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ORIGINAL PAPER

Digestibility of Quinoa (*Chenopodium quinoa* Willd.) Protein Concentrate and Its Potential to Inhibit Lipid Peroxidation in the Zebrafish Larvae Model

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Abstract Quinoa protein concentrate (QPC) was extracted and digested under *in vitro* gastrointestinal conditions. The protein content of QPC was in the range between 52.40 and 65.01% depending on the assay used. Quinoa proteins were almost completely hydrolyzed by pepsin at pH of 1.2, 2.0, and 3.2. At high pH, only partial hydrolysis was observed. During the duodenal phase, no intact proteins were visible, indicating their susceptibility to the *in vitro* simulated digestive conditions. Zebrafish larvae model was used to evaluate the *in vivo* ability of gastrointestinal digests to inhibit lipid peroxidation. Gastric digestion at pH 1.2 showed the highest lipid peroxidation inhibition percentage (75.15%). The lipid peroxidation activity increased after the duodenal phase. The digest obtained at the end of the digestive process showed an inhibition percentage of 82.10%, comparable to that showed when using BHT as positive control (87.13%).

Keywords Quinoa protein · Simulated gastrointestinal digestion · Peptides · Inhibition of lipid peroxidation · Zebrafish larvae model

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Introduction

Quinoa (*Chenopodium quinoa* Willd.) is a native pseudocereal of the Andean region in South America, belonging to the *Chenopodiaceae* family [1]. Because of its stress-tolerant characteristics, its gluten-free nature, and its nutritional and biological properties, quinoa has been described as 'one of the grains of the 21st century [2–4].

Oxygen may be partially reduced during normal metabolism to yield reactive molecules termed reactive oxygen species (ROS). An excess of these species can cause cellular changes, oxidation of membrane lipids, apoptosis, and damage to enzymes and DNA. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are used as food additives. However, because of the possible toxic and carcinogenic effects associated to these two antioxidants, their use is legally restricted [5]. Nowadays, there is great interest in finding new natural compounds as an alternative to the use of synthetic antioxidants. In the last years, food protein-derived hydrolysates and peptides with antioxidant activity have attracted the interest [6]. As it has been reported for other activities, *in vitro* simulated gastrointestinal digestion has an important effect increasing the antioxidant activity of food proteins [7–9].

Zebrafish (*Danio rerio*) is an emergent vertebrate model used to study toxicological events, such as neurochemical alterations promoted by heavy metals, new drugs and chemicals [10]. Richetti et al. [11] used the zebrafish larvae model to evaluate lipid peroxidation produced in presence of heavy metals [11]. Recently, this model has been applied to evaluate the antioxidant activity of food compounds. Carrillo et al. [12] reported the lipid peroxidation inhibitory activity of peptides isolated from hen egg white lysozyme using the TBARS method in the zebrafish larvae model [12]. Lipids are biomolecules with great possibilities to undergo oxidation resulting in

the release of different secondary molecules, mainly aldehydes, capable to increase oxidative damage. Malondialdehyde is the most studied product of polyunsaturated fatty acid peroxidation. Letelier et al. [13] studied the inhibition of lipid peroxidation of polyphenol thiol components of quinoa seeds using the TBARS method [13]. However, to our knowledge, no data about the quinoa proteins potential to release inhibitory peptides of lipid peroxidation in *in vivo* models can be found. The aim of this study was to obtain QPCs from *Chenopodium quinoa* Willd. and to evaluate their digestibility in *in vitro* conditions simulating gastrointestinal digestion. The cytotoxicity and the inhibition of lipid peroxidation in the zebrafish larvae model of gastroduodenal digests were also studied.

Materials and Methods

Obtention of Quinoa Protein Concentrate (QPC)

Quinoa protein concentrate (QPC) was prepared according to Martínez and Añón [14] with some modifications. The defatted flour was suspended in water (1:10, *w*/*v*) adjusting the pH to 8.0. The suspension was stirred for 1 h and then, centrifuged at 4,500 x *g* for 30 min at 25 °C. The supernatant was adjusted to pH 4.0, and centrifuged for 20 min at 4,500 x *g*. The pellet was collected, the pH adjusted with 0.1 M NaOH, lyophilized and kept at −20 °C. The QPC protein content was determined using Biuret, BCA (Pierce, Rockford, IL, USA), and Dumas methods [15–17].

