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Full-length Article

Prebiotics Mitigate the Detrimental Effects of High-Fat Diet on memory, anxiety and microglia functionality in Ageing Mice

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ABSTRACT

Ageing is characterised by a progressive increase in systemic inflammation and especially neuroinflammation. Neuroinflammation is associated with altered brain states that affect behaviour, such as an increased level of anxiety with a concomitant decline in cognitive abilities. Although multiple factors play a role in the development of neuroinflammation, microglia have emerged as a crucial target. Microglia are the only macrophage population in the CNS parenchyma that plays a crucial role in maintaining homeostasis and in the immune response, which depends on the activation and subsequent deactivation of microglia. Therefore, microglial dysfunction has a major impact on neuroinflammation. The gut microbiota has been shown to significantly influence microglia from birth to adulthood in terms of development, proliferation, and function. Diet is a key modulating factor that influences the composition of the gut microbiota, along with prebiotics that support the growth of beneficial gut bacteria. Although the role of diet in neuroinflammation and behaviour has been well established, its relationship with microglia functionality is less explored. This article establishes a link between diet, animal behaviour and the functionality of microglia. The results of this research stem from experiments on mouse behaviour, i.e., memory, anxiety, and studies on microglia functionality, i.e., cytochemistry (phagocytosis, cellular senescence, and ROS assays), gene expression and protein quantification. In addition, shotgun sequencing was performed to identify specific bacterial families that may play a crucial role in the brain function. The results showed negative effects of long-term consumption of a high fat diet on ageing mice, epitomised by increased body weight, glucose intolerance, anxiety, cognitive impairment and microglia dysfunction compared to ageing mice on a control diet. These effects were a consequence of the changes in gut microbiota modulated by the diet. However, by adding the prebiotics fructo- and galacto-oligosaccharides, we were able to mitigate the deleterious effects of a long-term high-fat diet.

1. Introduction

Ageing is a complex process characterised by a number of interrelated factors such as genomic instability, mitochondrial dysfunction, cellular senescence, stem cell depletion, altered intercellular communication and progressive increase in systemic inflammation (Villalobos et al., 2022). In the CNS, cellular ageing and dysfunction is exacerbated by chronic neuroinflammation (Megur et al., 2020; Mou et al., 2022; Müller et al., 2019). This inflammatory response is orchestrated by the over production of pro-inflammatory factors such as

Abbreviations: BSA, Bovine serum albumin; DAPI, 4′, 6-diamidino-2-phenylindole; DI, Discrimination index; DIO, Diet induced obesity; DMEM, Dulbecco's modified Eagle's medium; FBS, Faetal bovine serum; FOS, Fructo-oligosaccharides; GM-CSF, Granulocyte-macrophage colony-stimulating factor; GOS, Galacto-oligosaccharides; HFD, High-fat diet; M−CSF, Macrophage colony-stimulating factor; PBS, Phosphate-Buffered Saline; ROS, Reactive oxygen species; SA-β-gal, Senescence-associated Beta galactosidase; TNF-α, Tumour necrosis factor alpha.

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cytokines TNF-α, IL-1β, reduced expression of anti-inflammatory factor TREM2 (Fantuzzi et al., 1999; Singh et al., 2021). Chronic neuroinflammation propels cellular dysfunction evidence by the reduced production of chemokine CCL2 (Leng & Edison, 2021) and it subsequent receptor CCR2 (Komiya et al., 2020), increased senescent markers like p16 and β-galactosidase (Rayess et al., 2012; Valieva et al., 2022), excessive production of reactive oxygen species (ROS) and diminished expression of NADPH oxidase enzyme subunits NOX1 and NOX2 associated with oxidative stress (Haslund-Vinding et al., 2017). Meanwhile, microglia, the resident immune cells of the brain, play a critical role in neuroinflammation and brain functions (Hickman et al., 2018; Kwon & Koh, 2020; Subhramanyam et al., 2019). Therefore, any dysfunction of these cells can potentially impact neuroinflammation and contribute to the development of neurodegenerative diseases (Ho, 2019; Leng & Edison, 2021; Subhramanyam et al., 2019). It is therefore crucial to maintain and modulate the functionality of these cells. The microbiota is an ecological community of commensal microorganisms that live symbiotically and pathogenically in our body and are also proactively involved in multiple biological processes and the development of diseases (Adak & Khan, 2019; Burokas et al., 2015; Zmora et al., 2019).

Recent studies have demonstrated a bidirectional communication between the brain and the gut microbiota, such that dysbiosis of the gut microbiota can significantly affect brain function through neuroinflammation (Bairamian et al., 2022; Solanki et al., 2023). Indeed, microbial dysbiosis can lead to impaired gut barrier function, allowing microbial metabolites such as lipopolysaccharides to enter the circulation to activate microglia and induce neuroinflammation, suggesting that modulation of the gut microbiome can have a significant impact on microglia induced neuroinflammation (Al-Asmakh et al., 2012; Kalyan et al., 2022). Furthermore, the gut microbiota has also been shown to significantly influence microglia from birth to adulthood in terms of development, proliferation, maturation, and functionality (Agirman et al., 2021; De Vos et al., 2022). It is therefore crucial to maintain and modulate a healthy gut microbiota.

Diet plays an important role in shaping and modulating the composition and functions of the microbiota (Kolodziejczyk et al., 2019; Zmora et al., 2019). Complex interactions between nutrients and microorganisms determine the positive or negative effects on host health. The impact of diet on microbial composition and diversity have been extensively studied, but the influence of different diets on the microbiota is still quite unclear. For example, a high-fat diet (HFD) with increased calorie intake leads to obesity (Burokas et al., 2018; J. Li et al., 2020), followed by glucose intolerance (Espinosa-Carrasco et al., 2018a; Malesza et al., 2021), overstimulation of the sympathetic nervous system, low-grade systemic inflammation as well as overexpression of immune cells (Tan & Norhaizan, 2019). Excessive consumption of HFD is generally associated with systemic inflammation and age-related diseases (Christ et al., 2019). Furthermore, studies have also shown that animals can exhibit learning deficits after prolonged HFD exposure, with deviant behaviour in terms of learning impairment/inflexibility, elevated stress, reinforcement, impulsivity and compulsivity (Burokas et al., 2015; Espinosa-Carrasco et al., 2018).

However, it is possible to negate the effects of HFD through the consumption of certain dietary supplements. Prebiotics are fibre-rich foods that support the growth and/or proliferation of healthy microbiota (Kolodziejczyk et al., 2019; Megur et al., 2022; Sanders et al., 2019a). Galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) (Megur et al., 2022) are some classic examples of prebiotics that are indigestible and resistant to degradation by gastric acid and enzymes and thus serve as food for the stimulation and growth of beneficial bacteria (Hijová et al., 2019; Sanders et al., 2019b). GOS and FOS have been used extensively in brain research and have been shown to promote anti-inflammatory mechanisms (Hong et al., 2016; Sabater-Molina et al., 2009), improve cognition, reduce anxiety and modulate the secretion of the gut-brain axis (Burokas et al., 2017a; Megur et al., 2022).

Although the role of diet in systemic inflammation is well established, its relationship to microglial function is far less explored. In this study, we demonstrated the effects of HFD and/or prebiotic consumption on microglial functions, brain functions such as cognition and behaviours such as anxiety and related these parameters to the microbiota in ageing mice. Finally, we revealed that the detrimental effects of HFD on brain and microglial functions can be mitigated by the consumption of prebiotics GOS+FOS.

2. Materials and methods

2.1. Animals

For this study, 56 young, 2-month old male C57BL/6J mice (Janvier Laboratories, France) were used. The permission of the Lithuanian State Food and Veterinary Service to perform the experiment was given (No. G2–104), and the husbandry and experiments complied with the requirements of Directive 2010/63/EU. The animals were kept (6 per cage) under controlled conditions (22 °C±1, humidity 40 % food and water supply *ad libidum*) and under veterinary supervision. The animals were culled using cervical dislocation.

2.2. Diet and dietary supplements

In this study, the effect of diet and dietary supplements on the animals were analysed in short and long term. For the short-term study, 24 young animals were used. The animals were divided into 4 groups (6 animals per group). These animals were fed the diet and supplements for 1 month. In the long-term, 24 animals were fed the same diet and supplements for 10 months. The groups are as follows: control diet + H_2O (control); control diet + Prebiotics (control + P); High-fat diet + H_2O (HFD); High-fat diet + Prebiotics (HFD+P) as described in Fig. 1A. The control diet was composed of a carbohydrate diet (3.87 kcal/g) and the HFD was composed of 2 types of saturated fatty acids (stearic acid and palmitic acid) and where 60 % of calories come from fats, 24 % from carbohydrates and 16 % from proteins (5.21 kcal/g) (Altromin; Germany). For the prebiotics, a combination (1:1 ratio) of 3 % GOS (King-Prebiotics®; New Francisco (Yunfu City) Biotechnology Corporation Limited; China) and 3 % FOS (Beneo; Germany) in water was added to the housing bottles and the bottles were changed twice a week.

2.3. Animal behaviour experiments

The animals were acclimatised for 1 h in the respective experimental rooms before the behavioural experiments. The animals were allowed to rest for one day. After the glucose tolerance test, the animals were rested for 5 days before culling procedure. The timing of the experiments is shown in Fig. 1B.

- **Open field test**

To study anxiety-like behaviour in mice, the animals were placed in the centre of the open field arena (the walls of the maze were made of white opaque Plexiglas; 40 cm long × 40 cm wide × 30 cm high) at ground level under 500 lx illumination (measured at floor level in the centre of the arena) for 10 min (Kulesskaya & Voikar, 2014). A video recording was carried out during the experiment. The percentage time and number of entries were counted when the animal moved into the centre of the maze, which was virtually marked as a “small central square” (10 cm × 10 cm). The videos were analysed using the computer software (Viewer; Biobserve; Germany).

- **Light- dark box test**

The experimental set-up consisted of a “dark box” (black, opaque Plexiglas box, 20 cm long × 20 cm wide × 35 cm high) with dull lighting (25 lx, measured at the level of the maze floor), which was directly

