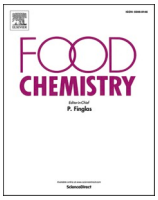
Food Chemistry 439 (2024) 138124

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



Sunflower seed-derived bioactive peptides show antioxidant and anti-inflammatory activity: From *in silico* simulation to the animal model

Federica Tonolo [a,b,1], Sara Coletta [c,1], Federico Fiorese [a], Alessandro Grinzato [d], Marica Albanesi [e,f], Alessandra Folda [a], Stefania Ferro [a], Agnese De Mario [a], Ilaria Piazza [a], Cristina Mammucari [a], Giorgio Arrigoni [a], Oriano Marin [a], Giulia Cestonaro [g], Luigi Nataloni [h], Enrico Costanzo [g], Claudia Lodovichi [e,f,i], Maria Pia Rigobello [a,*], Marina de Bernard [c]

[a] *Department of Biomedical Sciences, Via Ugo Bassi 58/B, 35131 Padova, Italy*
[b] *Department of Compared Biomedicine and Food Science, Viale dell'Università 16, 35020 Legnaro, PD, Italy*
[c] *Department of Biology, Via Ugo Bassi 58/B, 35131 Padova, Italy*
[d] *ESRF: European Synchrotron Radiation Facility, 71 Avenue des Martyrs, 38000 Grenoble, France*
[e] *Veneto Institute of Molecular Medicine (VIMM), Padova, Italy*
[f] *Padova Neuroscience Center, Università degli Studi di Padova, Padova, Italy*
[g] *Cereal Docks S.p.A. - Research & Innovation Department, Via Innovazione 1, 36043 Camisano Vicentino, VI, Italy*
[h] *Cereal Docks S.p.A, Via Innovazione 1, Camisano Vicentino, VI 36043, Italy*
[i] *Institute of Neuroscience, Consiglio Nazionale delle Ricerche (CNR), Padova, Italy*

ARTICLE INFO

Keywords:
Sunflower
Antioxidants and anti-inflammatory molecules
Bioactive peptides
Circular economy
Keap1/Nrf2

ABSTRACT

The evolving field of food technology is increasingly dedicated to developing functional foods. This study explored bioactive peptides from sunflower protein isolate (SPI), obtained from defatted flour, a by-product of the oil processing industry. SPI underwent simulated gastrointestinal digestion and the obtained peptide-enriched fraction (PEF) showed antioxidant properties *in vivo*, in zebrafish. Among the peptides present in PEF identified by mass spectrometry analysis, we selected those with antioxidant properties by *in silico* evaluation, considering their capability to interact with Keap1, key protein in the regulation of antioxidant response. The selected peptides were synthesized and evaluated in a cellular model. As a result, DVAMPVPK, VETGVIKPG, TTHTNPPPEAE, LTHPQHQQQGPSTG and PADVTPEEKPEV activated Keap1/Nrf2 pathway leading to Antioxidant Response Element-regulated enzymes upregulation. Since the crosstalk between Nrf2 and NF-κB is well known, the potential anti-inflammatory activity of the peptides was assessed and principally PADVTPEEKPEV showed good features both as antioxidant and anti-inflammatory molecule.

1. Introduction

Food demand is increasing worldwide, driven by population growth and longer lifespans. In this context, plant-derived proteins have gained significant attention as a growing class of food ingredients with a wide range of potential applications. This global trend is associated with a lower environmental impact compared to animal-based proteins and the increasing concerns of consumers about health issues related to the high consumption of animal products, such as obesity and cardiovascular disease (Leeming, 2021; Schiermeier, 2019).

Sunflower (*Helianthus annuus L.*) seeds are achieving interest as a protein source because of their high protein content, well-balanced amino acid profile, and low environmental impact (Hadidi et al., 2024). Sunflower seeds, mainly owing to their polyphenol activity, were found to possess significant antioxidant capacity (Guo et al., 2017; Karamać et al., 2012). Additionally, this property is regulated by the activity of antioxidant enzymes such as superoxide dismutase (SOD), guaiacol peroxidase, and catalase (De'Nobili et al., 2021; Guo et al., 2017). Defatted sunflower flour is a by-product of oil extraction from seeds usually used as animal feed. However, they represent an abundant and cost-effective source of proteins for human consumption. The two most abundant storage proteins in sunflower seed are 11S globulins

* Corresponding author.
E-mail address: mariapia.rigobello@unipd.it (M.P. Rigobello).
[1] Equality contribution as first author.

https://doi.org/10.1016/j.foodchem.2023.138124
Received 17 August 2023; Received in revised form 9 November 2023; Accepted 29 November 2023
Available online 2 December 2023

(60–80 %) and 2S albumins (20–30 %).

