Supplementary Table 1).

2.2. Animals and experimental design

All animal protocols were approved by the UNED Ethics Committee, followed the 'Principles of Laboratory Animal Care' and were carried out in accordance with the European Union Directive (2010/63/EU). This investigation conforms to the ARRIVE guidelines (Percie du Sert, et al., 2020).

Three-month-old adult male Wistar rats (n = 30) were purchased from Charles River Laboratories (Barcelona, Spain) and were kept pair-housed in transparent Plexiglas cages with *ad libitum* pellet food and water and maintained under a 12/12 h light/dark cycles distributed from 8 a.m.- 8p.m., with constant temperature and humidity conditions, following the standards proposed by the European Community (86/609/EEC).

When the animals reached 15 months of age, they were randomly allocated to two groups (of fifteen rats each). 1) Control diet group (CT) only fed Eurorodent Diet 22 % (LabDiet, Spain), characterized by its low polar lipid (PL) content, and 2) the treated group (BMC) fed the same diet but supplemented daily with MFGM cookie piece (BMC) corresponding to 0.5 g / animal /day. After four months of diet supplementation, when animals reached 19 months of age, behavioral experiments were undertaken in order to evaluate cognitive abilities of the animals (Supplementary Fig. 1). At this age, we observed that one control rat has eyelid drooping and it was excluded from the experimental design since spatial learning and contextual fear conditioning require a proper functioning of vision that allows animals to see distal cues and recognize the context.

2.3. Cognitive assessment

All behavioral tests were carried out during the light phase (between 9.00 a.m. and 13.30p.m.) in order to avoid circadian influence.

2.4. Spatial reference learning and memory procedure

The Morris Water Maze (MWM) test was used to assess the spatial learning reference and memory, as previously described by Garcia Serrano et al. (2020). Spatial reference training consisted of a block of four trials per day for four consecutive days. Spatial memory test was performed 24 h after the last training session and consisted of a 60 s transfer test (free swim without platform). In this test, several parameters were analyzed, i.e.); total distance swam to reach the platform (cm); latency to find the platform (s); swim speed (cm/s); and, during the transfer memory test, the percentage of time spent by the rat swimming at the place where the platform was placed at training.

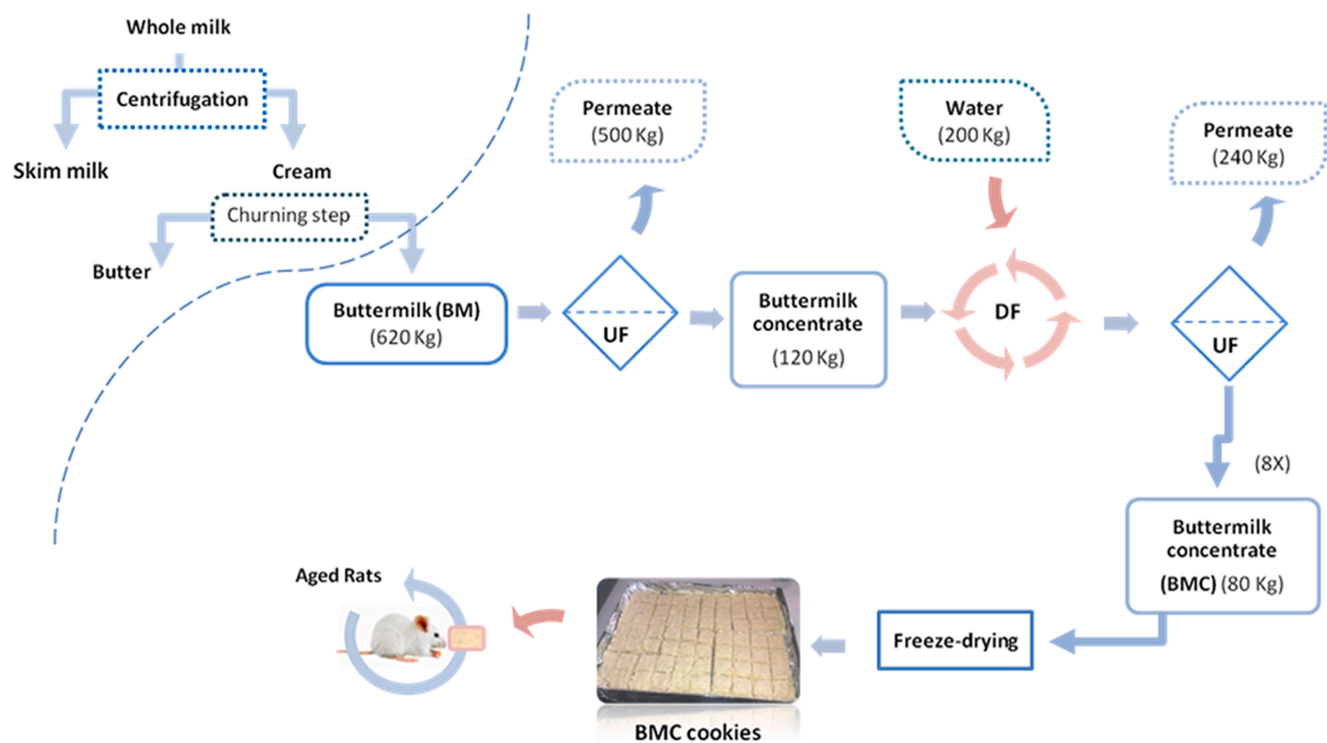

Fig. 1. Preparation of buttermilk MFGM concentrate (BMC) as a diet supplement.

2.5. Spatial working memory procedure

Another MWM protocol was used to assess spatial working memory. The test assesses the correct functioning of the frontal cortex and consisted of a paired sample task. The task was developed in four sessions that were carried out on four consecutive days. Each session consisted of two trials (sample and retention) in which the platform was placed at the same location and hidden 2 cm below the level of the water. The sample trial consisted of releasing the animal from one of the four starting points of the pool and letting it swim until it reached the hidden platform or 90 s elapsed. If the animal did not reach the hidden platform, it was guided to the platform and kept there for 15 s. Then, the animal was removed from the maze and the retention trial started after 5 s. The task requires a recall of the position occupied by the platform during the sample trial. The starting and retention positions of the platform were the same for each one-day session but varied on the different days. The mean latency to reach the platform was recorded for the sample and retention trials.

2.6. Fear conditioning

The fear conditioning procedure took place in a rodent observation cage (30 × 37 × 25 cm), placed in a sound-attenuating chamber and using a shock generator (model LI100-26 Shocker, LETICA I.C., Madrid, Spain) as described by García-Serrano, et al. (2020). After 160 s of habituation period, the animal received three tone-shock pairings with an inter-shock interval of 60 s. The tone (85 dB sound at 1000 Hz) sounded for 20 s and, at the end of each tone, an electric shock was delivered (1 s, 0.4 mA, constant current). Each rat was removed from the conditioning chamber 30 s after the final shock presentation and returned to the home cage. Thus, a conditioning session lasted 330 s. Animals were tested for contextual fear conditioning (24 h after training) by placing them back in the same chamber used for conditioning, but in the absence of shocks or tone, for an 8 min context test. Auditory fear conditioning testing (48 h after training) was performed according to the protocol described by García-Serrano, et al. (2020) and the scores for the pre-tone and tone periods were also considered separately. After each test, the apparatus was cleaned with a 1 % acetic acid solution and dried.

2.7. Preparation of tissues homogenates

After the end of the cognitive assessment, the animals were kept for 2 weeks within the corresponding diet group to minimize the potential influence of transient brain biochemical alterations induced by behavioral testing. Following a 12-hour fast, rats were anesthetized with isofluorane (4 %) before being sacrificed by decapitation. Blood was collected in heparinized tubes and plasma and erythrocytes were separated by centrifugation. HP, FC, occipital and temporal cortex (OC, TC) and cerebellum (CB) were quickly separated, washed in PBS, snap-frozen in liquid nitrogen, and stored at −80 ℃.