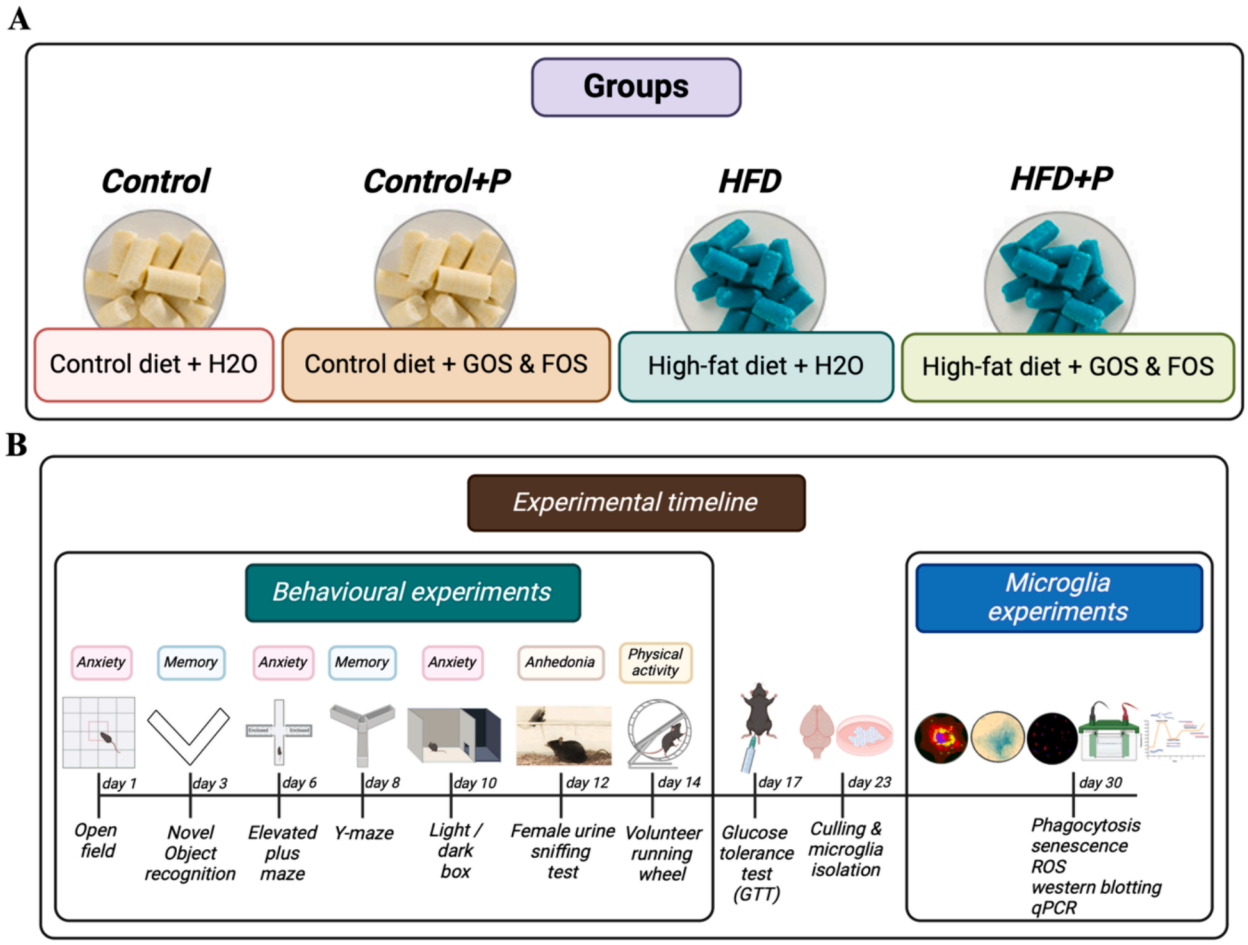

Fig. 1. Overview diagram of the research. (A) Schematic representation of animal diets with prebiotics and water supplementation. (B) Schematic representation of the entire experimental timeline.

connected by a narrow tunnel (7 cm long × 7 cm wide × 7 cm high) to a "light box" (white opaque Plexiglas box, 30 cm long × 20 cm wide × 35 cm high) with intense lighting (500 lx, measured at the level of the maze floor) (Kulesskaya & Voikar, 2014). Video recordings were carried out during the experiment. The mice were placed individually in the "dark box" facing the tunnel at the beginning of the 5-minute observation session. The number of entries and the percentage of time spent in the "light box" were analysed in our experimental conditions.

- **Elevated plus maze**

The mice were placed in a black, opaque Plexiglas apparatus centre with 4 arms (35 cm long × 6 cm wide), 2 arms open and 2 arms closed. The apparatus was elevated 30 cm above the floor and was illuminated (30–35 lx in the open arms and 4–6 lx in the closed arms; light intensity was measured at the maze level) (Filgueiras et al., 2014). Video recordings were carried out during the experiment. A five-minute test session was performed per animal, and the percentage of time and number of entries into the open arms were used as an indicator of anxiety.

- **Novel object recognition test**

The V-shaped maze test was conducted as previously reported (Burokas et al., 2014; Busquets-Garcia et al., 2011). The V-shaped maze consisted of two perpendicular arms (made of black opaque Plexiglas, dimensions of the arms: 25 cm long × 5 cm wide × 15 cm high) with dull lighting (15 lx, measured at the top of the maze). The experiment consisted of 3 phases: acclimatisation (day 1), training (day 2) and testing (day 2). A 3-hour period left between the training and test phases to evaluate the short-term memory in the mice. This test is based on the spontaneous tendency of rodents to spend more time exploring a novel object than a familiar one assigned as discrimination index (DI). The DI was calculated using the following formula:

$$DI = \frac{(T_R - T_L)}{(T_R + T_L)}$$

where T_R represented the exploration time devoted to the new object and T_L represented the exploration time devoted to the old object. A DI of 0.4 and above was considered as a standard for a good memory and a value below 0.2 was considered as memory impairment.

- **Y- maze**

The mice were kept in a grey, opaque Plexiglas apparatus with 3 identical arms (40 cm long × 9 cm wide × 15 cm high). The apparatus was a closed Y-shaped maze and was illuminated from the top (15 lx, measured at the end of the arms) (Kraeuter et al., 2019). Ten-minute test sessions were performed, and the number of arms visited in alterations by the mice was counted and recorded. Entry was considered to have occurred when all four limbs of the mice were within an arm zone of the maze.

- **Female urine sniffing test**

For the test, urine collected from female mice in oestrus. Male mice

were placed in a quiet, dimly lit room before the test and acclimatised to an empty cotton-tipped applicator inserted into their home cage. One hour later, the animal was presented with a cotton swab dipped in sterile water for three minutes and the sniffing time was measured. After a 45-minute interval in which the mice were not disturbed, they were presented with a cotton bud soaked in 60 µl of fresh urine from a female mouse in oestrus for three minutes and the sniffing time was measured (Scheggi et al., 2018). The DI was calculated using the following formula:

$$DI = \frac{(T_U - T_W)}{(T_U + T_W)}$$

where T_U represented the sniffing time on the cotton with urine, and T_W represented the sniffing time on the cotton with water.

- **Voluntary running wheel**

The animals were housed individually in standard cages with a running wheel (metal wheel diameter 12 cm), provided with their respective food and supplemented with water (Scheggi et al., 2018). Voluntary physical activity was measured by counting the number of rotations by the running wheel during a 24-hour period: 12-hour day cycle (cage lighting 80–100 lx, light intensity measured at the top of the cage) and 12-hour night cycle (no lighting). Physical activity was recorded using specialized recording system with PowerLab hardware and LabChart-8 software (ADInstruments Ltd, UK) was used to measure the number of wheel rotations.

2.4. Animal weight and glucose tolerance test

The animals were weighed at the end of each month. At the end of the final time point (for young animals 1 month, and for aged animals 10 months after following the diets) a glucose tolerance test (GTT) was performed to check insulin resistance. During the GTT, glucose was measured at different time points up to 2 h. The animals were not fed for 14 h before the experiment but were provided with water. The dose of 2 g/kg glucose was injected intraperitoneally and 10 µl of blood was collected from the snipped tail to measure glucose levels using a blood glucose meter (Contour next gen, USA).

2.5. Isolation and maintenance of microglial cells

Mice were culled and brains were quickly harvested for microglial cell isolation as described in (Vijaya et al., 2023). Briefly, cells were seeded in a T25 culture flask in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12; ThermoFisher Scientific, Lithuania) with GlutaMAX™ supplement (ThermoFisher Scientific, Lithuania) containing 10 % faetal bovine serum (FBS) and 1 % penicillin/streptomycin (10,000 units). On day 2, the medium was supplemented with macrophage colony-stimulating factor (M−CSF; 50 ng/mL; R&D Systems, UK) and granulocyte–macrophage colony-stimulating factor (GM-CSF; 50 ng/mL; R&D Systems, UK). Microglia were cultured for 7 days, and the medium was changed every 3 days. After reaching confluence, the cells were detached from the flasks using 0.05 % trypsin and 0.5 mM EDTA and seeded into 48-well plates for the experiments. The time course of the experiments is described in Fig. 1B.

2.6. Immunocytochemistry

- ***Phagocytosis***

The phagocytic capacity of microglia was determined by measuring the uptake of fluorescent latex beads with a diameter of 1 µm (Schroeder & Kinden, 1983) (Sigma Chemical Co., USA). 100,000 cells/well were seeded into 48-well plates. After 24 h, cells were incubated with 0.025 % (w/w) fluorescent latex beads in media for 4 h at 37 °C and 5 % CO_2. Cells were then rinsed twice with PBS, fixed for 15 min with freshly prepared 4 % (w/v) paraformaldehyde in PBS, and permeabilized (PBS+1% Triton X-100) for 5 min. Cells were blocked with 3 % bovine serum albumin (BSA) and 10 % FBS prepared in PBS for 30 min. Next, the cells were incubated with a primary conjugated antibody (anti-CD11b; 1:150; PE, Thermo Fisher Scientific; Lithuania) prepared in a blocking solution for 1 h at room temperature. The cell nuclei were counterstained with the DAPI dye (5 µg/mL), and the fluorescence was visualised using a fluorescence microscope (Olympus IX51; Japan). Analyses were performed using ImageJ software (version 1.53f51) and GraphPad software (version 9.3.1; USA). The percentage of $CD11b^+$ cells that have taken up $>$ 20 latex beads was counted.

- ***Oxidative stress (ROS)***

To assess ROS, 100,000 cells were seeded into a 48-well plate. ROS generation was measured by staining with CellRox Deep Red (Thermo Fisher; USA) according to the manufacturer's instructions. Briefly, CellRox Deep Red solution was added to the media after treatment and incubated for 30 min in the dark at 37 °C and 5 % CO_2. The cells were then washed and fixed with 4 % paraformaldehyde for 15 min. The cell nuclei were counterstained with the DAPI dye (5 µg/mL) and the fluorescence was visualised using a fluorescence microscope (Olympus IX51; Japan) at 20x magnification. The analysis was performed using ImageJ software (version 1.53f51).

- ***Senescence***

Microglial senescence was evaluated by determining the activity of β-galactosidase. 100,000 cells/well were seeded in a 48-well plate. After 24 h, the activities of senescence-associated β-galactosidase enzyme (SA-β-gal) was determined using the Senescence Cells Histochemical Staining Kit (Sigma-Aldrich; USA) according to the manufacturer's instructions. Briefly, cells were fixed (7 min in fixative solution), washed, incubated with β-galactosidase staining solution (overnight, 37 °C), and visualised under a light microscope (Olympus IX51; Japan) at 20X magnification. The analysis was performed with ImageJ software (version 1.53f51). The number of β-galactosidase+ (blue coloured) microglia was assessed and expressed as a percentage of the total number of cells.

2.7. Protein expression analysis

Microglial cells were lysed, and the proteins were extracted using the protein isolation kit (Macherey-Nagel; Germany). The total protein volume of extracted proteins was 40 µL. SDS PAGE was performed to separate the proteins according to their molecular weight in the 15 % acrylamide gel. The proteins from the gel were transferred to a nitrocellulose membrane using sem-dry transfer (25 V for 35 min). The membranes were blocked with 3 % bovine serum albumin (BSA; Sigma) in TBST (Tris buffer saline tween) (50 mmol/L Tris, pH 7.6; 0.9 % NaCl; and 0.1 % Tween-20) for 1 h at room temperature (RT). The membranes were incubated overnight at 4 °C with the primary antibodies (Suppl. Material, Table S1). After washing three timed with TBST, the membranes were incubated with the corresponding secondary antibodies (Suppl. Material, Table S1) for 1 h at RT. After incubation with the secondary antibodies, the membranes were incubated for (5 min) with Pierce Enhanced Chemiluminescence Substrate (Thermo Fisher Scientific; Lithuania) for the detection of HRP (Thermo Fisher Scientific; Lithuania). Imaging and quantification of the bands were performed with the Alliance Q9 software (UK).

2.8. Gene expression analysis

RNA was extracted from microglial cells together with proteins using

a High Pure RNA Isolation kit (Macherey-Nagel, Germany) according to the manufacturer's protocol, and subjected to cDNA synthesis using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA). Real-time PCR was performed using the QuantStudio Real-Time PCR System (Thermo Fisher Scientific; Lithuania) with SYBR green reaction mixture (Thermo Fisher Scientific; Lithuania). Standardisation was performed using alpha-tubulin as a reference gene. The primers were designed with the Primer Blast software and the primers were used for quantification of gene expression (Suppl. Material, Table S2).

