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Research article

Dietary synergy of chub mackerel and quinoa in reducing cognitive decline and retinal degeneration in the 5XFAD Alzheimer's mouse model

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ABSTRACT

Alzheimer's disease (AD) is marked by cognitive decline and retinal degeneration. This study aimed to explore the potential of dietary chub mackerel (*Scomber colias*), rich in docosahexaenoic acid (DHA) and vitamin B12, and quinoa, as a source of vitamin B9, to prevent or delay these effects in a 5XFAD mouse model of AD. Thirty-two five-week-old male mice were divided into four groups: control, 10% chub mackerel, 5% quinoa, and 10% chub mackerel plus 5% quinoa, over 28 weeks. After 28 weeks, behavioural tests confirmed that none of the tested diets induced confounding effects related to anxiety-like behaviour or motor impairment. The diet combining chub mackerel and quinoa improved selected memory-associated outcomes and protected retinal integrity ($p < 0.05$), particularly within the neural layers. This diet also improved the brain n-3/n-6 PUFA ratio, primarily by reducing n-6 PUFA levels rather than by a significant increase in DHA. These benefits likely result from the complementary effects of key nutrients, including DHA

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and vitamins B9 and B12. The preservation of retinal thickness observed with the combined diet supports the use of retinal layer assessment as a potential non-invasive readout of AD-associated neurodegeneration. These findings indicate that combining chub mackerel and quinoa may help mitigate selected neurodegenerative and retinal alterations in the 5XFAD model, positioning them as promising components of a dietary strategy to support neuroprotection and retinal health during AD-related decline.

1. Introduction

Alzheimer's disease (AD) is a multifactorial and multigenic neurodegenerative disorder that accounts for the majority of dementia cases worldwide, posing significant challenges to healthcare systems, families and societies globally [1]. As life expectancy rises and birth rates decline, the median age of the European population is increasing, leading to a sharp rise in the prevalence of neurodegenerative diseases. By 2050, the number of people aged over 60 years is expected to double, significantly increasing the incidence of conditions like AD [2].

Retinal health has recently emerged as a significant area of interest in AD research [3]. The retina, an extension of the central nervous system, reflects the pathological changes occurring in the brain. Retinal degeneration, characterised by thinning of retinal layers, is increasingly recognised as an early indicator of neurodegenerative diseases such as AD. The accessibility of the retina and its direct connection to the brain make it a promising non-invasive biomarker for detecting and monitoring AD progression. Changes in retinal thickness, particularly in the neural layers, could provide valuable insights into cognitive decline and overall brain health [4].

Docosahexaenoic acid (DHA, 22:6n-3), an essential n-3 long-chain polyunsaturated fatty acid (n-3 LC-PUFA), is uniquely concentrated in the brain and is crucial for normal neurological development and optimal brain function [5–7]. A deficiency in DHA has been shown to adversely affect cognition and synaptic plasticity [8] and is strongly associated with the onset of neurological disorders, including AD [8]. Given the critical role of DHA in brain health, increasing its intake is essential for preventing cognitive decline. In addition to DHA, vitamins B9 (folic acid) and B12 (cobalamin) are essential for proper nervous system function and for maintaining healthy neuronal activity [9–12]. Vitamin B9 is crucial for DNA biosynthesis, while vitamin B12 plays a key role in the production of nerve cells. Both vitamins are integral to maintaining nervous system health and preventing neurological decline.

International nutritional guidelines have long recommended increasing DHA intake to support brain health [13]. Oily fish, traditionally considered the best source of n-3 LC-PUFA, have been the primary means of achieving this intake [7]. However, the sustainability of current n-3 LC-PUFA sources is in question, as existing supplies of wild and farmed fish are insufficient to meet the recommended intake values [14]. Therefore, alternative sources of n-3 LC-PUFA are needed to enhance DHA bioavailability beyond conventional, overexploited species like salmon, tuna, trout or sardine [15]. Atlantic chub mackerel (*Scomber colias*), or tinker mackerel, is an underutilised and undervalued species with a sustainable DHA and vitamin B12 source [16]. Quinoa (*Chenopodium quinoa*), rich in vitamin B9 [17,18], also presents a sustainable option for neuroprotection.

This study aimed to investigate the potential of dietary interventions with chub mackerel, quinoa or their combination, to prevent or delay cognitive decline and retinal dysfunction associated with AD in the 5XFAD mouse model. Specifically, the study hypothesised that these diets, rich in neuroprotective agents like DHA, vitamin B9 and vitamin B12, can mitigate the effects of ageing on cognitive and retinal health. Additionally, it explored the potential of retinal layers as non-invasive indicators of overall brain health in AD. To achieve these objectives, the 5XFAD transgenic mice, a well-established AD model with five familial AD mutations (three in the APP gene and two in the PS1 gene) [19], were used to investigate the effects of these dietary interventions on cognitive function and retinal health. The study employed a multidisciplinary approach, including behavioural evaluation, blood metabolite analysis, brain fatty acid profiling, histological and immunohistochemical assessment of brain lesions, and retinal layer examination.

2. Materials and methods

2.1. Ethics statement

This animal investigation strictly adhered to the regulatory framework stipulated by the European Union (Directive 2010/63/EU). The study received requisite approvals from the Animal Welfare Committee of the National Veterinary Authority (Direção Geral de Alimentação e Veterinária [DGAV], Portugal) with the reference 0421/000/000/2021 (at 13 July 2021), and the Organization Responsible for Animal Welfare at the Faculty of Veterinary Medicine of the University of Lisbon (ORBEA-FMVULisboa) with the reference 006/2021 (at 10 May 2021). The ARRIVE guidelines 2.0 have been followed in the mouse trial (https://arriveguidelines.org/arrive-guidelines). The sample size established for this *in vivo* experiment met the ethical guidelines and the 3Rs principle, particularly the principle of Reduction, minimising the number of animals used for the return of scientifically robust information.

2.2. Animals, experimental design and diets

Thirty-two 5XFAD male mice, aged five weeks, were purchased from Charles River Laboratories (L'Arbresle, France). The mouse strain used for this research project, B6SJL-Tg(APPSwFlLon, PSEN1M146LL286V)6799Vas/Mmjax, RRID, was obtained from the Mutant Mouse Resource and Research Centre (MMRRC) at The Jackson Laboratory, an NIH-funded strain repository. This strain was

donated to the MMRRC by Robert Vassar, Ph.D., Northwestern University. Although it should be acknowledged that the 5XFAD mouse model may primarily reflect the pathological features associated with familial AD, it is widely used and well-established for investigating key AD–related pathological mechanisms, including amyloid pathology, neuroinflammation, and synaptic dysfunction, which are also relevant to sporadic forms of AD. Besides, while the underlying causes of AD are not yet fully understood, there is a clear sex-related difference in disease prevalence, with women accounting for approximately two-thirds of cases worldwide [20]. Given this disparity, including female mice in the study could introduce an additional source of variation beyond the diet. As such, in the present study, only male mice were used in order to reduce biological variability and allow a more controlled assessment of the effects under investigation.

Upon arrival, all animals were single-housed in the animal facility of the Faculty of Veterinary Medicine, University of Lisbon, under controlled conditions: temperature of 22 ± 2°C, relative humidity of 55 ± 5%, and a 12/12-h light-dark cycle (lights on at 8:00 a.m.) [21]. During the first week, mice were fed a standard purified diet (AIN-93M, Envigo, Spain) to minimise stress and stabilise metabolic conditions.

After this acclimation period, 32 mice were assigned to four body-weight-matched dietary groups of 8 each: the control diet (non-supplemented group), the 10% chub mackerel diet, the 5% quinoa diet, and the 10% chub mackerel plus 5% quinoa diet. Target doses were established following a comprehensive review of the literature, with particular reference to studies conducted by other research groups using mouse models of AD. Accordingly, the target DHA dose was defined as 1.2 g/100 g of diet. For vitamin B9, the human recommended daily intake of 400 μg/day was used as a reference. Applying the Human Equivalent Dose (HED) approach and accounting for mice daily intake, this corresponds to a target dose of 47 μg/100 g in the mouse diet. The supplementation levels for chub mackerel and quinoa were adjusted based on these targets and the specific nutrient concentrations in each ingredient. The diets were manufactured by Sparos (Olhão, Portugal). Diets chub mackerel and chub mackerel plus quinoa were also added with 6.2% chub mackerel oil. The proximate chemical composition of the diets was determined according to AOAC AOAC Official [22], and the fatty acid composition was assessed as described by Bandarra et al. [23] (Table 1). Vitamins B9 and B12 were quantified using a liquid chromatographer linked to a mass spectrometer detector, as reported by Švarc et al. [24] and Rego et al. [25], respectively.

All diets were based on the AIN-93M purified diet (Envigo, Spain) with modified lipid fractions. Each diet contained the following components (g/100 g dry matter): casein (14.0), corn starch (46.6), maltodextrin (15.5), sucrose (10.0), cellulose (5.0), soybean oil (4.0), L-cystine (0.18), AIN-93M mineral mix (3.5), AIN-93M vitamin mix (1.0), choline bitartrate (0.25), and *tert*-butylhydroquinone (0.0008).

Throughout the trial, mice had free access to water and food. Body weight and food intake were measured and recorded weekly.

Table 1
Proximal and nutrient composition of experimental diets were provided to mice throughout the trial.