To create value-added molecules from food by-products, researchers have shifted their focus toward repurposing bioactive molecules, such as peptides, exploiting them in the production of new foods (Hadidi et al., 2024). Bioactive peptides have attracted significant attention in the last decade due to their beneficial effects on the well-being of organisms (Zaky et al., 2022). Typically composed of 2–20 amino acid residues, bioactive peptides can originate from animal sources (milk, eggs and meat) or plant sources such as soybeans or cereals (Manzoor et al., 2022; Zaky et al., 2022). Enzymatic hydrolysis, fermentation, food processing, and gastrointestinal digestion are the main mechanisms by which bioactive peptides can be generated (Manzoor et al., 2022; Zaky et al., 2022). Bioactive peptides are able to enter the cell where they carry out their biological functions as extensively reported in the literature (Akbarian et al., 2022; D'Opazo et al., 2023). These peptides, inactive until they are integral parts of native proteins, can exert various functions, including antihypertensive, anti-inflammatory, anticancer, antimicrobial, immunomodulatory, and antioxidant properties (Duffuler et al., 2022; Manzoor et al., 2022; Zaky et al., 2022). Antioxidant peptides can act directly as free radical scavengers or can activate specific signaling pathways. Notably, Nuclear factor erythroid 2 related factor 2 (Nrf2) and Kelch-like ECH-associated protein 1 (Keap1) represent the main system that modulates the cellular antioxidant response. In basal conditions, Nrf2 is usually sequestered by Keap1 in the cytosol and degraded by the proteasome. Once Keap1 is modified by electrophiles, oxidants, or other stimuli, Nrf2 migrates to the nucleus interacting with a specific promoter called antioxidant response element (ARE). Nrf2-ARE complex modulates the expression of antioxidant and phase II enzymes, such as heme-oxygenase 1 (HO-1), thioredoxin reductase (TrxR), or NADPH quinone oxydoreductase 1 (NQO1) (Baird & Dinkova-Kostova, 2011).

Sunflower seeds are widely used for oil extraction, yet their potential as a protein source for human consumption remains largely untapped. In this study, we introduce an innovative method developed by Cereal Docks S.p.A for obtaining a sunflower seed protein isolate (SPI) that demonstrates promising health benefits for humans. This effect is attributed to the biological activity of specific peptides, known as potential ingredients in functional foods (Manzoor et al., 2022; Rivero-Pino, 2023).

2. Materials and methods

2.1. Extraction of proteins from sunflower seeds

The sunflower protein isolate (SPI) was provided by Cereal Docks S.p.A. (Camisano Vicentino VI, Italy). Different varieties of sunflower (*Helianthus annuus L.*) seeds, with varying oil content (total fat content between 40 and 50 %), were used as starting material. The seeds showed a black, thin hull adhering to the kernel and displayed the following average chemical composition, characterized according to official EU guidelines: moisture 5.7 %, total fats 44.6 %, protein 16.2 %, and crude fiber 22.6 %. To obtain SPI, a proprietary process developed by Cereal Docks S.p.A. (Italian patent application number IT102022000016812) was used. Briefly, the seeds were cooled, then milled and defatted using propane. Subsequently, dehulled seeds were kept at 3 °C and microsliced in particles in between 600 and 800 μm (Urschel Lab, Chesterton, IN, USA), and then propane was used for oil extraction at a pressure higher than 4.5 atm, in a temperature range between 20 °C and 35 °C (Exalt R&D, LLC, USA). After defatting, the sunflower flour appeared white and displayed the following chemical composition: moisture 9.1 %, total fats 9.8 %, protein 45.6 %, crude fiber 12.4 %. Next, the defatted flour was processed with wet extraction that relies on the solubility properties and isoelectric point of the proteins and involves various steps. First, the defatted sunflower seed flour was solubilized in water, at pH 9 in the presence of sodium sulfite (≤5% w/w), centrifuged and the supernatant was discarded. The solid residue was resuspended in water, centrifuged again and at the end, the supernatant was collected. After, the proteins of the supernatants were precipitated at pH 4.25 and neutralized. The solution was pasteurized and subjected to spray drying, obtaining a protein isolate (moisture 1.9 %, proteins 84.4 %, fats 10.7 % and 0.4 % crude fiber). The protein recovery, expressed as isolate proteins/flour proteins, was 63.9 %, with a process yield, expressed as isolate weight/flour weight, of 36.8 %.