2.9. Microbiota analysis

During the culling of the mice (young and aged), caecal microbiota was collected and dispatched in dry ice for shotgun sequencing (CeGaT; Germany). After receiving the data, an analysis was performed for microbial profiling of the experimental animals. The web tool ClustVis (Metsalu & Vilo, 2015) was used to perform the beta diversity analysis.

2.10. Statistical analysis

The data are presented as mean ± SEM, with the number of replicates indicated in each case. For data normal distribution and homogeneous variance, ANOVA followed by Tukey comparison test was used to determine differences between groups. In the case of non-normally distributed data or non-homogeneous variance, the data were analysed using the non-parametric Kruskal Wallis test with subsequent Dunn posthoc comparison test to determine differences between the groups. The significance level was set at $p < 0.05$. GraphPad Prism was used for ANOVA statistical analysis (version 9.3.1; USA). The Spearman correlation matrix analysis was performed using JASP 0.17.2.1 software (Amsterdam, Netherlands). The R programming language (version 4.1.2) was used for the correlation matrix and the principal component analysis.

3. Results

3.1. Animal weight and glucose tolerance test

To evaluate the effects of age and diet on the animals, weight and glucose tolerance were considered as important parameters and measured at the end of their dietary periods (Fig. 2A). Age and diet had a combined effect on the weight of the animals ($F_{(3,40)} = 52.57$, $p < 0.001$). Aged mice weighed significantly more than young mice in the HFD ($p < 0.001$) and HFD+P groups ($p < 0.001$). No significant difference in weight was determined in the young animals in all groups. This is due to a relatively short exposure of the animals to the respective diets. However, a significant increase in body weight was observed in the older animals due to the longer dietary exposure (HFD mice ($p < 0.01$) and HFD+P mice ($p < 0.001$) compared to control mice and control + P mice, respectively).

Glucose intolerance is one of the main symptoms of diabetes. The results of the GTT showed that animals become more susceptible to DIO with increasing their age ($F_{(3,40)} = 52.57$, $p < 0.001$). Compared to, a decrease in blood glucose tolerance was observed in young mice ($F_{(15,312)} = 2.702$, $p < 0.01$), particularly in HFD animals ($p < 0.01$) at 30 min and ($p < 0.001$) at 45 min and 60 min compared to control animals, and HFD ($p < 0.05$) at 45 and 60 min compared to control mice (Fig. 2B). Similar significance was found in HFD+P ($p < 0.001$) at 45 min and ($p < 0.05$) at 60 min compared to control + P mice. In the aged animals ($F_{(15,294)} = 2.693$, $p < 0.01$), the data once again showed a significant decline in blood glucose tolerance in HFD mice ($p < 0.05$) at

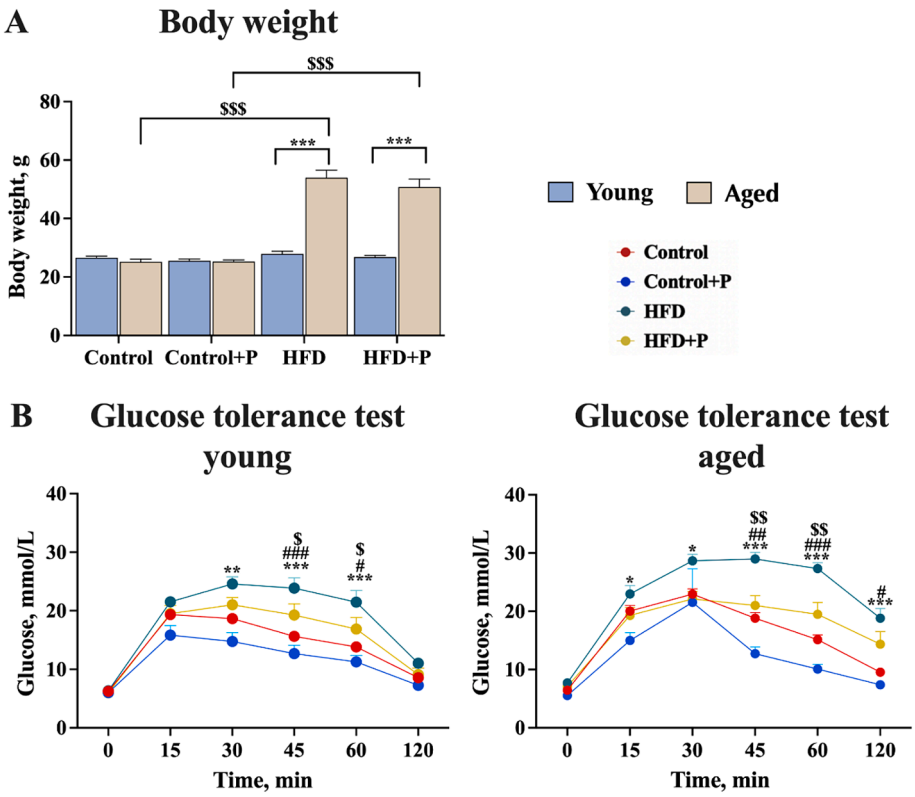

Fig. 2. Weight and glucose tolerance test for young and aged animals. (A) Graphical representation of animal weight at the end of the diet period of 1 month for young and 10 months for aged animals. Data presented as ± SEM, $^{***}p < 0.001$ among young vs aged, $\$p < 0.001$ among diet groups in aged animals; N=6. (B) Graphical representation of glucose measurement at the end of the diet period of 1 month for young and 10 months for aged animals. Data presented as ± SEM, $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ among young animals, $^{\$}p < 0.05$; $p < 0.01$; $\$p < 0.001$ among diet groups in aged animals; N=6. Control vs HFD (*), control + P vs HFD+P (#) and HFD vs HFD+P ($).

15 min, 30 min and ($p < 0.001$) from 45 min to 120 min compared to control mice. Comparable results were observed in the HFD group ($p < 0.001$), in which the blood glucose levels of the mice decreased more slowly compared to HFD+P. In addition, HFD+P animals also showed a decline in blood glucose tolerance ($p < 0.01$) at 45 min and ($p < 0.001$) at 60 min and 120 min compared to the control mice (Fig. 2B).

3.2. Anxiety-like behaviour

3.2.1. Open field test

This test was performed to evaluate anxiety-like behaviour in mice. No significant differences were found between all groups, young and aged mice, in the number of visits to the small square (centre of the maze) ($F_{(3,36)} = 0.5684$; $p = 0.0178$). However, compared to the aged animals, the mice in the HFD group visited the small square less frequently, indicating a possible increase in anxiety-like behaviour (Fig. 3A). Similar results were observed when evaluating the percentage of total active time in the small square: no significant difference was determined between age groups or between diets at their respective ages for anxiety-like behaviour ($F_{(3,40)} = 3.775$; $p = 0.0696$). Nevertheless, aged animals showed higher levels of anxiety compared to young animals, although not significantly. Among aged mice, the HFD groups tended to demonstrate higher anxiety levels among diets (Fig. 3B). In addition, these data overlap with the number of visits to the small square, as fewer visits correlate with less time spent in the square.

3.2.2. Light-dark box

The aim of this experiment was to assess anxiety-like behaviour in mice. Age and diet had no significant combined effect on anxiety-like behaviour in mice ($F_{(3,40)} = 2.546$; $p = 0.0696$). However, age ($F_{(1,40)} = 49.93$, $p < 0.001$) and diet ($F_{(3,40)} = 8{,}567$, $p = 0.001$) influenced anxiety-like behaviour in mice. No anxiety-like behaviour was determined in the young animals. In contrast, the aged mice in the HFD group visited the light compartment the least often compared to the control mice ($p < 0.001$). This behaviour was possibly restored by prebiotic supplementation, as the HFD+P mice made significantly more visits

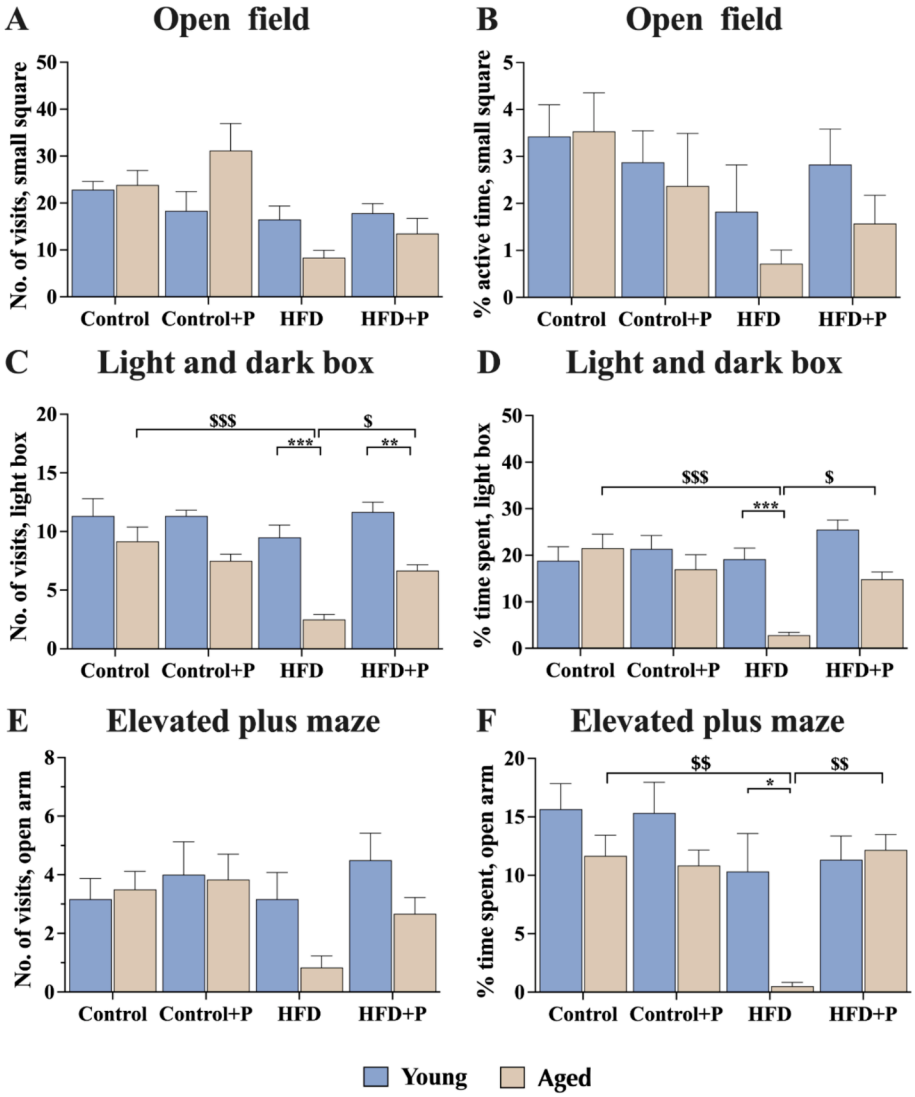

Fig. 3. Animal behaviour experiments for anxiety-like behaviour. (A) Open field experiment: graphical representation of number of visits, small square; N=6. (B) Open field experiment: graphical representation of percentage time spent, small square; N=6. (C). Light & dark box: graphical representation of number of visits, light box. Data presented as ± SEM, $^{**}p < 0.01$; $^{***}p < 0.001$ among young vs aged; $\$p < 0.05$; $\$p < 0.001$ among diet groups in aged animals; N=6. (D) Light & dark box: graphical representation of percentage time spent, light box. Data presented as ± SEM, $^{**}p < 0.01$; $^{***}p < 0.001$ among young vs aged; $\$p < 0.05$; $\$p < 0.001$ among diet groups in aged animals; N=6. (E) Elevated plus maze: graphical representation of number of visits, open arm. Data presented as ± SEM, N=6. (F) Elevated plus maze: graphical representation of percentage time spent, open arm. Data presented as ± SEM; $^{*}p < 0.05$; among young vs aged; $p < 0.01$ among diet groups in aged animals; N=6.

than the HFD mice ($p < 0.05$), indicating a lower level of anxiety (Fig. 3C). When considering the interaction between age and diet, a significant difference was determined ($F_{(3,40)} = 5.368$; $p = 0.0033$). With increasing age of animals, a significant reduction in time spent in the light box was observed only in aged HFD mice ($p < 0.001$), whereas no differences were observed between diets in the groups of young mice. However, in the aged mice, the HFD animals were more anxious as they spent less time in the light compartment compared to control mice ($p < 0.0001$) and mice in the HFD+P group ($p < 0.05$), confirming the effects of prebiotic supplementation (Fig. 3D).