	Control	CM	Q	CM + Q
Gross energy (kcal/kg)	406.9 ± 0.2	411.4 ± 0.2	411.2 ± 0.3	433.2 ± 0.5
Proximate composition (g/100 g)				
Dry matter	87.4 ± 0.0	90.0 ± 0.0	89.5 ± 0.0	94.9 ± 0.1
Crude protein	22.7 ± 0.1	22.7 ± 0.1	22.5 ± 0.1	23.4 ± 0.4
Crude fat	10.8 ± 0.0	9.9 ± 0.0	9.8 ± 0.1	10.3 ± 0.2
Carbohydrates[a]	51.7 ± 0.1	54.8 ± 0.1	55.1 ± 0.0	58.6 ± 0.7
Ash	2.2 ± 0.0	2.6 ± 0.0	2.0 ± 0.0	2.6 ± 0.0
Fatty acid composition (mg/100 g)				
16:0 anteiso	ND	3.6 ± 0.1	ND	ND
16:0	1148.7 ± 0.3	1468.1 ± 6.6	914.2 ± 1.2	1340.1 ± 2.3
18:0 anteiso	ND	ND	ND	ND
18:0	361.2 ± 1.8	481.5 ± 0.4	274.7 ± 0.3	420.1 ± 2.5
SFA	1614.1 ± 2.2	2486.8 ± 12.3	1272.7 ± 2.1	2241 ± 3.5
16:1n-7	12.0 ± 0.0	153.9 ± 4.0	9.4 ± 0.9	152.2 ± 0.7
18:1n-9	2060.1 ± 5.4	1585.4 ± 1.5	1625.1 ± 0.5	1446.4 ± 2.8
18:1n-7	116.1 ± 0.2	233 ± 1.9	101.4 ± 0.2	209.7 ± 0.3
MUFA	2225.4 ± 11.1	2245.4 ± 3.6	1761.1 ± 11.5	2065 ± 23.4
18:2n-6	5218 ± 0.6	1813.2 ± 1.5	4104.2 ± 7.9	1572.6 ± 4.2
18:3n-3	669.9 ± 1.9	271.6 ± 0.2	531.3 ± 0.8	242.4 ± 1.1
20:4n-6	ND	93.6 ± 0.4	ND	82.0 ± 0.6
20:5n-3	ND	477.4 ± 2.2	ND	416.3 ± 1.9
22:6n-3	ND	1007.8 ± 8.7	ND	878.5 ± 0.5
PUFA	5913.8 ± 0.7	4116.6 ± 16.1	4641.1 ± 8.9	3538.1 ± 8.4
n-3 PUFA	673.7 ± 1.9	2044.4 ± 12.9	531.3 ± 0.8	1770.7 ± 4.3
n-6 PUFA	5233.7 ± 0.8	1982 ± 3.0	4107 ± 8.0	1726 ± 3.8
n-3/n-6	0.1 ± 0.0	1.0 ± 0.0	0.1 ± 0.0	1.0 ± 0.0
Vitamins (μg/100 g)				
Vitamin B9	172.7 ± 11.24	203.3 ± 9.53	315.9 ± 15.26	355.5 ± 11.24
Vitamin B12	13.8 ± 1.77	118.1 ± 6.77	19.8 ± 1.11	128.1 ± 5.94

[a] Carbohydrates were determined by difference, as described by FAO (2003). Control: AIN-93M diet; CM: 10% chub mackerel diet; Q: 5% quinoa diet; CM + Q: 10% chub mackerel plus 5% quinoa diet. ND: not detected; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; n-3/n-6: ratio obtained through the division of n-3 PUFA by n-6 PUFA. Results are expressed as the mean ± standard deviation of 3 replicates.

After 28 weeks of the feeding trial, mice were fasted for 12 h and then euthanised by a mechanical-physical method. Mice were placed in a chamber and anaesthetised using a mixture of 20% isoflurane in propylene glycol (v/v) for 30 s, followed by decapitation with a small animal guillotine.

Trunk blood was collected into lithium heparin tubes (Sarstedt, Nümbrecht, Germany) and centrifuged (10 min, 1500 ×g, room temperature) to isolate plasma. Following blood collection, half of the brain was excised and fixed by immersion in 10% neutral buffered formalin (Merck, VWR International, PA, USA) for 24 h and then processed for paraffin embedding (Microscopy Histosec, Merck, VWR International, PA, USA). The other half of the brain was collected for fatty acids profiling and kept at −80°C until analysis. Eyes were removed and placed in a 4% formaldehyde solution (pH = 7.4) in pre-prepared and identified test tubes.

2.3. Behavioural testing

Behavioural tests were conducted 27 weeks after the diet intervention. In this regard, the review article published by Pádua et al. [19] provides valuable insights about the way 5XFAD recapitulate Alzheimer's disease progression, starting with the increased levels of β-amyloid and intraneuronal deposits at the age of 1.5 months until the moment when these model mice exhibit a significant cognitive impairment at 4 months old. Yet other authors identified behavioural impairments between 4 and 6 months old [26]. Based on this, and considering that a nutritional approach proposed in this investigation may be more beneficial in the earlier stages of the disease than in the advanced stages, the authors selected 7 months of age as the optimal time to conduct both the behavioural and *ex vivo* tests. The tests included the T-Maze spontaneous alternation test, the open-field habituation task (OF), and the novel object recognition test (NOR). Before testing, mice were habituated to handlers and the testing room once a day for two to three consecutive days. Habituation to handlers consisted of 5 min of daily handling. To acclimatise them to the testing environment, mice were placed in the testing room at least 1 h before testing. All apparatuses were cleaned with 30% ethanol between mice and sessions. The researchers conducting the behavioural analysis were blind to the treatment groups to ensure unbiased evaluation.

2.3.1. T-maze test

The spatial working memory was evaluated using the spontaneous alternation T-maze test, following a protocol similar to that reported by d'Isa et al. [27]. The testing room was dimly lit with an intensity of 25 lx in each maze arm. During the sample trial, mice were placed in the starting arm facing the southern end of the maze. The timer started when the mice turned to the centre of the maze, and the time taken to choose an arm was recorded. An arm choice was defined when all paws and the tail were inside one of the goal arms. Mice were confined to the chosen arm for 30 s to adapt. This process was repeated for trials 1-6 with an inter-trial interval of 35 s. The working memory index was calculated using the formula: (total number of correct alternations/5) × 100. Correct alternation was defined as choosing the arm not visited in the previous lap. Choice latency and defecation index (number of faecal boli/time) were also recorded to ensure no motor impairments or emotional instability influenced the results.

2.3.2. Open-field test

The open-field test was used to assess baseline motor activity and anxiety behaviour before the Novel Object Recognition test. Mice were placed individually in a closed arena (28 cm height, 28 × 28 cm width and length) and allowed to explore for 5 min. The room was dimly lit with an apparatus intensity of 20 lx. Behavioural parameters were counted, including rears, grooms, jumps, and sniffing instances. The exploratory movement of the animals was video-tracked with a camera installed at the top of the apparatus. An automated system (ANY-maze software, Stoelting CO., Wood Dale, Illinois, USA) measured the speed of movements, distance travelled, and the time spent in the centre and periphery of the field.

2.3.3. Novel object recognition test

Recognition memory was assessed using the Novel Object Recognition Test (NOR test), following the protocol reported by Bevins and Besheer [28]. This test was conducted in the same apparatus used for the OF test under the same lighting conditions and consisted of two phases separated by a 24-h interval. On the first day, during the familiarisation phase, animals were exposed for 3 min to two identical objects (F and F′) placed in the back corners of the box. The next day, during the test phase, which lasted 3 min, mice were placed in the arena containing one novel object and the previously encountered familiar object (N and F). Mice were returned to their home cage between phases. The exploratory movement during both phases was recorded with a video camera installed at the top of the apparatus.

Variables measured included the frequency of rearing and grooming, and the exploration time and frequency of both familiar and novel objects. Object exploration was defined as the nose of the mouse either touching or being directed to the object at a distance of less than 1 cm. Climbing or sitting on an object was not recorded as exploration unless the nose was directed towards it. Mice displaying excessive freezing behaviour, corresponding to less than 10 s of interaction, were excluded from the analysis (1 mouse per group: control, G1, G2, G3). Lack of side bias was also ensured, with no animals showing a side preference. For memory and cognitive analysis, the total time of object interaction was considered, and the discrimination index was evaluated by the time spent exploring the novel object relative to the familiar object, calculated as the difference in time spent exploring the novel (TN) and familiar (TF) objects.

2.4. Plasma metabolites

Plasma metabolites, including glucose, insulin, urea, creatinine, total protein, triacylglycerols (TAG), total cholesterol, HDL-

cholesterol, and LDL-cholesterol, as well as the activities of ALT (EC 2.6.1.2), AST (EC 2.6.1.1) and GGT (EC 2.3.2.13), were measured using diagnostic kits from Roche Diagnostics' Modular Hitachi Analytical System (Mannheim, Germany). The original formula by Friedewald et al. [29] and the derived formula by Covaci et al. [30] were applied to calculate VLDL-cholesterol and total lipids, respectively. C-reactive protein was quantified via immunoturbidimetry (Roche Diagnostics, Meylan, France). Folic acid, vitamin B12 were both determined by electrochemiluminescence immunoassay (ECLIA). Interleukin-6 (IL-6) levels were measured using an ECLIA, as well as with a lower detection limit of 1.5 ng/L. Insulin-like growth factor-1 (IGF-1) was quantified using a chemiluminescence immunoassay (CLIA), with a sensitivity below 7.00 μg/L.

2.5. Light microscopy and immunohistochemistry in the brain

Serial tissue sections (3 μm thick) from each of the paraffin-embedded specimens were cut using a Minot microtome (Leica RM2135, Nussloch, Germany). Sections were stained with Harris haematoxylin (Bio-Optica, Milan, Italy) and eosin-floxin (Bio-Optica, Milan, Italy) to assess morphology under a light microscope (Olympus BX51 equipped with Olympus DP21 microscope digital camera system, Olympus, Tokyo, Japan). Neurons were counted in 40 fields per section at a magnification of ×200 using ImageJ software tools.