2.2. Simulation of the gastrointestinal digestion of SPI and production of a peptide enriched fraction (PEF)

SPI underwent simulated gastrointestinal digestion following the protocol described by Brodkorb et al. (2019), with modifications by Tonolo, Moretto, et al. (2020). Briefly, freshly prepared simulated gastrointestinal fluids were used to mimic the physiological conditions of human digestion. The digestion was carried out by three sequential phases. In the oral phase, 0.5 g of SPI were mixed with 4 mL of the simulated salivary fluid, SSF (15.1 mM KCl, 3.7 mM KH_2PO_4, 13.6 mM $NaHCO_3$, 0.15 mM $MgCl_2(H_2O)_6$ and 0.06 mM $(NH_4)_2CO_3$) and 1.5 mM $CaCl_2(H_2O)_2$. After reaching pH 7.0, water was added to a final volume of 20 mL and the mixture was incubated at 37 °C for 2 min in an orbital shaker. Subsequently, the obtained oral bolus was added with 16 mL of simulated gastric fluid, SGF (6.9 mM KCl, 0.9 mM KH_2PO_4, 25 mM $NaHCO_3$, 47.2 mM NaCl, 0.1 mM $MgCl_2(H_2O)_6$ and 0.5 mM $(NH_4)_2CO_3$ and 0.15 mM of $CaCl_2(H_2O)_2$) and 1 mL of pepsin solution (1:20 enzyme to substrate ratio). The pH was lowered to 2.5 with 6 M HCl and the volume was brought to 40 mL with water. After 90 min at 37 °C at 150 rpm, gastric chyme was mixed with 17 mL of simulated intestinal fluid SIF (6.8 mM KCl, 0.8 mM KH_2PO4, 85 mM $NaHCO_3$, 38.4 mM NaCl, 0.33 mM $MgCl_2(H_2O)_6$), 0.6 mM of $CaCl_2(H_2O)_2$) and pancreatin stock solution (1:20). After the neutralization, the mixture was incubated for 2 h at 37 °C at 150 rpm. At the end, digestive enzymes were inactivated by heating the digested SPI at 85 °C for 15 min. Samples were maintained at −20 °C until extraction of peptides. Using the solid-phase extraction technique outlined by Tonolo, Fiorese, et al. (2020), two fractions enriched with peptides from the digested SPI were obtained. The fraction eluted with 5–30 % acetonitrile (ACN), referred to as PEF throughout the text, was selected for further analysis due to its high peptide content.

2.3. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis and peptide identification

PEF was dissolved with 3 % ACN/0.1 % formic acid (FA) and subjected to LC-MS/MS analysis using a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), connected with a nano-HPLC Ultimate 3000 (Dionex–Thermo Fisher Scientific, Waltham, MA, USA) (Aebersold & Mann, 2003; Han et al., 2008; Heck & Krijgsveld, 2004). Briefly, the solution was loaded onto a 10 cm homemade pico-frit chromatographic column (75 μm internal diameter, 15 μm tip, New Objective, Littleton, MA, USA) packed with C18 material (Aeris Peptide 3.6 μm XB-C18, Phenomenex, Torrance, CA, USA). Peptides were separated with a gradient of ACN/0.1 % FA increasing linearly from 3 % to 40 % in 20 min. A data-dependent acquisition (DDA) method was applied. Raw data files were analyzed with the Proteome Discoverer software (version 1.4, Thermo Fisher Scientific, Waltham, MA, USA) connected to a Mascot Search engine (version 2.2.4, Matrix Science, London, UK) against the UniProt database (Sunflower section, version October 202184477 sequences) concatenated with a database of common contaminants found in proteomics experiments. For the unrestricted search (no enzyme), peptide and fragment tolerance were set at 10 ppm and 0.6 Da, respectively. All identified peptides were reported in Supplementary Data section.

2.4. Molecular docking analysis

The identified peptides were screened using an *in silico* molecular docking approach to identify those that may have antioxidant effects. The Kelch domain of Keap1, which binds to a conserved DxETGE motif located in the Neh2 domain of Nrf2 (PDB 2FLU), was chosen as target for the docking analysis (Lo et al., 2006). The Nrf2 peptide present in the crystal structure was deleted, and the resulting model was equilibrated and protonated in preparation for molecular docking. The peptide-protein interaction was assessed using two different docking methods, CABS-Dock and GalaxyPepDock, simultaneously (Kurcinski et al., 2015; Lee et al., 2015). According to the docking protocols used, the procedure is unbiased regarding the pre-selection of the possible binding site. However, in agreement with the internal binding score of each software, the peptides that bound with the Neh2 binding site of Nrf2 were preferred. The docking results were then analyzed using PISA (Proteins, Interfaces, Structures and Assemblies), and the peptides were scored based on their binding affinity and the quality of the docking prediction (Dominguez et al., 2003; Tonolo, Moretto, et al., 2020).

2.5. Peptide synthesis

Starting from the list of peptide sequences identified by nLC-MS/MS, the selection of the peptides was based on the highest scores obtained from the molecular docking analysis and on their length. Eight peptides, chosen from the list reported in Table S1, were synthesized employing a solid-phase technique performed on a fully automated peptide synthesizer (Syro II, MultiSynTechGmbh), as previously reported in Tonolo, Folda, et al. (2020).

They were **D-8-K** (DVAMPVPK) from translation elongation factor 1, **V-9-G** (VETGVIKPG) from elongation factor 1 alpha (Fragment), **I-10-A** (IIVDDVNNPA) from Putative 11-Seed storage protein, plant, RmlC-like jelly roll fold protein, **A-10-D** (ANINDASRPD) from Cruciferina-like protein, **Q-11-T** (QGIMLPGCPET) from Putative glutelin type-B 1, **T-11-E** (TTHTNPPPEAE) from Putative temperature-induced lipocalin, **P-12-V** (PADVTPEEKPEV) from Putative histone H5 and **L-14-G** (LTHPQHQQQGPSTG) from Oleosin. All the synthesized peptides belonged to reference proteins of *Helianthus annuus l.* proteome.