2.8. Brain-derived neurotrophic factor (BDNF) quantitation

The BDNF quantitation protocol was carried out on brain tissue homogenates, both FC and HP. Tissue samples were homogenized with ten strokes in a Potter homogenizer with 0.5 ml cold isolation buffer containing distilled water (dH2O), 137 mM NaCl, 20 mM Tris-HCl, 1 % Triton X-100, and 10 % glycerol. To maximize the free protein yield, homogenized samples were acidified with 1 N HCl and neutralized with 1 N NaOH. Before starting the protocol, Tris-buffered Tween 20 saline buffer (dH2O, 20 mM Tris-HCl, 150 mM NaCl, and Tween 20) was prepared for use as a washing agent before each major incubation step. The samples were analyzed in duplicate using a commercially available enzyme-linked immunosorbent assay (BDNF Rapid^{TM}ELISA Kit, Biosensis Australia) with an expected lower detection limit of 2 pg/mL and intra- and inter-assay precision of 5.1 and 6.7 %.

2.9. Isolation of synaptosomes

Synaptosomes from hippocampus (Synapt-HP) and frontal cortex (Synapt-FC) were isolated. Briefly, rat HP and FC were dissected in ice, washed twice in cold PBS then homogenized in 5 mM HEPES, pH 7.4,

320 mM sucrose, 1 mM EDTA using a Dounce homogenizer with 10 S at 200–250 rpm. The homogenate was centrifuged at 3,000xg for 10 min at 4 °C and the supernatant was recovered and again centrifuged at 14,000xg for 12 min at 4 °C. The resulting pellet was carefully re-suspended in Krebs-Ringer buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM EDTA), Percoll solution was added (final concentration 45 % v/v) and the solution was mixed gently by inverting the tube. After centrifugation at 13,000 xg for 2 min at 4 °C, the synaptosomal fraction was recovered at the surface of the flotation gradient and carefully re-suspended in Krebs-Ringer buffer. The pellet resulting from another centrifugation at 13,000 g for 30 *sec* at 4 °C, represented the synaptosomal preparation. These samples were stored at −80 °C.

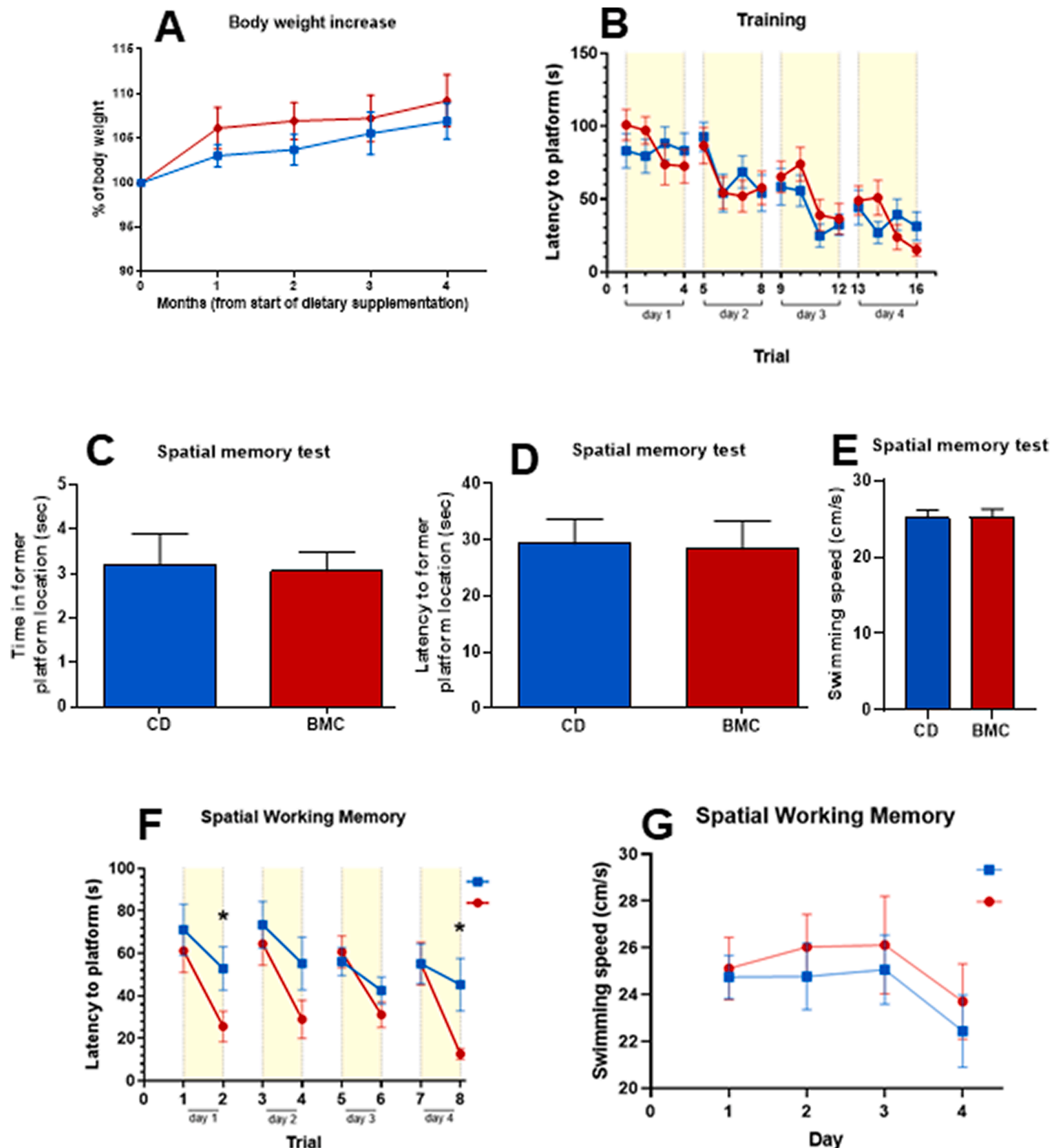

Fig. 2. Body weight increase and spatial learning and memory abilities in the Morris Water Maze. Body weight increase during BMC supplementation (**A**). Spatial reference learning (**B**); time in target platform location in the spatial reference memory test (**C**); latency to former platform location in the spatial memory test (**D**); Average swimming speed in spatial reference memory (**E**); time to platform in spatial working memory (**F**): Swimming speed in Spatial Working memory (**G**), from aged rats fed control diet (blue) or supplemented with MFGM concentrate (BMC) diet (red). Data expressed as means ± SD. *Statistically significant differences between experimental groups ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.10. Lipidomic analyses

Plasma, erythrocyte, tissue and synaptosome samples were dissolved in methanol and sonicated prior to lipid extraction, which was performed using the procedure described by García-Serrano et al., [33]. The lipid extracts were evaporated under a nitrogen stream, weighted, and stored at −35 °C. Lipid classes were analyzed by HPLC-ELSD, FAMEs were analyzed by GC–MS, and cholesterol (Chol) was analyzed by GC-FID as previously described by Castro-Gomez, Montero, & Fontecha (2017).

