Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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Shedding light on the toxicity of SARS-CoV-2-derived peptide in non-target COVID-19 organisms: A study involving inbred and outbred mice

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ABSTRACT

Despite advances in research on the vaccine and therapeutic strategies of COVID-19, little attention has been paid to the possible (eco)toxicological impacts of the dispersion of SARS-CoV-2 particles in natural environments. Thus, in this study, we aimed to evaluate the behavioral and biochemical consequences of the short exposure of outbred and inbred mice (male Swiss and C57Bl/6 J mice, respectively) to PSPD-2002 (peptide fragments of the Spike protein of SARS-CoV-2) synthesized in the laboratory. Our data demonstrated that after 24 h of intraperitoneal administration of PSPD-2002 (at 580 μg/kg) the animals did not present alterations in their locomotor, anxiolytic-like, or anxiety-like behavior (in the open field test), nor antidepressant-like or depressive behavior in the forced swimming test. However, the C57Bl/6 J mice exposed to PSPD-2002 showed memory deficit in the novel object recognition task, which was associated with higher production of thiobarbituric acid reactive substances, as well as the increased suppression of acetylcholinesterase brain activity, compared to Swiss mice also exposed to peptide fragments. In Swiss mice the reduction in the activity of superoxide dismutase and catalase in the brain was not associated with increased oxidative stress biomarkers (hydrogen peroxide), suggesting that other antioxidant mechanisms may have been activated by exposure to PSPD-2002 to maintain the animals' brain redox homeostasis. Finally, the results of all biomarkers evaluated were applied into the "Integrated Biomarker Response Index" (IBRv2) and the principal component analysis (PCA), and greater sensitivity of C57Bl/6 J mice to PSPD-2002 was revealed. Therefore, our study provides pioneering evidence of mammalian exposure-induced toxicity (non-target SARS-CoV-2 infection) to PSPD-2002, as well as "sheds light" on the influence of genetic profile on susceptibility/resistance to the effects of viral peptide fragments.

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1. Introduction

It is known that since the emergence of coronavirus disease-19 (COVID-19) in Wuhan Province (China) in late 2019 (Wang et al., 2020), and it is subsequent worldwide spread, the COVID-19 has led to a dramatic loss of human life worldwide (WHO, 2022) and presents an unprecedented challenge to public health (Aslam, 2022), food systems (Cable et al., 2021; Béné et al., 2021), and the world of work (ILO monitor, 2022). The economic and social disruption caused by the pandemic is devastating (Nicola et al., 2020; Das et al., 2022). Estimates by United Nations (UN) show that COVID-19 poses a real challenge to the UN Sustainable Development Goal of ending poverty by 2030 (UN, 2022) because global poverty could increase for the first time since 1990 and could potentially represent a reversal of approximately a decade in the world's progress in reducing poverty. According to, in some countries, the negative impacts could result in poverty levels like those recorded 30 years ago. Therefore, this scenario demonstrates that the extent of transmission of the novel coronavirus [pioneered by Nishiura et al. (2020)] still constitutes a public health emergency of international concern. Unfortunately, the extraordinary advance observed in recent years in the development of vaccines (Eroglu et al., 2022; Zheng et al., 2022) and therapeutic measures for COVID-10 (Salasc et al., 2022), has not yet been sufficient to decree the end of the pandemic.

In addition, recent studies have shown that the impacts of COVID-19 may also cover the environment and wildlife. Although the pandemic situation has significantly improved air quality (Agarwal et al., 2021), reduces greenhouse gases emission (Kumar et al., 2022), reduces the pressure on the tourist destinations (which may assist with the restoration of the ecological system) (Gössling et al., 2020), the increase of plastic (C.J. Silva et al., 2021; A.L.P. Silva et al., 2021) and medical wastes (Parikh and Rawtani, 2022), haphazard use and disposal of disinfectants, mask, and gloves (Amuah et al., 2022); and burden of untreated wastes (Rume, Islam, 2020), imply significant environmental risks. If this were not enough, the detection of viral particles of SARS-CoV-2 in aquatic environments, especially from domestic and hospital sewage (Gonçalves et al., 2021; Achak et al., 2021; Crank et al., 2022; Amoah et al., 2022; Domokos et al., 2022; Galani et al., 2022), has imposed new challenges on environmental and health managers. These studies raise not only the possibility of river resources acting as secondary sources of transmission of the disease among individuals (Liu et al., 2020; Giacobbo et al., 2021; Thakur et al., 2021), as well as warn about the potential threat of the dispersion of the new coronavirus or its fragments to the biota (Charlie-Silva and Malafaia, 2022). On this aspect, our research group recently reported some negative effects arising from the exposure of amphibians, fish, and insects to distinct protein fragments of the Spike protein of SARS-CoV-2 (Malafaia et al., 2021; Mendonça-Gomes et al., 2021; Charlie-C.J. Silva et al., 2021; Charlie-A.L.P. Silva et al., 2021; Gonçalves et al., 2022; Fernandes et al., 2021). In *Physalaemus cuvieri* tadpoles, the increase in several biomarkers predictive of oxidative stress and the alteration in acetylcholinesterase (AChE) activity demonstrated that the short exposure (24 h) to these protein fragments was sufficient to affect the health of tadpoles (Charlie-C.J. Silva et al., 2021; Charlie-A.L.P. Silva et al., 2021). In Mendonça-Gomes et al. (2021)), we showed that short-term exposure de *Culex quinquefascitus* larvae to protein fragments of the Spike protein of SARS-CoV-2 induced alterations in the locomotor system and in the olfactory behavior, which were associated with increased production of reactive oxygen species (ROS) and AChE activity. In addition, exposure to these fragments alter the behavior of fish (*Poecilia reticulata*), induce redox imbalance, affect the growth and development of these animals (Malafaia et al., 2021) and induce genomic instability and DNA damage (Gonçalves et al., 2022), as well as several morphological alterations in zebrafish *(Danio rerio*) (Fernandes et al., 2021). On the other hand, intranasal delivery of SARS-CoV-2 Spike protein was sufficient to cause olfactory damage, inflammation, and olfactory dysfunction in zebrafish (Kraus et al., 2022). Therefore, taken together, our data reinforce that the (eco)toxicological risks arising from the presence of SARS-CoV-2 Spike protein peptides in freshwater environments cannot be neglected.

However, in mammals (non-humans), studies have focused on the susceptibility of different species to viral infection and their roles in the dissemination of COVID-19 (Shi et al., 2020; Tiwari et al., 2020; Rockx et al., 2020; Audino et al., 2021; Mathavarajah et al., 2021a,2021b; Gryseels et al., 2021; Delahay et al., 2021; Patel et al., 2021; Melo et al., 2022). Although Rhea et al. (2021)) have recently demonstrated that intravenously injected radioiodinated S1 (I-S1) (S1 subunit of Spike protein of SARS-CoV-2) readily crossed the blood-brain barrier in male mice, was taken up by brain regions, and entered the parenchymal brain space, the consequences of this translocation have not been evaluated and therefore remain unknown. Thus, seeking to broaden our knowledge about the possible effects of mammalian (non-human) exposure to peptide fragments of the Spike protein of SARS-CoV-2, we questioned whether the short exposure of mice to these fragments would be able to induce neurotoxicity. For this, two rodent strains of distinct genetic profiles (Swiss and C57BL/6 J mice) were exposed to the peptide fragment PSPD-2002 [one of the fragments synthesized by Charlie-A.L.P. Silva et al. (2021); Charlie-C.J. Silva et al. (2021))], assuming that this implies redox imbalance and cerebral cholinesterasic, as well as behavioral changes. Furthermore, a molecular docking analysis was performed to assess the affinity of this peptide to key protein binding sites.

2. Materials and methods

2.1. Peptide fragments of the SARS-CoV-2 Spike protein

The synthesis, cleavage, purification, and characterization of the protein fragments of the Spike protein of SARS-CoV-2 used in our study (called PSPD-2002) were performed according to methods described in detail by Charlie-A.L.P. Silva et al. (2021); Charlie-C.J. Silva et al. (2021). Briefly, the synthesis was conducted using the solid phase peptide synthesis method (SPPS) following the Fmoc strategy (Raibaut et al., 2014; Behrendt et al., 2016). The resin used in this process was Fmoc-Thr-Wang (sequence: Gln-Cys-Val-Asn-Leu-Thr-Thr-Thr-Thr-COOH; MW: 1035.18 g/mol). At the end of the synthesis, this resin made it possible to obtain peptides with a carboxylated C-terminal end. After coupling all the amino acid residues of the peptide sequence, the chains were removed from the solid support utilizing acid cleavage using trifluoroacetic acid, similarly to Guy and Fields (1997). The crude compounds were purified by high-performance liquid chromatography (HPLC) with a reverse-phase column using different purification methods according to the retention time obtained in a gradient program of 5–95% in 30 min (exploration gradient) in analytical HPLC [similarly to Klaassen et al. (2019)]. Only compounds with purity equal to or greater than 95% were considered for in vivo evaluation, following the rules determined by the National Health Surveillance Agency (ANVISA/Brazil) and Food and Drug Administration (FDA/USA).