2.6. In vivo model: Evaluation of the effects of PEF on the capacity of zebrafish larvae to cope with a stress condition

Zebrafish larvae were raised in fish-water at 28 °C until 5–6 d post fertilization (dpf) on a 12 h light /12 h dark cycle using standard procedures. On the day of the experiment, the larvae were divided into two groups: a control group maintained at 28 °C and a group placed in 10 °C water for 5 min. The latter group was further divided into 3 experimental groups: an acute group tested immediately after the cold stress, and two recovery groups tested after a 1-h recovery period. One of the two recovery groups received PEF treatment at a final concentration of 0.05 mg/mL in the cold water. For each test, a standard 24-well plate was utilized with one larva placed in each well. Each plate contained different experimental groups. The test was conducted using a DanioVision system (Noldus, Wageningen, the Netherlands). The protocol included an initial phase of light lasting 10 min, followed by alternating periods of light and darkness, each lasting 10 min. Python was used to preprocess the DanioVision datasets. Kernel Density estimation for the fish position probability was estimated using Scipy software (version 1.9.3, Virtanen et al., 2020).

2.7. Evaluation of bioactive peptides effects on Caco-2 cells

For cellular tests, Caco-2 cells (human colon-rectal adenocarcinoma) were cultured in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10 % FBS, at 37 °C with 5 % CO_2 atmosphere.

2.7.1. MTT assay

Caco-2 cells (1×10^4) were seeded in a 96-well plate and treated with either PEF or synthetic peptides (0.05 mg/mL) to assess cell viability (Tonolo et al., 2018; van Meerloo et al., 2011). After the treatment, the medium was removed, and 0.1 mL of MTT solution (0.5 mg/mL) in PBS (1×) was added. The plate was then incubated in the dark at 37 °C and 5 % CO_2 for 3 h. The MTT solution was then discarded, and 0.1 mL of a solution containing isopropanol and DMSO in a ratio of 9:1 was added to stop the reaction. The plate was further incubated for 15 min at 37 °C in the dark. The absorbance was followed ($Abs_{595-690}$) using a plate reader (Tecan Infinite® M200 PRO, Männedorf, Switzerland). Where specified, oxidative stress was induced by treating the cells with 250 μM *tert*-butyl hydroperoxide (TbOOH) for 18 h after the pre-treatment with peptides or PEF.

2.7.2. Estimation of ROS levels in Caco-2 cells

ROS levels in Caco-2 cells were measured using 5-(and 6)-chloromethyl-2′,7′-dichlorohydrofluorescein diacetate (CM-H2DCFDA, Molecular Probes, Thermo Fisher Scientific, Waltham, MA, USA) (Tonolo, Folda, et al., 2020). Caco-2 cells (1×10^4) were seeded in a 96-well plate and treated with synthetic peptides (0.05 mg/mL) for 24 h. The cells were then rinsed with 0.1 mL of PBS (1×) containing 10 mM glucose and incubated with 10 μM of the CM-H2DCFDA probe. After 20 min in the dark, the probe was removed, and the cells were washed as mentioned above. To induce oxidative stress conditions, 250 μM of TbOOH was used. The increase in fluorescence was measured using a TECAN plate reader at 485 nm (excitation wavelength) and 527 nm (emission wavelength) over a period of 90 min. The reported results are expressed as the percentage of the fluorescence at 90 min respect to the values observed in untreated group (Cnt).

2.7.3. Lipid peroxidation assay

Lipid peroxidation was assessed following the method described by Tonolo, Folda, et al. (2020). Caco-2 cells (5×10^5) were seeded in a 6-well plate in the presence of peptides (0.05 mg/mL). After 24 h, 250 μM TbOOH was added to induce oxidative stress for a duration of 1 h and 30 min. Cells were then washed with 1 mL of PBS (1×) and treated with a mixture of 1 mL of 0.1 N H_2SO_4 and 150 μL of 10 % phosphotungstic acid. Samples were centrifuged at 15,600 g for 10 min and this process was repeated twice. The resulting dry pellets were dissolved in a solution containing 0.35 % IGEPAL, 0.014 % BHT, and 0.23 % thiobarbituric acid in a H_2O/acetic acid mixture (1:1) in a final volume of 0.25 mL. Samples were then incubated at 95 °C for 60 min. Subsequently, the cooled samples were centrifuged at 15,600 g for 10 min. Supernatants were treated with 400 μL of *n*-butanol, vigorously mixed, and centrifuged again for 15 min. The upper phase containing the thiobarbituric acid reactive substances (TBARS), which were by-products of lipid peroxidation, was measured fluorimetrically at 530 nm (excitation wavelength) and 590 nm (emission wavelength) using a TECAN plate reader.