3.2.3. Elevated plus maze

Similar to the previous test, the results for anxiety-like behaviour showed no significant differences in the interaction between age and diet on anxiety levels ($F_{(3,40)} = 1.304$; $p = 0.2864$). No significant differences were determined between age groups for any of the diets on open arm visits. Furthermore, similar differences between diets were also observed in both young and aged animals. However, in the aged animals, there were a tendency for the HFD groups to show increased anxious behaviour as they made the fewest visits compared to the control groups (Fig. 3E). No significant differences were found in the interaction effects of age and diet on anxiety levels ($F_{(3,40)} = 2.302$; $p = 0.0917$). However, age ($F_{(1,40)} = 9.263$; $p = 0.0041$) and diet ($F_{(3,40)} = 6.967$; $p = 0.0007$) had an independent effect on the anxiety levels of the animals. Results showed a significant reduction in the percentage of active time spent in the open arm in aged HFD mice ($p < 0.001$) compared to young HFD mice. In young animals, no difference was determined between diets in terms of anxiety levels. In aged mice, HFD animals were more anxious as they spent less time in the open arm compared to control mice ($p < 0.0001$) and HFD+P group mice ($p < 0.05$), confirming the recovery effects of prebiotic supplementation (Fig. 3F).

3.3. Memory

3.3.1. Novel object recognition test.

In order to assess short-term memory in mice, the novel object recognition test was carried out. The DI measured for age and diet effects showed a significant decrease in cognition $F_{(3,35)} = 3.743$, $p = 0.0197$). A significant age-related decrease in memory performance was only determined in HFD mice ($p < 0.01$). Young animals exposed to a short-term diet showed no signs of cognitive impairment on any of the diets. However, in the aged animals, HFD mice showed significant cognitive impairment with reduced short-term memory compared to control mice ($p < 0.01$), with prebiotics attenuating this effect in HFD+P mice ($p < 0.01$) (Fig. 4A).

3.3.2. Y-maze.

This experiment was conducted to evaluate spacial memory in mice. It was found that the interaction between age and diet was significant ($F_{(3,35)} = 7.682$, $p = 0.0004$). When counting the number of alteration patterns between age groups, the data showed a significant decline in spatial memory only in HFD aged mice ($p < 0.05$) compared to young mice. Though, no such differences were observed between diets in young animals. However, in the aged animals, HFD mice showed a significant cognitive impairment in spatial memory compared to control mice ($p < 0.001$). Interestingly, HFD+P aged mice were able to recover this deterioration in cognitive abilities with prebiotics compared to HFD mice ($p = 0.0448$) (Fig. 4B).

3.4. Female urine sniffing test

Anhedonia-like behaviour was evaluated by the female urine sniffing test. The interaction between age and diet was found to be significant ($F_{(3,40)} = 3.520$, $p = 0.0235$). When determining the DI between age, we

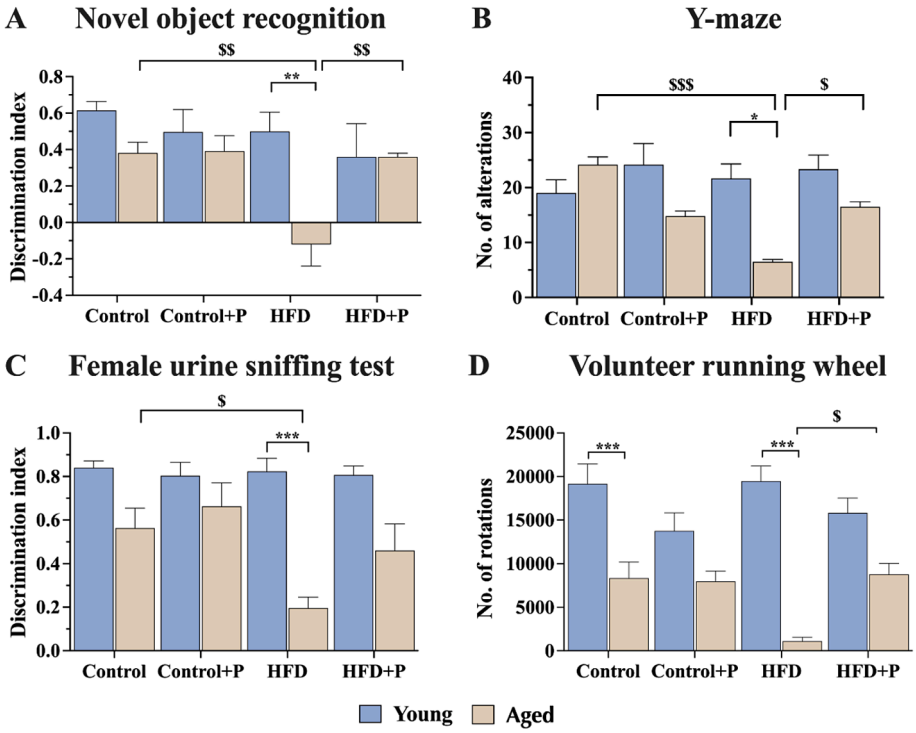

Fig. 4. Animal behaviour experiments for cognition. (A) Novel object recognition to assess short term memory: graphical representation of discrimination index; Data presented as ± SEM, $^{**}p < 0.01$; among young vs aged; $p < 0.01$ among diet groups in aged animals; N=6. (B) Y maze to evaluate spatial memory: graphical representation of number of alterations; Data presented as ± SEM, $^{*}p < 0.05$; among young vs aged; $\$p < 0.05$; $\$p < 0.001$among diet groups in aged animals; N=6. (C) Female urine sniffing test to assess anhedonia: graphical representation of discrimination index; Data presented as ± SEM, $^{***}p < 0.001$; among young vs aged; $\$p < 0.05$ among diet groups in aged animals; N=6. (D) Volunteer running wheel to evaluate total physical activity: graphical representation of number of rotations; Data presented as ± SEM, $^{***}p < 0.001$; among young vs aged; $\$p < 0.05$ among diet groups in aged animals; N=6.

observed a significant loss of sensitivity to pleasure in the HDF group ($p < 0.001$) of aged mice compared to young HFD mice. No such pleasure impairment between diets was determined in the young animals. However, in the aged animals, the HFD animals had significantly lost the ability to experience pleasure compared to the control animals ($p < 0.05$). Interestingly, such a decline was not observed in aged HFD+P (Fig. 4C).

3.5. Voluntary physical activity

In this experiment, the total voluntary activity of the animals was determined. The total number of rotations measured for the interaction of age and diet was significant ($F_{(3,40)} = 5.803$, $p = 0.0022$). With increasing age of the animals, a significant loss of physical activity was observed in the control mice ($p < 0.001$) and the HFD aged mice ($p < 0.001$) compared to their younger counterparts. Young animals were physically active and therefore showed no differences between diets. In contrast, the aged animals, especially the HFD animals, were severely physically impaired and therefore had significantly lower voluntary activity compared to HFD+P mice ($p < 0.05$), suggesting a possible beneficial effect of the prebiotics (Fig. 4D).

3.6. Phagocytosis

To investigate the phagocytic capability of microglia, the efficacy of uptake of fluorescent latex beads was evaluated. The data show that age and diet together have a significant effect on the phagocytic activity of microglia ($F_{(3,32)} = 60.02$; $p < 0.001$). Phagocytic efficiency decreased significantly with age in all diet groups ($p < 0.001$). In contrast, no such difference in phagocytosis was observed in the young animals receiving different diets. However, in the aged animals, phagocytosis was more effective in the microglia of the control mice than the HFD mice ($p <$