Analysis of the molecular species of phospholipid and sphingolipid present in Synapt-HP and Synapt-FC was performed by UPLC-QToF-MS, according to the procedure reported by Castro-Gomez, et al. (2017). External standards of PE(12:0/12:0), PI(8:0/8:0), and PC(10:0/10:0) from Sigma-Aldrich (Madrid, Spain) were used to draw the respective correlation curves of chromatographic peak area to known standard concentration (μg/mL) for quantification. SM, ceramides, and sphingosines were quantified using the coefficients of the correlation curve drawn for PC, whereas PS were quantified using the coefficients of the correlation curve drawn for PE. Chol values are given as the peak area value per mg of fat. Negative ion mode data were used to identify the acyl chains from the MS/MS (MSE) spectrum, whereas data from positive ionization mode were used for quantification. All the lipidomics analysis were carried out on all rats individually.

2.11. Statistical analyses

Cognitive assessment data are reported as mean ± SEM and were analyzed using analyses of variance (ANOVA) or with repeated measures (including treatment and 'training day' in the water maze as a repeated measure). Dunnett's multiple comparison test was used for post hoc analyses. Data from FAME and lipid classes analyses were analyzed by a U-Mann–Whitney non-parametric test, using the SPSS package (version 22.0 for Windows, SPSS Inc. IBM, Armark, New York, USA). Comparison of lipid species measured in isolated synaptosomes was made using two tail *t*-test considering the proper modification whether the sample sets for treated animals and controls had the same or different variance according to the F-test. The statistical abilities of the Microsoft Excel software were used.

3. Results

Rats were weighed monthly during the experimental period. There were no significant differences in body weight between control and BMC before (647.9 ± 15.1 g and 649.1 ± 15.7 g respectively; $t(27) = 0.591$, $p > 0.55$) or after ($t(27) = 0.591$, $p > 0.55$) the diet supplementation (Fig. 2A). A time-based increase in body weight was observed for both experimental groups ($F(1.603, 54, 49) = 6.997$; $P = 0.004$), but there were no differences in body weight gain between control and BMC-supplemented animals (F (DFn, DFd) ($F(1, 27) = 1.243$; $P = 0.272$) and no time interaction was observed ($F(4, 108) = 0.152$; $P = 0.962$).

3.1. Cognitive assessment

The spatial learning abilities of the animals were tested in the Morris Water Maze (MWM) and results are shown in Fig. 2.

During the acquisition phase, BMC-supplemented and control rats progressively learned the location of the platform as the training proceeded ($F(15.405 = 9.271$, $P < 0.0001$), but no effect of group ($F(1.27) = 0.437$; $P = 0.501$) or time per group interaction ($F(15.405) = 0.9924$; $P = 0.517$) was observed (Fig. 2 B). In the probe trial, the controls and treated rats spent similar time in the target zone where the platform was previously located on the training days $t(27) = 1.070$; $P = 0.301$) (Fig. 2C). Additionally, no significant differences in latency to the former location of the platform were observed between the groups $t(27) = 1.070$, $P = 0.301$ (Fig. 2D). The swimming speed in the spatial memory test was similar in both groups, $t(27) = 0.878$, $P = 0.393$ (Fig. 2E). Repeated measures ANOVA, for total time spent to find the platform on the retention trial of each spatial working memory training day, showed significant differences in treatment $F(1.27) = 10.958$, $P = 0.003$. Further post-hoc analysis revealed statistically significant differences between groups with the BMC-supplemented group performing significantly better on day 1 and day 4 compared to control animals ($U = 53.5$, $P = 0.0235$ and $U = 57.5$, $P = 0.037$; respectively). No significant effect was observed for time $F(1.27) = 0.701$, $P = 0.557$ or time × treatment interaction $F(3) = 0.528$, $P = 0.666$) (Fig. 2F). Swimming speed in the Spatial working memory task was similar across the four training days ($F(1.27) = 1.075$, $P = 0.315$ (Fig. 2G).

BMC-supplemented and control groups showed similar freezing behavior after the auditory-cued fear conditioning training $t(27) = 0.223$, $P = 0.819$. One day after training, both groups of animals showed similar contextual fear conditioning memory $t(27) = 1.049$, $P = 0.304$. A tone-cued fear conditioning test was performed two days after conditioning training in a different chamber. The analysis of the freezing behavior indicated that there were no significant differences between the groups for the new context $t(27) = 0.353$, $P = 0.727$ or after the tone exposure $t(27) = 0.408$, $P = 0.686$ (Fig. 3).

3.2. Brain-derived neurotrophic factor (BDNF)

The evaluation of BDNF levels in homogenates from the hippocampus (HP) and frontal cortex (FC) did not differ significantly (FC, $U = 79$, $P = 0.265$ and HP; $t(22) = 0.0190$, $P = 0.985$) between the group supplemented with BMC and the control.

3.3. Lipidomic analysis

3.3.1. Tissues: Fatty acid composition and lipid classes profile

Both subcutaneous tissue (SAT) and visceral adipose tissue (VAT) showed a significantly lower fat content after BMC supplementation (Supplementary Table 2), but a similar fatty acid (FA) profile, with linoleic (LA), oleic (OA) and palmitic acids (PA) as main compounds (Supplementary Table 3). Remarkable amounts of palmitoleic (C16:1 *cis*9) and *cis*-vaccenic (C18:1 *cis*11) acids were also found. Both tissues differed mainly in their PUFA content, which was higher in SAT than in VAT (43 *vs* 36 % respectively). Their FA profiles were not substantially modified by BMC supplementation and only the C20:3 n6 (DGLA) content decreased slightly in VAT. The composition of FA in the liver showed a predominance of unsaturated acids (MUFA + PUFA approximately 68 %), with a notable presence of long- and very long- chain FA (LCFA and VLCFA). Arachidonic (AA) was the most abundant FA (25 %), and DHA was also abundant (7 %). Only the content of DGLA was significantly affected by the BMC diet ($P < 0.05$). Regarding the profile of the lipid classes although, as stated before, the total fat content was significantly decreased in both tissues (SAT and VAT) by the BMC diet, triglycerides (TAG) content remained the main lipid class accounting for 99 % of the total fat (Supplementary Table 4). In the liver, no major changes were observed in response to the incorporation of BMC in the diet, TAG content was around 38 %, while total polar lipid (PL) content was around 42–45 %, with PC and phosphatidylethanolamine (PE) as major components.

The FAME composition of plasma and erythrocytes are shown in Supplementary Table 3. Red blood cell lipids are a reliable indicator of overall fatty acid status, while plasma lipids are used as short-term indicators of dietary intake (Jackson, & Harris, 2018). In plasma, non-significant increases in EPA, docosapentanoic (DPA), and DHA concentrations were observed in rats of the BMC supplemented group, while the only significant change recorded in erythrocytes was a higher content of oleic acid (C18:1) content in the treated group, which explained an increase in the level of MUFA. Distribution of lipid classes in the plasma and erythrocytes of rats was clearly affected by the BCM supplement (Supplementary Table 4), and significant reductions in Chol

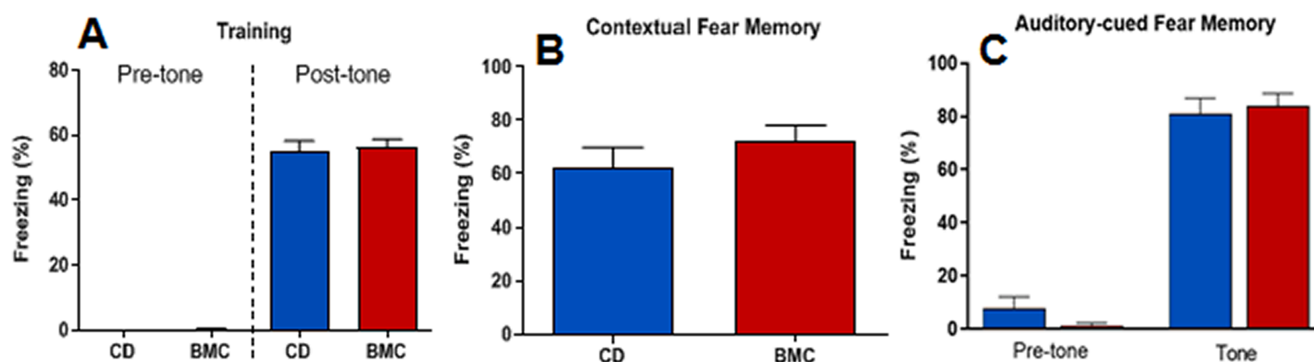

Fig. 3. Percentage of freezing behavior from aged rats fed control diet (**blue**) or MFGM concentrate supplemented diet (**red**) in training (**A**); contextual fear conditioning test (**B**) and during the auditory cued fear-conditioning test (**C**).* Statistically different from control ($P < 0.05$). Data are mean percentages ± SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and Chol esters (CE) contents were observed. The decrease in Chol concentration found in the erythrocytes of the treated group could be related to the elevated presence of SM and its known anti-cholesterolemic activity (Cohn, Kamili, Wat, Chung, & Tandy, 2010).