2.7.4. Preparation of nuclear fraction

Caco-2 cells were seeded in T25 flasks and treated with peptides at a concentration of 0.05 mg/mL for 24 h (Tonolo et al., 2022; Tonolo, Fiorese, et al., 2020). To obtain nuclear and cytosolic fractions, the following procedure was performed: cells were lysed on ice for 15 min using 100 μL of a solution containing 10 mM Hepes/Tris pH 7.9, 0.1 mM EGTA, 0.1 mM EDTA, 0.1 mM PMSF, 10 mM KCl, 1 mM NaF, and a protease inhibitor cocktail (Merck, Darmstadt, Germany). Subsequently, IGEPAL was added to the final concentration of 5 %, and the samples were mixed for 15 *sec* before being centrifuged at 1,000 g for 10 min at 4 °C. The resulting pellet, representing the nuclear fraction, was then dissolved in a solution containing 20 mM Hepes/Tris pH 7.9, 1 mM EGTA, 1 mM EDTA, 0.4 M NaCl, 0.1 mM PMSF, 1 mM NaF, and protease inhibitors. The mixture was mixed every 2 min for 10–15 *sec*. Samples were centrifuged at 20,000 g for 10 min at 4 °C. Nuclear proteins (30 μg) were loaded onto a 10 % SDS-PAGE and subjected to Western blot

analysis to determine the levels of Nrf2, NF-κB and IκB with PCNA and GAPDH being used as the loading control (Tonolo et al., 2022; Tonolo, Fiorese, et al., 2020). Densitometry evaluation was performed using the Nine Alliance software (Mini 9 17.01 version, Uvitec Alliance, Cambridge, UK).

2.7.5. RNA extraction and qRT-PCR

To evaluate the anti-inflammatory effect of bioactive peptides, Caco-2 cells were plated (4.5×10^5 per well in a 6-well plate) and exposed to a concentration of 0.05 mg/mL for each peptide. After 22 h, the cells were stimulated with 2 ng/mL of TNF-α for additional 2 h. Total RNA was extracted using TRIzol reagent (ThermoFisher, Waltham, MA, USA) following the manufacturer's protocol. Total RNA (1 μg) was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA), according to the manufacturer's instructions. The obtained cDNA (5 μg) was used in a qRT-PCR reaction with PowerUp SYBR Green (Applied Biosystems). Data for each sample were normalized to the endogenous reference gene β-actin. Primers used were as follows: β-actin forward 5′-TGA-GATGCGTTGTTACAGGA-3′, reverse 5′-ACGAAAGCAATGCTATCA-3′; IL-6 forward 5′-AACCTGAACCTTCCAAAGATGG-3′, reverse 5′-TCTGGCTTGTTCCTCACTACT-3′; IL-8 forward 5′-TTGGCAGCCTTCCT-GATT-3′, reverse 5′-AACTTCTCCACAACCCTCTG-3′; TNF-α forward 5′-ATGAGCACTGAAAGCATGATCC-3′, reverse 5′-GAGGGCTGATTAGA-GAGAGGTC-3′. Data analysis was performed using the ΔΔCt method.

2.7.6. Oxygen consumption rate (OCR) measurements

For OCR measurements, cells were plated in XF24-well cell culture microplates (Agilent, Santa Clara, CA, USA). After 24 h, cell medium was replaced with 2 % FBS DMEM in the presence of PEF-derived bioactive peptides (0.05 mg/mL). After 90 min, medium was replaced with DMEM (Sigma Aldrich), supplemented with 5 mM glucose, 33 mM NaCl, 25 mM HEPES, in the presence of SPI-derived bioactive peptides (0.05 mg/mL). Then, 30 min later OCR measurements were performed. Thus, total peptide incubation lasted 2 h. OCR was assessed in realtime with the XF24 Extracellular Flux Analyzer (Agilent). A titration was performed to utilize the concentration of carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (CCCP, 1 μM) that maximally increases OCR. OCR was measured in basal condition and following the addition of 2 μM oligomycin, to assess ATP-linked respiration, 1 μM CCCP, to assess maximal respiration, and 1 μM rotenone/1 μM antimycin A to assess non-mitochondrial respiration. The OCR was normalized for the protein content.

2.8. Statistical analysis

Values were indicated as mean ± SD of at least 3 independent experiments. The analysis of variance was performed with multiple comparison test through the Tukey-Kramer method. OriginPro software (OriginLab Corporation, Northampton, MA, USA) was utilized for the analysis. Only differences with $p < 0.05$ were considered significant.