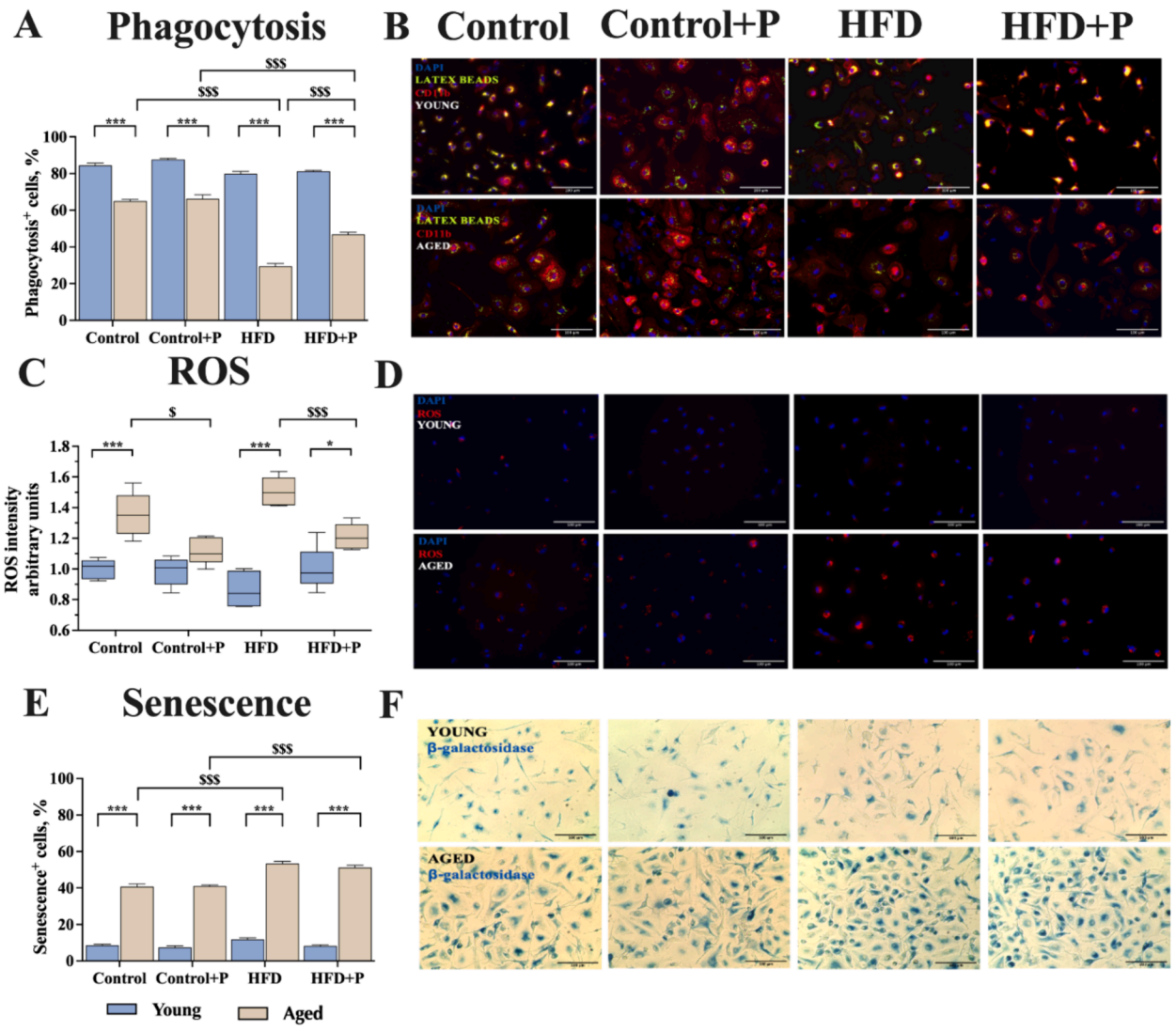

Fig. 5. Characterisation and evaluation of microglia functionality. (A) Assessment of phagocytic activity of microglia: graphical representation of percentage of phagocytic$^+$ cells; Data presented as mean ± SEM, ***$p < 0.001$; among young vs aged; $$p < 0.001$ among diet groups in aged animals; N=6. (B) Phagocytosis: depicted merged image of microglia incubated with fluorescent latex beads (green), immunolabelled for CD11b to identify the cell soma (red), and nuclei stained with DAPI (blue). Scale bar: 100 μm. (C) Assessment of ROS production for oxidative stress: graphical representation of ROS intensity, normalised to $\log_{10}$ standardized to young control animals; Data presented as box plot; *$p < 0.05$; ***$p < 0.001$; among young vs aged; $$p < 0.05$; $$p < 0.001$ among diet groups in aged animals; N=6. (D) ROS (intensity): depicted merged images of microglia incubated with fluorescent ROS stain (red), and nuclei stained with DAPI (blue). Scale bar: 100 μm. (E) Senescence (cellular aging): Estimation of microglia senescence by β-galactosidase activity: graphical representation of percentage of SA-β-gal$^+$ cells; Data presented as mean ± SEM, ***$p < 0.001$; among young vs aged; $$p < 0.001$ among diet groups in aged animals; N=6. (F) Senescence: depicted image of microglia stained with SA-β-gal (blue). Scale bar: 100 μm.

0.001), suggesting a loss of function in the HFD mice, and similar results were obtained in the microglia of the control + P and HFD+P mice ($p <$ 0.001). Interestingly, the loss of function was mitigated by the addition of prebiotics, as shown in the microglia of HFD+P animals compared to the microglia of HFD mice ($p < 0.001$) (Fig. 5A).

3.7. Oxidative stress

To evaluate the oxidative stress of microglia, ROS production was estimated. The interaction between oxidative stress, age and diet was significant in relation to ROS production ($F_{(3,32)} = 98.50$; $p < 0.001$). The significant increase in ROS production is apparent between age as in control aged mice ($p < 0.001$) and HFD group aged mice ($p < 0.001$ and $p < 0.05$) compared to aged mice. Although not significant, we could observe the same trend in control + P ($p = 0.08543$). No significant difference was found in the microglia in either of the diets, so ROS levels were similar. ROS levels increased significantly with increasing age. Interestingly, the effect of the prebiotics significantly reduced oxidative stress in microglia of control + P and HFD+P mice compared to control ($p < 0.05$) and HFD ($p < 0.001$) mice respectively (Fig. 5C).

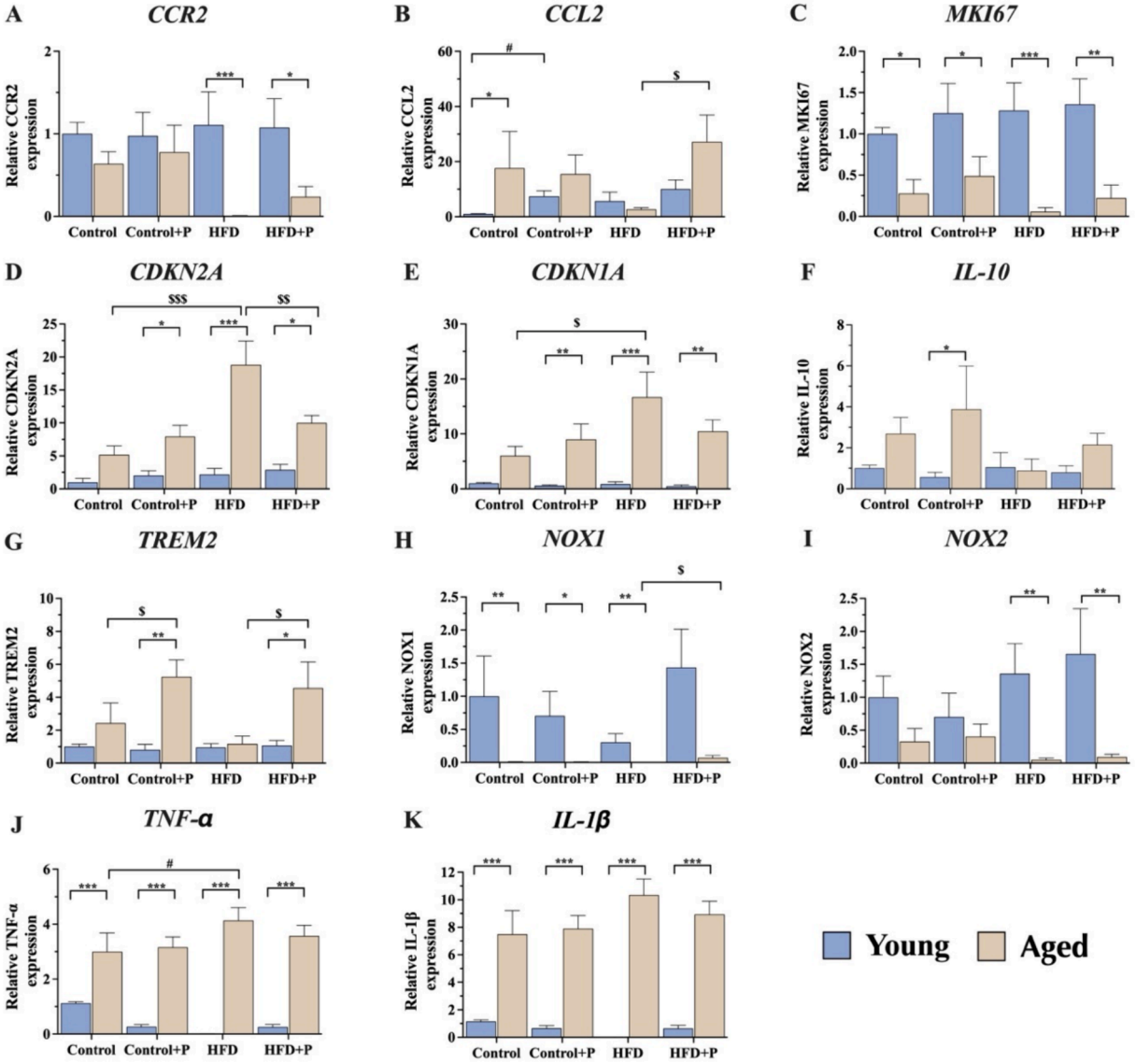

Fig. 6. Evaluation of the effects of diet on microglial gene expression during ageing. (A) *CCR2* (expression): graphical representation of *CCR2* gene expressions; Data presented as mean ± SEM, $^{*}p < 0.05$, $^{***}p < 0.001$ among young vs aged; N=6. (B) *CCL2* (expression): graphical representation of *CCL2* gene expressions; Data presented as mean ± SEM, $^{*}p < 0.05$ among young vs aged; $^{\#}p < 0.05$ among diet groups in young animals; $\$p < 0.05$ among diet groups in aged animals; N=6. (C)) *MKI67* (expression): graphical representation of *MKI67* gene expressions; Data presented as mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ among young vs aged; N=6. (D) *CDKN2A* (expression): graphical representation of *CDKN2A* gene expressions; Data presented as mean ± SEM, $^{*}p < 0.05$, $^{***}p < 0.001$ among young vs aged; $p < 0.01$, $\$p < 0.001$among diet groups in aged animals; N=6. (E) *CDK1NA* (expression): graphical representation of *CDK1NA* gene expressions; Data presented as mean ± SEM, $^{**}p < 0.01$, $^{***}p < 0.001$ among young vs aged; $\$p < 0.05$ among diet groups in aged animals; N=6. (F) *IL-10* (expression): graphical representation of *IL-10* gene expressions; Data presented as mean ± SEM, $^{*}p < 0.05$, among young vs aged; N=6. (G) *TREM2* (expression): graphical representation of *TREM2* gene expressions; Data presented as mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$ among young vs aged; $\$p < 0.05$ among diet groups in aged animals; N=6. (H) *NOX1* (expression): graphical representation of *NOX1* gene expressions; Data presented as mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$ among young vs aged; $\$p < 0.05$ among diet groups in aged animals; N=6. (I) *NOX2* (expression): graphical representation of *NOX2* gene expressions; Data presented as mean ± SEM, $^{**}p < 0.01$ among young vs aged; N=6. (J) *TNF-α* (expression): graphical representation of *TNF-α* gene expressions; Data presented as mean ± SEM, $^{***}p < 0.001$, $^{\#}p < 0.05$, among young vs aged; N=6. (K) *IL-1β* (expression): graphical representation of *IL-1β* gene expressions; Data presented as mean ± SEM, $^{*}p < 0.001$, among young vs aged; N=6.

3.8. Senescence

Cellular senescence / ageing was evaluated using the senescence marker β-galactosidase. A significant effect in senescence is observed in the interaction between age and diet ($F_{(3,32)} = 15.76$, $p < 0.001$). The senescence of microglia increased with age in all diet groups ($p < 0.001$). No signs of senescence were determined in microglia of young animals, as hypothesised, while a significant acceleration of senescence is observed in aged mice, particularly in the microglia of the HFD groups compared to the control groups ($p < 0.001$) (Fig. 5E).

3.9. Gene expression analysis in microglia cells

3.9.1. CCR2

The expression of the *CCR2* gene was used as a marker to evaluate microglial chemotaxis. The interaction between age and diet did not report an increase in the expression of *CCR2* gene, however, age independently had a significant effect ($F_{(1,32)} = 22.93$; $p < 0.001$). A significant downregulation was only observed with age in HFD ($p < 0.001$) and HFD+P ($p < 0.05$). Among young and aged animals, no difference in gene expression levels in diet group was observed. Interestingly, a tendency of severe downregulation was reported in aged HFD mice with almost no expression compared to all other dietary groups. However, increased *CCR2* expression was detected in HFD+P aged mice compared to HFD aged mice, indicating a possible recovery of chemotactic microglia functionality (Fig. 6A).

3.9.2. CCL2

Similar to the expression of the CCR2 receptor, *CCL2* cytokine gene expression levels were determined to evaluate microglia recruitment capability. No significant effect on CCL2 protein expression was determined in the interaction between age and diet ($H_{(3)} = 1.027$, $p = 0.3385$). However, age had an effect on *CCL2* expression ($H_{(1)} = 4.367$, $p = 0.0447$). With increasing age of the animals, *CCL2* expression increased in all groups except the HFD mice ($p < 0.05$). In young animals receiving a control diet, the addition of prebiotics ($p < 0.05$) led to approximately 10-fold higher expression of *CCL2* gene, while effect of prebiotics was not as prominent in animals receiving HFD. In aged mice, however, the effect of prebiotics was only detectable in the HFD groups. The addition of prebiotics ($p < 0.05$) also increased *CCL2* gene expression by about 10-fold (Fig. 6A). These data suggest a loss of function of HFD microglia in chronic neuroinflammation that could be mitigated by the administration of prebiotics (Fig. 6B).

3.9.3. Ki-67 (MKI67)

Expression of the *MKI67* gene was used as a marker to evaluate cellular proliferation in ageing microglia. The interaction between age and diet did not report an increase in the expression of *MKI67* gene, however, age independently had a significant effect ($F_{(1,32)} = 32.58$; $p < 0.001$). With increase age of the animals, a significant downregulation was observed in all dietary groups comparing with control groups ($p < 0.05$), HFD ($p < 0.001$) and HFD+P ($p < 0.01$). Among young and aged animals, no difference in gene expression levels was observed in diet groups. However, a tendency was noted in the aged HFD mice were *MKI67* gene expression was the most downregulated compared to all other dietary groups with prebiotic supplementation to some extent reducing the effects of HFD with regards to cellular proliferation (Fig. 6C).