For immunohistochemistry studies, brain sections were subjected to antigen retrieval with citrate buffer (pH 6.0) at 96°C for 20 min using PTLink equipment (DAKO, LusoPalex, Portugal). Sections were washed twice with distilled water for 5 min, treated with H_2O_2 to quench endogenous peroxidase activity (Envision FLEX Kit, catalogue #K8000, DAKO, LusoPalex, Portugal) for 15 min, and then washed twice in PBS for 5 min. Sections were incubated with β-amyloid recombinant rabbit monoclonal antibody (H31L21) (1:500, Invitrogen, ThermoScientific, Waltham, MA, USA, catalogue #700254, RRID: AB_2532306), IBA1 polyclonal antibody (1:100, Invitrogen, ThermoScientific, Waltham, MA, USA, catalogue #PA5-27436, RRID: AB_2544912), or TAU polyclonal antibody (1:100, Invitrogen, ThermoScientific, Waltham, MA, USA, catalogue #PA5-27287, RRID: AB_2544763) for 1 h. Following incubation, sections were washed twice in PBS for 5 min, incubated with donkey biotinylated anti-rabbit IgG (H + L) cross-absorbed secondary antibody (1:500, Invitrogen, ThermoScientific, Waltham, MA, USA, catalogue #31458, RRID: AB_228213) at room temperature for 30 min, washed twice in PBS for 5 min, and developed with DAB (1:20, Envision FLEX Kit, DAKO, catalogue #K8000, LusoPalex, Portugal) for 5 min. Sections were washed twice with distilled water for 5 min, stained with Harris haematoxylin (Bio-Optica, Milan, Italy) for 1 min, washed twice with distilled water for 5 min, dehydrated with increasing concentrations of ethanol, cleared with xylene and covered with Entellan mounting reagent (Merck, VWR International, PA, USA).

Immunohistochemistry analysis using antibodies against Aβ, IBA1, and TAU allowed for the quantification of β-amyloid plaque count and area, IBA1 count and area, and TAU count in 40 fields per section at a magnification of ×200 using the ImageJ software tools. The entire histologic protocol was adapted from Christensen and Pike [31].

2.6. Brain fatty acid profile

Lipids were extracted according to Folch et al. [32]. Fatty acid methyl esters (FAME) were prepared according to Bandarra et al. [23] by adding 5 mL of a 5% acetyl chloride-methanolic solution (freshly prepared before use). Tubes were left to react for 1 h in a water bath at 80°C. After cooling, 1 mL of Milli-Q water and 2 mL of n-heptane were added, the tubes were agitated, and the layers were separated by centrifugation (3 min at 3000 ×g). The organic phase was collected and filtered through anhydrous sodium sulphate. The final extract was analysed by gas chromatography (GC) using a Scion 456-GC gas chromatograph (West Lothian, UK) equipped with a 30 m × 0.25 mm i.d (film thickness, 0.25 μm) DB-WAX capillary column (Agilent Technologies, Santa Clara, CA, USA). The separation of FAME was carried out with helium as the carrier gas, using a temperature program starting at 180°C for 5 min, increasing to 220°C at 4 °C/min, and holding for 24 min. Fatty acids were identified based on their retention times using a standard mix (PUFA-3, Menhaden oil, Sigma-Aldrich) as a reference.

2.7. Retinal layers assessment

During necropsy, the eyes of the mice were removed and preserved in a 4% formaldehyde solution (pH 7.4) in pre-prepared and identified test tubes.

For retinal imaging, multiple images were obtained using Spectral Domain Optical Coherence Tomography (SD-OCT) with a commercially available device (SPECTRALIS OCT; Heidelberg Engineering). The different retinal layers were assessed using these images. After positioning the eye on the platform, the SD-OCT was centred on the pupillary axis, and two volume scans along different axes (0° and 90°) were performed to obtain transverse images of the retina.

The volume scan consisted of SD-OCT acquisitions (15° × 3°, 13 high-resolution B-scans, 25 frames per scan), 9 raster horizontal B-scans with 768 A-scans per B-scan, and a depth resolution of 3.9 μm. These scans were centred on the optic nerve to ensure an anatomical landmark for the study and measurements. The retinal SD-OCT images were segmented and measured using HEYEX v.1.7.1 software. Two measurements along different axes (0° and 90°) were taken, and the average value was analysed. To ensure data consistency, all OCT measurements were performed by the same investigator.

For total retinal thickness, the inner limiting membrane (ILM) and the lower boundary of the retinal pigment epithelium and Bruch's membrane (RPE/BM) were segmented. The evaluation of neural layers included the nerve fibre layer, ganglion cell layer, and inner plexiform layer. For the outer retina assessment, the outer plexiform layer up to the retinal pigment epithelium was measured. The ratio of neural layers to total retinal thickness (neural/retinal ratio) was also studied.

2.8. Statistical analysis

Statistical analysis was performed using the generalized linear mixed (GLM) model in the Statistical Analysis System (SAS) software, version 9.4 (SAS Institute Inc., Cary, NC, USA). A selection of statistical tests was used, based on the data's specific characteristics and the goals of each analysis, to ensure the most accurate and reliable results. The p-values were automatically generated as part of those statistical procedures.

A post hoc power analysis using SAS was conducted to assess the adequacy of the sample size in this study. The analysis was conducted assuming a fixed-effects model with four experimental groups, a significance level (α) of 0.05, and a desired statistical power of 0.80. Based on a moderate-to-large effect size assumption (RMSSE = 0.35), the estimated required total sample size was 31 animals, supporting the adequacy of the sample size used in the present study. In addition, the sample size used in our study follows is in line the estimates of sample sizes and effect sizes for typical behavioural and neuropathological outcome measures in 5xFAD mice by Faisal et al. [33], as well as with previous reports employing the same mouse model [34–36].

Regarding the ANOVA assumptions, normality was evaluated using the Kolmogorov–Smirnov test, and homogeneity of variances was assessed with Levene's test. After confirming that the variances were homogeneous among the different dietary groups, we performed the ANOVA using pooled variance and reported the pooled standard error of the mean (SEM).

Post hoc multiple comparisons were conducted using the PDIFF option adjusted with the Tukey-Kramer method to identify statistical differences among dietary groups. This method controls for Type I errors associated with multiple testing by providing