3. Results and discussions

3.1. Sunflower PEF ameliorates the capacity of zebrafish larvae to cope with a stress condition

With the intention of assessing the bioactive potential of the sunflower peptide enriched fraction (PEF), we initially examined its beneficial effect on a model animal's well-being, with the final goal of identifying the peptides responsible for this outcome and characterizing their molecular mechanism. To explore the potential of PEF in alleviating the consequences induced by cold exposure, we chose to study the thermal responses in zebrafish (*Danio rerio*), a suitable model for this purpose (Chou et al., 2008; Reid et al., 2022; Tseng et al., 2011; Wu et al., 2015). Indeed, thermal stress can have adverse effects on animal well-being and health and various evidence indicate that cold stress induces mitochondrial metabolic disorders and increases production of ROS, which, in turn, may result in oxidative stress, and inflammation in the brain and in several other tissues (Donaldson et al., 2008; Lu et al., 2019; Reid et al., 2022; Tseng et al., 2011). Zebrafish larvae (5–6 dpf) were immersed in cold water (10 °C) and the motor behavior was assessed by an automatic device (DanioVision) which tracks the motility of the larvae (Fig. 1A). In agreement with previous studies (Bai et al., 2016; Reid et al., 2022), we found that larvae exposed to cold water exhibited statistically significant decrase in motility, measured as average distance travelled and reduced average speed in respect to controls (which were immersed in water in physiological conditions, about 28 °C), as shown in Fig. 1. These results agree with the metabolic alterations induced by cold temperature, which causes reduced availability of oxygen and energy required for proper motor activity. To assess the effect of PEF on larvae motility upon cold exposure, a second group of larvae was immersed in cold water in presence of PEF, while the control group was immersed in cold water without PEF. Both groups were then placed at 28 °C for about 1 h. As reported in Fig. 1 (C and D), which shows the motility of the larvae and the statistics related to the different experimental conditions, the group of zebrafish larvae exposed to cold water + PEF recovered completely the motility, travelling for an average distance and at a speed which were similar to controls (larvae maintained at 28 °C). On the contrary, the larvae immersed in cold water without PEF exhibited only a small increase in the average distance and speed with respect to controls (see the video in SM showing representative larva's motility in each experimental condition). Excessive oxidative stress and altered energy metabolism are assessed as the primary reasons for fish mortality at low temperatures, while reduced oxidative stress has been found to enhance tolerance to acute cold stress. Considering the effect of cold temperature on oxidative stress and metabolism, as reported by Kyprianou et al. (2010), along with our findings, we deduced that the positive impact of PEF on cold-exposed animals stemmed from its antioxidant and anti-inflammatory properties. In accordance, Caco-2 cells treated with PEF exhibited enhanced resistance to the oxidative stress induced by TbOOH (Fig. S1).

3.2. Identification and molecular docking analysis of bioactive peptides present in sunflower PEF

Based on the evidence obtained *in vivo*, our investigation aimed to explore the presence of antioxidant peptides in PEF. The primary focus was on the Keap1/Nrf2 pathway, which plays a crucial role in the cellular antioxidant capacity (Tonolo et al., 2023; Tonolo, Fiorese, et al., 2020; Tonolo, Folda, et al., 2020). The sequences of peptides present in PEF were identified using nano liquid chromatography/tandem mass spectrometry. Several peptides from sunflower proteins were confidently identified (Supplementary Data) and their sequences are reported in Table S1. First, peptides were selected based on their length (less than 20 amino acids). Subsequently, to optimize the selection of peptides with antioxidant properties, a molecular docking approach was utilized to assess the activation of the Keap1/Nrf2 system. In our previous works, we demonstrated that this signaling cascade stimulates the expression of various antioxidant enzymes, and peptides derived from fermented milk have been found to exhibit antioxidant capacity through this pathway (Tonolo, Fiorese, et al., 2020; Tonolo, Folda, et al., 2020). The *in silico* screening for interaction of peptides with the Kelch domain of Keap1 was recently suggested as a new methodological approach to identify potential antioxidant peptides (Tonolo et al., 2023).

As shown in Table S2, a score was assigned to each selected peptide, indicating the probability of interaction with Keap1. The docking score is based on the specific amino acidic interaction, the calculated dissociation energy of the system, and the overall quality of the predicted complex. To facilitate readability, this probability is represented using a chromatic scale from green to red, where green indicates a higher probability and red indicates a lower probability (Table S2). Peptides **D-**