2.2. Animals and experimental design

In this study, we used two strains of male mice (Swiss and C57BL/6 J), which were obtained and maintained in the bioterium of the Biological Research Laboratory of the Federal Institute Goiano Campus Urutaí (IF Goiano, Urutaí, Goiás, Brazil). Males were chosen to avoid any influence of hormonal peaks commonly observed in females during the short estrous cycle (Chari et al., 2020; Lovick, Zangrossi, 2021) on the biomarkers evaluated. While Swiss mice (outbred) present very similar heterozygosity average to estimates for wild mouse and human populations (Rice and O'Brien, 1980), C57BL/6 J mouse colonies are genetically identical within each strain, making them free of genetic differences that could impact research results. Inbred mouse strains exhibit a high degree of uniformity in their inherited characteristics, or phenotypes, which include appearance, behavior, and response to

experimental treatments (Sacca et al., 2013). All animals were kept under controlled laboratory conditions (temperature of 23–25 °C; relative humidity of ± 45%; light/dark 12 h photoperiod) throughout the experimental period. The animals were kept in collective standard mice polypropylene crates (30 ×20 x 13 cm) with latticed galvanized wire caps with antioxidant treatment with a maximum of three animals each. The crates were cleaned three times a week, with the change of sawdust and food. The standard rodent diet and water were offered ad libitum.

After weaning (21 days after birth) the animals were relocated to acclimatization boxes until they completed 60 days of life. Then, 24 Swiss mice [45.69 g ± 1.823 g – mean ± standard deviation (SD)] and 24 C57BL/6 J mice (20.57 g ± 0.6501 g – mean ± SD) were distributed in different experimental groups. Mice that were not exposed to PSPD-2002 constituted the control groups and those exposed to protein fragments of the Spike protein of SARS-CoV-2 constituted the group "PSPD-2002". Each group was composed of 4 replicates (n = 3 animals/replica). In both strains, exposure to PSPD-2002 occurred intraperitoneally, and each animal received a single injection of 12 μg PSPD-2002/mouse diluted in phosphate-buffered saline (PBS, pH 7.2), making a dose of 580 μg PSPD-2002/kg of body biomass. The animals of the control groups received only PBS (i.e.: vehicle used in the dilution of peptide fragments). This dose was defined based on the study by Rhea et al. (2021) who, to evaluate whether S1 protein of SARS-CoV-2 would be able to cross the blood-brain barrier in male CD-1 mice, intravenously administered 10 μg radioiodinated S1/animal. After 24 h, the animals were submitted to different behavioral tests and biochemical evaluations. The short exposure period evaluated in our study was also defined based on Rhea et al. (2021), which showed that within a few minutes the S1 protein of SARS-CoV-2 was able to reach the central nervous system (CNS) of the animals.

2.3. Toxicity biomarkers

2.3.1. Hippocratic screening

Aiming to evaluate the effect of peptide fragment administration on parameters related to general activity, consciousness, motor coordination, muscle tone, reflexes, central and autonomic nervous system activities, a Hippocratic screening (Malone, 1977, 1983) was performed during 15 min, after intraperitoneal injection at 0.5, 1, 2, 4, 8, 12, 24 h. The following signs were evaluated: general activity, vocal frantic, irritability, touch response, tail grip response, contortion, posterior train position, straightening reflex, body tone, force to grasp, ataxia, auricular reflex, corneal reflex, tremors, convulsions, anesthesia, lacrimation, ptosis, urination, defecation, piloerection, hypothermia, breathing, cyanosis, hyperemia, and death. All these signs were evaluated by behavioral observation and systematic clinical examination of the mice, like examine the studies by Moreira et al. (2021) and Brígido et al. (2021)). A score for Hippocratic screening was set from 0 (absent) to 4 (intense), according to the observation of animal activity parameters.

2.3.2. Traditional behavioral paradigms

To evaluate whether PSPD-2002 would be able to cause damage to the behavior of animals in traditional behavioral paradigms, the mice were submitted to a battery of sequential tests. In these tests, we were able to evaluate possible changes in the locomotor abilities of the animals, as well as induction of anxiety-like and depressive-like behaviors. In addition, possible short-term memory deficit caused by exposure to PSPD-2002 was also evaluated. All tests were performed in a specific room with acoustic insulation, temperature, and controlled luminosity. In addition, the tests were performed on the same day, adopting the "triple tests" model, described in Ramos (2008) and Souza et al. (2018), with some modifications (see sections 2.3.1.1 to 2.3.1.3). The animals were sequentially submitted to the open-field test, novel object recognition test, and forced swimming test (from most stressful to least stressful). The behavior of the animals was analyzed in Plus MZ v1 software. In addition, all animals were allowed to acclimate to the test room for 30 min, before the tests.

2.3.2.1. Open field test. The open-field test (OFT) was used to evaluate the possible induction of locomotor alterations and anxiety-like behavior (Gould et al., 2009) by exposure to PDPD-2002. This test is based on conflicting innate tendencies of avoidance of bright light and open spaces (that ethologically mimic a situation of predator risk) and of exploring a novel environment. When placed into a brightly lit open field for the first time, rats and mice tend to remain in the periphery of the apparatus or against the walls (thigmotaxis) (Gould et al., 2009). In the present study, we adopted the procedures described in Estrela et al. (2021)), with minor modifications. Briefly, the test consisted of introducing the animals individually into a rectangular arena (16.5 cm height x 31 cm width x 40.5 cm length), of white opaque walls, which were filmed for 5 min. The total crossings of the quadrants plotted virtually on the computer screen were used to infer the impact of PSPD-2002 peptides on the locomotor activity of the animals. The proportion between the length of stay in the central zone of the device and the total time of the test (5 min = 300 s) was used to evaluate the possible induction of anxiolytic- or anxiety-like behaviors. Conform discussed by Prut and Belzung (2003), a lower percentage of crosses between quadrants in the central zone and, consequently, a high percentage of crosses in the lateral quadrants can be used as an anxiety index. Between each session, the parades were cleaned with 70% alcohol.

2.3.2.2. Basso mouse scale for locomotion. During the OFT we also evaluated the possible effect of PSPD-2002 on biomechanical aspects of animal locomotion. For this, the animals were assessed through the Basso Mouse Scale for Locomotion (BMS), proposed by Basso et al. (2006) and used in the (eco)toxicological study of Mendes et al. (2017). Locomotion events include assessing the forelimb and hindlimb coordination during sustained locomotion, trunk instability, paw orientation, and tail position, among others.

2.3.2.3. Novel object recognition test. After the end of the OFT, the novel object recognition test (NORT) was performed, similarly to the procedures adopted by Rabelo et al. (2016). As discussed by Antunes and Biala (2012), The NORT can be evaluated by the differences in the exploration time of the novel and familiar objects. Its application is not limited to a field of research and enables various issues can be studied, such as memory and learning, the preference for novelty, the influence of different brain regions in the process of recognition, and even the study of different drugs and their effects. Briefly, the test performed in our study consisted of two steps. In the first, the animals were submitted to a “training session", which consisted of introducing two identical objects into the instrument, called familiar objects (F1 and F2), and recording the exploration time in each object, for 5 min. Such objects (Lego toys) had the same color, shape, and size. Then the animal was removed from the test equipment and taken to the bioterium. An hour later, one of the familiar objects was replaced by a different object [called a novel object (N)]. This object was of the same color, but different size and shape than the ones that were utilized in the "training session". Subsequently, the animals were reintroduced into the device and the time of exploration of the objects (N and F) was recorded for 3 min. It is emphasized that a cross-drawing was used in all test sessions, so that the novel and familiar objects were placed in alternating positions, to exclude the potential preference of the animals for a certain spatial location of the objects in the apparatus. It was considered exploitation when the animal touched the object intentionally with the paws or when it smelled at a distance < 2 cm (Rajagopal et al., 2014). For each animal, the index of recognition of the objects (Eq. 1) was calculated, as described in Pietá-Dias et al. (2007).

2.3.2.4. Forced swimming test. After the NORT, the animals were submitted to the forced swimming test (FST), according to the procedures

described in Silva et al. (2018)), with minor modifications. As one of the most widely used behavioral tests to assess depressive-like behavior in animal models and for the pharmaceutical screening of potential antidepressant treatments, the FST is based on the principle that animals develop an immobile posture in a non-escapable cylinder filled with water (Petit-Demouliere et al., 2005). Briefly, the test consisted of introducing the animal into a cylindrical glass tank (diameter 20 cm), containing 2 L of water (25 °C), and filming it for 5 min. After the test, each animal was introduced into a box containing dry towel paper under heated lighting. During the analysis of the videos, the immobility time was recorded, which has been broadly used to identify depressive-like behaviors in studies involving FST and rodents (Holanda et al., 2022; Gumus et al., 2022; Sofidiya et al., 2022).

2.3.3. Biochemical assessment

Seeking to associate possible behavioral changes with biochemical alterations, different biomarkers of toxicity were evaluated in the brains of the animals. For this, after the behavioral tests (see previous items) the animals were euthanized (via cervical displacement) and a craniotomy was performed for brain extraction. Then, the organs were transferred to previously sterilized microtubes, containing 1 mL of PBS, for subsequent maceration and homogenization. Then, the samples were centrifuged at 13,000 rpm for 10 min at 4 °C, and the supernatant was used for the analysis of the discriminated biomarkers in Table 1.