In Vitro Gastrointestinal Digestion of QPC

QPC (5 mg/mL) was subjected to gastric digestion using simulated gastric fluid (0.35 M NaCl) at different pH: 1.2, 2.0, 3.2, 4.5, and 5.5 [18]. Porcine pepsin (EC 3.4.23.1, 4,500 U/mg protein, Sigma-Aldrich, St. Louis, MO, USA) was added with an enzyme/substrate (E/S) ratio of 2000 U/mg. The digestion was performed at 37 °C for 120 min. Aliquots of gastric digests were withdrawn inactivating the enzyme by heating at 80 °C for 5 min. The pH of digest obtained at the end of gastric phase (pH 3.2) was adjusted to 7.0, and intestinal phase started after addition of 0.125 M bile salt mixture (Sigma-Aldrich), 1 M $CaCl_2$ (7.6 mM final concentration), and pancreatin (100 U/mg) dissolved in 20.3 mM Bis-Tris buffer. Reactions were carried out at 37 °C for 120 min, and stopped by heating at 80 °C for 5 min.

Characterization of QPC Digests by SDS–PAGE Electrophoresis and RP-UHPLC

SDS-PAGE of QPC and its gastric and gastroduodenal digests was carried out using 4 and 12% polyacrylamide gels in a Mini-Protean electrophoresis system (Bio-Rad, Hercules, CA, USA). Polypeptide bands were stained with Coomassie Brilliant Blue G-250 for 12 h. Relative molecular masses of protein were determined by comparing them with molecular weight markers (Bio-Rad). Characterization was also carried out by RP-UHPLC using an Agilent 1200 infinity series UHPLC system (Agilent Technologies, Waldbron, Germany). The wavelength detector was 214 nm. The column used was Zorbax EC C18 (Agilent Poroshell 120, 4.6 × 50 mm × 2.7 μm of particle size). Samples were eluted at 1.0 mL/min with a lineal gradient from 0 to 70% of solvent B (acetonitrile and trifluoroacetic acid, TFA, 0.270% *v*/v) in solvent A (water and TFA, 0.370% *v*/v) [19].

Zebrafish Larvae Collection and Toxicity Test

Adult zebrafish was kept on 16 h light and 8 h dark cycles. Embryos were obtained by photo-induced spawning over green plants and then, cultured at 28 °C in a fishbowl. Early larvae were maintained according to Nagel [20]. Post fertilization early larvae zebrafish were maintained according to Kimmel et al. [21]. Five days post fecundation larvae (30 larvae per well) were incubated in 24-well plates. QPC digests were added at three different concentrations (5.0, 2.5, and 1.25 mg/mL) in 200 μL of water. The effect of digests on larvae was measured after 4, 24 and 48 h. At the end of incubation time, larvae mortality and morphologic changes were observed, determining the percentage of dead larvae. Stereoscopic microscope images were taken to obtain a registration of the morphological effects of digests on larvae anatomy and compared to controls.

Thiobarbituric Acid Reactive Substances (TBARS) in Zebrafish Larvae Model

TBARS method was used as described by Carrillo et al. [12]. Larvae were incubated in 24-well plates (30 larvae/well) with 2.0 mg/mL of QPC digests. Groups of 30 larvae/well in aquarium water were used as controls. Lipid peroxidation was initiated by adding 1 mL of 500 μM H_2O_2 and incubated for 8 h at 28 °C. Then, H_2O_2 was removed and 500 μL of Tween 0.1% were added. Larvae were mixed and homogenized with a homogenizer (T25 basic Ultra Turrax IKA, Thermo Fisher Scientific, Germany). 1 mL of 1% TBA was added and subsequently, the solution was heated at 95 °C for 1 h, and then cooled down for 15 min. Absorbance of the final solution containing zebrafish larvae and QPC digests was measured at 532 nm using a spectrophotometer (Thermo Scientific Evolution 200, Germany). The values of antioxidant activity were expressed as the percentage of inhibition of lipid peroxidation in larvae homogenate as follows: % Inhibition of lipid peroxidation = [1-(Ab - As)/Ab × 100], where Ab is the absorbance of blank and As is the absorbance of the sample.