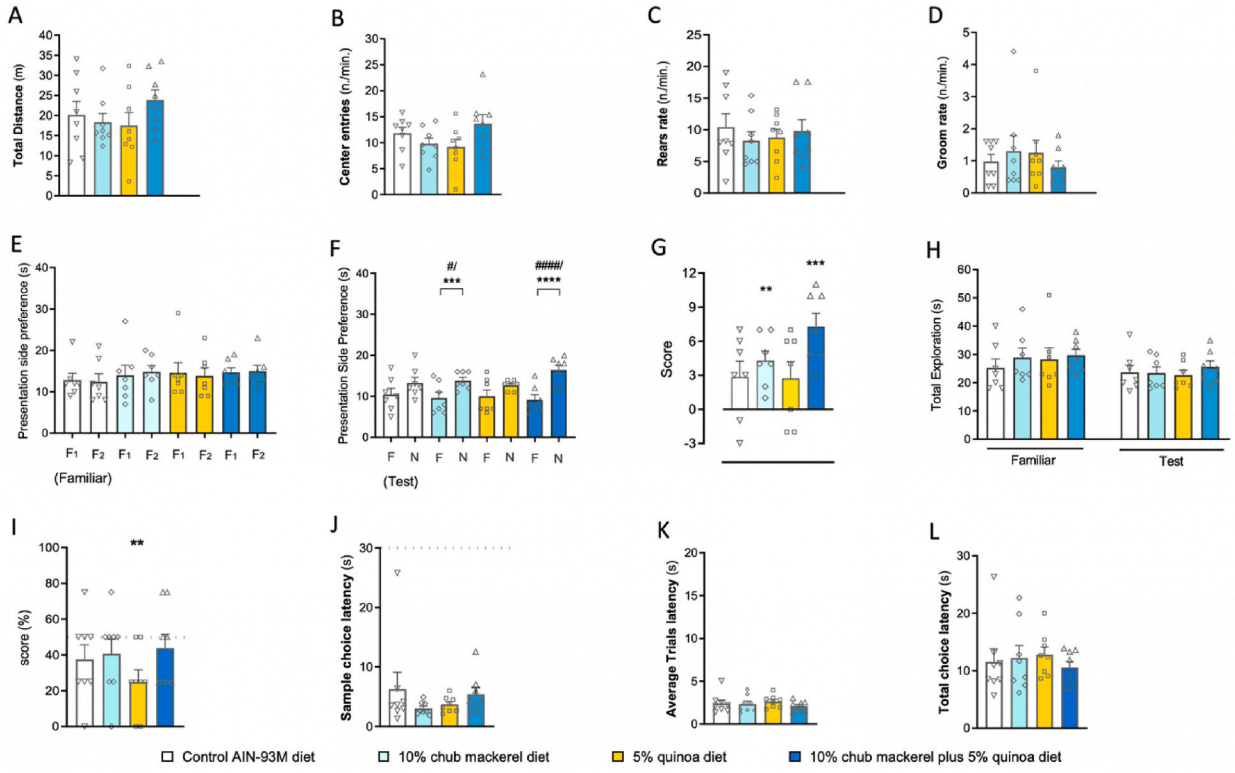

Fig. 1. Exploratory behaviour, recognition memory and spatial working memory of 7-month-old 5XFAD mice fed chub mackerel and quinoa diets. Panels A to D display the results from the Open Field test, assessing exploratory behaviour: (A) distance travelled, (B) centre entries, (C) rearing rate, and (D) grooming rate. The data indicate no significant differences between groups, although mice fed the chub mackerel plus quinoa diet exhibited a trend toward increased exploratory activity. Panels E to H present the results of the Novel Object Recognition (NOR) test: (E) exploration times during the familiarisation phase show no preference for either object, validating the absence of side bias; (F) during the test phase, mice fed chub mackerel and chub mackerel plus quinoa diets demonstrated a clear preference for the novel object, indicating sustained recognition memory; (G) the discrimination score (novel object - familiar object) confirmed that these diets helped preserve recognition memory, while (H) total exploration times remained consistent across groups in both phases, suggesting motivation was not a factor. Panels I to L illustrate the Spatial Working Memory as measured by the spontaneous alternation T-maze test: (I) spontaneous alternation scores within the first five trials were significantly below the chance level in mice fed the quinoa diet, with a similar trend observed in the control group ($p = 0.1$); (J) group average choice latency during the sample trial was below the 30-s threshold across all groups, indicating appropriate engagement with the task; (K) test trial latencies were low and consistent across groups, and (L) similar total choice latencies suggest no significant differences in motor function or anxiety levels, corroborated by the defecation index in supplementary information. The dashed line represents the 50% chance level in panel I and the cut-off latencies in panels J and K. Data are presented as mean ± standard error of the mean, with control AIN-93M diet (white); 10% chub mackerel diet (blue light); 5% quinoa diet (yellow); 10% chub mackerel plus 5% quinoa diet (dark blue).

corrected p-values for multiple comparisons, thereby ensuring accurate identification of group differences. Data are presented as the mean and SEM. A p-value of less than 0.05 was considered statistically significant.

Behavioural test data were analysed using one-way ANOVA with Tukey's post-hoc tests to compare behaviours within each treatment group. Additionally, alternation scores in the T-maze and discrimination indices (DI) in the NOR test were analysed using one-sample t-tests, comparing each group's alternation score or DI to the chance level of 50% or chance exploration level of 0, respectively. Object exploration time was analysed using repeated-measures one-way ANOVA, corrected for multiple comparisons with Benjamini's post-hoc tests, or by paired t-tests to compare object exploration within each treatment group.

3. Results

3.1. Body weight and feed intake

Animal groups' initial average body weight had no significant differences (control = 22.8 g, chub mackerel = 23.2 g, quinoa = 22.4 g, chub mackerel plus quinoa = 22.4 g, standard error of the mean (SEM) = 0.638 g, p = 0.769). During the experimental assay, no significant effects of the diet were observed on final body weight (control = 35.3 g, mackerel = 38.9 g, quinoa = 32.4 g, chub mackerel plus quinoa = 34.1, SEM = 2.10 g, p = 0.183), daily feed intake (control = 3.45 g, chub mackerel = 3.56 g, quinoa = 3.42 g, chub mackerel plus quinoa = 3.46, SEM = 0.086 g, p = 0.677), and body weight gain (control = 12.5 g, mackerel = 15.8 g, quinoa = 10.1 g, chub mackerel plus quinoa = 11.7, SEM = 1.88 g, p = 0.205).

3.2. Behavioural tests

The effects of the experimental diets on spontaneous exploratory and memory-associated behaviours in 5XFAD mice are shown in Fig. 1. In the Open-Field (OF) test, which assessed anxiety-like behaviours through distance travelled, centre entries (horizontal exploratory activity), rearing events (vertical exploratory activity), and grooming, no significant differences were observed between the diet groups in horizontal activity (distance travelled: p = 0.415; centre entries: p = 0.121) or vertical exploratory activity (p = 0.801), nor in grooming behaviour (p = 0.706) (Fig. 1A–D). However, 5XFAD mice fed the chub mackerel plus quinoa diet travelled 19% farther and made 16% more centre entries compared to controls, while those fed quinoa showed reductions of 13%, 22%, and 16% in distance travelled, centre entries, and vertical exploratory activity, respectively (Fig. 1A–C). Grooming frequency decreased by 16% in the chub mackerel plus quinoa group, whereas it increased by approximately 30% in the chub mackerel and quinoa groups compared to controls (Fig. 1D).

Memory-associated behaviour was assessed using the Novel Object Recognition (NOR) and T-maze tests. In the NOR test, which evaluates recognition memory, no differences were found during the adaptation phase in the exploration of identical objects (p =

Table 2
Plasma metabolites of 5XFAD mice fed the experimental diets. Control: AIN-93M diet; CM: 10% chub mackerel diet; Q: 5% quinoa diet; CM + Q: 10% chub mackerel plus 5% quinoa diet. ALP: alkaline phosphatase (EC 3.1.3.1); ALT: alanine aminotransferase (EC 2.6.1.2); AST: aspartate aminotransferase (EC 2.6.1.1); GGT - glutamyltransferase (EC 2.3.2.13); HDL - high-density lipoproteins; LDL: low-density lipoproteins; VLDL: very low-density lipoproteins; TAG: triacylglycerols. Total lipids = 2 × [total cholesterol] + [TAG] + 150, as derived from the formula described by Covaci et al. [30]. VLDL-cholesterol = 1/5 [TAG], as described by Friedewald et al. [29]. SEM: standard error of the mean. Different superscript letters within a row indicate a significant difference (p < 0.05). When two or more groups share the same letter, this indicates that there are no statistically significant differences between them for that parameter. For example, both the control and CM groups share the letter "b" for glucose, indicating that there is no significant difference in glucose levels between these two groups. On the other hand, groups with different letters (e.g., "a" and "b") represent significant differences.

	Control	CM	Q	CM + Q	SEM	p-value
Glucose (mg/dL)	116^{b}	115^{b}	150^{a}	139^{a}	3.92	<0.001
Insulin (mU/L)	<0.4	<0.4	<0.4	<0.4	-	-
Urea (mg/dL)	53.4^{b}	52.6^{b}	57.0^{b}	65.4^{a}	1.8	<0.001
Creatinine (mg/dL)	0.090	0.119	0.092	0.089	0.018	0.546
Cholesterol (mg/dL)	84.8	79.0	73.1	76.0	3.86	0.156
LDL-CHR (mg/dL)	7.75^{b}	11.0^{a}	6.75^{b}	11.4^{a}	0.518	<0.001
HDL-CHR (mg/dL)	73.9^{a}	63.3^{ab}	69.3^{ab}	57.0^{b}	3.29	0.005
VLDL-CHR (mg/dL)	11.9^{ab}	15.7^{a}	10.2^{b}	15.8^{a}	1.04	0.000
Total lipids (mg/dL)	379	386	347	381	11.8	0.078
Triacylglycerols (mg/dL)	59.9^{ab}	78.3^{a}	51.1^{b}	79.0^{a}	5.20	0.000
ALT (U/L)	34.8^{a}	24.9^{b}	24.1^{b}	35.4^{a}	1.78	<0.001
AST (U/L)	241^{b}	143^{c}	159^{c}	261^{a}	5.19	<0.001
GGT (U/L)	1.00^{b}	1.86^{a}	1.13^{b}	1.00^{b}	0.143	<0.001
Total protein (g/dL)	8.70^{a}	8.67^{a}	8.53^{b}	8.61^{ab}	0.035	0.006
C-reactive protein (mg/dL)	0.003^{ab}	0.006^{a}	0.002^{b}	0.001^{b}	0.001	0.028
Vitamin B9 (μg/L)	>20.0	>20.0	>20.0	>20.0	-	-
Vitamin B12 (ng/L)	>2000	>2000	>2000	>2000	-	-
IGF-1 (μg/L)	<7.00	<7.00	<7.00	<7.00	-	-
IL-6 (ng/L)	<1.5	<1.5	<1.5	<1.5	-	-

0.792) or side preference (p = 0.962) (Fig. 1E and H left). However, in the test phase, all groups showed a preference for the novel object over the familiar one (p = 0.0013) (Fig. 1F). Mice fed the chub mackerel or chub mackerel plus quinoa diets explored the novel object significantly more than controls (p = 0.024 and p = 0.003, respectively). The discrimination index (DI) indicated a trend towards improved recognition memory in these groups, with DI values 1.5 to 2.6 times higher than controls (p = 0.0514) (Fig. 1G). Notably, mice fed the control and quinoa diets did not discriminate above zero, confirming a recognition memory deficit, while the chub mackerel and chub mackerel plus quinoa groups demonstrated a clear preference for the novel object (p = 0.0026 and p = 0.0008, respectively). Spontaneous behaviours such as rearing and grooming showed no significant differences between diet groups during both familiarisation (p = 0.367, p = 0.701) and test trials (p = 0.780, p = 0.649) (Supplementary Fig. S1). These results suggest that 5XFAD mice fed the chub mackerel plus quinoa diet, and, to a lesser extent, the chub mackerel diet, retained memory for the novel object, unlike those on control or quinoa diets.