2.3.3.1. Determination of the protein level. All results of biochemical analyzes were expressed in "unit/mg" of protein in the samples. Thus, we used a commercial kit (Bioténica Ind. With. LTD, Varginha, MG, Brazil, code #10.009.00), whose total protein levels were determined based on the colorimetric reaction resulting from the reaction of Cu^{2+} ions and peptide bonds of proteins, giving rise to a blue color detected in an ELISA reader at 492 nm.

2.4. Docking molecular

To predict the mode of binding and affinity of the bonds between PSPD-2002 and the protein structures of the enzymes SOD, CAT, and AChE, a molecular docking analysis was performed. This analysis was important to predict the possible mechanisms of action of peptides and their relationship with behavioral changes. For this, the structure of PSPD-2002 was modeled using the Web server PEP-FOLD3 (https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/). As protein structures (targets) de mouse (*Mus musculus*) ([acetylcholinesterase (code Uniprot: P21836), catalase (code Uniprot: P24270), superoxide dis (code Uniprot: P08228)] was obtained by the homology construction technique by the SWISS-MODEL server (https://swissmodel.expasy.org/) with structural similarity values between 91.21% and 99.70% compared to structures of *Homo sapiens*. The validation of the structures was verified through the SAVES v.6.0 (https://saves.mbi.ucla.edu/) server. For molecular docking simulations, AutoDock Tools (ADT) v4.2 were used to prepare ligands and targets (Morris et al., 2009) and AutoDock Vina 1.1.2 to perform the calculations (Trott and Olson, 2010). Binding affinity and interactions between residues were used to determine better molecular interactions. The results were visualized using ADT and UCSF Chimera X (Pettersen et al., 2021).

Table 1
General information about biochemical biomarkers evaluated in brain samples Swiss and C57Bl/6 J mice exposed or not to peptide fragments (PSPD-2002).

Biomarkers	References (methods)
Nitrite	Bryan, Grisham (2007)
Reactive oxygen species (ROS)	Maharajan et al. (2018)
Hydrogen peroxide (H_2O_2)	Elnemma (2004)
Thiobarbituric acid reactive species	Pothiwong et al. (2007)
Catalase	Sinha (1972)
Superoxide dismutase (SOD)	Del-Maestro & McDonald (1985)
Acetylcholinesterase (AChE)	Ellman et al. (1961)
Butyrylcholinesterase (BChE)	A.L.P. Silva et al. (2021); C.J. Silva et al. (2021)

2.5. Integrated biomarker response index

To demonstrate the toxicity of the treatments, the results of all biomarkers evaluated were applied into the "Integrated Biomarker Response Index" (IBRv2), which is based on the principle of reference deviation between a disturbed and undisturbed state. For this, we adopted the procedures described in Beliaeff and Burgeot (2002), with some modifications proposed by Sanchez et al. (2013). The deviation among biomarkers measured in mice exposed to PSPD-2002 (of each strain) was compared to those measured in animals from the "control" groups. For each experimental group, the ratio among the mean value obtained at each biomarker was evaluated and the respective reference control value was log-transformed (Yi). In the next step, a general mean (μ) and standard deviation (sd) were calculated, considering the Yi values of a given biomarker measured in each group. Subsequently, Yi values were standardized by Eq. 1 and the difference between Zi and Z0 ("control" group) was used to define the biomarker deviation index (A). To obtain integrated multiple biomarker responses, similarly to García-Medina et al. (2022), the value of A of each biomarker was calculated for every exposed group, and IBRv2 was calculated for each group by the sum of the absolute values of A. The area above 0 reflects biomarker induction, and the area below 0 indicates biomarker.

$$Zi = (Yi - \mu)/sd \quad (1)$$

2.6. Statistical analysis

2.6.1. Comparison means

Initially, all data obtained were evaluated regarding the assumptions for using parametric models. For this, we used the Shapiro-Wilk test to assess the distribution of residual data and the Bartlett test was used to assess the homogeneity of variances. Data on biochemical biomarkers and those obtained in open field tests and forced swimming were submitted to two-way ANOVA, with Tukey's post-test, considering the factors "strain" (Swiss and C57Bl/6 J) and "treatment" (control and PSPD-2002). On the other hand, data referring to the "test session" of NORT were submitted to three-way ANOVA, with Tukey's post-test, considering the factors "strain " (Swiss and C57Bl/6 J), "treatment" (control and PSPD-2002), and "objects" (novel and familiar). In addition, the data obtained in the "training session" of the NORT were submitted to the Student t-test.

2.6.2. Principal component analysis

The PCA was performed to explore correlations between treatments, based on the average value of each biomarker evaluated. For this, before performing the PCA, the variables were log-transformed to adjust distribution to normality. Then, the values were centered at zero for PCA and an ellipse was drawn to indicate the 95% confidence interval assuming a student distribution of the principal components (PCs). After PCA, we used the proportion of variance plot, scree plot, loading plot, PC1 score plot, as well as loading values and correlation (or covariance) matrix between variables generated in GraphPad Prism Software Version 9.0. Ward's hierarchical clustering method was also used to identify groups distribution according to the variables on the PCA results (Eszergár-Kiss and Caesar, 2017). Significance levels were set at Type I error (p) values lower than 0.05. GraphPad Prism Software Version 9.0 and PAST (PALaeontology STatistic) software were used to perform the statistical analyzes.

3. Results

Initially, our data did not contain any visible signs of toxicity (Hippocratic screening) after the administration of PSPD-2002 peptide fragments, such as contortion, muscle tone, tremors, convulsions, straub, hypnosis, lacrimation, ptosis, urination, piloerection, cyanosis, among others (see item "2.3.1 "). In addition, the animals of all experimental groups received a maximum score in the evaluation performed via BMS (in the OFT), which indicates that the PSPD-2002 did not affect biomechanical parameters of the locomotion of the animals. We also did not record the death in the experimental groups and no behavioral alteration suggestive of locomotor dysfunction and or anxiolytic-or anxiety-like behavior was observed in the OFT. Neste test, we observed only that the responses of the animals were influenced by the factor "strain", having the C57Bl/6 J mice presented higher locomotor activity (Fig. 1 A) and the Swiss mice, higher anxiety index (Fig. 1B).

Regarding the NORT, the recognition indices for "familiar objects" (F1 and F2) in the "training session" of the controls groups differed from zero and did not show a significant difference (Fig. 2 A), which validates the test performed, once it demonstrates that the random exploration of the objects in the "training session" resulted in an equal exploration of both objects, besides excluding potential preference for a certain spatial location of the objects placed in the test box. Furthermore, the "controls" groups (Swiss and C57B6/6 J mice) and "PSPD-2002 Swiss" group yielded higher recognition indices for the "novel object" in the "test session", compared to the indices of the "familiar object", indicating success in retaining the memory of the "familiar object" (Fig. 2B). However, this result was not observed in the animals of the "PSPD-2002 C57Bl/6 J" group. In addition to the time of exploration not having deferred between the "novel" and "familiar" objects, the recognition index of the "familiar object" of these animals was higher than that observed in the other experimental groups (Fig. 2B). In the forced swimming test, we did not observe significant differences between the "control" and "PSPD-2002" groups within each evaluated strain. However, we observed that the animals of the "PSPD-2002 C57Bl/6 J" group remained longer immobile (>125%) in the FST when compared to Swiss mice also exposed to peptide fragments (Fig. 3).

Regarding the predictive biochemical evaluations of oxidative stress, our statistical analyses revealed the effect of the interaction between the factor's "strain" and "treatment" for most biomarkers evaluated. The levels of nitrite (Fig. 4A), hydrogen peroxide (Fig. 4B), and ROS (Fig. 4C) in the brain of Swiss mice were higher than those observed in c57Bl/6J mice. However, we observed a suppressive effect of nitrite and hydrogen peroxide production only in the Swiss mice exposed to PSPD-2002, when compared to their respective "control" group (Fig. 4A-B, respectively). ROS production was not altered by exposure to peptide fragments in the rodent strain evaluated (Fig. 4C). On the other hand, we noticed that the production of TBARs was significantly higher in the animals exposed to PSPD-2002, whose increase about their respective "control" groups was 161.8% and 175.7% for the Swiss and C57Bl/6 J scans, respectively (Fig. 4D). In this case, such results indicate the effect only of the factor "treatment" on the TBARs brain levels.