Statistical Analysis

Results are presented as means ± standard deviation from three replicates of each experiment. Differences between mean values were determined by the analysis of variance (ANOVA). The *post hoc* analysis was performed by the Tukey test. All tests were considered significant at $P < 0.05$ using the software package Prism 4.

Results and Discussion

Characterization of QPC Digested under Simulated Gastrointestinal Digestion

QPC was obtained using an isoelectric precipitation method with water at pH 4.0. The protein content value of QPC, measured by Biuret, Dumas, and BCA methods, were 65.01, 52.4, and 55.50%, respectively. The statistical differences ($p < 0.05$) could be due to the different level of sensitivity of each method. Since the protein extraction method used in our study did not include a lipid extraction phase, residual lipids present in QPC could interfere on the Biuret measurement, increasing the protein value determined by this method. This interference had been previously reported [22]. Therefore, the protein value obtained by Dumas method was used to prepare the samples for further analyses.

QPC and its digests were analysed using SDS-PAGE. As shown in Fig. 1a, the protein profile of QPC showed bands with molecular weights between 6.5 to 200 kDa. The most intense bands were those ranged from 15 to 40 kDa corresponding to 7S and 11S globulins, and 2S albumin from quinoa. These results are in agreement with Abugoch et al. [23] that indicated the presence of quinoa globulins with molecular weight between 20 to 50 kDa, and albumins with molecular weight lower than 20 kDa [23]. Previously, Brinegar et al. 24 identified 7S globulin with molecular weight of 50 kDa, 11S globulin (acid subunit 22–23 kDa and basic subunit 32–39 kDa), and quinoa albumins with molecular weight of 8–9 kDa [24]. In Fig. 1a, quinoa proteins were completely hydrolysed by pepsin when incubation was performed at pH 1.2, 2.0, and 3.2. However, no hydrolysis of quinoa proteins is appreciable at pH 4.5 and pH 5.5. This could be due to the lower enzyme activity shown by pepsin at high pH values. After incubation with pancreatic enzymes, quinoa proteins were totally hydrolysed (Fig. 1b), indicating the high digestibility of these proteins under conditions simulating physiological digestion. No small peptides could be observed because of the composition of the gel (12% acrylamide) used to separate proteins. Gels with 16–20% of acrylamide would allow visualizing bands corresponding to small peptides released during gastrointestinal digestion [25].

Analysis of quinoa protein hydrolysates was also carried out by RP-UHPLC to observe the changes in the molecular weight and hydrophobicity of peptides released during the digestive process. The results obtained are shown in Fig. 2. Four zones, named as F1, F2, F3 and F4 with retention times of 0–1.0, 1.0–4.0, 4.0–5.0 and 5.0–8.0 min, respectively, were clearly identified in the chromatogram for non-digested QPC (Fig. 2a). F1 was constituted by a major peak, which an area corresponding to 36% of the total chromatogram area. F2 showed several small peaks, representing 12% of the total area, and F3 presented two major peaks representing 32% of the total chromatogram area. Finally, two major peaks, which area corresponded to 20% of the total area, were visible in fraction F4. The chromatograms of gastric (at different incubation pH) and gastroduodenal digests are shown in