The T-maze test, which assesses spatial working memory, involved placing the mouse at the end of an open arm of the maze and recording the latency to enter one of the other two arms and the alternation between arms. All groups had choice latencies below 30 s in the sample trial, with no significant differences among the groups (p = 0.444) (Fig. 1J). Test trial latencies were also similar across groups (p = 0.6746) (Fig. 1K), and total choice latencies showed no significant differences (p = 0.831) (Fig. 1L), indicating that the observed exploratory behaviour was not due to a lack of motivation. Alternation scores, which indicate working memory, showed that mice fed the quinoa diet scored 33% lower than controls, suggesting a working memory deficit (p = 0.0072) (Fig. 1I). In contrast, mice on the chub mackerel plus quinoa diet scored 17% higher than controls, although these differences were not statistically significant (p = 0.535). When compared to chance level (50%), all diet groups scored below this threshold, with the quinoa group showing a significant deficit (p = 0.007) and the control group showing a deficit trend (p = 0.170). These findings suggest that spatial working memory is slightly protected in mice fed the chub mackerel plus quinoa diet, and to a lesser extent in those fed chub mackerel alone, while a cognitive deficit is evident in the quinoa group.

3.3. Plasma metabolites

The blood biochemistry of 5XFAD mice fed the experimental diets is displayed in Table 2. Although insulin concentration was below the detection limit of the equipment (<0.4 mU/L), glucose levels were significantly increased in mice fed quinoa and the combination of chub mackerel plus quinoa compared to those fed control and chub mackerel diets (p < 0.001). The chub mackerel plus quinoa diet also resulted in higher urea levels relative to the other dietary groups (p < 0.001), without affecting creatinine levels (p =

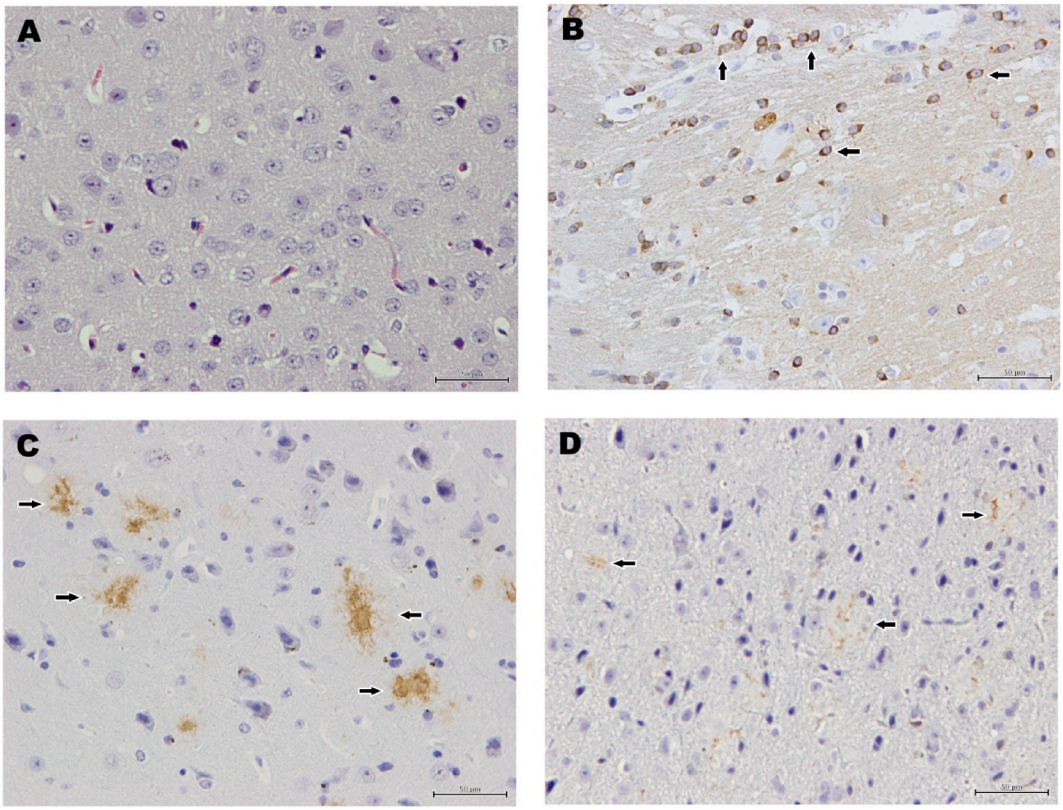

Fig. 2. Illustrative images of H&E (A) staining and immunohistochemistry markers for TAU (B), β-amyloid plaques (C), and IBA1 (D) marked with arrows in the cerebral cortex of 5×FAD mice (magnification ×200, scale bar = 50 μm).

0.546).

While total lipids ($p = 0.078$) and cholesterol levels ($p = 0.156$) remained unchanged across dietary groups, LDL-cholesterol ($p < 0.001$), VLDL-cholesterol ($p = 0.000$) and triacylglycerols ($p = 0.000$) reached the highest levels in mice fed chub mackerel plus quinoa. Conversely, HDL-cholesterol was reduced in this same dietary group ($p = 0.005$). Additionally, the chub mackerel plus quinoa diet increased alanine aminotransferase (ALT, $p < 0.001$) and aspartate aminotransferase (AST, $p < 0.001$) levels, while reducing gamma-glutamyltransferase (GGT, $p < 0.001$). Total protein levels were decreased in mice fed quinoa compared to control and chub mackerel diets ($p = 0.006$). Notably, C-reactive protein levels were reduced in mice fed quinoa and chub mackerel plus quinoa compared to those fed only chub mackerel ($p = 0.028$).

Vitamins B9 and B12 were above 20 μg/L and 2000 ng/L, respectively, in all dietary groups. Regarding the inflammatory response, both IGF-1 (<7.00 μg/L) and IL-6 (<1.5 ng/L) were below the minimum detection limit, suggesting no inflammation in transgenic 5XFAD mice from any dietary group.

3.4. Neuronal density and immunohistochemistry markers in the brain

Fig. 2 illustrates H&E staining (A) and immunohistochemistry markers for TAU (B), β-amyloid plaques (C), and IBA1 (D) in the cerebral cortex of 5XFAD mice.

Classical H&E staining was used to evaluate neuronal density, with no significant variations observed in the number of neurons across dietary groups ($p = 0.104$).

Immunohistochemistry using antibodies directed against β-amyloid plaques revealed no significant differences in plaque area ($p = 0.092$) or count ($p = 0.899$) among the dietary groups (Table 3).

The histological detection of IBA1, a marker for microglial activation, showed no significant differences in either the area ($p = 0.176$) or the number of IBA1-positive cells ($p = 0.314$) between dietary groups (Table 3).

Immunostaining with TAU antibody indicated no significant differences in the number of TAU-positive cells across the dietary groups ($p = 0.799$) (Table 3).

3.5. Fatty acid profile in the brain

Table 4 presents the detailed fatty acid composition in the brains of mice fed the experimental diets. Significant variations were observed in fatty acids under the influence of chub mackerel and quinoa, individually or combined. The sum of saturated fatty acids (SFA) was increased in mice fed quinoa and the combination of chub mackerel and quinoa relative to the control and chub mackerel dietary groups ($p < 0.001$). This increase was primarily due to variations in major fatty acids, such as palmitic acid (16:0) ($p < 0.001$) and stearic acid (18:0) ($p < 0.001$), which are the predominant SFA.

The sum of monounsaturated fatty acids (MUFA) was not influenced by the diets ($p = 0.078$), reflecting the non-variation in 18:1n-7 ($p = 0.699$) and 20:1n-9 ($p = 0.571$) fatty acids. However, 18:1n-9 reached higher levels in mice fed chub mackerel alone or combined with quinoa compared to mice fed only quinoa ($p = 0.027$).

Total PUFA were increased in the chub mackerel diet relative to the quinoa or the combination of quinoa with chub mackerel ($p < 0.001$), mainly due to the changes observed in n-3 PUFA ($p < 0.001$), specifically DHA ($p < 0.001$), which represented almost the totality of n-3 PUFA identified in mice brain. Conversely, the sum of n-6 PUFA was lower in mice fed chub mackerel alone or in combination with quinoa compared to the other diets ($p < 0.001$). This decrease was reflected in the variations observed for 18:2n-6 ($p < 0.001$), 20:4n-6 ($p < 0.001$), and 22:4n-6 ($p < 0.001$) fatty acids.

Reflecting the changes in n-3 and n-6 PUFA, the n-3/n-6 ratio was increased in the chub mackerel diet alone or combined with quinoa compared to the other two diets ($p < 0.001$).

Table 3
Effect of diet on neuronal density (H&E) and immunohistochemistry markers in the cerebral cortex of mice. A total of 40 photos/fields were used per section at ×200 magnification (real area of each photo at ×200 magnification = 94308 μm^2). Control: AIN-93M diet; CM: 10% chub mackerel diet; Q: 5% quinoa diet; CM + Q: 10% chub mackerel plus 5% quinoa diet. IBA1: Ionised calcium-binding adapter molecule 1. SEM: standard error of the mean.

	Control	CM	Q	CM + Q	SEM	p-value
Neural density (Number)	2804	2317	2875	3142	229	0.104
β-amyloid plaques						
Number	102.0	86.4	93.0	106.0	19.9	0.899
Area (%)	7.45	6.72	6.51	14.40	2.46	0.092
IBA1						
Number	102.0	87.4	104.0	144.0	21.9	0.314
Area (%)	3.59	4.66	2.61	5.58	0.97	0.176
TAU						
Number	489	378	439	452	80	0.799

Table 4
Main fatty acids determined in the mouse brain (results are expressed as % of total fatty acids). Control: AIN-93M diet; CM: 10% chub mackerel diet; Q: 5% quinoa diet; CM + Q: 10% chub mackerel plus 5% quinoa diet. ND: not detected; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; n-3/n-6: ratio obtained through the division of n-3 PUFA by n-6 PUFA. SEM: standard error of the mean. Different superscript letters within a row indicate a significant difference ($p < 0.05$).