Concerning the activity of the enzymes evaluated in our study, we observed that exposure to PSPD-2002 induced suppression of SOD (Fig. 5 A) and catalase activity (Fig. 5B) in the brain of Swiss mice (Fig. 5 A), as well as increased catalase activity in C57Bl/6 J mice (Fig. 5B). In addition, we observed that the "PSPD-2002" groups (for both strains) showed a significant reduction in AChE activity (Fig. 5 C)

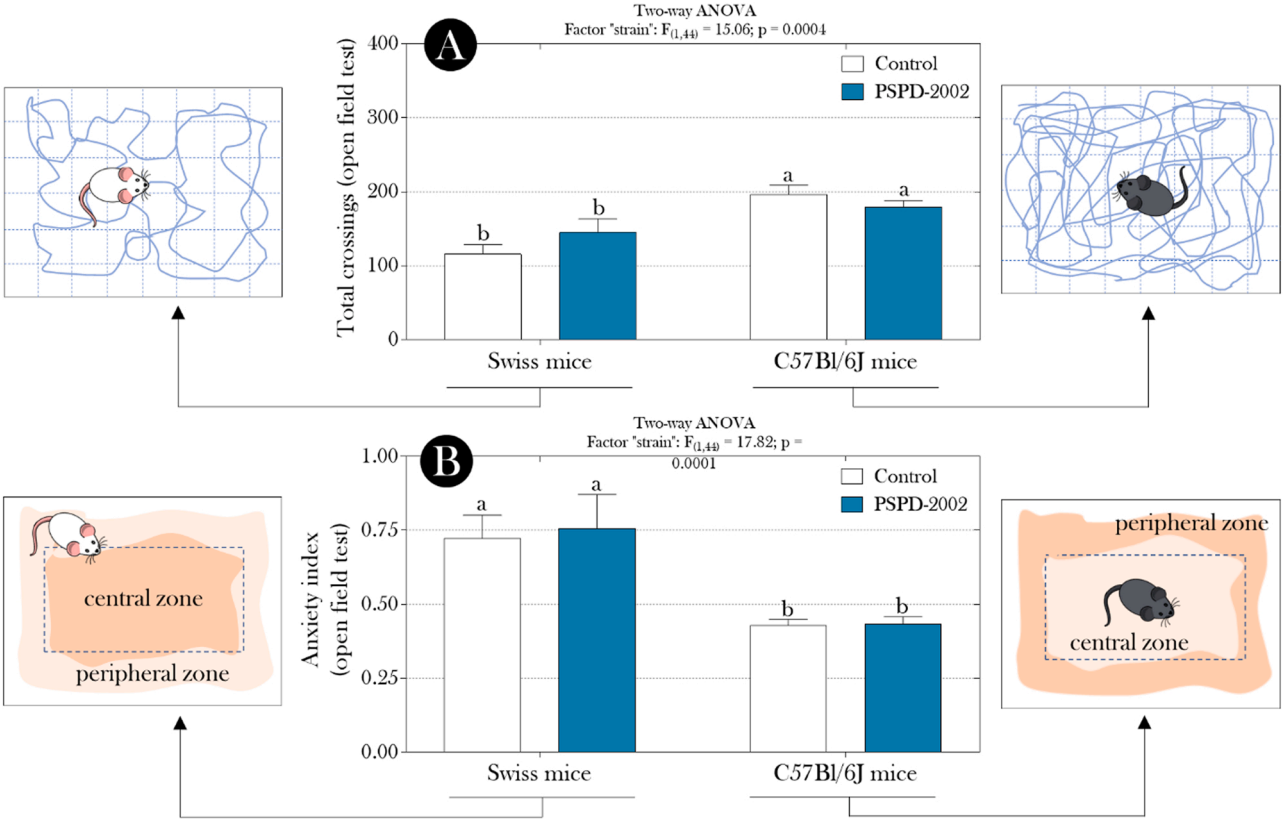

Fig. 1. . (A) Total crossings and (B) anxiety index of Swiss and C57Bl/6 J mice exposed or not to peptide fragments PSPD-2002 in the open field test (OFT). The bars indicate the mean + SD (n = 12 animals/group), whose data were submitted to two-way ANOVA, with Tukey's post-test, up to 5% probability (see the summary of statistical analyses at the top of the graphs). Distinct lowercase letters indicate significant differences between groups. PSPD-2002 refers to groups of mice (Swiss or C57Bl/6 J) that received an intraperitoneal injection containing the peptide fragment PSPD-2002 (at 580 μg/kg). The central zone corresponds to 70% of the total area of the arena used in the OFT. For the behavioral assessment, all animals in the replicas were subjected to behavioral tests, totaling 12 animals/group. All statistical comparisons were based on the averages of each individual replicate tank (i.e.: 4 replica/group).

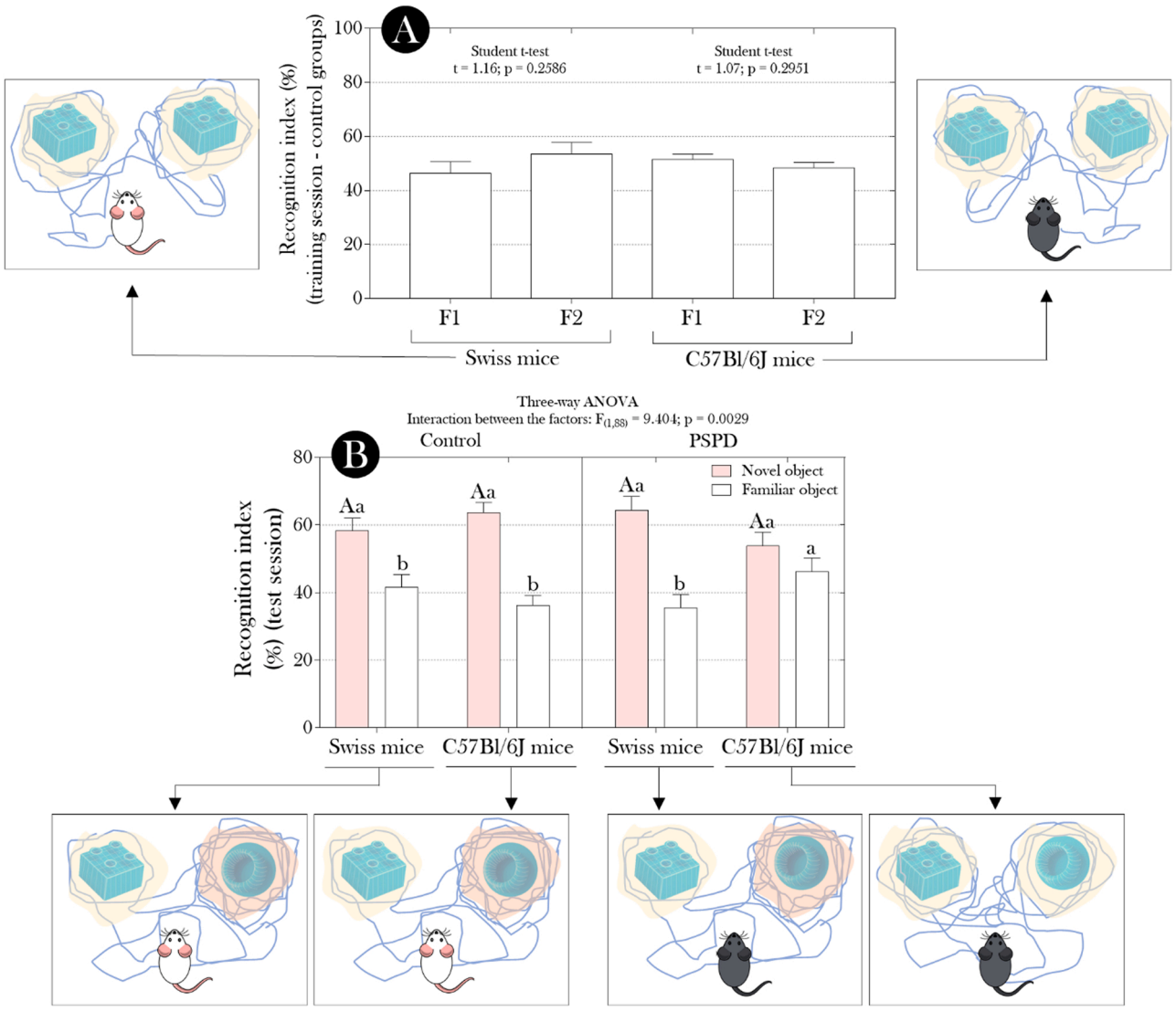

Fig. 2. Object recognition indices of Swiss and C57Bl/6 J mice exposed or not to peptide fragments PSPD-2002 in the novel object recognition test (NORT). (A) Recognition index of the "familiar objects" of the "controls" groups in the "training session" and (B) in the "test session" ("control" and "PSPD-2002" groups). The bars indicate the mean + SD (n = 12 animals/group). In "A", The Student's t-test was applied, at 5% probability. In "B", the data were submitted to the Three-way ANOVA, with Tukey's post-test, also at 5% probability (see the summary of statistical analyses at the top of the graphs). Equal capital letters indicate the absence of differences in the "novel object" recognition indexes between the different experimental groups. On the other hand, the distinct lowercase signs indicate significant differences between the recognition indexes of the novel vs. "familiar" objects of each experimental group. PSPD-2002 refers to groups of mice (Swiss or C57Bl/6 J) that received an intraperitoneal injection containing the peptide fragment PSPD-2002 (at 580 μg/L). For the behavioral assessment, all animals in the replicas were subjected to behavioral tests, totaling 12 animals/group. All statistical comparisons were based on the averages of each individual replicate tank (i.e.: 4 replica/group).

compared to their respective "control" groups. While in the Swiss mice this reduction was 40.1%, in the C57Bl/6 J mice was higher than 65.8%. However, we did not observe a suppressive or stimulator effect induced by peptide fragments in the BChE brain activity of the evaluated animals (Fig. 5D).