In the cerebellum (CB), temporal cortex (TC) and occipital cortex (OC), the FA profile was mainly composed of palmitic (PA), stearic (SA), oleic acids (OA) and AA and DHA (Supplementary Table 5). Overall, this distribution agrees with data reported by García-Serrano, et al. (2020) and Rahman, et al. (2010); however, these samples contained higher levels of AA and DHA and lower ones of saturated FA (SFA). BMC supplementation markedly altered the FA profile of the TC. It should be noted that the temporal lobe exhibited significantly higher amounts of ω3 FA compared to the control group, namely α-linolenic acid (ALA), EPA, DPA, and DHA. A reduction in SFA content was also found in the treated group, mainly due to the decrease in palmitic acid content. In contrast, the FA profile both in OC and CB, was affected to a lesser extent by the provision of the BMC supplement. Thus, only significant increases in the DPA and LA contents were observed in OC and CB, respectively. Plasmalogens were determined as their dimethylacetal derivatives (DMA) by GC–MS in lipid extracts, and no differences in the total content of DMA in CB, TC, and OC were observed in the BMC group compared to the control. Consistent with data recently reported by other authors (García-Serrano, et al., 2020; Dawson, 2015), the total PL content in the CB and brain areas, analyzed (OC and TC) was approximately 50 % of total lipids (Supplementary Table 6). The distribution of PL was similar in the three tissues, with 41–47 % PE, ~38 % PC, and 10–13 %, PS being the most abundant compounds. In agreement with the FA profile, diet markedly affected PL concentration in TC. Thus, there was a significant increase in PL content, while we recorded a lower presence of SM and Gangliosides (Gang) ($P < 0.05$) in that cortical area (Fig. 4). However, only a significant decrease of phosphatidylinositol (PI) in OC and PS in CB were observed in the BMC group (Supplementary Table 6).

3.3.2. *Lipidomic analysis of Synapt-HP and Synapt-FC*

The FA composition of isolated synaptosomes from aged rats fed CD or BMC are shown in Table 1. Both HP and FC synaptosomes in the BMC group showed ω3 FA contents higher than those found in the control group, which is especially significant in Synapt-HP (14 % *vs* 19 %). The lipid profile of both Synapt-HP and Synapt-FC, markedly changed in the BMC supplemented animals. Furthermore, we recorded a significantly lower concentration of SFA (mainly palmitic acid, C16:0) in Synapt-HP in the BMC group compared to the control. A significant increase in dimethylacetal derivatives (C16:0 DMA, C18:0 DMA and C18:1 DMA) and therefore the total plasmalogen content was also observed (Fig. 5).

In parallel, we also found a significant increase in VLCFA of ω3 series, EPA and DHA. Particularly, the increase in DHA was highly correlated ($P < 0.01$) to the rise in plasmalogens level, which fits with the notion that they predominantly contain AA or DHA in their sn-2 position (Farooqui, & Horrocks, 2001; Farooqui, Farooqui, & Horrocks, 2008). Therefore, the changes induced by the BMC-supplemented diet in the composition of Synapt-FC followed a pattern similar to that of Synapt-HP where a significant increase in plasmalogen content was also detected. Concerning ω3 FA, a significant increase of EPA was found.

Molecular species of the phospholipids PC and PE are shown in Figs. 6 and 7. The total PE content was higher than the PC content in both Synapt-HP and Synapt-FC, but significant differences between treated and control rats were only shown in Synapt-HP, where a high increase in PE species was measured in the treated group compared to the control group (48.5 *vs* 34.6). In Synapt-HP, the most abundant PC species were (16:0/18:1) > (16:0/20:4) >(18:0/18:1)> (16:0/22:6); while the most abundant PE species were (18:0/20:4) > (18:0/22:6) > (P-16:0/22:6) > (P-18:0/22:6) > (16:0/22:6).The AA/DHA ratio was higher than one for PC species, but lower than one for the PE species.

In Synapt-FC, the most abundant PC species were (16:0/18:1) > (16:0/16:0) > (18:0/18:1) ~ (16:0/20:4) > (16:0/22:6); whereas the

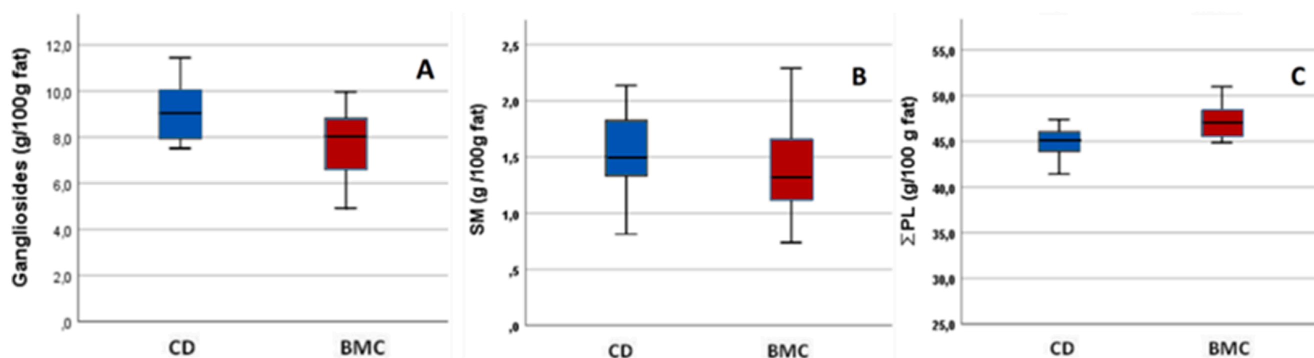

Fig. 4. Concentration of **(A)** gangliosides, **(B)** sphingomyelin (SM), and **(C)** polar lipids (PL) in the temporal cortex (TC) from aged rats fed the control diet (CD) or the MFGM concentrate supplement (BMC). Data expressed as means ± SD. Level of significance ($P < 0.05$).

Table 1
Fatty acid composition (g/100 g fat) of hippocampal synaptosomes (Synapt-HP) and frontal cortex synaptosomes (Synapt-CF) from aged rats fed control diet (CD) or MFGM concentrate supplement (BMC). Data expressed as means ± SD.