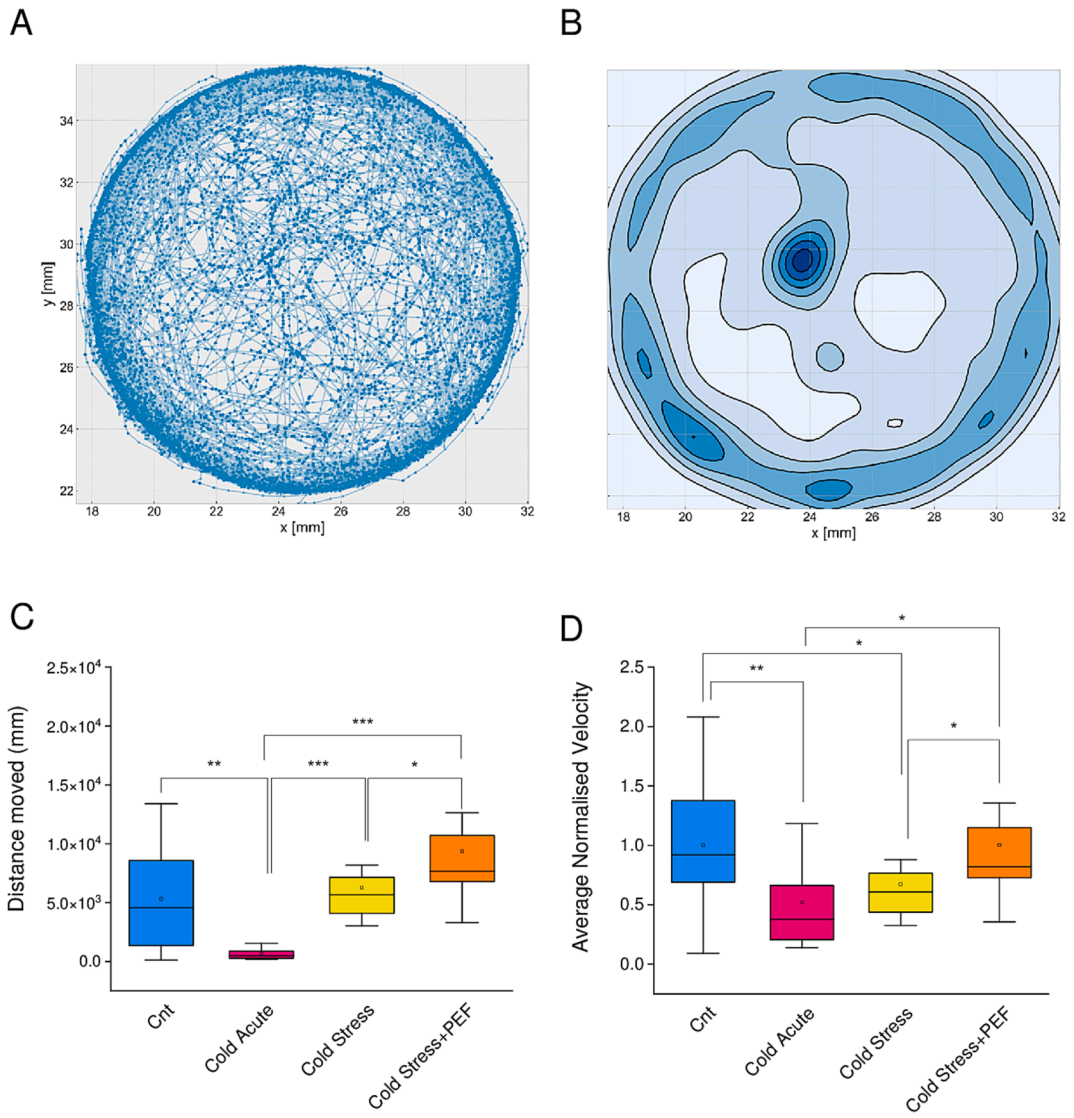


Fig. 1. Effects of PEF on zebrafish larvae under cold stress conditions. (A) Example of the movement traces of a zebrafish larva freely swimming in well filled with water at 28 °C (control); (B) Gaussian probability density estimation to find the larva in a certain point of the space (i.e. the well). Darker colors indicate higher probability. (C) Zebrafish larvae placed in cold water (10 °C) exhibited dramatically reduced motility, measured as average distance (red box) compared to control larvae immersed in normal temperature water (28 °C) (blue box). After acute cold stress, the addition of PEF to cold water favored complete recovery of larvae motility after 60 min at 28 °C (orange box) compared to larvae immersed in cold water without PEF (negative controls) (yellow box). (D) Cold water also significantly reduced the average swimming speed of zebrafish larvae (red box) compared to controls (blue box). Similarly to what is observed for motility, PEF rescued the average speed of zebrafish larvae compared to controls, at the end of the recovery period of 60 min at 28 °C (orange box). (n = 44, distributed as 16 controls, 12 acute cold, 8 cold stress and 8 cold stress + PEF), *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

8-K, **V-9-G, I-10-A, Q-11-T**, **T-11-E**, **P-12-V** and **L-14-G** were identified as the most likely interactors of Keap1 (Fig. 2). Consequently, these peptides were selected and synthesized for the subsequent evaluation of their antioxidant activity in a cell model. Moreover, we included an additional peptide (**A-10-D**) in our assays as a negative control owing to its negative score.

3.3. Antioxidant capacity of PEF peptides in Caco-2 cells

Peptides selected as described above, were tested for their capability to inhibit the interaction between Keap1 and Nrf2 in Caco-2 cells. The choice of a cell line that mimics intestinal cells was made because these cells are the first ones which encounter the ingested peptides and play a vital role in coordinating the immune response. First, these peptides, tested for cell viability, exhibited no cytotoxic effects (Fig. S2). Furthermore, to evaluate their antioxidant activity, cells were pre-treated with the peptides for 24 h and, after induction of oxidative stress with 250 μM TbOOH, ROS production was determined. In presence of peptides alone, ROS values were similar to the basal production (Fig. S3). However, as reported in Fig. 3A, when cells were pre-treated with **D-8-K**, **T-11-E**, **P-12-V** and **L-14-G** and then exposed to oxidative stress the amount of ROS production was significantly reduced, while pre-treatment with the other peptides did not show the ability to reduce induced oxidative stress. Subsequently, the antioxidant action of the peptides was confirmed by the lipid peroxidation assay. Caco-2 cells were pre-treated with the peptides for 24 h and, then 250 μM TbOOH was added to cause lipid peroxidation. As reported in Fig. 3B, especially **D-8-K**, **V-9-G**, **T-11-E**, **L-14-G,** and **P-12-V** were able to prevent the generated lipid peroxidation, bringing back to baseline values as in the absence of the peroxidative stimulus (the untreated control).