	Control	CM	Q	CM + Q	SEM	p-value
16:0 anteiso	1.5^{a}	1.3^{b}	0.5^{c}	0.3^{d}	0.04	<0.001
16:0	19.2^{a}	18.6^{b}	21.7^{a}	22.2^{a}	0.42	<0.001
18:0 anteiso	2.8^{a}	2.5^{a}	0.9^{b}	0.5^{b}	0.07	<0.001
18:0	18.8^{b}	18.9^{b}	20.8^{a}	20.8^{a}	0.16	<0.001
SFA	44.2^{b}	43.0^{b}	47.3^{a}	47.4^{a}	0.49	<0.001
16:1n-7	0.5^{c}	0.7^{a}	0.5^{c}	0.6^{b}	0.01	<0.001
18:1n-9	18.7^{ab}	19.0^{a}	17.4^{b}	19.1^{a}	0.41	0.027
18:1n-7	2.8	2.9	2.9	2.9	0.05	0.699
20:1n-9	1.3	1.3	1.4	1.2	0.08	0.571
MUFA	25.0	25.3	23.9	25.2	0.42	0.078
18:2n-6	1.4^{a}	1.1^{b}	1.3^{a}	1.1^{b}	0.06	<0.001
18:3n-3	ND	ND	ND	ND	-	-
20:4n-6	7.8^{a}	6.1^{b}	7.3^{a}	5.5^{c}	0.15	<0.001
20:5n-3	ND	0.2	ND	0.2	0.01	<0.001
22:4n-6	2.2^{a}	1.2^{b}	2.2^{a}	1.2^{b}	0.07	<0.001
22:6n-3	12.3^{b}	15.4^{a}	11.4^{b}	12.2^{b}	0.50	<0.001
PUFA	25.1^{ab}	25.5^{a}	23.2^{bc}	21.2^{c}	0.57	<0.001
n-3 PUFA	13.0^{b}	16.4^{a}	11.8^{b}	12.9^{b}	0.52	<0.001
n-6 PUFA	12.1^{a}	9.1^{c}	11.3^{b}	8.3^{d}	0.17	<0.001
n-3/n-6	1.1^{b}	1.8^{a}	1.0^{b}	1.5^{a}	0.05	<0.001

3.6. Retinal layers

Table 5 provides a detailed comparison of retinal layer metrics across the four dietary groups, while Fig. 3 illustrates examples of images obtained after positioning the eye on the platform and the SD-OCT centred on the pupillary axis and retinal images from individuals from the groups fed with (b) control AIN-93M diet; (c) 10% chub mackerel diet; (d) 5% quinoa diet; (e) 10% chub mackerel plus 5% quinoa diet.

The control group exhibited a decrease in several retinal thickness measures compared to the other dietary groups. Specifically, mice fed a combination of chub mackerel and quinoa showed a significantly greater retinal thickness in horizontal scans, with an average value of 369.1 μm ($p = 0.043$). This group also demonstrated the highest mean retinal thickness (369.6 μm), although this increase did not reach statistical significance ($p = 0.101$).

When examining the neural retina layers, the chub mackerel and quinoa groups, both individually and combined, exhibited significantly higher thickness values in horizontal scans compared to the control group ($p = 0.015$). The combined diet of chub mackerel and quinoa resulted in a neural retina layer thickness of 99.0 μm (mean), which was significantly higher than the control group's 87.9 μm ($p = 0.046$).

The ratio of neural retina thickness to total retinal thickness was also higher in the intervention groups, particularly in horizontal scans. The control group had a ratio of 0.232, while the chub mackerel, quinoa, and combined diet groups showed ratios of 0.278,

Table 5
Comparison of retinal layers metrics of intervention groups. Control: AIN-93M diet; CM: 10% chub mackerel diet; Q: 5% quinoa diet; CM + Q: 10% chub mackerel plus 5% quinoa diet. h: measured with horizontal b-scan; v: measured with vertical b-scan; Neural retina: includes nerve fibre layer, ganglion cell layer and inner plexiform layer; INL: inner nuclear layer; Outer retina: outer plexiform layer until retinal pigment epithelium. SEM: standard error of the mean. Different superscript letters within a row indicate a significant difference ($p < 0.05$).

	Control	CM	Q	CM + Q	SEM	p-value
Retinal thickness h (μm)	348.7^{b}	354.1^{b}	352.5^{b}	369.1^{a}	4.78	0.043
Retinal thickness v (μm)	371.8	373.4	362.1	370.1	5.85	0.524
Retinal thickness mean (μm)	360.3	363.8	357.3	369.6	4.30	0.101
Neural retina layer h (μm)	81.3^{b}	98.1^{a}	97.9^{a}	98.2^{a}	2.47	0.015
Neural retina layer v (μm)	94.5	100.9	101.6	99.8	2.17	0.145
Neural layer mean (μm)	87.9^{b}	99.5^{a}	99.8^{a}	99.0^{a}	2.08	0.046
INL h (μm)	53.8	56.8	56.8	57.3	1.75	0.892
INL v (μm)	66.0	61.3	57.1	54.9	1.52	0.174
INL mean (μm)	59.9	59.1	56.9	56.1	1.23	0.534
Outer retina h (μm)	213.0	199.2	197.9	213.6	4.20	0.144
Outer retina v (μm)	211.3	211.2	203.3	215.4	5.81	0.551
External mean (μm)	212.5	205.2	200.6	214.5	4.19	0.385
Ratio neural/retina h	0.232^{b}	0.278^{a}	0.280^{a}	0.265^{a}	0.007	0.026
Ratio neural/retina v	0.252	0.272	0.280	0.270	0.007	0.627
Ratio neural/retina mean	0.244^{b}	0.274^{a}	0.281^{a}	0.270^{a}	0.006	0.027

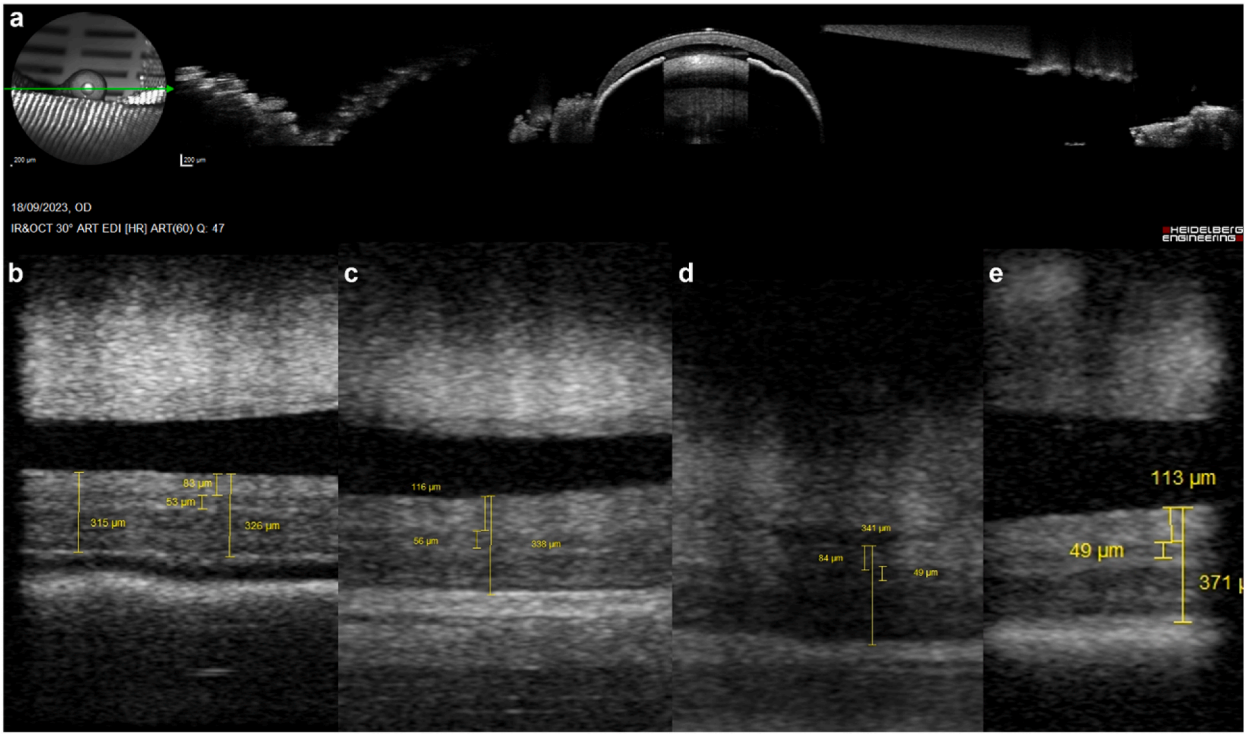

Fig. 3. Examples of horizontal B-scans (768 A-scans per B-scan, depth resolution of 3.9 µm) and measurements in the dietary groups: (a) image obtained after positioning the eye on the platform and the SD-OCT centred on the pupillary axis; (b) control AIN-93M diet; (c) 10% chub mackerel diet; (d) 5% quinoa diet; (e) 10% chub mackerel plus 5% quinoa diet.

0.280 and 0.265, respectively ($p = 0.026$). This indicates that all the intervention diets contributed to a greater preservation of the neural retina layers relative to the overall retinal thickness.

No significant differences ($p > 0.05$) were observed in the inner nuclear layer (INL) or outer retina thickness across the dietary groups, suggesting that the observed protective effects were more pronounced in the neural retina layers.