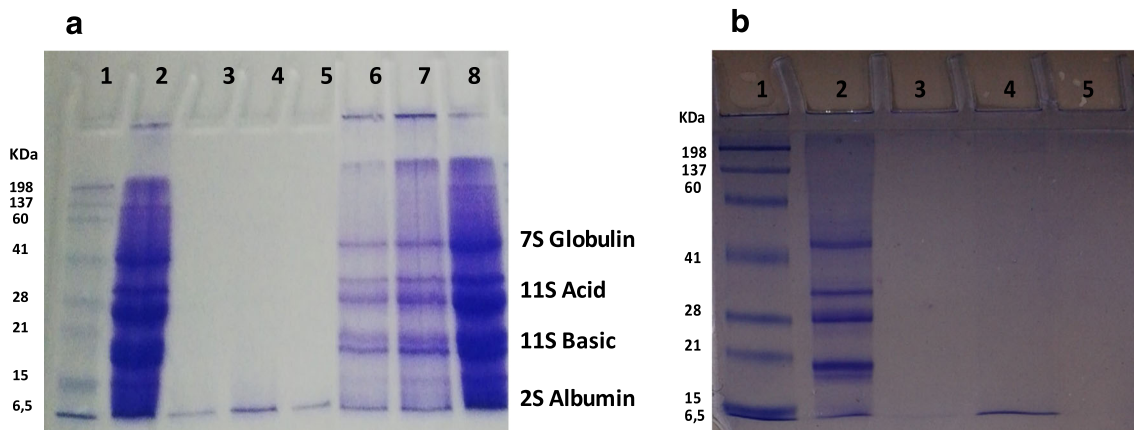

Fig. 1 SDS-PAGE electrophoresis of quinoa protein concentrate (QPC) and its gastric and gastroduodenal digests. **a** Lanes 1: molecular weight marker, lanes 2 and 8: QPC, lane 3: gastric digest obtained at pH 1.2, lane 4: gastric digest obtained at pH 2.0, lane 5: gastric digest obtained at pH 3.2, lane 6: gastric digest obtained at pH 4.5, lane 7: gastric digest obtained at pH 5.5. **b** Lane 1: molecular weight marker, lane 2: QPC, lane 3: gastric digest obtained at pH 3.2, lane 4: gastroduodenal digest, lane 5: water