	Synapt-HP						Synapt-FC					
	CD			BMC			CD			BMC		
C14:0	0,85	±	1,74	0,52	±	0,45	0,78	±	0,66	0,29	±	0,09
C15:0	0,10	±	0,14	0,12	±	0,07	0,14	±	0,08	0,10	±	0,03
C16:0 DMA	1,14	±	0,52	1,61	±	0,32	0,87	±	0,29	1,24	±	0,18
C16:0	37,43	±	13,87	29,04	±	3,81	29,33	±	9,48	22,85	±	3,00
C16:1*cis9*	0,06	±	0,07	0,08	±	0,05	0,16	±	0,09	0,20	±	0,06
C17:0	0,16	±	0,15	0,10	±	0,04	0,18	±	0,10	0,15	±	0,09
C18:0 DMA	1,77	±	0,87[a]	2,35	±	0,51[b]	1,97	±	0,75	2,86	±	0,42
C17:1	0,14	±	0,13[a]	0,21	±	0,11[b]	0,18	±	0,10[a]	0,38	±	0,15[b]
C18:1DMA	0,19	±	0,17[a]	0,27	±	0,14[b]	0,25	±	0,12[a]	0,48	±	0,19[b]
C18:0	23,31	±	6,35	18,63	±	3,84	18,20	±	3,26	17,14	±	1,11
C18:1*cis9*	7,54	±	4,37	10,11	±	1,91	9,47	±	2,84	10,77	±	0,93
C18:1*cis11*	1,32	±	0,88	1,88	±	0,82	2,13	±	0,76	2,55	±	0,44
C18:2n6 (LA)	0,15	±	0,25	0,15	±	0,12	0,22	±	0,12	0,26	±	0,09
C20:3n6	0,26	±	0,20	0,17	±	0,14	0,30	±	0,26	0,17	±	0,10
C20:4 (AA)	11,51	±	5,79	15,75	±	1,79	13,67	±	2,05	14,99	±	0,85
C20:5n3 (EPA)	1,95	±	1,21[a]	2,56	±	0,85[b]	3,04	±	0.98[a]	4,11	±	0,38[b]
C24:0	0,24	±	0,20[a]	0,38	±	0,20[b]	0,59	±	0,33[a]	0,86	±	0,23[b]
C22:5n3 (DPA)	0,04	±	0,10	0,07	±	0,10	0,09	±	0,08	0,11	±	0,05
C22:6n3 (DHA)	11,84	±	5,58[a]	16,00	±	3,25[b]	18,42	±	5,10	20,49	±	2,35
ΣDMA	3,10	±	1,54[a]	4,24	±	0,92[b]	3,09	±	1,11[a]	4,58	±	0,76[b]
ΣSFA	62,09	±	19,21	48,79	±	5,82	49,22	±	11,62	41,39	±	3,83
ΣMUFA	9,06	±	5,33	12,29	±	2,77	11,93	±	3,66	13,90	±	1,15
ΣPUFA	25,75	±	12,64	34,69	±	4,69	35,75	±	8,12	40,13	±	3,16
ΣMCFA	0,95	±	1,72	0,64	±	0,50	0,91	±	0,67	0,39	±	0,12
ΣLCFA	70,11	±	13,23	60,20	±	5,12	59,87	±	8,72	54,31	±	3,55
ΣVLCFA	25,85	±	12,71	34,93	±	4,83	36,12	±	8,34	40,73	±	3,28
Σn6	11,92	±	5,99	16,07	±	1,78	14,20	±	2,16	15,42	±	0,85
Σn3	13,83	±	6,73[a]	18,62	±	3,49[b]	21,55	±	6,21	24,71	±	2,66

DMA, dimethylacetals; DHA, docosahexaenoic acid; AA, arachidonic acid; LA, linoleic acid; LCFA, long chain fatty acids; MCFA, medium chain fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; VLCFA, very long chain fatty acids.
[a,b] Means with different superscript letters within a row are significantly different ($P < 0.05$).

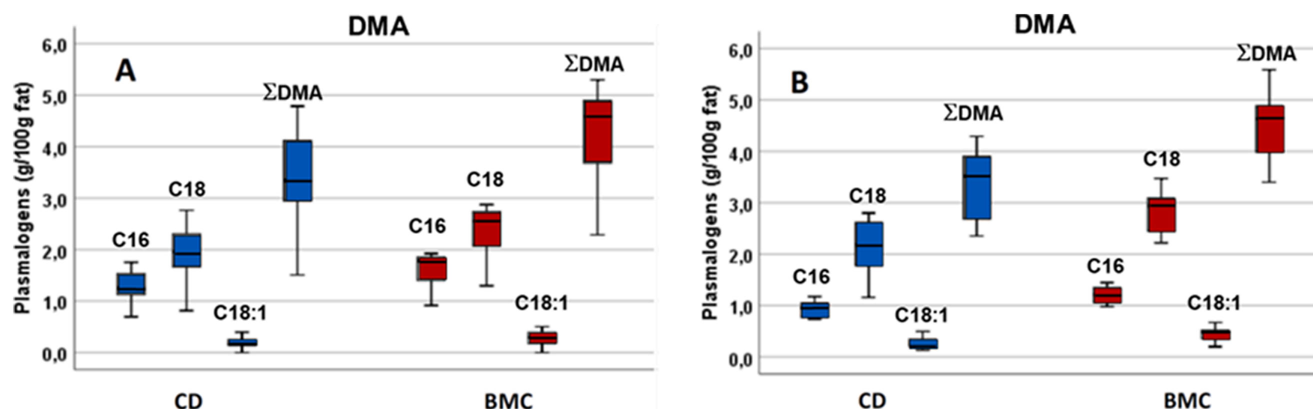

Fig. 5. Plasmalogen concentration, meassured as dimethylacetals (DMA), in synaptosomes from **(A)** hippocampus (Synapt-HP) and **(B)** frontal cortex (Synapt-FC) from aged rats fed control diet (CD) or MFGM concentrate supplement (BMC).

most abundant PL species especially in those PC and PE species containing AA or DHA were higher in the treated animals than in the controls in Synapt-HP. Conversely, no significant differences were found in the lyso-PC and lyso-PE (except Lyso-PE 22:5) content in Synapt-FC. Most lyso-PE and lyso-PC (16:0) showed a higher content in the treated rats than in controls. However, other PC and PE species did not show significant differences or their content was higher in controls (i.e PC (16:0/22:5)) than in treated rats, apart from PE (P-18:0/18:1), PE (38:5) and PE (P-16:0/22:4) (Figs. 6 and 7).

A small but significant increment of the Chol content in the treated animals was observed in both Synapt-HP and Synapt-FC (Table 2). Some SM species were also enhanced in Synapt-FC treated animals, but there were no significant differences in Synapt-HP samples between treated rats and controls. Different PS and PI species were detected, but only those shown in Table 2could be quantified, the other species being present in trace amounts. PS18:0/22:6) was the most abundant PS in both Synapt-HP and Synapt-FC samples, with minimal contents of PS (18:1/18:1) and PS(40:5) (likely PS(18:0/22:5). The PS content was comparable in the controls of both Synapt-HP and Synapt-FC apart from PS(18:1/18:1), which was weakly but significantly higher ($P < 0.01$) in Synapt-HP. Although PI(16:0/20:4) and PI(18:1/20:4) were detected, only the PI(16:0/18:1) species could be quantified which exhibited higher values in both treated animals and controls of Synapt-FC than of Synapt-HP. All PS and PI contents were higher in Synapt-FC of treated animals; however, in Synapt-HP, only PS(18:1/18:1) and PI(16:0/18:1) showed higher values in treated rats than in controls.