Assuming that the biochemical effects observed could be related to the possible binding of peptide fragments (PSPD-2002) to the protein structures of the enzymes SOD, catalase, and AChE, we performed a molecular docking analysis. In this case, all the interactions evaluated presented acceptable affinity data exceeding the low-quality limit (−6.0 kcal/mol). The affinity values for SOD and catalase were − 7.9 kcal/mol (for both) and AChE was − 8.1 kcal/mol. The interactions between PSPD-2002 and AChE involved glu223, SER224, GLY141, SER145, TYR144, ASP300, SER304, PHE355, and TYR354. With SOD and catalase, the residues "ASN92, GLU76, ALA179, LYS30, MET27, GLY155, ILE140, ARG142, and ALA179" and "ALA384, ARG382, ASN142, ASN238, SER337 and MET339", respectively, were involved in the interaction with the peptide fragments. The results of the couplings between peptides and the active sites of enzymes are shown in Fig. 6.

Considering the behavioral and biochemical (joint) responses of the animals, we observed that the "PSPD-2002 C57Bl/6 J" group presented an IBRv2 value 30.17% higher than that obtained in the Swiss mice exposed to peptide fragments (Fig. 7 A). The star graph (polygon) of A values obtained for each compound (Fig. 7B) shows how each biomarker contributed to the IBRv2 index for the groups that received PSPD-2002 (Swiss and C57Bl/6 J). In general, the graph shows a suppressive trend of most biomarkers evaluated in the "PSPD-2002 Swiss" group and, in the C57Bl/6 J mice, a stimulator effect. On the other hand, in both strains, the AChE and TBARs values were discriminant, denoting suppressive and stimulator effects, respectively (Fig. 7B).

In PCA, we observed that the first two principal components (PC1 and PC2) cumulatively explained 91.16% of the total variation (Fig. 8A), with the eigenvalues of PC1 and PC2 being higher than 2.6 (Fig. 8B). The

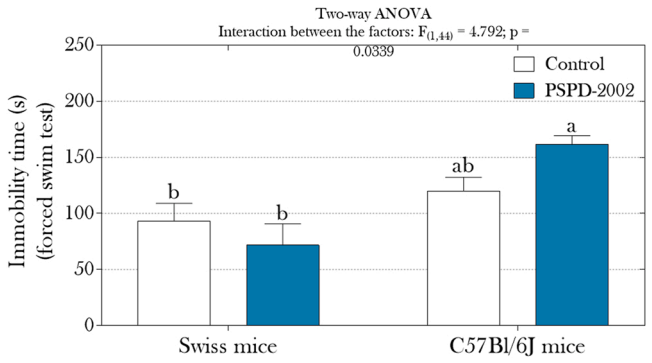

Fig. 3. Swiss and C57Bl/6 J mice immobility tempo exposed or not to PSPD-2002 peptide fragments in a forced swimming test. The bars indicate the mean + SD (n = 12 animals/group), whose data were submitted to two-way ANOVA, with Tukey's post-test, up to 5% probability (see the summary of statistical analyses at the top of the graphs). Distinct lowercase letters indicate significant differences between experimental groups. PSPD-2002 refers to groups of mice (Swiss or C57Bl/6 J) that received an intraperitoneal injection containing the peptide fragment PSPD-2002 (at 580 µg/kg). For the behavioral assessment, all animals in the replicas were subjected to behavioral tests, totaling 12 animals/group. All statistical comparisons were based on the averages of each individual replicate tank (i.e.: 4 replica/group).

loadings plot (which shows the relationship between the PCs and the original variable – Fig. 8 C) and Table S1 show that most biomarkers were negatively associated with PC1 and that PC2 was negatively determined only by the variables AChE, a total of crosses and recognition of the novel object. As expected, the biomarkers of oxidative and nitrosative stress (nitrite, ROS, hydrogen peroxide, SOD, and catalase) were strongly correlated, considering the proximity between their vector loads in the negative upper quadrant of PC1, as well as the high values of correlation coefficients observed in Table S2. We also note that the rodent strains evaluated (exposed or not to PSPD-2002) were separated by PC1, and the Swiss mice groups were positioned in the negative quadrants of PC1 and those of the C57Bl/6J mice, in the positive quadrants of this PC (Fig. 8D). The hierarchical clustering analysis that is carried out confirms this trend (Fig. 8E). On the other hand, the positioning of the groups "control" and "PSPD-2002" (for both strains) in opposite quadrants in PC2 confirms that the response of the animals (inferred by the biomarkers evaluated in this study) was influenced by exposure to peptide fragments. The greater distance between the PC scores of the groups "control C57Bl/6J" and "PSPD-2002 C57Bl/6J" groups in PC2 shows, particularly, a greater effect of exposure to PSPD-2002 on the C57Bl/6 J strain, as also pointed out by the IBRv2 (Fig. 7A).

4. Discussion

Although it is very incipient that there is a prognosis on the ecological risks associated with the dispersion of SARS-CoV-2 or its particles/fragments in natural environments, previous studies of our group have shown that these risks cannot be neglected (Charlie-Silva et al., 2022). The effects of the exposure of representatives of insect groups (Mendonça-Gomes et al., 2021), amphibians (Charlie-C.J. Silva et al., 2021; Charlie-A.L.P. Silva et al., 2021), and fish (Malafaia et al., 2022; Gonçalves et al., 2022) peptide fragments of the Spike protein of SARS-CoV-2 raise concerns related to the possible effects of PSPD-2002 on mammals, not only because man is part of this taxonomic group, but also due to the importance of these animals in ecosystem balance. In this sense, our study extends the knowledge about the (echo)toxicological potential of these viral fragments by demonstrating that the short exposure of mice to PSPD-2002 was able to induce alterations that affect the health of animals, which constitutes, therefore, the "big picture" of our study.

Initially, our data pointed to the absence of induction of hyper-/hypoactivity or anxiogenic-/anxiety-like behavior by exposure to PSPD-2002, which departs from the reported effects for *C. quinquefasciatus*

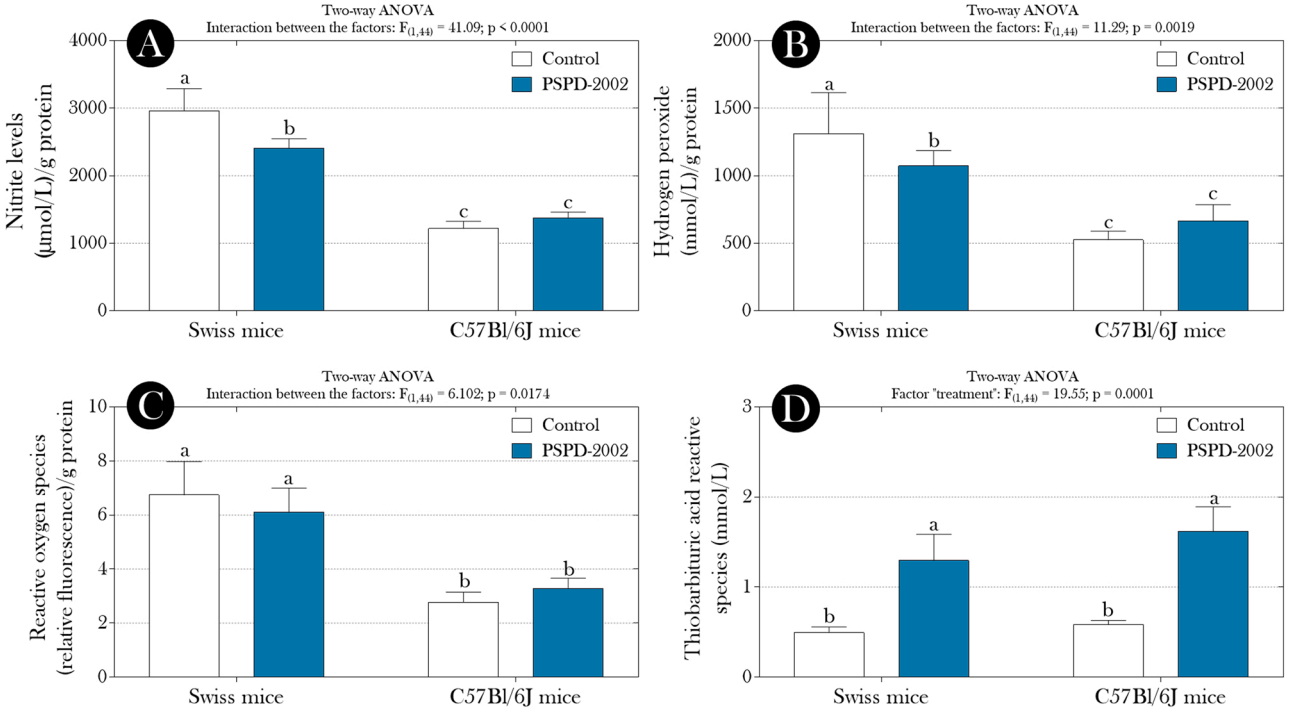

Fig. 4. . (A) Nitrite, (B) hydrogen peroxide, (C) reactive oxygen species, and (D) thiobarbituric acid reactive species levels in the brain of Swiss and C57Bl/6 J mice exposed or not to peptide fragments PSPD-2002. The bars indicate the mean + SD (n = 12 animals/group), whose data were submitted to two-way ANOVA, with Tukey's post-test, up to 5% probability (see summary of statistical analyses at the top of the graphs). Distinct lowercase letters indicate significant differences between groups. PSPD-2002 refers to groups of mice (Swiss or C57Bl/6 J) that received an intraperitoneal injection containing the peptide fragment PSPD-2002 (at 580 µg/kg).