3.9.4. P16 (CDKN2A)

The expression of the *CDKN2A* gene was used as a marker of cellular senescence in microglia. The interaction between age and diet showed a significant increase in the expression of *CDKN2A* gene ($F_{(3,32)} = 5.834$; $p < 0.01$). Significant upregulation was observed in all dietary groups expect for control mice during ageing with control + P and HFD+P ($p < 0.05$) and HFD mice ($p < 0.001$). In young animals, no difference in gene expression levels was determined in any of the diet groups. However, in aged animals, HFD mice showed significantly higher *CDKN2A* upregulation than control animals ($p < 0.001$) and HFD+P ($p < 0.01$), correlating with accelerated senescence demonstrated by HFD mice (Fig. 6D).

3.9.5. P21 (CDKN1A)

The expression of *CDKN1A* gene was also used as a marker of cellular senescence in ageing microglia. The interaction between age and diet did not show an increase in the expression of *CDKN1A* gene, however, age had a significant effect independently ($F_{(1,32)} = 42.63$; $p < 0.001$). Significant upregulation was reported in all dietary groups except for control mice during ageing with control + P and HFD+P ($p < 0.01$) and HFD mice ($p < 0.001$). In young animals, no difference in gene expression levels was determined in any of the diet groups while in aged animals only HFD mice showed significantly higher *CDKN1A* levels compared to control mice ($p < 0.05$). The cellular senescence data all showed similar expression in ageing animals, especially in HFD mice while the addition of prebiotics slowed this accelerated effect (Fig. 6E).

3.9.6. IL-10

The expression of *IL-10* gene was used as a marker to evaluate anti-inflammatory function of microglia. The interaction between age and diet did not show a significant increase in the expression of *IL-10* gene, but age independently had a significant effect ($F_{(1,32)} = 5.160$; $p < 0.05$). With increasing age, a significant increase in *IL-10* level was found only in control + P aged mice ($p < 0.05$) and the only downregulation was determined in HFD aged mice, whereas HFD+P aged mice reported an upregulation of *IL-10* gene, suggesting a beneficial effect of the addition of prebiotics. Among young and aged animals, no difference in gene expression was observed in any of the diet groups (Fig. 6F).

3.9.7. TREM2

In addition to the results of TREM2 protein levels, *TREM2* gene expression was also evaluated. A significant effect on *TREM2* expression was observed in the interaction between age and diet ($H_{(3)} = 1.027$, $p = 0.0385$). Furthermore, age upregulated *TREM2* gene expression ($H_{(1)} = 16.24$, $p = 0.0003$) only in the prebiotic- supplemented control + P ($p < 0.01$) and HFD+P ($p < 0.05$) groups. In the young animals, all diets had similar *TREM2* expression levels (Fig. 6G). This shows that supplementation with prebiotics can have positive effects on microglial function during ageing, independent of diet.

3.9.8. NOX1 and NOX2

The enzyme subunits *NOX1* and *NOX2* are associated with increased ROS production and are therefore used to assess oxidative stress. No significant effect on *NOX1* gene expression was observed in the interaction between age and diet ($H_{(3)} = 0.9303$, $p = 0.4374$). In ageing mice, however, *NOX1* expression was dramatically reduced in all groups ($H_{(1)} = 13.09$, $p = 0.0010$). A significant downregulation of *NOX1* expression was observed in aged control mice ($p < 0.01$), control + P ($p < 0.05$) and HFD ($p < 0.01$) compared to young animals. Although not significant, the same trend was found for HFD+P group of animals. No differences in *NOX1* levels were observed in the young animals, but expression tended to be reduced in the young HFD mice. Interestingly, aged HFD+P ($p < 0.05$) mice exhibited significant upregulation compared to aged HFD mice (Fig. 6H).

No significant effect on *NOX2* gene expression was observed in the interaction between age and diet ($H_{(3)} = 1.354$, $p = 0.2745$). However, a substantial decrease in *NOX2* gene expression was found with increasing age of the animals ($H_{(1)} = 14.96$, $p = 0.0005$). Ageing animals appeared to show a down-regulation of the *NOX2* gene, which was seen in all aged animals, but was particularly significant in aged HFD mice ($p < 0.01$) and HFD+P ($p < 0.01$) compared to their younger counterparts. No such differences in *NOX2* expression levels were observed among young and aged animals in either of the diets (Fig. 6I).

3.9.9. *TNF-α*

To evaluate pro-inflammation, the gene expression of the cytokine *TNF-α* was evaluated. The results showed a significant interaction of age and diet ($H_{(3)} = 3.531$, $p = 0.0257$). In addition, age ($H_{(1)} = 150.1$, $p < 0.001$) had an independent effect on *TNF-α* expression, as significant overexpression of TNF-α was observed in all diet groups ($p < 0.001$). No such difference in *TNF-α* expression was determined in young mice. Interestingly, HFD aged mice ($p < 0.05$) had a significant upregulation of *TNF-α* expression compared to control mice, whereas no such effect was observed in aged HFD+P mice (Fig. 6J).

3.9.10. *IL-1β*

IL-1β is a potent pro-inflammatory cytokine and therefore gene expression was determined. The data showed a significant interaction between age and diet ($H_{(3)} = 1.857$, $p = 0.1541$). The effect of age ($H_{(1)} = 167.1$, $p < 0.001$) also led to a significant upregulation of *IL-1β* expression in all diet groups ($p < 0.001$). However, no such differences in *IL-1β* expression levels were found in young or aged mice in any of the diet groups (Fig. 6K).

3.9.11. *Principal component analysis*

Two separate principal component analyses were performed for the animal behaviour (Fig. 7A) and the cellular (Fig. 7B) data. In the principal component analysis of animal behaviour (Fig. 7A), principal component 1 explained 41 % of the data. All behaviour parameters taken together showed significant differences only in HFD aged mice compared to the rest of the animals, suggesting significant change behaviour compared to the normal behaviour. In contrast, microglia principal component analysis (Fig. 7B), which explained 53.7 % of the data variance, showed significant differences between young and aged animals of all diets. Furthermore, there was an additional separation in the aged animals, particularly in the HFD mice, which was not observed in the young animal groups. This suggests that HFD mice exhibit cellular changes with increasing age that impair microglia functionality and are reduced by administration of the prebiotics FOS and GOS.

3.9.12. *Correlation matrix*

The correlation results presented in (Fig. 7C) show a combined correlation matrix between the behaviour of the animals and the cellular analysis. Behavioural experiments related to anxiety scores (A,B,C,D,E,F,G) were positively correlated with inflammatory markers (M,N,O,P,Q,R,S,TU), while behavioural experiments related to memory scores (H,I) were inversely correlated with inflammatory markers. Furthermore, the loss of phagocytic function of microglia (L) is directly / positively related to increased oxidative stress (M) and senescence (N) and also positively correlates with declining memory (H,I) and increased anxiety levels (A,B,C,D,E,F,G). All declining traits could be the result of DIO, glucose intolerance, and/or systemic inflammation. In addition, any behavioural trait may have a direct or indirect effect on microglia and vice-versa, e.g. mice groups, especially (HFD) with reduced cognition and increased anxiety levels also showed dysfunctional microglia exhibiting decreased phagocytosis associated with alarming ROS levels and increased senescence.

3.9.13. *Microbiota analysis*

- ***Differences in the diversity of the gut microbiota of young and aged mice***

The gut microbiota sequencing was performed to examine the changes to microbiota composition during ageing and to determine the effects of diet as well as to assess the modulation capability of prebiotics GOS+FOS. Samples were collected from the cecum and sent for shot gun sequencing. A significant difference in beta diversity was identified between the mice groups. The gut microbiota of the mouse groups was clustered together suggesting that the gut microbiota was more similar within the groups (Fig. 8A).

- ***Impact of diet on the diversity of the microbiota of young and aged mice***

To study the impact of diet on the gut microbiota, both old and young mice were fed with HFD and prebiotics. The consumption of HFD and prebiotics increased the differences in the diversity of the gut microbiota of the mice. Analysis of the gut microbiota showed that the beta diversity of each group differed from the other in both young and aged mice (Fig. 8B). However, the gut microbiota of the aged mice after feeding was more diverse than that of the young mice, as the gut microbiota of the young mice was more clustered towards the centre of the two principal components.

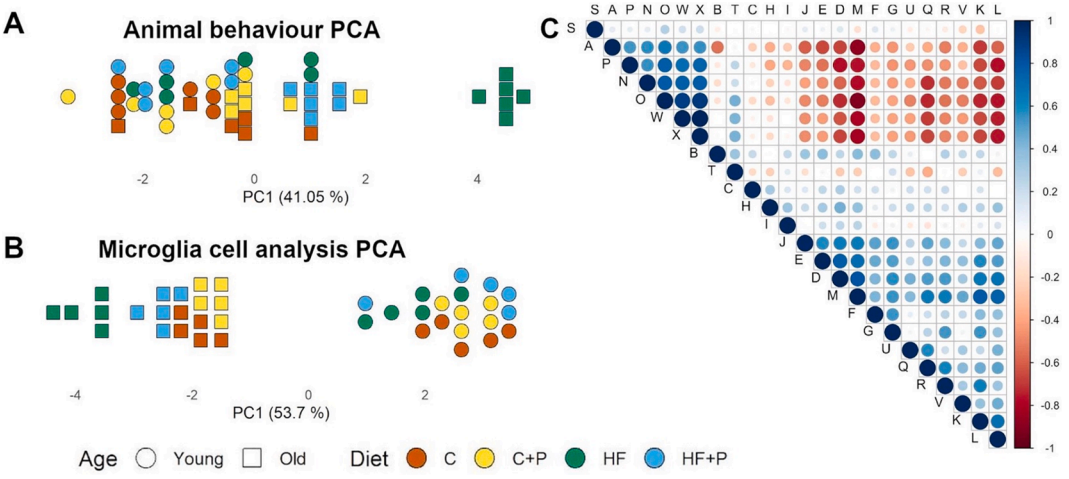

Fig. 7. (A) principal component analysis for animal behaviour data between diets among young and aged (n = 6). Accounting for 41 % of the variance. (B) principal component analysis for cellular data between diets among young and aged (n = 6). Accounting for 41 % of the variance. (C) Correlation matrix between animal behaviour data and cellular data represented as coloured dots, red (negative correlation) and blue (positive correlation). Correlation matrix alphabet representation: A: weight, B: Open field: visits to small square, C: Open field: percent time in small square, D: Light and dark box: visits to light box, E: Light and dark box: percent time spent in light box, F: Elevated plus maze: visits to open arm, G: Elevated plus maze: percent time spent in open arm, H: Hidden + Partially hidden marbles, I: Short-term memory, J: Y maze, K: Sniffing test, L: Running wheel, M: Phagocytosis, N: ROS, O: Senescence, P: p16 protein, Q: CCR2 protein, R: TREM2 protein, S: *CCL2* gene, T: *TREM2* gene, U: *NOX1* gene, V: *NOX2* gene, W: *TNF-α* gene, X: *IL-1β* gene.