4. Discussion

Throughout the 28 weeks of nutritional trial, no significant effects of diet were observed on the initial and final body weight, body weight gain or average daily feed intake of the 5XFAD mice. This suggests that dietary interventions with chub mackerel, quinoa or their combination did not impact energy intake or body weight changes, indicating no effect on the overall caloric balance or growth parameters of mice. In detail, in terms of average daily feed intake of DHA, and vitamins B9 and B12, mice from the reference group consumed 5.96 ± 0.770 µg of vitamin B9 and 0.476 ± 0.062 µg of vitamin B12 but no DHA. Regarding the chub mackerel dietary group, these mice consumed per day 35.9 ± 5.08 mg of DHA, 7.23 ± 1.32 µg of vitamin B9 and 4.20 ± 0.594 µg of vitamin B12. Concerning mice fed only quinoa, they consumed per day 10.8 ± 1.42 µg and 0.677 ± 0.089 µg of vitamins B9 and B12, respectively, but no DHA. Lastly, mice fed the combination of chub mackerel and quinoa ingested per day 30.4 ± 3.61 mg of DHA, 12.3 ± 1.46 µg of vitamin B9 and 4.43 ± 0.526 µg of vitamin B12. Overall, the daily ingestion of these compounds by mice from the different dietary groups reflects the nutritional chemical composition of experimental diets.

The 5XFAD mouse model, which exhibits cognitive and spatial memory loss, displays amyloid pathology and neuroinflammation from an early age [37]. These mice also typically present with memory deficits and learning impairments starting at around four months of age [26,37–40]. The dietary interventions in this study did not introduce confounding factors such as anxiety, motivation or motor impairments, allowing for an accurate assessment of memory-associated behaviour in the 5XFAD mice. While spontaneous exploratory behaviour remained unaffected, the chub mackerel and chub mackerel plus quinoa diets significantly improved memory-associated behaviours. Mice fed these diets retained recognition memory and demonstrated better working memory performance compared to those on a standard diet. These findings align with previous research highlighting the cognitive benefits of n-3 PUFA-rich diets, particularly in mitigating cognitive decline in degenerative conditions [41]. The observed cognitive benefits are likely attributed to the combined key nutritional components of chub mackerel and quinoa, which are recognised for their anti-inflammatory and antioxidant properties [42,43].

Blood metabolites often reflect dietary patterns [44]. In this study, mice fed quinoa exhibited elevated glucose levels, even though their insulin concentrations remained unchanged. This paradoxical result may be influenced by the 5XFAD genotype, which is known

to exhibit impaired insulin signalling and glucose dysregulation. In addition, quinoa has a unique nutritional profile, which includes a higher fat content and a lower carbohydrate content compared to typical cereals. Normally, a lower carbohydrate intake would be expected to reduce glucose levels, but the higher fat content in quinoa might alter glucose metabolism, leading to increased blood glucose levels without a corresponding rise in insulin. Although quinoa is generally known for its moderate glycaemic index and its potential benefits in glucose regulation [45,46], the elevated glucose levels observed in 5XFAD mice may reflect an interaction between dietary composition and the inherent metabolic phenotype of this transgenic model. It is important to note that reference values for fasting glucose, insulin, and hepatic enzyme activities in 5XFAD mice are limited or inconsistently reported in the literature. This hampers direct comparisons and limits the interpretation of potential metabolic stress or toxicity. The lipid profile was particularly affected by the chub mackerel plus quinoa diet, leading to increases in LDL-cholesterol, VLDL-cholesterol, and triacylglycerols while reducing HDL-cholesterol. Despite these changes, total cholesterol and total lipids remained unaffected. The combination diet also elevated urea levels, but stable creatinine levels indicated that renal function was not compromised. The increased levels of hepatic markers ALT and AST, along with a reduction in GGT, suggest that hepatic homeostasis was maintained, despite the metabolic changes induced by the chub mackerel plus quinoa diet. A significant reduction in C-reactive protein levels in the quinoa and chub mackerel plus quinoa groups, compared to the chub mackerel-only group, suggests quinoa's potential anti-inflammatory effects. Additionally, systemic IGF-1 and IL-6 levels were below detection limits, indicating an absence of inflammatory responses across all dietary groups. This supports the notion that optimal nutrition, such as that provided by a Mediterranean diet, is crucial for brain function and the prevention of neurodegenerative diseases [47].

Quinoa is a rich source of B vitamins, including folic acid [17,18]. B vitamins play crucial roles in DNA methylation and the pathogenesis of neurological diseases [48]. Their beneficial effects on cognitive function are associated with homocysteine metabolism, which, when disrupted, contributes to cardiovascular risk and cognitive impairment [49]. Deficiencies in B vitamins can lead to the accumulation of homocysteine, reducing S-adenosylmethionine levels and inducing DNA demethylation, which is linked to AD [50,51]. Vitamin B12 has been associated with protective effects against Aβ-induced cytotoxicity and oxidative stress. For instance, treatment with B12 in the SH-SY5Y neuroblastoma cell line led to a dose-dependent reduction in H_2O_2-induced apoptosis and enhanced cell viability [52]. Additionally, vitamin B12 has demonstrated the ability to increase levels of phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, and plasmalogens in SH-SY5Y cells. This is particularly relevant given the extensive lipid changes observed in AD, namely in these lipid classes, which, being rich in n-3 LC-PUFA like DHA, are highly susceptible to oxidative damage [53]. Despite this, our study's vitamin B12 and folate data were inconclusive due to equipment detection limits. Since an immunological detection method was used for vitamin B12 and folate quantification, the samples' dilution is not recommended as this could compromise the epitopes' precision and selectivity and thereby affect results reliability. Nonetheless, adequate intake of B vitamins can lower homocysteine levels, indirectly protecting against the development of AD.

Analysis of brain lesions in the cerebral cortex of 5XFAD mice through classical histology and immunohistochemistry showed no significant differences across dietary groups regarding neural density, Aβ deposits, or hyperphosphorylated TAU. Gliosis, characterised by reactive astrocytes and microglia around dense amyloid plaques, is a hallmark of AD [54,55]. The levels of IBA1, a marker for reactive microglia [56], remained consistent in both area and number across the cortex, suggesting that the dietary interventions, including the combined diet of chub mackerel and quinoa, did not significantly alter the progression of these specific AD-related brain lesions. This outcome may seem inconsistent with the observed benefits of the combined diet on cognitive function and retinal health. However, it is possible that the neuroprotective effects of DHA, and vitamins B9 and B12, provided by the dietary interventions, primarily support synaptic function, neuroplasticity and retinal integrity rather than directly altering the established amyloid and tau pathology in the brain. The improvements in cognitive behaviour and retinal preservation may reflect enhanced neuronal resilience or compensatory mechanisms that occur independently of the traditional markers of AD pathology. Therefore, while the combined diet did not reduce the physical manifestations of AD in terms of amyloid plaques or hyperphosphorylated tau, it may still offer functional benefits that contribute to the preservation of cognitive and retinal health.

N-3 long-chain polyunsaturated fatty acids (LC-PUFAs), particularly DHA, are vital for brain development and function, protecting against age-related cognitive decline [5]. DHA plays a key role in neurogenesis, neuroplasticity and the overall health of neurons [8], which are essential for synaptic transmission, learning, and memory [57]. In this study, the brain fatty acid profiles of the 5XFAD mice reflected their dietary intake, consistent with findings from a previous study [15]. Dietary supplementation with chub mackerel increased DHA levels in the brain, while EPA was undetected, likely due to its conversion into DHA [58]. The n-3/n-6 fatty acid ratio was particularly favourable in the groups fed chub mackerel alone or in combination with quinoa, highlighting the beneficial effects of these diets on brain lipid metabolism, which is often altered in AD. These results suggest that the cognitive benefits observed in the chub mackerel and chub mackerel plus quinoa groups may be attributed to the reduction of the pro-inflammatory n-6 PUFA, along with the anti-inflammatory and neuroprotective effects of n-3 PUFA, namely DHA [5,6,59,60].

Increasing evidence demonstrates a strong correlation between AD progression and irreversible neuronal lesions, with retinal degeneration emerging as a key aspect of this neurodegenerative process [61,62]. Retinal degeneration in AD is often characterised by abnormal extracellular deposits of amyloid β (Aβ) and intracellular tau aggregates, leading to an exacerbated inflammatory response in the retina [61]. This degeneration not only reflects the pathological changes occurring in the brain but also serves as a potential early biomarker for AD. Optical Coherence Tomography (OCT) has revolutionised the study of retinal changes in neurodegenerative diseases, including AD, by providing detailed, *in vivo* images of the retina. The retina, being an extension of the central nervous system, mirrors the pathological processes occurring in the brain, making it an invaluable biomarker for early diagnosis and monitoring the progression of AD [4,63]. In particular, changes in retinal thickness and integrity, especially within the neural layers, have been increasingly associated with cognitive decline and the progression of AD. These changes particularly affect the inner retinal layers, including retinal ganglion cells (RGCs), which are known to transport amyloid precursor protein and are vulnerable to β-amyloid (Aβ)

accumulation. Their degeneration may reflect early apoptotic events associated with neurodegenerative cascades in the brain [4,64,65].