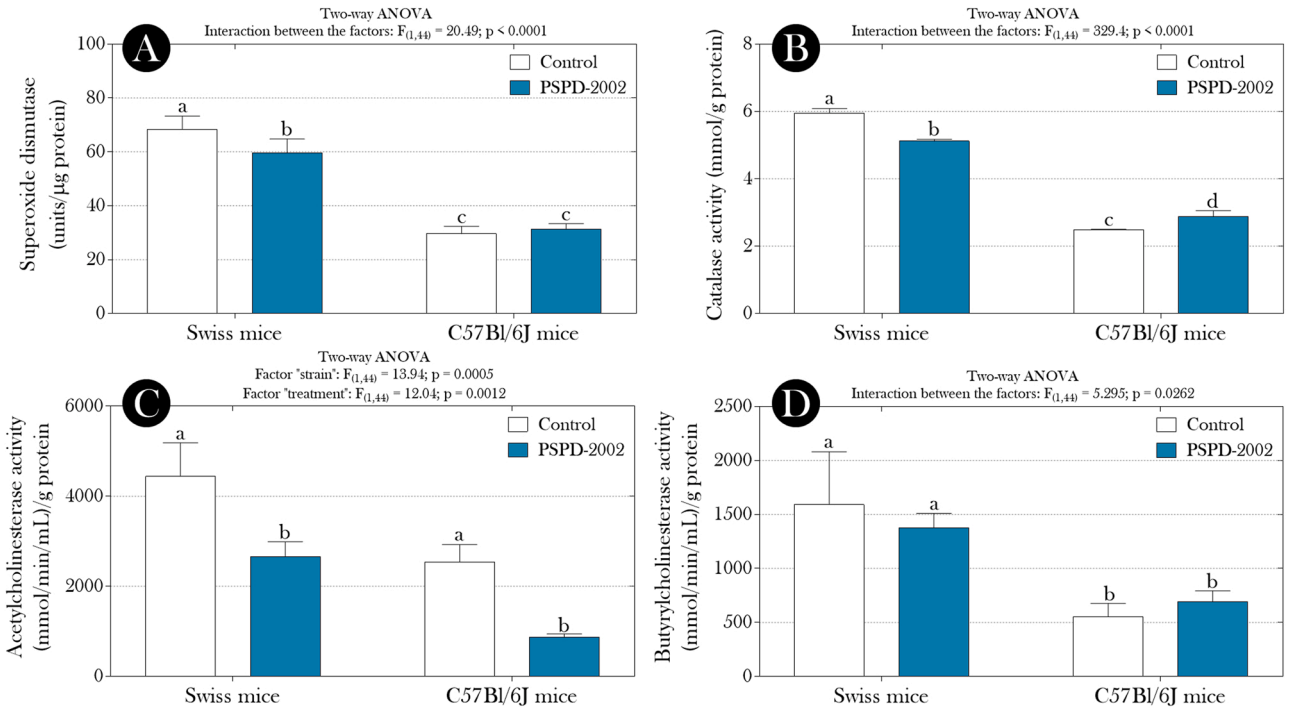

Fig. 5. . (A) Superoxide dismutase, (B) catalase, (C) acetylcholinesterase, and (D) butyrylcholinesterase activity in the brain of Swiss and C57Bl/6 J mice exposed or not to peptide fragments PSPD-2002. The bars indicate the mean + SD (n = 12 animals/group), whose data were submitted to two-way ANOVA, with Tukey's post-test, up to 5% probability (see summary of statistical analyses at the top of the graphs). Distinct lowercase letters indicate significant differences between groups. PSPD-2002 refers to groups of mice (Swiss or C57Bl/6 J) that received an intraperitoneal injection containing the peptide fragment PSPD-2002 (at 580 µg/kg).

larvae and *P. reticulata* juveniles exposed to the same peptides. In insects, aquatic exposure to PSPD-2002 (at 40 µg/L), for 48 h, induced a significant increase in the locomotor activity of larvae (Mendonça--Gomes et al., 2021), and in fish, we demonstrated that although habituation learning has not been altered by the peptides (40 µg/L, 35 days exposure), the animals exposed exhibited anxiety-like behavior (Malafaia et al., 2022). At the time, we evidenced a close relationship between the stimulation of the cholinergic system in animals exposed to peptides and the induction of reported behavioral changes, which was not observed in our study. Conversely, we evidenced in both strains of mice tested that exposure to PSPD-2002 induced a significant suppression of ache brain activity (Fig. 5C) without, however, having eaten alterations in locomotor abilities (Fig. 1A), anxiety-like behavior (Fig. 1B), or depressive-like behavior of animals (Fig. 3). Although the activity of AChE plays a fundamental role in the central cholinergic synapses and neuromuscular junctions necessary for the maintenance of the physiological homeostasis of locomotion (Holschneider et al., 2011; Mille et al., 2021) or influential role in anxiety and depression (Suarez-Lopez et al., 2019), its reduction in the evaluated mice does not seem to have been sufficient to induce behavioral changes of these natures. In this case, it is tempting to speculate that the reduction threshold of this enzyme capable of inducing changes in the neurophysiological mechanisms that regulate the emotionality of animals in the OFT and FST may not have been reached in the short period of exposure to PSPD-2002 (i.e.: 24 h).

On the other hand, we observed in the C57Bl/6 J mice a significant effect of exposure to PSPD-2002 on their performances in NORT, considered the hallmark method used in assessing non-spatial object memory in rodents (Cohen and Stackman, 2015). The similarity between the exploit times of the "novel" and "familiar" objects in the animals of the "PSPD-2002 C57Bl/6 J" group (Fig. 2B), showed significantly reduced discriminating ability compared to their "control" group. As discussed by Rabelo et al. (2016) and Guimarães et al. (2017)), results such as these suggest a memory deficit induced by the treatments, since an increase in the time of exploration of the "novel" object would indicate that a memory trace for the "family object" was properly coded, consolidated and then recovered to guide the rodent's behavior during the "test session" of the NORT. The pioneering of our study restricts our ability to elucidate the mechanisms that mediated the effect of PSPD-2002 on the neurobiological mechanisms responsible for this memory deficit. However, our study provides evidence that the memory deficit observed in c57Bl/6 J mice may be related to higher production of TBARs (≅ 10%) and greater suppression of AChE activity (≅ 65%) observed in the brain of these animals, compared to Swiss mice also exposed to PSPD-2002 (Figs. 4D and 5A, respectively). Although these results have not differed statistically from a biological point of view, these percentage differences cannot be overlooked.

It is known that NORT requires the hippocampus for encoding, consolidation, and retrieval (Mumby et al., 2002; Haettig et al., 2011) thus providing a measure of hippocampus-dependent spatial memory (Vogel-Ciernia, Wood, 2014). Therefore, it is possible that the increase in lipid peroxidation (LPO)-induced processes, induced by PSPD-2002, may have caused cytotoxic changes in hippocampal neurons, which would have potentially affected the performance of C57Bl/6 J mice in NORT. Studies such as those of De-Lima et al. (2005), Tang et al. (2013), Mamiya et al. (2013) and Pondugula et al. (2021)), by demonstrating a strong association between the occurrence of LPO in the hippocampus of rodents (who received different substances/chemical compounds) and a memory deficit in NORT, reinforce this hypothesis. However, interestingly, the increase in TBARs levels in animals exposed to PSPD-2002 does not seem to be related to the induction of oxidative stress in the brain of mice, as observed in other animals exposed to the same peptides (Charlie-C.J. Silva et al., 2021; Charlie-A.L.P. Silva et al., 2021; Mendonça-Gomes et al., 2021; Malafaia et al., 2022; Gonçalves et al., 2022), since the levels of hydrogen peroxide and ROS did not differ between the "control C57Bl/6 J" and "PSPD-2002 C57Bl/6 J" groups (Fig. 4B-C, respectively). In this case, it is plausible to assume that the increase in TBARs in c57Bl/6 J mice is part of an adaptive response to try to counterbalance the oxidative stress induced by PSPD-2002. As discussed by Morales and Munné-Bosch (2019) and Rangasamy et al. (2022)),