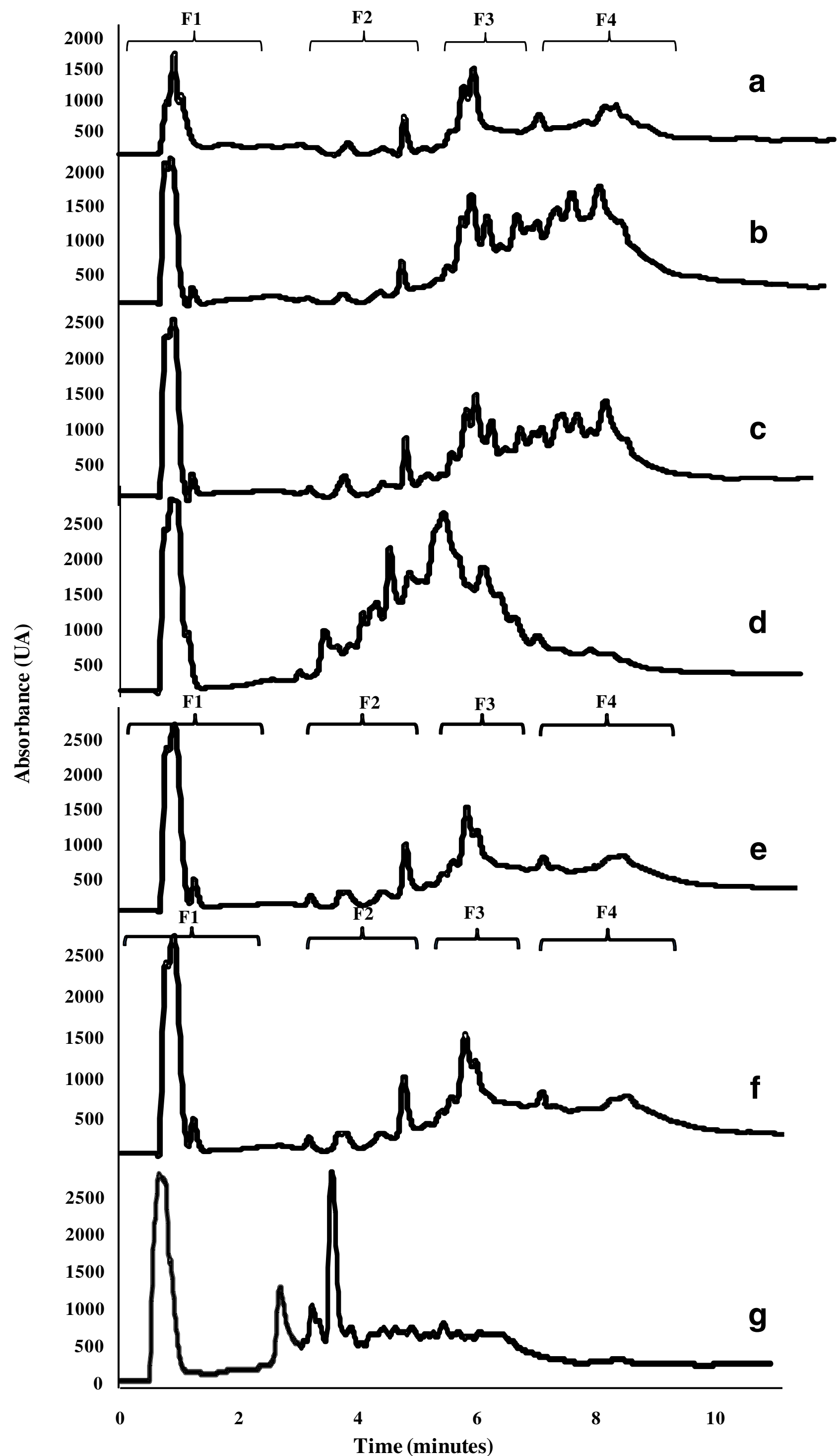

Fig. 2 RP-UHPLC analysis of quinoa protein concentrate (QPC) and its gastric and gastroduodenal digests. (**a**) QPC, (**b**) gastric digest obtained at pH 1.2 (**c**) gastric digest obtained at pH 2.0 (**d**) gastric digest obtained at pH 3.2 (**e**) gastric digest obtained at pH 4.5 (**f**) gastric digest obtained at pH 5.5 (**g**) gastroduodenal digest

Fig. 2b-g. The profiles of gastric digests obtained at pH 4.5 and 5.5 were similar to those obtained for non-digested QPC. This result is in agreement with those found by SDS-PAGE, indicating that no hydrolysis of quinoa proteins by pepsin was appreciable at high incubation pH. However, at lower pH, proteins were hydrolysed by pepsin, and profiles of fractions F2, F3, and F4 notably changed. Mainly, an increase in the intensity of the peaks contained in F3 and F4 was observed in comparison with the intensity of non-digested QPC fractions. The chromatogram obtained at the end of simulated gastrointestinal digestion showed that whereas F1 was conserved, new peaks corresponding to small and hydrophilic peptides appeared after the action of pancreatin (Fig. 2g).

Antioxidant Activity of Quinoa Protein Digests

The cytotoxicity of QPC digests was evaluated using the *in vivo* zebrafish larvae model. After 48 h of exposure to different concentrations (5.0, 2.5, and 1.25 mg/mL) of digests, the zebrafish larvae exhibited a similar physiology than the larvae control (non-treated) (Fig. 3a). These results indicate that the digests did not show cytotoxic effects. Then, the zebrafish larvae model was used to evaluate the *in vivo* lipid peroxidation inhibitory capacity of QPC digests as it had been previously used for peptides derived from hen egg-white lysozyme [12]. This capacity was determined as the protective effect of hydrolysates against lipid peroxidation induced by hydrogen peroxide in the animal model. The inhibition percentages for the gastric and gastroduodenal digests are shown in Fig. 3b. Among gastric digests, those obtained at lower pH showed higher inhibitory capacity than those obtained at high pH. The inhibition percentage of the digest obtained at pH 1.2 was of 75.15% while the percentage of digest at pH 5.5 was of 16.08%. This fact could be due to the highest hydrolysis of quinoa proteins observed when pepsin acted at its optimum pH, resulting in the release of high number of peptides with a potent ability to inhibit lipid peroxidation. These peptides could be further hydrolysed by pancreatin releasing new fragments with higher antioxidant activity. Thus, the hydrolysate obtained at the end of the digestive process showed the strongest inhibitory capacity with a percentage of inhibition of 82.10%, similar to the positive control (BHT) used in the assay (87.13%).