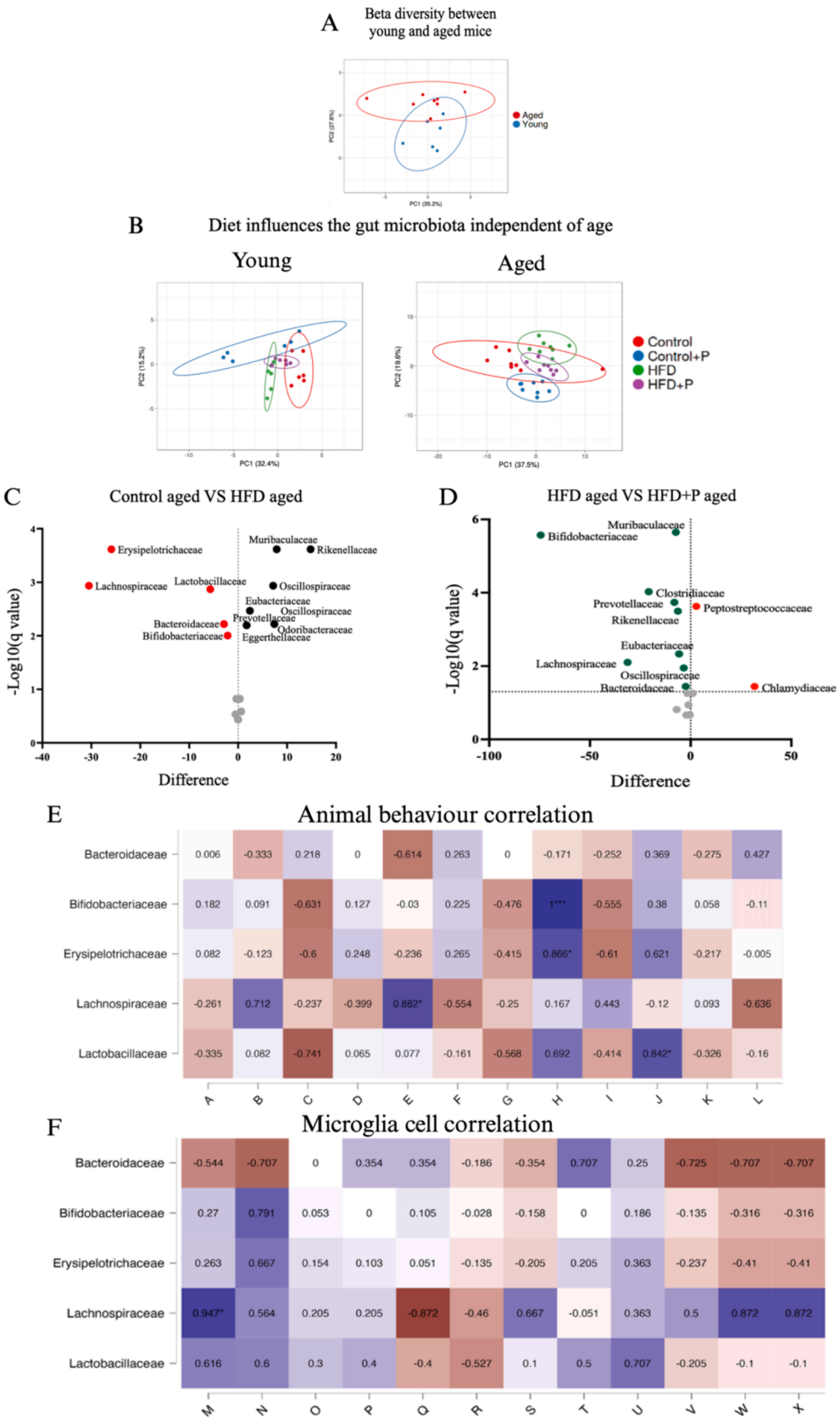

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Fig. 8. Gut microbiota beta diversity analysis of control aged mice compared to control young mice. Principal component analysis based on Bray–Curtis distance matrix, performed in ClustVis web tool showed a marked separation between the gut microbiota communities of aged and young mice. Data for aged mice and young mice (n = 6–8) were plotted on two principal components. The coloured circles cover individuals near the centre of gravity for each cluster. (B) Effects of diet on gut microbiota beta diversity in young and aged mice. Principal component analysis based on Bray–Curtis distance matrix, performed in ClustVis web tool showed a marked separation between the gut microbiota communities of A. Young and B. Aged mice. Data for young mice and aged mice were plotted on two principal components. The coloured circles cover individuals near the centre of gravity for each cluster. (C) Volcano plot of differential caecal microbiota associated with HFD feeding and control mice. The fold change (difference) associated with the groups and the -log q-values were plotted for each function. Significantly different metabolites (q = 0.05) are coloured in red (high in HFD mice) or black (high in control mice). Grey dots represent metabolites that are not significantly different among the mice groups. (D) Volcano plot of differential caecal microbiota associated with HFD+P and HFD consumption. The fold change (difference) associated with the groups and the -log q-values were plotted for each function. Significantly different metabolites (q = 0.05) are coloured in red (HFD mice) or green (HFD+P mice). Grey dots represent metabolites that are not significantly different among the mice groups. (E) Association between significantly altered gut microbial composition in HFD feeding and their behaviour. A spearman's correlation matrix showing positive (blue) and negative (red) associations between significantly altered gut bacterial family and mice behaviour of HFD mice. Significant correlations are marked with asterisks. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$, Spearman's rho correlation analysis with 95 % confidence interval. (F) Association between significantly altered gut microbial composition in HFD consumption and biomarkers of aging in aged mice. A spearman's correlation matrix showing positive (blue) and negative (red) associations between significantly altered gut bacterial family and phagocytosis, senescence, reactive oxygen species, P16 and CCR2 proteins in HFD mice. Significant correlations are marked with asterisks. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$, Spearman's rho correlation analysis with 95 % confidence interval.

- ***Differences in the gut microbiota of control and HFD fed aged mice***

Since the gut microbial beta-diversity of aged mice was more diverse after feeding different diets, we investigated the microbial changes in the gut of HFD-fed mice and control mice to ascertain which bacteria were significantly enriched after HFD feeding and which were enriched only in control mice. As shown in (Fig. 8C), bacteria of the families *Erysipelotrichaceae, Lactobacillaceae, Lachnospiraceae, Bacteroidaceae* and *Bifidobacteriaceae* were overrepresented in HFD fed mice.

- ***Effects of HFD+P on the microbiota of HFD aged mice***

To determine the eubiotic effect of prebiotics during HFD feeding, we co-administrated HFD and prebiotics to mice and studied the changes in gut microbiota compared to mice fed HFD only. As shown in (Fig. 8D), the populations of *Muribaculaceae, Prevotellaceae, Rikenellaceae* and *Oscillospiraceae*, which were initially present in control mice, were maintained but decreased when the mice were fed HFD only.

- ***Relationship between the gut microbiota of HFD mice and the behaviour and ageing of mice***

The Spearman correlation matrix was used to establish a relationship between changes in the mice behaviour and changes in the gut microbiota during obesity. As shown in (Fig. 8E), there was a strong correlation between *Lachnospiraceae* and the time spent in open arms and RW-total activity. Meanwhile, there was a strong positive correlation between *Bacteroidaceae* and phagocytic cells, senescent cells, ROS intensity, p16 expression and CCR2 expression in HFD mice (Fig. 8F).

4. Discussion

The interaction of age and diet plays an important role in influencing systemic problems that are often associated with inflammation (Hata et al., 2023; McGrattan et al., 2019). Ageing is epitomised by an increase in chronic inflammation in the CNS and is closely associated to deterioration in brain function and the onset of neurodegenerative diseases (Kwon & Koh, 2020; Lisboa et al., 2016). Moreover, diet has a significant impact on brain functionality. A suboptimal diet, characterised by a high calorie content increases fat and glucose levels, contributes to cognitive impairment, stress, and anxiety (Arnoriaga-Rodríguez et al., 2020; Ekstrand et al., 2021; Meeusen, 2014). Furthermore, diet plays a pivotal role in shaping the gut microbiota (Cryan et al., 2019), with the intake of prebiotics such as oligosaccharides from fruits and vegetables actively promoting the growth and activity of beneficial microorganisms (Kao et al., 2016). In this study, we therefore aimed to evaluate the interplay between ageing, diet, and neuroinflammation in mice. Animals fed with HFD demonstrated accelerated ageing, characterised by a decline in cognitive abilities and increased anxiety, and that prebiotics GOS+FOS negated the detrimental effects of HFD.

It was found that the HFD groups gained the most weight regardless of supplementation, which has also been demonstrated in previous research (Skinner et al., 2018; Woo et al., 2020). In addition, obesity and higher fat consumption are associated with diabetes caused by insulin resistance. (Aras et al., 2021; Vega-López et al., 2018). As hypothesised, the GTT of HFD mice showed a significant glucose intolerance not only between age groups but also within dietary groups. Although blood glucose levels were higher in the HFD+P mice group, the control groups showed lower glucose intolerance between the age groups and within the diet groups compared to the HFD mice. In addition, endotoxin in the blood was evaluated to detect signs of systemic inflammation. LPS released from the gut enters the blood stream and eventually the brain to elicit a long-term neuroinflammation. A short-term diet does not modulate gut microbiome and therefore, young animals usually possess a healthy system and are devoid of endotoxins (Leeming et al., 2019). Therefore, the level of LPS endotoxins was evaluated only in aged mice. HFD aged mice demonstrated the highest levels of LPS compared to all other dietary groups, indicating leaky gut syndrome. Administration of prebiotics reduced the LPS concentration in the blood of HFD+P aged mice (Suppl. material Fig S1). Given the evidence from metabolic pathologies and endotoxin levels, exploring the long-term HFD effects on ageing related issues to behaviour (anxiety and memory) and brain (microglia functionality and inflammation) of the mice was the next step.

Age and diet are the driving factors in most pathological problems. Long-term HFD in many studies has been reported to accelerate ageing and behaviour impairment. Aged mice fed with HFD demonstrated significant anxiety-like behaviour compared to the control group. Such an increase in anxiety due to HFD accelerating ageing was reported in (Y. Li et al., 2022). In addition, the HFD aged mice showed a significantly lower level of voluntary physical activity compared to the other groups. An unmotivated and obese animal is less likely to participate in any activity (Leventhal, 2012a). The urine sniff test in female mice showed a significant decline in motivation or anhedonia in HFD aged mice compared to the control mice. This could also explain the lack of exploratory motivation in anxiety tests. There is evidence that cognition is severely impaired in obese mice models, including short-term (W. Zhang et al., 2022) and spatial memory (Medrano-Jiménez et al., 2019). Our study is in line with this research, as HFD aged mice showed clear signs of cognitive defects and impaired spatial memory compared to their control counterparts. Although the behaviour of HFD+P and HFD aged mice sometimes overlap, HFD+P aged mice demonstrated less anxiety and cognitive impairment compared to HFD aged mice. Moreover, we attribute the positive effect in the reduction in anxiety levels and cognitive impairment to the additional administration of the prebiotics GOS+FOS, which has been shown in previous studies (Ansari

et al., 2020; Burokas et al., 2017b; Holscher, 2017). In contrast, young animals were largely unaffected by the diet and prebiotics, which may be due to the fact that feeding for 1 month is not sufficient to have an effect on animal behaviour, which is consistent with previous reports (Messa et al., 2020).