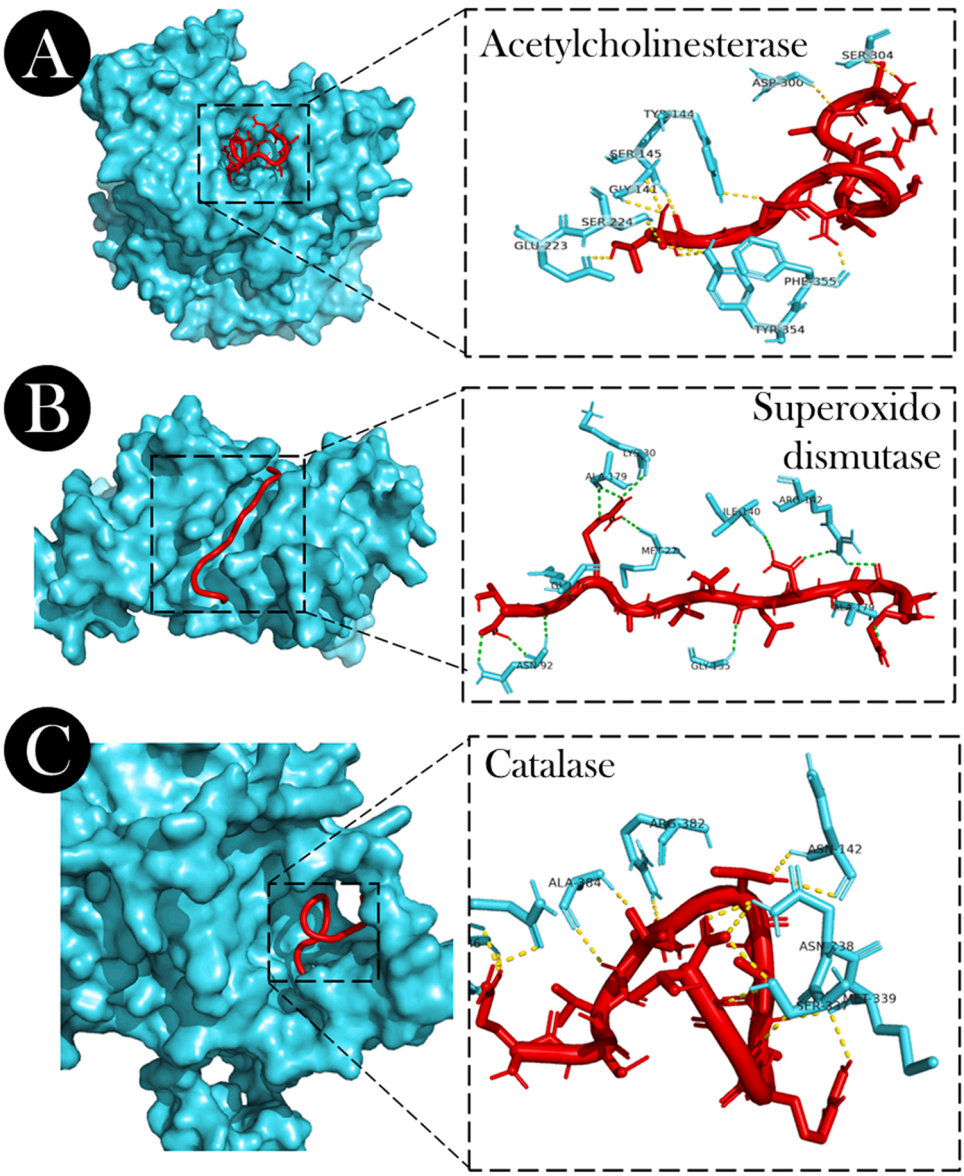

Fig. 6. Three-dimensional surface-ligand coupling of peptide fragment PSPD-2002 in the active sites of the (A) acetylcholinesterase, (B) superoxide dismutase, and (C) catalase enzymes and their interactions.

TBARs increases may represent acclimation processes rather than damage, since TBARs can exert a positive role by activating regulatory genes involved in animal defense and development and granting cell protection under oxidative stress conditions. Anyway, this presumption needs to be validated by future studies.

On the other hand, studies have shown that a decrease in cholinergic function in the central nervous system can result in cognitive dysfunction and memory loss (Araujo et al., 2005; Maurer and Williams, 2017; Fahimi et al., 2021), which reinforces the hypothesis that the greatest anticholinesterase effect induced by PSPD-2002 in C57Bl/6J mice (Fig. 5C) is also associated with memory deficit in these animals (Fig. 2B), which has also been evidenced in previous studies. In Haider et al. (2014), for example, the authors showed that the impaired memory exhibited by the aged rats may be attributed to the observed decreases in AChE activity and increased LPO in plasma and brain. In addition, Paul and Borah (2017) observed a strong association with the suppression of AChE activity in the brain and the significant reduction in discriminating ability of hypercholesterolemic mice in NORT.

In our study, in particular, the hypothesis of the anticholinesterase effect observed in the brain of animals is a consequence of direct interactions between the PSPD-2002 and AChE is supported by molecular docking analysis (Fig. 6), different from the findings of Charlie-A.L. P. Silva et al. (2021); Charlie-C.J. Silva et al. (2021), Mendonça-Gomes et al. (2021) and Malafaia et al. (2022), in which opposite effect (cholinesterasic stimulation) was observed *in P. cuvieri* tadpole, *C. quinquefasciatus* larvae and *P. reticulata* juveniles, respectively. In these studies, two possible mechanisms have been proposed for an increase in AChE activity. In the first situation, the increase in AChE activity would characterize a compensatory mechanism in response to the catalytic deficit induced by the peptides. In this case, it is possible that the peptides would bind AChE instead of the natural ligand and thus reduce the catalysis of acetylcholine ACh. In the second, the increase would be explained by a more efficient response of the enzyme to the increase in the release of ACh in the synaptic clefts via activation of the cholinergic anti-inflammatory pathway (CAP). However, in mice exposed to PSPD-2002, such assumptions do not seem to be applied, considering factors involving physiological differences between the evaluated models, the sites (organs/tissues) where the AChE activity was measured, the time sands, and the routes of exposure to peptide fragments. Therefore, future studies will be useful to understand

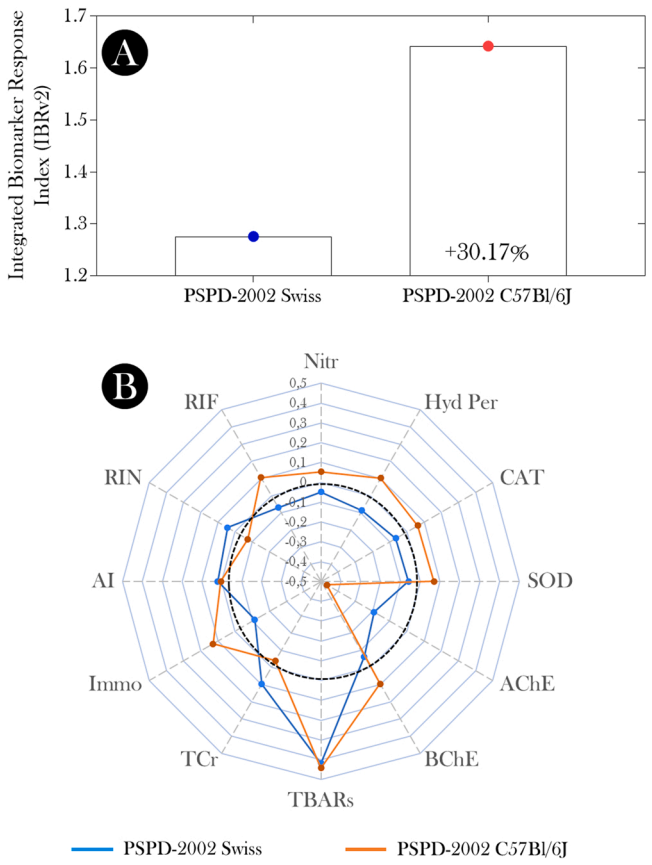

Fig. 7. (A) Results of Integrated biomarker responses index (IBRv2) calculations and (B) star graph (polygon) of A values obtained with the IBRv2 method for the "PSPD-2002 Swiss" and "PSPD-2002 C57Bl/6 J" groups. **Legend**: Nitr: nitrite; Hyd Per: hydrogen peroxide; CAT: catalase; SOD: superoxide dismutase; AChE: Acetylcholinesterase; BChE: Butyrylcholinesterase; TBARs: thiobarbituric acid reactive substances; TCr: total crossing; Immo: immobility time; AI: anxiety index; RIN: recognition index (novel object); RIF: recognition index (familiar object); and ROS: reactive oxygen species.

whether possible interactions between PSPD-2002 and AChE in mice would culminate in the suppression of AChE activity due to changes in the mechanisms of association and catalysis or the reduction of enzymatic efficiency caused by decreased affinity of the substrate for the active site of the enzyme. Alternatively, investigations on the influence of PSPD-2002 on the activation/inhibition of the cholinergic anti-inflammatory approach (CAP) may elucidate whether the reduced AChE activity is the result of the simple decrease in the release of ACh in the synaptic clefts by the inactivation of the cholinergic anti-inflammatory pathway (CAP) by the peptides. Moreover, the hypothesis that the reduction of AChE activity in these animals is associated with negative regulation of the AChE gene by the peptide of the Spike protein of SARS-CoV-2 is an interesting investigative perspective to be addressed in the future.