Previous studies have reported the ability of peptides derived from different food sources to inhibit lipid peroxidation. As an example, Chen et al. [26] reported this ability for fish and shellfish protein hydrolysates [26]. Even though the exact mechanism of peptides to act as antioxidant is not clearly known, the size and the presence of some aromatic amino acids and histidine are reported to play a vital role in this activity [12, 27]. It is expected that hydrophobic peptides can protect linoleic acid easily by donating photons to hydrophobic peroxyl radicals. On the other hand, Mendis et al. [28] have described the ability of hydrolysates derived from jumbo squid (*Dosidicus gigas*) to inhibit linoleic acid peroxidation in an emulsified model system [28]. The fraction lower than 3 kDa obtained by ultrafiltration showed an inhibition percentage of 82.0% indicating that small peptides released during hydrolysis were the main responsible for the observed effects. Sequences Phe-Asp-Ser-Gly-Pro-Ala-Gly-Val-Leu and Asn-Gly-Pro-Leu-Gln-Ala-Gly-Gln-Pro-Gly-Glu-Arg were identified as the major contributors of the antioxidant activity, with inhibition percentages of 66 and 72%, respectively [28]. The presence of amino acids Phe and Leu has been suggested important to exert the antioxidant effects. Quinoa proteins are known to be rich in amino acids Leu, Hys, Phe and Arg, thus peptides containing these amino acids might be released during digestive processes and exert antioxidant activity through inhibiting lipid peroxidation [29]. Leu and Arg were also present in antioxidant sequences reported by Carrillo et al. [12] obtained from hen egg lysozyme hydrolysate by pepsin [12].

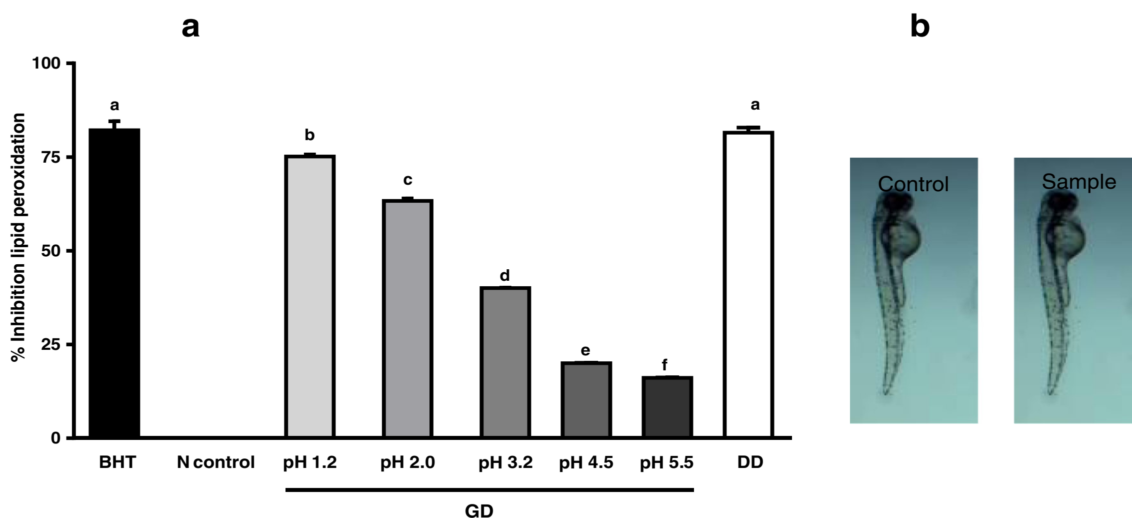

Fig. 3 **a** TBARS activity. BHT: positive control (BHT 0.1 mg/mL); N control: negative control (water); GD: gastric digests obtained at pH 1.2, 2.0, 3.2, 4.5, and 5.5 (concentration of 2.0 mg/mL). DD: gastroduodenal digest (concentration of 2.0 mg/mL). **b** Effect on cell morphology of zebrafish larvae. Zebrafish were incubated for 48 h with 5.0 mg/mL gastroduodenal digest (sample) in comparison with control. Magnification 40× of stereoscopic microscopy

Conclusion

In summary, results of this study demonstrate, for the first time, that quinoa protein hydrolysates obtained during an *in vitro* simulated gastrointestinal digestion are capable of inhibiting lipid peroxidation in the *in vivo* zebrafish model. Therefore, quinoa is a promising source of antioxidant peptides after its gastrointestinal digestion that could exert a preventive effect against oxidative stress-associated disorders. Moreover, quinoa derived hydrolysates might be used as natural additive and/or preservative in foodstuff to control the lipid oxidation responsible for deterioration of food during production and storage. Further studies would be needed to identify the peptides responsible for the antioxidant activity, and to study in depth the *in vivo* mechanisms of action.

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Compliance with Ethical Standards All animal work conformed to ethical guidlines and was approved by relevant local animal ethics committees.

Conflict of Interest The authors declare that they have no conflict of interest.

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