Furthermore, HFD also accelerates cellular ageing, propagating cellular dysfunction in the context of inflammation. We have also demonstrated the effects of diet on the functionality of the brain's immune system and on the defence mechanisms of the CNS in relation to microglia. The characterisation of microglia was undertaken to gain new insights into their functioning and cellular mechanisms. In parallel to the mice behaviour, in which a short-term diet of 1 month had no significant effects, we observed the same in terms of microglia functions and cellular status. Aged animals that received the diet over a 10-month period showed differences in microglia functionality, particularly in phagocytosis and immune cell recruitment. HFD aged microglia had significantly lost the ability to phagocytose compared to other aged diet groups, as well as a significant downregulation of the *TREM2* and *IL-10* gene with a concomitant reduction in TREM2 protein expression. Activation of *TREM2* and *IL-10* has an anti-inflammatory effect (Chen et al., 2020), which is retained in aged HFD+P microglia due to the supplementation of prebiotics. In addition, we showed that both protein and gene expression for the crucial cytokine CCL2 and its receptor CCR2 were significantly downregulated only in HFD aged microglia, which is in contrast to other aged diet groups. The inhibition of CCR2 expression and the parallel increase in CCL2 secretion may represent a feedback mechanism in the regulation of the chemotactic response of macrophages (Fantuzzi et al., 1999; Singh et al., 2021). Loss of functional and recruitment capacity is a hallmark of a poor cellular state caused by oxidative stress. Our experiments show that HFD aged microglia produce an excess of intracellular deleterious ROS, which increases oxidative stress, while releasing less extracellular defence ROS. In addition, a significant downregulation of *NOX1* and *NOX2* genes was also observed compared to other dietary groups, indicating cellular dysfunctionality. Interestingly, the administration of prebiotics was able to correct this imbalance, as HFD+P aged microglia demonstrated similar ROS levels to the control aged groups as well as a significant upregulation of the *NOX1* gene compared to HFD aged microglia. Previous studies have shown a negative feedback mechanism in which accumulated intracellular ROS were responsible for the downregulation of genes related to NADPH oxidase subunits (El-Benna et al., 2009; Wang et al., 2017). HFD microglia showed the lowest expression of *NOX1* (undetectable) and *NOX2,* which would result in the animal being unable to fight extracellular pathogens and thus maintain homeostasis (Chéret et al., 2008; Hu et al., 2021). Prebiotics mitigated this loss of function, at least in part, by increasing the expression of *NOX1* (Kao et al., 2016). Given the increased production of ROS and loss of phagocytic capacity in aged microglia regardless of diets, we focussed on assessing cellular ageing accelerated by HFD. As suspected, significant ageing was observed in all dietary groups, but HFD microglia showed significantly greater ageing, also described (Hou et al., 2022; Rubio-Tomás et al., 2022) by accumulating high levels of β-galactosidase and upregulating *p16* and *p21* gene compared to other aged groups. Interestingly, the supplementing HFD with prebiotics delayed microglia senescence by downregulating *p16* and *p21*. Additionally, cellular proliferation was significantly declined in all aged mice, however, HFD mice had the least proliferation *MKI-67* expression while HFD+P was able to recover cellular proliferation to some extent. Cellular ageing with simultaneous loss of proliferation is closely related and the downregulation of one impacts the other. The negative effects of HFD on aged microglia may have been a consequence of systemic inflammation and, in particular, chronic neuroinflammation in the brain (Pizza et al., 2011; Sikora et al., 2021). IL-1β and TNF-α are key cytokines that are upregulated during neuroinflammation (Alasmari et al., 2018). HFD aged microglia were severely affected by chronic neuroinflammation and therefore showed significant overexpression of the inflammatory cytokine genes *IL-1β* and *TNF-α* compared to other dietary groups. Although aged HFD microglia showed loss of function, excessive ROS production and accelerated senescence due to chronic neuroinflammation, the effect was less severe, non-significant and comparable to the aged control diet microglia with the supplementation of prebiotics GOS+FOS. This is similar to a previous study in which we demonstrated that the administration of GOS+FOS can reduce chronic stress-induced levels of pro-inflammatory cytokines (Burokas et al., 2017b). The current study shows that prebiotics reduce ROS levels, indicating a possible antioxidant ability and delay microglia senescence, emphasising their potential anti-ageing potential, as reported in other studies (Fang et al., 2021; Sharma, 2022). The overwhelming data suggest that prebiotics reverse the deleterious effects of long-term HFD as aged HFD+P microglia did not have the same consequences as only HFD aged microglia. GOS and FOS are known to promote immune response (Akatsu, 2021) and maintain immune cells (Ashaolu, 2020). Thereby, by adding of prebiotics, we were able to curb the negative effects of HFD, which is consistent with the results we have observed in animal behaviour.

When all the data in the PCA are combined, only aged HFD mice demonstrated a drastic separation in behavioural experiments compared to the other. This is in line with previous research, as long-term HFD has negative effects on the system as a whole (Duan et al., 2018; Liang et al., 2023). This would explain the altered behavioural patterns of HFD aged mice, such as increased levels of anxiety and stress concomitant with cognitive impairment, as defined in various other research (Patki et al., 2013; Sinoff & Werner, 2003). In addition, there is a link between lack of motivation and low physical activity, which are consequences of DIO, and overall brain damage (Eisenberger et al., 2010; Leventhal, 2012b; Soini et al., 2024). On the other hand, there is also a visible separation in microglia functionality between young and aged mice and particularly in HFD aged mice, which is in agreement with previous studies (Baufeld et al., 2016; Spencer et al., 2019). This supports our hypothesis that microglia dysfunction was a consequence of exposure to long-term HFD and its effects on the CNS (Freeman et al., 2014; Kim et al., 2019). To get a clearer view of how the individual parameter is influenced by the other, a correlation matrix was performed. The strong association was found between microglia dysfunctionality (decreased phagocytosis, increase in ROS and simultaneous accelerated senescence) and animal behaviour (increased stress and increased cognitive impairment), suggesting that HFD consumption may have triggered chronic neuroinflammation leading to deterioration in brain health as visually depicted in Fig. 9. Chronic neuroinflammation was assessed by the rapid increase in proinflammatory cytokines and oxidative stress, which is in line with previous studies (Y. K. Kim et al., 2016; Solleiro-Villavicencio & Rivas-Arancibia, 2018). Furthermore, these factors are most commonly studied in research showing that a disproportionate increase in ROS production is usually associated with increased cellular senescence and cellular dysfunction (Mittal et al., 2014; Victorelli & Passos, 2019).

Age and diet are well known to influence the composition and functions of the gut microbiome, which affects host physiology, brain functions and behaviour (Berding et al., 2021; Dinan & Cryan, 2017). HFD promotes the growth of pathogenic microbes that disrupt the local environment in the gut and promotes inflammation (Tang et al., 2024; Wei et al., 2024). Inflammation in the gut causes damage to epithelial layer and makes it loose and permeable leading to "leaky gut syndrome" (Camilleri, 2019). Additionally, the presence of endotoxin LPS in the blood plasma is a confirmation of leaky gut as bacterial LPS was able to cross the epithelial barrier of the gut and enter the blood stream (Candelli et al., 2021; Mohammad & Thiemermann, 2021). For this reason, we assessed the impact of the different diets on the gut microbiota. HFD feeding promoted the growth of pro-inflammatory bacterial families such as *Erysipelotrichaceae* (Dinh et al., 2015; Schaubeck et al., 2016) and *Lachnospiraceae* (Bolte et al., 2021) and considering that HFD promotes gut permeability, the abundance of these bacteria may have contributed to the high levels of proinflammatory factors detected in the

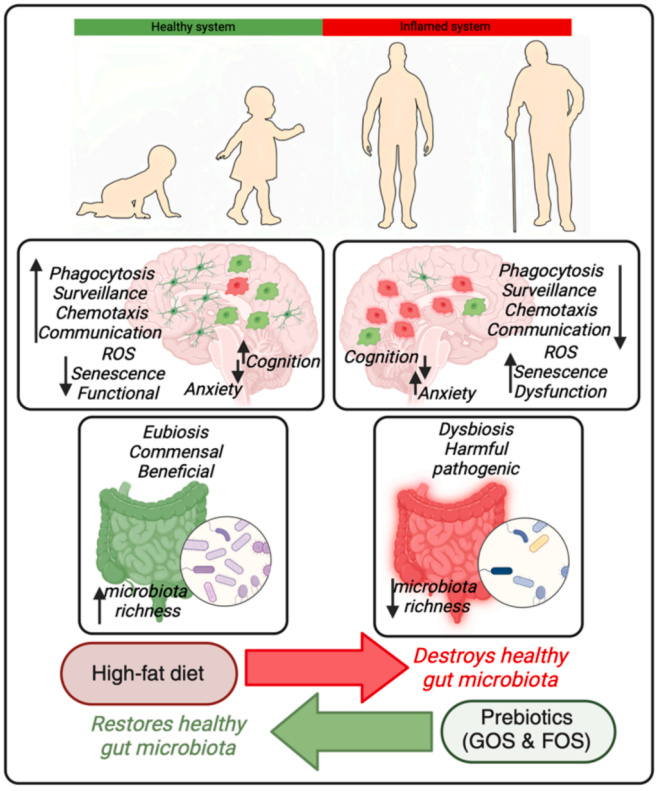

Fig. 9. Visual representation of the discussion.

HFD aged group. This is supported by the strong association between *Lachnospiraceae* and proinflammatory cytokines, phagocytotic cells in aged mice fed with HFD found in this study. In addition to the proinflammatory potential of *Lachnospiraceae,* the strong association of the bacteria with anxiety-like behaviour (Fig. 8) also suggests their potential role in brain functions. Taken together, our results are consistent with previous studies reporting that long-term HFD consumption enhances the proliferation of pathobionts and can affect host behaviour (Seguella et al., 2021).

Meanwhile, supplementation of HFD with the prebiotic GOS+FOS suppressed the levels of these pro-inflammatory bacteria families and enhanced the growth of anti-inflammatory and short chain fatty acid producing families, including *Prevotellaceae* (Y. Chen et al., 2022), *Muribaculaceae* (Hao et al., 2021), *Rikenellaceae* and *Oscillospiraceae* (X. Chen et al., 2021) and improved mice behaviour. In fact, a previous study has shown that boosting *Prevotellaceae* populations through dietary intervention can improve HFD associated brain impairments (M. Zhang et al., 2023). Short-chain fatty acids not only suppress systemic inflammation in high fat fed mice, but also inhibit neuroinflammation and promote behaviour and brain function (Dalile et al., 2019). It is therefore likely that these bacterial families play a key role in mitigating the deleterious effects of HFD in aged mice.

5. Conclusions

Based on the results this study, we can conclude that diet consumed by young animals over a short period of time (1 month) has no effect on the behaviour of the mice or brain function due to a similar profile of the gut microbiota. However, a long-term diet (10 months) in aged animals altered the composition of the microbiota in all dietary groups and consequently affected brain function. In particular, the composition of the gut microbiota was most altered in HFD aged animals and therefore showed impaired behaviour and brain functionality. The negative effects on the brain are caused by increased neuroinflammation, which ultimately affects cognition, anxiety and the microglial immune response. Furthermore, the alteration of the gut microbiota may have played a key role in neuroinflammation, as only HFD aged mice had the healthy bacterial strains eliminated. It has been shown that the administration of prebiotics GOS+FOS can reverse the negative effects of long-term HFD. Administration of prebiotics to HFD aged mice reduced anxiety, improved glucose tolerance, improved cognitive impairment and prevented loss of microglial functions. This is evidenced by the fact that certain beneficial bacterial families were preserved in HFD+P aged mice compared to HFD aged mice. Consequently, we can infer that the prebiotics GOS+FOS indeed have beneficial effects on the gut microbiota and that this effect has a positive impact on reducing neuroinflammation and overall brain health during ageing. Finally, this study also emphasises the importance of potential new therapeutic targets in the field of nutritional neuropsychopharmacology.

6. Data availability statement

The original contributions presented in the study are included in the article/ supplementary material, further inquiries can be directed to the corresponding author/s.

7. Ethics statement

The animal study was reviewed and approved by the Lithuanian State Food and Veterinary Service (No. G2-104).

8. Author contributions

DB and AB designed the experiments, AKV conducted the behavioural, *in-vitro* experiments and wrote the manuscript. SK, ES, JM, KM, RB, and EBMD participated in the experiments, data analysis. AB, DB, and RM revised the manuscript; AB conceived the project. All authors contributed to the article and approved the submitted version.

9. Publisher's note

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Funding acquisition, Conceptualization.

Declaration of Competing Interest

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

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.bbi.2024.08.022.

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