On the other hand, the possible reduction of SOD and catalase activity caused by their strong interactions with PSPD-2002 suggested by molecular docking analysis (Fig. 6) did not culminate in the increase of oxidative stress biomarkers evaluated in our study, which suggests that other antioxidant mechanisms may have acted to prevent the occurrence of oxidative and nitrosative processes. The increase in the production of hydrogen peroxide, ROS, and nitrite was not observed in any of the mouse strains exposed to PSPD-2002 (Fig. 5), which also diverges from previous findings of our group involving aquatic models exposed to the peptide fragments of the Spike protein of SARS-CoV-2. Although SOD and catalase are considered first-line antioxidants defenses that are important for preventing physiological oxidative stress (Ighodaro and Akinloye, 2018), in specific situations other components of the antioxidant system can be activated as a result of a mechanism that aims to compensate for dysfunctions in these enzymes. This would include enzymatic antioxidants such as glutathione peroxidases (GPx) [which can use glutathione (GSH) as a reductant to catalyze hydrogen peroxide or organic hydroperoxides (He et al., 2017)], the components of the thioredoxin (Trx) system, which is composed of NADPH, thioredoxin reductase (TrxR), and Trx (Nordberg and Arnér, 2001; Lu and Holmgren, 2014), as well as peroxiredoxins (Prxs), are a very large and highly conserved family of peroxidases that reduce peroxides (Rhee, 2016). In addition, a large number of low molecular weight compounds are considered to be antioxidants of biological importance, including vitamins C and E, different selenium compounds, lipoic acid, and ubiquinones (Grune et al., 2005). Therefore, there is a range of possibilities to be investigated which would explain the non-observance of increased hydrogen peroxide and ROS in mice exposed to PSPD-2002, even though the activities of SOD and catalase brain activity have been reduced.

Finally, is important to point out that although our study gathered evidence on the negative effects of peptide fragments of the Spike protein of SARS-CoV-2 on object recognition memory (in C57Bl6J mice), SOD and catalase activity (in Swiss mice), cholinesterase homeostasis, and on the mechanisms that regulate or support the processes of LPO (in both rodent strains), many issues still need to be investigated. From our study, questions are raised about the role of the genetic background in the response of animals to exposure to peptide fragments of the Spike protein of SARS-CoV-2, especially when we evidenced (based on the set of biomarkers evaluated) that c57Bl/6 J mice (inbred) were more affected by exposure to PSPD-2002, compared to Swiss mice (outbred) (Figs. 7 and 8). While the inbred animals are genetically homogeneous and there is a very little variation or heterogeneity within a pure inbred strain (Watkins-Chow and Pavan, 2008), the outbred are bred specifically to genetic maximize diversity and heterozygosity within a population (Rice and O'Brien, 1980). Therefore, this question "sheds light" on the possible influence of genetic profile of individuals on susceptibility/resistance as peptide fragments of the Spike protein of SARS-CoV-2, similarly to the genetic determinism of resistance or susceptibility of humans to COVID-19 (Khan, 2020; Andreakos et al., 2021). In addition, assessments of the toxicity of these peptides, as well as other SARS-CoV-2 particles, at different concentrations and exposure periods and different stages of animal life constitute some future investigative perspectives. Equally important will be to expand the list of biomarkers to be evaluated (e.g.: histopathological, molecular, endocrine, among others), as well as the environmental representativeness of the animal models to be studied, since the sensitivity to viral peptides can be different between non-host organisms of the new coronavirus. Monitoring the effects observed in the adult life of animals, as well as their consequences at the population level and on their ecological roles, should also be the focus of further studies.

5. Conclusion

In conclusion, our study confirms the hypothesis that the exposure of mice to peptide fragments (PSPD-2002) of the Spike protein of SARS-CoV-2 induces alterations involving redox and cholinesterasic homeostasis in the brain of animals, especially in C57Bl/6 J mice, whose IBRv2 value was higher than that observed in Swiss mice. Therefore, our study reinforces the importance of evaluating not only the susceptibility of different mammal species to viral infection and their roles in the dissemination of COVID-19 but also their responses to exposure to viral particles. We believe that approaches of this nature will be useful for a better understanding of the extent of the environmental/ecological impact of the COVID-19 pandemic, whether in the short, medium or long term.

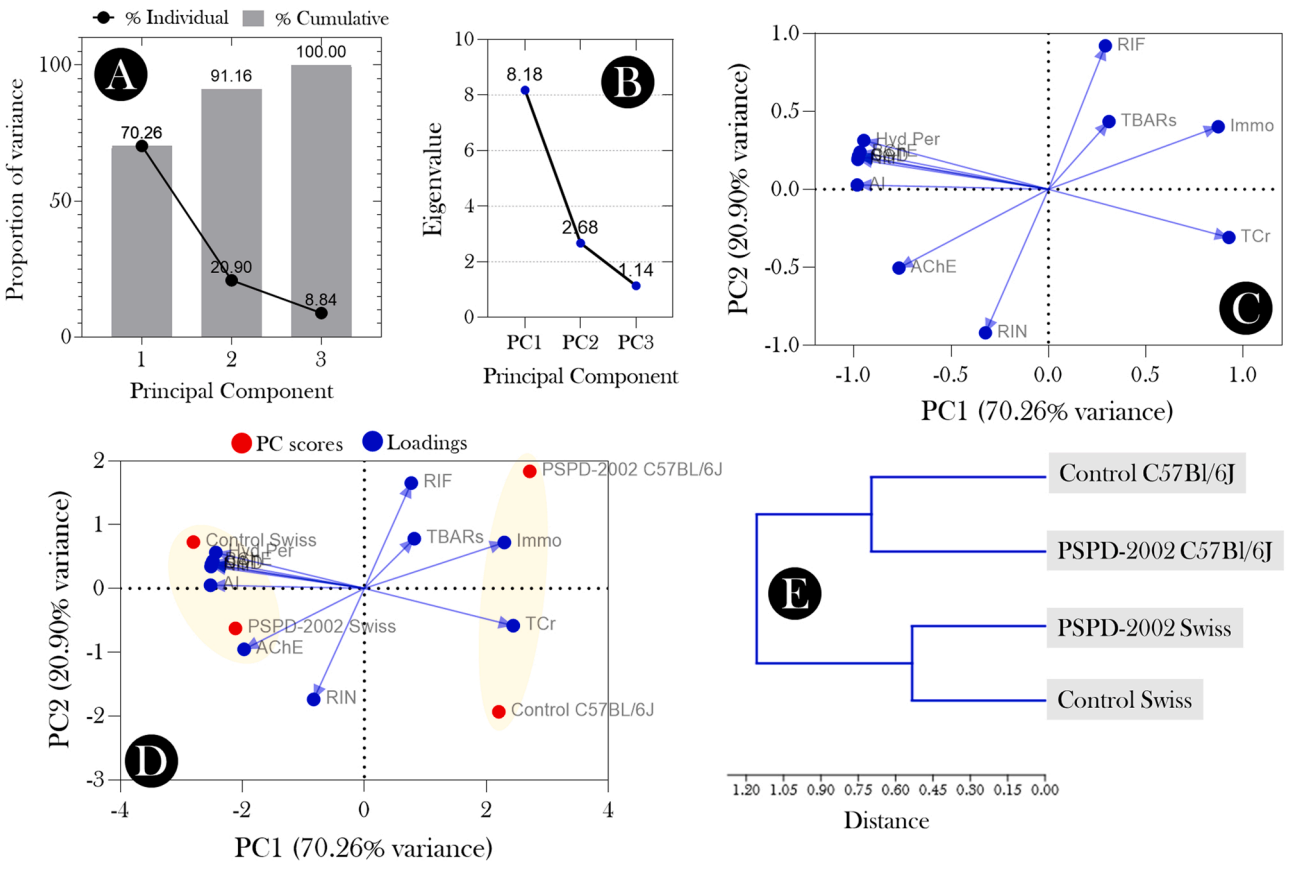

Fig. 8. (A) Proportion of variance and (B) eigenvalues of the principal components, (C) loadings plot of the variables investigated, (D) PCA biplot of the first two principal components that simultaneously shows PC scores of experimental groups (red points) and loadings of explanatory variables (vectors – blue arrows) and (E) cluster analysis dendrogram. PSPD-2002 refers to groups of mice (Swiss or C57Bl/6 J) that received an intraperitoneal injection containing the peptide fragment PSPD-2002 (at 580 μg/kg). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article)

Author contribution statements

Thiarlen Marinho da Luz: study conception and design, data collection, analysis, and interpretation of results, and draft manuscript preparation. **Amanda Pereira da Costa** Araújo: data colletion. **Fernanda Neves Estrêla Rezende**: data colletion. **Abner Marcelino Silva**: data colletion. **Ives Charlie-Silva**: analysis, and interpretation of results, and draft manuscript preparation. **Helyson Lucas Bezerra Braz**: data colletion. **Paulo R.S. Sanches**: data colletion. **Md. Mostafizur Rahman**: analysis, and interpretation of results, and draft manuscript preparation. **Damià Barceló:** analysis, and interpretation of results, and draft manuscript preparation. **Guilherme Malafaia:** conceived of the presented idea, collected the data, provided. funding, analysis, and interpretation of results, and drafted manuscript preparation.

All authors reviewed the results and approved the final version of the manuscript.

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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Declaration of competing interest

We confirm that there are no known conflicts of interest associated with this work and there has been no significant financial support for this work that could have influenced its outcome. We confirmed that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. Due care has been taken to ensure the integrity of the work.

Ethical aspects

All experimental procedures were performed in accordance with the ethical standards for animal experimentation and meticulous efforts were made to ensure that the animals suffered as little as possible and to reduce external sources of stress, pain, and discomfort. The current study has not exceeded the number of animals needed to produce reliable scientific data. This article does not refer to any study with human participants performed by any of the authors.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.neuro.2022.03.012.

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