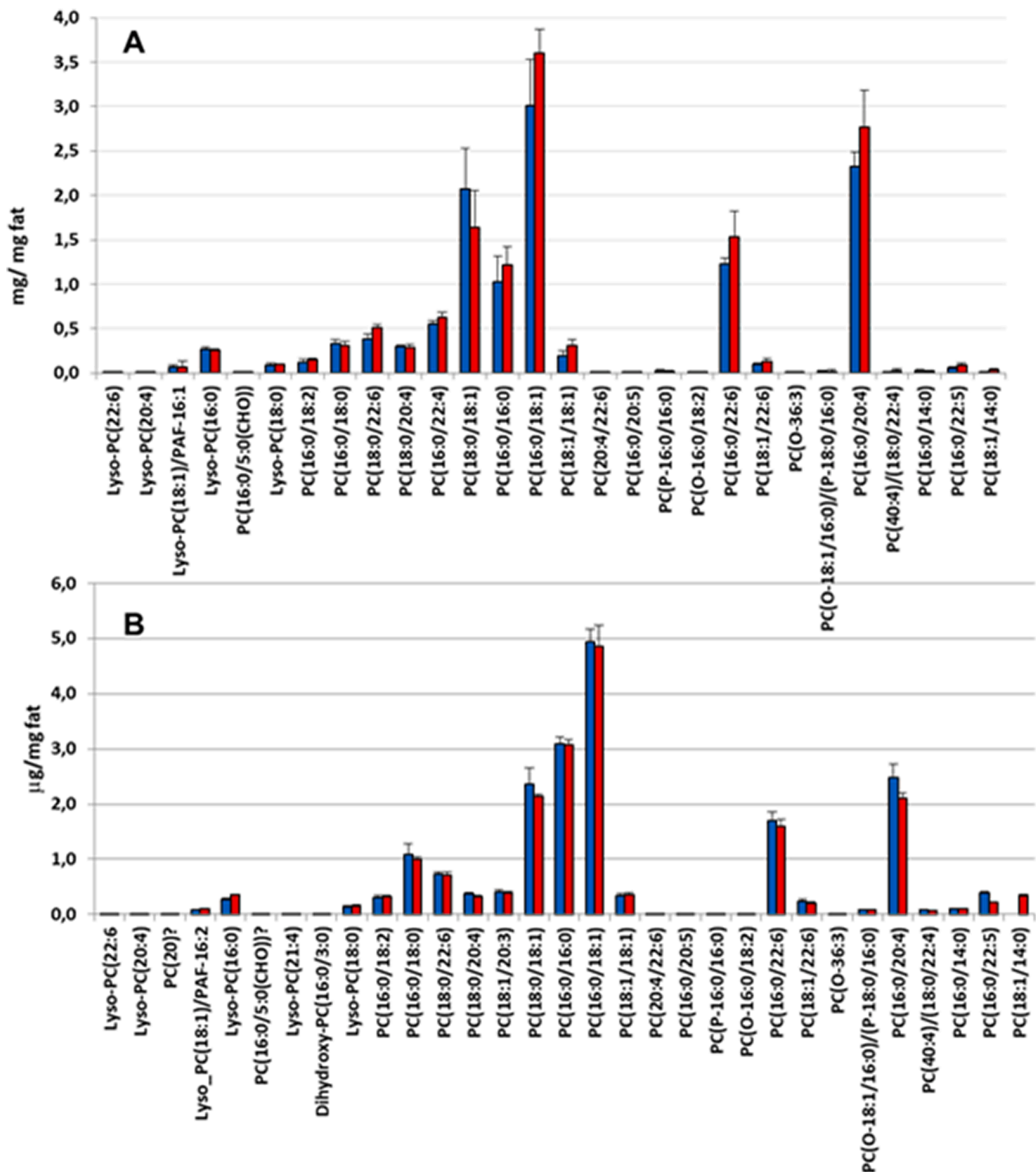

Fig. 6. The acyl chain compositions of the molecular species of phosphatidylcholine (PC) in the hippocampal synaptosomes (Synapt-HP) **(A)** and in the frontal cortex synaptosomes (Synapt-FC) **(B)** from aged rats fed control diet (CD) or MFGM concentrate supplement (BMC) as determined by UPLC/QToF-MS.

4. Discussion

Dietary supplementation with a MFGM concentrate supplement (BMC) improved the spatial working memory in aged-rats while the reference spatial memory and emotional memory were not affected. Since these two types of memory are based on different brain structures (Smith, & Kosslyn, 2007; Jo, et al., 2007), we speculate that BMC supplementation could differentially affect the HP and the FC. HP is a complex structure, broadly connected throughout the brain (Maller, et al., 2019) and it is known that the hippocampal-prefrontal cortex interactions play a crucial role in cognition and behavior (Sigurdsson, & Duvarci, 2016), and that disruption of functional connectivity in such circuits might be a common pathogenetic element of neurodegenerative disorders (Li, Long, & Yang, 2015).

A modulation of brain BDNF as possible explanation of changes on spatial working memory was discarded since we observed similar BDNF

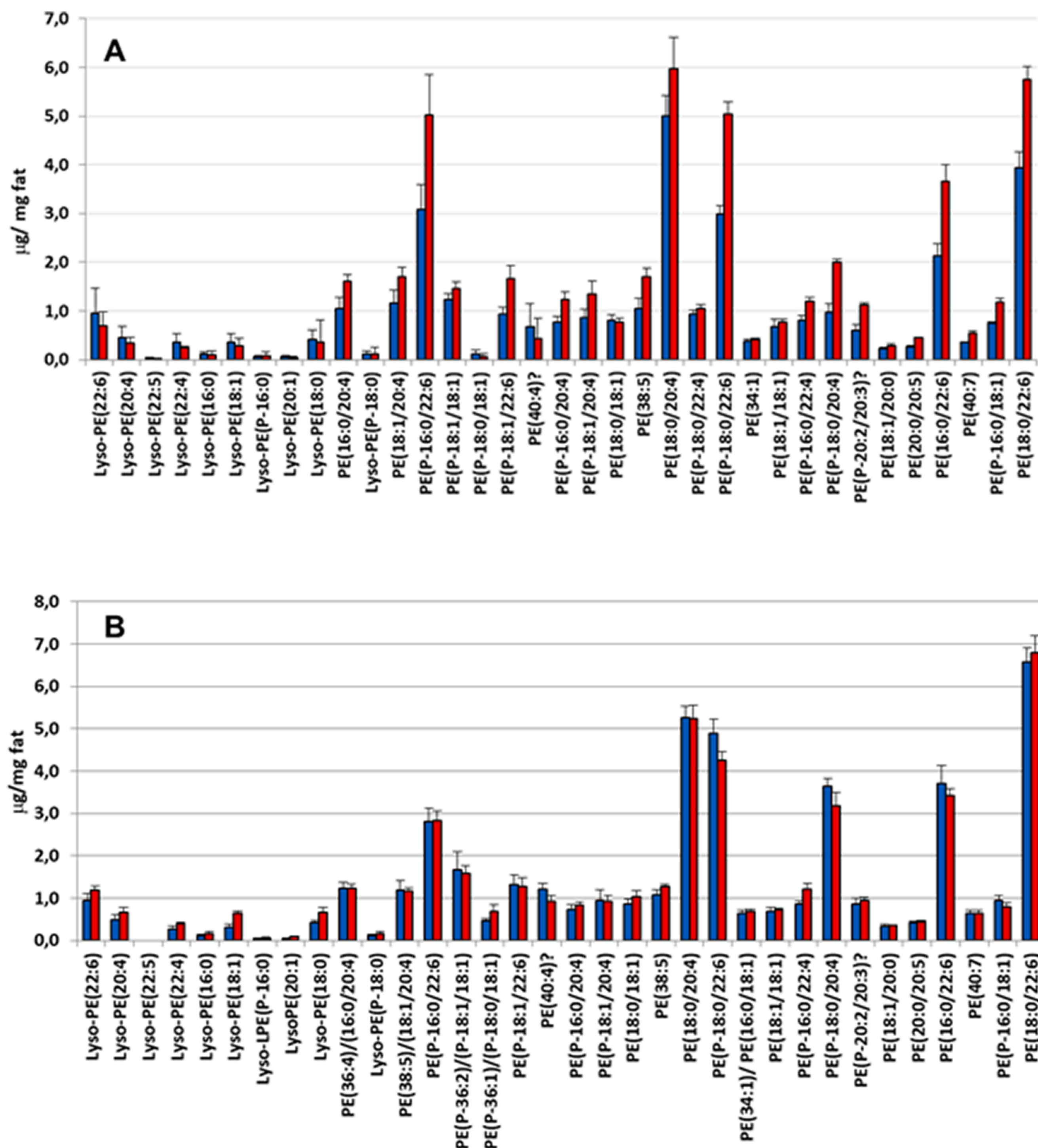

Fig. 7. The acyl chain compositions of the molecular species of phosphatidylethanolamine (PE) in the hippocampal synaptosomes (Synapt-HP) **(A)** and in the frontal cortex synaptosomes (Synapt-FC) **(B)** from aged rats fed control diet (CD) or MFGM concentrate supplement (BMC) as determined by UPLC/QToF-MS.