In our study, mice fed a diet enriched with chub mackerel and quinoa exhibited significantly greater retinal thickness compared to controls, in line with what was observed in previous studies [66]. This observation suggests a protective effect against retinal degeneration, likely due to the potent antioxidant and anti-inflammatory properties inherent in chub mackerel and quinoa. Indeed, photoreceptor dysfunction and structural damage to the outer retina have been functionally associated with abnormal scotopic ERG responses, supporting the idea that preservation of the outer retina may contribute to behavioral and visual stability in early AD stages [67]. The preservation of retinal thickness, especially in the neural layers, is critical, as inner retinal degeneration may be more directly associated with cognitive decline, while outer layer damage is more likely to impair visual perception and functional behaviour. Although the relationship between retinal structural changes and cognitive function is complex, this layer-specific vulnerability offers a valuable framework for interpreting functional decline in AD and should be further explored. This preservation aligns with previous research demonstrating the efficacy of dietary antioxidants in preventing the loss of retinal ganglion cells and maintaining retinal integrity in degenerative conditions [68,69]. Moreover, the observed improvement in the neural/retinal thickness ratio in the dietary intervention groups underscores the potential of these diets in maintaining retinal health. This is particularly relevant as the ratio of neural to total retinal thickness has been proposed as a more sensitive indicator of retinal health and may help distinguish neurodegeneration from inflammatory conditions, although further comparative studies are required. While some retinal changes may overlap across diseases, sectoral thinning patterns in AD differ from those observed in glaucoma and other inflammatory disorders. For instance, AD-related thinning tends to occur in specific macular sectors following a horizontal pattern, contrasting with the typical superior–inferior RNFL loss observed in glaucomatous optic neuropathy [64]. The ability to preserve or even enhance this ratio through dietary means could represent a significant step forward in non-invasive biomarkers for the early detection and monitoring of AD.

Overall, our findings suggest that combining chub mackerel and quinoa in the diet may provide dual benefits in the context of AD by both slowing cognitive decline and preserving retinal integrity, where DHA, vitamin B12 and vitamin B9 are likely to exert their protective effects. DHA is known to play a crucial role in neurogenesis, synaptic function, and anti-inflammatory pathways. It enhances membrane fluidity, modulates signalling pathways, and reduces the production of pro-inflammatory cytokines, all of which contribute to neuroprotection. Additionally, DHA reduces amyloid-beta accumulation and tau phosphorylation, both of which are hallmarks of AD pathology. Although no evident changes in these hallmarks were depicted in our study, an improved cognitive behaviour was observed. One possible explanation for the improved brain function could have been the modulation of brain derived neurotrophic factor (BDNF), the neurotrophin essential for neurogenesis, neuroplasticity, and neuroprotection, which is known to play a critical role in cognitive function [70]. Several studies have shown that one of the functional effects of increasing DHA in the brain, either by fish or krill oil diets, is the increase of BDNF levels in the brain [41,42]. The exact mechanism by which increases in brain DHA, seen in our study and in above mentioned studies, leads to increase in BDNF levels is not clear. Some authors have reported a direct effect of DHA on the expression of BDNF and have proposed that it may be partially related to GPR40 (free fatty acid receptor 1) signalling [70]. According to Sona et al. [70] when GPR40 signalling was blocked in the brain, DHA could no longer improve memory or increase BDNF in the hippocampus, suggesting that DHA effects partly depend on activating GPR40. Another possible mechanism, very likely to explain this study reported synergistic effect of DHA sources and vitamins B12 and B9 in improving brain function of an Alzheimer model, is that since DHA is known to induce antioxidant enzyme and vitamins B12 and B9 known for antioxidant properties, and since BDNF levels are negatively correlated with oxidative stress [41], the decrease of the oxidative stress will result in the increase of BDNF levels. The neuroprotection effect of an increase in brain DHA has also been confirmed by the reported increase in Synaptic Vesicle Glycoprotein 2A (SV2A), a synaptic vesicle protein, which has been correlated with improved cognitive behaviour [71]. In addition, vitamins B12 and B9 are essential for maintaining the methylation cycle, which regulates homocysteine levels. Elevated homocysteine has been strongly associated with neurodegenerative processes, including amyloid plaque formation and tau hyperphosphorylation. By lowering homocysteine levels, these vitamins indirectly protect against AD-related neurodegeneration. Furthermore, B vitamins are critical for DNA synthesis, repair, and maintaining the integrity of the nervous system, all of which support cognitive function. This dual action supports a more holistic approach to managing and potentially slowing the progression of AD. These results underscore the critical role of nutritional strategies in the early intervention of neurodegenerative diseases, highlighting retinal biomarkers as a valuable tool for gaining insight into the broader neurodegenerative processes occurring in the brain. In extrapolating the values of chub mackerel and quinoa from the 5XFAD mouse model to humans, we can use different scaling methods, such as metabolic rate adjustments and caloric intake. When adjusting for human caloric intake, an adult human on a 2000-calorie diet would need about 98 g of chub mackerel and 83 g of quinoa daily to match the proportions used in the mouse study. Alternatively, metabolic rate scaling, considering that mice have a higher metabolic rate relative to body size, suggests that a human would need around 128 g of chub mackerel and 64 g of quinoa daily to achieve similar neuroprotective effects observed in the Alzheimer's mouse model.

5. Limitations of the study

Several limitations of the present study should be acknowledged. First, the absence of a wild-type control group limits direct comparison with physiological baseline conditions and prevents assessment of whether the dietary interventions restored behavioural, retinal, biochemical, or brain fatty acid parameters towards non-transgenic values. Second, only male 5XFAD mice were used, precluding evaluation of potential sex-dependent responses. Third, although the sample size was consistent with ethical reduction principles and adequate to detect moderate-to-large effects, smaller dietary effects may not have been detected.

In addition, although the study identified behavioural, retinal, biochemical, and brain fatty acid changes associated with the tested

diets, the underlying molecular mechanisms were not thoroughly examined. Finally, caution is required when translating findings from the 5XFAD model to human AD, as this model does not fully capture the complexity of sporadic disease. Future studies, including wild-type controls, sex-balanced cohorts, larger sample sizes, longitudinal retinal assessments, and mechanistic analyses, will help to validate and extend these findings.

6. Conclusion

This study demonstrates that the combination of chub mackerel and quinoa provides relevant benefits in preserving selected memory-associated behaviours and protecting retinal integrity in the 5XFAD mouse model of AD. The dietary effects on brain fatty acid composition differed between chub mackerel alone and the combined diet. Chub mackerel alone significantly increased brain DHA levels, whereas the improved n-3/n-6 PUFA ratio observed with the combined chub mackerel plus quinoa diet was driven primarily by a reduction in n-6 PUFA levels rather than by a significant increase in DHA. The benefits of the combined diet are therefore likely to reflect the complementary actions of several key nutrients, including DHA and vitamins B9 and B12, rather than the effect of DHA alone. Furthermore, the preservation of retinal thickness with this diet, particularly within the neural layers, supports the potential of retinal layer assessment as a non-invasive readout of AD-associated neurodegeneration and cognitive decline.

Importantly, the dietary interventions did not negatively affect overall caloric balance, feed intake, body weight gain, or final body weight. Although blood biochemistry showed increased glucose, reduced C-reactive protein with quinoa-containing diets, and altered lipid-related parameters in the combined-diet group, these changes were not accompanied by evidence of impaired growth or overt systemic toxicity under the conditions tested. These findings support the tolerability of the dietary interventions and indicate that metabolic responses should be further monitored in future studies.

Overall, the results suggest that the combination of chub mackerel and quinoa, both sustainable and underutilised foods, may help mitigate selected neurodegenerative and retinal alterations in the 5XFAD model. This positions them as promising components of a dietary strategy to support neuroprotection and retinal health in the context of AD-related decline. However, these findings should be interpreted as dietary effects in the 5XFAD mouse model, not as direct evidence of clinical efficacy in humans.

Looking forward, these findings open new avenues for developing nutraceutical or functional food strategies aimed at preventing or reducing cognitive decline. Future research should focus on elucidating the molecular and cellular mechanisms by which these dietary components exert their protective effects, including pathways involved in lipid metabolism, neuroinflammation, oxidative stress, amyloid pathology, and retinal neurodegeneration. Further studies should also include wild-type controls, female or sex-balanced cohorts, larger sample sizes, and longitudinal retinal assessments to validate the use of retinal biomarkers for the early detection and monitoring of neurodegenerative diseases. In parallel, translational studies will be needed to determine whether similar dietary combinations can support neuronal and retinal health in individuals at increased risk of AD.

CRediT authorship contribution statement

Ana Gomes-Bispo: Writing – review & editing, Writing – original draft, Investigation. **Cristina Mello-Sampayo:** Writing – review & editing, Writing – original draft, Resources, Investigation, Funding acquisition, Conceptualization. **Pedro Camacho:** Writing – review & editing, Writing – original draft, Resources, Investigation, Funding acquisition, Conceptualization. **Beatriz Rego:** Writing – review & editing, Investigation. **Sandra Carvalho:** Writing – review & editing, Investigation. **Paula A. Lopes:** Writing – review & editing, Writing – original draft, Investigation. **Romina Gomes:** Writing – review & editing, Investigation. **Susana Oliveira:** Writing – review & editing, Investigation. **Maria Lourenço:** Writing – review & editing, Investigation. **Rui Pinto:** Writing – review & editing, Resources, Investigation. **Carla Motta:** Writing – review & editing, Writing – original draft, Resources, Investigation. **Jorge Correia:** Writing – review & editing, Writing – original draft, Resources, Investigation. **Cláudia Afonso:** Writing – review & editing, Investigation. **Carlos Cardoso:** Writing – review & editing, Writing – original draft, Investigation. **Narcisa M. Bandarra:** Writing – review & editing, Writing – original draft, Resources, Funding acquisition, Conceptualization. **José A.M. Prates:** Writing – review & editing, Writing – original draft, Resources, Funding acquisition, Conceptualization.

Data availability statement

The data generated for this study are included in the published article. All datasets produced during the experiment are available upon request from the corresponding author.

Declaration of competing interest

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

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Appendix A. Supplementary data

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