levels in the FC and the HP from both BMC-supplemented and control animals. In our previous study in aged rats (Tomé-Carneiro et al., 2018), dietary supplementation with PLs concentrates from BM and krill oil increased hippocampal BDNF levels, which might possibly be due to a synergistic effect in the concomitant administration of both PLs concentrates.

The improved spatial working memory might be consistent with the rise in the exploratory behavior or in the speed processing of spatial information, which usually declines during aging (Van Gerven, Van Boxtel, Meijer, Willems, & Jolles, 2007). However, indirect evidence that this is not solely the explanation for our results comes from the spatial reference learning and memory data (Fig. 2C and 2D) and from swimming speed during the four training sessions of the spatial working memory task (Fig. 2F) that were similar in both groups of rats. As this spatial working memory task combines spatial and executive components and BMC and control rats showed similar spatial reference learning and memory abilities, we suggest that the improved performance in spatial working memory displayed by BMC-supplemented animals might be due to an improved executive component. The significant difference between control and BMC groups observed on the

Table 2
Mean values of the acyl chain composition of the molecular species of cholesterol (Chol), ceramides (Cer), sphingomyielin (SM) phosphatidylserine (PS) and phosphatidylinosytol (PI) in the hippocampal synaptosomes (Synapt-HP) and frontal cortex synaptosomes (Synapt-FC) from aged rats fed control diet (CD) or MFGM concentrate supplement (BMC) as determined by UPLC/QToF-MS. *P* values in bold indicate statistically significant differences.

Molecular species	Synapt-HP CD		BMC		*P (t-test)*	Synapt-FC CD		BMC		*P (t-test)*
(mg/mg fat)	mean	SD	mean	SD		mean	SD	mean	SD	
C16:0-sphinganine	1.01E+00	4.34E-01	1.57E+00	7.37E-01	*0.140*	1.27E+00	4.57E-02	1.26E+00	7.49E-02	*0.76*
C17:0-sphinganine	8.30E-02	2.41E-02	1.26E-01	6.53E-02	*0.163*	1.42E-01	9.90E-03	1.22E-01	1.75E-02	***0.031***
Sphinganine	1.14E-02	8.12E-03	9.45E-03	1.58E-02	*0.794*	2.25E-02	1.82E-02	7.40E-03	1.08E-02	*0.111*
Sphingosine	1.29E-02	5.77E-03	4.67E-03	7.25E-03	*0.055*	1.88E-02	8.81E-03	5.81E-03	8.04E-03	***0.023***
Cer(d36:1)	1.59E-01	2.62E-02	1.38E-01	6.31E-03	*0.090*	1.45E-01	2.14E-02	1.21E-01	8.51E-03	***0.028***
SM(d18:1/20:0)/(d16:1/22:0)	9.59E-02	2.89E-02	5.88E-02	6.38E-03	***0.012***	7.04E-02	1.73E-02	1.23E-01	2.95E-02	***0.004***
SM(d18:1/16:0)	2.65E-02	2.91E-02	1.75E-02	2.32E-02	*0.567*	9.25E-04	8.41E-04	1.71E-03	8.36E-04	*0.135*
SM(d18:1/18:1)	8.34E-02	6.52E-02	9.34E-02	1.19E-02	*0.720*	1.30E-01	1.37E-02	1.20E-01	2.83E-02	*0.452*
SM(d18:1/18:0)	1.53E+00	3.70E-01	1.66E +00	9.81E-02	*0.401*	1.52E+00	1.61E-01	1.96E+00	1.73E-01	***0.001***
SM(d18:1/24:1)	7.10E-02	1.07E-02	6.29E-02	1.08E-02	*0.223*	7.03E-02	4.20E-03	7.80E-02	6.98E-03	***0.042***
Chol	6.17E-01	4.73E-02	7.69E-01	1.15E-01	***0.013***	6.10E-01	3.30E-02	6.59E-01	2.91E-02	***0.021***
oxo/epoxy-cholesterol						2.91E-02	1.30E-02	8.86E-02	5.91E-02	***0.037***
C24-Bile acid derivative	1.75E+00	1.45E-01	2.64E+00	1.10E+00	*0.080*	1.44E+00	5.53E-02	1.75E+00	3.07E-02	***0.001***
(µg/mg fat)										
PS(18:0/22:6)	1.36E+00	7.42E-02	1.09E+00	1.97E-01	***0.018***	1.35E+00	2.02E-01	1.80E+00	9.26E-02	***0.002***
PS(40:5)	6.90E-02	1.17E-02	4.93E-02	7.68E-03	***0.008***	7.22E-02	3.78E-03	1.56E-01	1.19E-02	***0.001***
PS(18:1/18:1)	3.96E-02	3.89E-03	7.19E-02	2.66E-02	***0.015***	3.16E-02	1.36E-03	3.63E-02	4.00E-03	***0.02***
PI(16:0/18:1)	1.78E-02	7.74E-03	4.19E-02	1.46E-02	***0.008***	2.88E-02	8.91E-03	4.61E-02	6.26E-03	***0.004***

first day of spatial working memory training, suggests improved spatial working memory acquisition. It may be postulated that BMC supplementation may have improved or preserved the efficiency of the direct input from HP to the pre-FC, that it is known to be crucial in the encoding of salient spatial cues during acquisition of spatial working memory (Spellman, et al. 2015). As the platform location was changed every day, a new spatial working memory acquisition was tested each training day and the absence of significant differences between groups in the second and third training days may be due to the distinct locations of the hidden platform, changes in attention and or motivation of the animals to find the submerged platform, etc. On the fourth day, the BMC supplemented animals showed improved spatial working memory compared to controls, suggesting that after three days, they were more efficient in acquiring and retrieving spatial working memory information.

On the other hand, BMC appears to bring about changes in lipid composition of some tissues, with synaptosomes being particularly affected. An efficient synaptic transmission depends on intact membranes (Gundersen, 2017) and among other factors the degeneration of its lipid composition appears to be involved in the loss of the neuroplasticity and synaptic function (Lauwers, Goodchild, & Verstreken, 2016); Egawa, Pearn, Lemkuil, Patel, & Head, 2016). Compared to their respective controls, higher contents of C17:1 and lignoceric acid (C24:0), a precursor of cerebrosides (Fonteh, Cipolla, Chiang, Arakaki, & Harrington, 2014; Pfeuffer, & Jaudszus, 2016), were found both in Synapt-HP and Synapt-FC from BMC group. As regards C17:1, similar results were reported by García-Serrano, et al. (2020) in HP. Odd chain FAs are found in minor amounts in body tissues and although they are preferably incorporated in brain sphingolipids (Fonteh, et al., 2014), their roles in cognition have been poorly investigated.

BMC supplementation did not significantly alter circulating ω3 LC-PUFAs in rats, but increased the levels of EPA and DHA in the Synapt-HP and Synapt-FC, which may have contributed to the improved spatial working memory found in the BMC supplemented animals, since that ω3 FA do not incorporate into the different brain areas in a random fashion (Lamaziere, et al., 2011). During aging there is a reduction in brain PLs and DHA levels (Delion, et al., 1997; Favrelère, et al., 2000). Interestingly, it has been reported that diet supplementation with fish oil increases the DHA content in several brain areas (including the Synapt-HP and the Synapt-FC) and improves reference and spatial working memory in rats (Chung, Chen, & Su, 2008), while a ω3 FA deficient diet impairs spatial learning and reduces brain DHA concentrations (Fedorova, & Salem, 2006; Fedorova, et al., 2007).

In Synapt-HP and Synapt-FC from BMC supplemented animals, an increase in the plasmalogen content also occurs that may have also exerted a nootropic effect. Synaptic vesicles are enriched in plasmalogens where it has been suggested that they play a role in facilitating trafficking and membrane fusion processes that are known to lead to efficient neurotransmitter delivery at the synapse (Brodde, Teigler, Brugger, Lehmann, Wieland, Berger, & Just, 2012; Dean, & Lodhi, 2018). Throughout life, the Gang content of the brain changes continuously, following a specific temporal and regional distribution pattern (Mlinac, & Bognar, 2010; Grassi, Giussani, Mauri, Prioni, Sonnino, & Prinetti, 2020). The diminution of Gang, and to a lesser extent of SM content found in TC might represent a compensatory mechanism in response to the increased concentration of PLs occurred in rats fed BMC supplement. The existence of a cross-talk between glycerophospholipids and sphingolipids in establishing plasma membrane asymmetry has been reported (Kihara, & Igarashi, 2004) and it appears that any change in one of the components activates a dynamic process that modifies the membrane lipid distribution and the composition of the lipid rafts. In model systems, the Gang-PL self-assembly was found to strongly depends on the proportions of the different lipid components in the mixture (Mojumdar, Grey, & Sparr, 2019). and that the co-existence of a variety of structures (vesicles, micelles, and discs) clearly affect the overall properties of the lipid system, including transport and solubilization.

Lipidomic analysis revealed a greater presence of those PE species containing VLCFAs and PE plasmenyls in Synapt-HP of BMC supplemented animals, which agrees with the FA profile obtained. In contrast, the composition of main PLs (PC and PE) did not show significant differences between treated rats and controls in Synapt-FC. Cone-shape lipids like PE, in addition to Chol, provide membranes with the required curvature for efficient fusion to take place between neurotransmitter-containing vesicles and synaptosomal membrane (Farooqui, et al., 2008; Bennett, et al., 2013; Ifuku, et al., 2012; Rohrbough, & Broadie, 2005). The small, but significant increment of the Chol content points to the restoration of membrane curvature and may have played a role in the improvement of cognitive functions observed in BMC supplemented rats. The higher presence of oxidized Chol found in the treated rats, could be indicative of enhanced Chol recycling pathway activity.

Sphingolipids associate with Chol in specific microdomains to promote increased PL hydrophobic chains order and packing (van Blitterswijk, van der Luit, Veldman, Verheij, & Borst, 2003). In this regard,

SM(d18:1/18:0) content was enhanced, while the Cer(d36:1), probably Cer(d18:1/18:0) level was reduced in Synapt-FC from supplemented rats. This suggests a decrease of sphingomyelinase activity after supplementation, with potential asymmetric accumulation of ceramide and concurrent enhancement of vesicle and membrane fusion at the Synapt-FC (Rohrbough, & Broadie, 2005).

Because encoding and performance of spatial working memory is dependent on the synaptic coupling between Synapt-HP and Synapt-FC (Spellman, et al., 2015; Tamura, Spellman, Rosen, Gogos, & Gordon, 2017), an increase of the SM content may have also contributed to the improvement of cognitive function in supplemented group. Enhanced activity of ceramidase to yield sphingosine is known to be a marker of aging in rats (Sacket, Chung, Okajima, & Im, 2009), and higher levels in controls than in treated mice were detected in both Synapt-HP and Synapt-FC, but only statistically significant in the later.

Consistent with the literature (Vance, 2018), PS(18:0/22:6) was the predominant PS in both Synapt-HP and Synapt-FC and its levels increased in Synapt-FC (but not in Synapt-HP) of BMC supplemented rats. PS species have been shown to play a "save-me" role in axonal regeneration (Neumann, Linton, Giordano-Santini, & Hilliard, 2019; Neumann, et al., 2015), increase Ca^{2+} uptake in synaptosomes (Floreani, Debetto, & Carpenedo, 1991), and have, among others, cholinergic effects (Pepeu, Pepeu, & Amaducci, 1996). Particularly PS(18:0/18:1) have considered to act in the membrane inner leaflet of the external signal transmission through inter-connection ("hand-shaking") with long chain SM located in the membrane outer leaflet (Skotland, & Sandvig, 2019) in exosomes from PC-3 cells. Among the possible mechanisms of action that may explain the nootropic effects of buttermilk diet supplementation reported here, PS should also be singled out. In aged rats, different studies have reported that chronic treatment with PS can improve the acquisition and memory of active and passive avoidance tasks and reference spatial memory in the Morris water maze (Drago et al., 1993). In addition, PS treatment also restored spontaneous alternation in the Y maze (Aporti et al., 1986), a task that measures spatial working memory (Albani et al., 2014). Several mechanisms of action have been postulated to mediate the rescue of impaired cognition observed by PS supplementation including restoring glutamatergic neurotransmission (Cohen and Müller, 1992), and re-establishment of brain cholinergic deficit in aged animals by increasing de novo acetylcholine synthesis and release (Pedata et al., 1985). In this sense, the reduced Ca^{2+} uptake in K^{+}-depolarized cortical synaptosomes from aged rats can be restored by PS treatment (Pepeu et al., 1986).

In this study, an increased presence of SM containing LCFA such as nervonic acid (C24:1) was observed in Synapt-FC, but not in Synapt-HP. However, PS (18:0/18:1) was detected at trace level, whilst PS(18:1/18:1) could be quantified and exhibited higher concentrations in Synapt-FC and Synapt-HP from supplemented animals. Perhaps this "hand-shaking" function is accounted for by PS(18:1/18:1) in synaptosomes, as the binding of the C18:1 in the sn-2 position seems to be the main requirement for it. Accordingly, it could be postulated that PS(18:1/18:1) along with SM is acting this way leading to restoration of the lost synaptic connections between Synapt-HP and Synapt-FC that might have occurred during aging.

In conclusion, the nootropic effect observed after BMC supplementation was accompanied by significant changes in the lipid composition of synaptic membranes, such as an increased presence of PS and PE molecular species, SM, DHA, EPA and plasmalogens. All these compounds are known to be involved in processes related to age-related cognitive decline, however further research is required to establish the underlying mechanism of action (individual or synergistic) that explains the results obtained.

Author contributions

JF, CV and FV contributed to conception and design of the study. MVC prepared BMC supplements. SB maintained subjects, provided dietary supplement and performed their cognitive assessment. SB, DP and CV performed BDNF analysis. OM carried out lipidomic analysis. SB and OM organized the database, performed statistical analysis and designed figures. MVC, CV and OM wrote the first draft of the manuscript. FV, and JF wrote sections of the manuscript. JF supervised the project. All authors contributed to manuscript revision, read, and approved the submitted version.

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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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.112163.

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