Therefore, we can conclude that the peptides selected with the *in silico docking* approach for their ability to interact with the Keap1

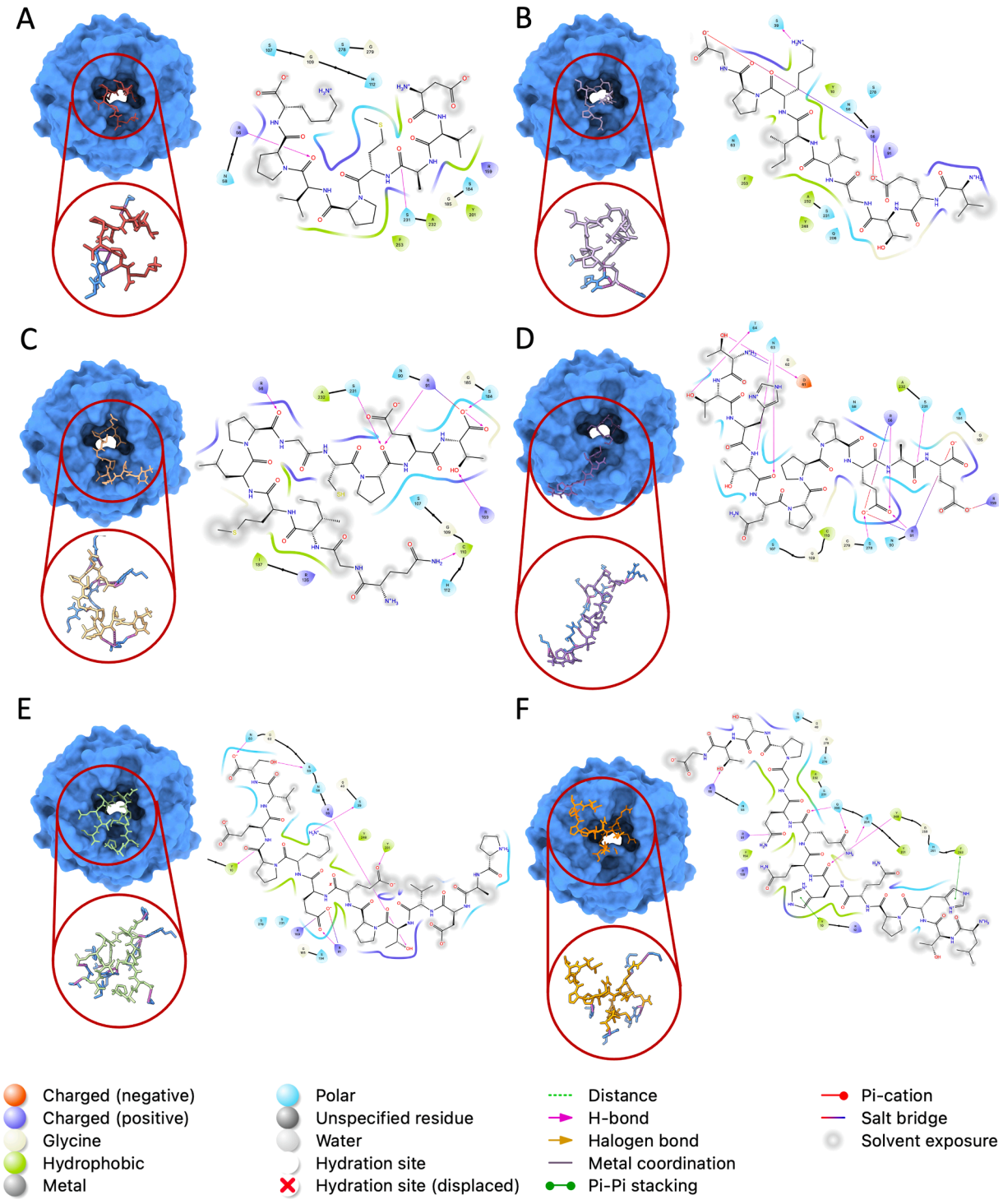


Fig. 2. Molecular docking analysis of the interaction between Keap1 and the peptides. Molecular docking analysis of the interaction between Keap1 and the following peptides: (A) D-8-K, (B) V-9-G, (C) Q-11-T, (D) T-11-E, (E) P-12-V, and (F) L-14-G. The analysis focuses on the interaction between the peptides and the Kelch domain of Keap1 (shown in light blue), which illustrates the binding geometry. Magnification highlights the H-bond interactions represented in red, and protein-peptide interaction diagrams are also provided. Charged negative: red; charged positive: blue; hydrophobic: light green; polar: light blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

domain, were capable of reducing induced oxidative stress in the cellular context. Notably, **A-10-D**, chosen for its inability to interact with the Kelch domain *in silico*, did not show a reduction in ROS production, nor was it able to prevent lipid peroxidation in Caco-2 cells.

3.4. PEF peptides activate the Keap1/Nrf2 pathway

To unravel the molecular mechanism by which sunflower PEF-derived bioactive peptides induced an antioxidant response in human cells, we extracted cell nuclear fractions from Caco-2 cells following treatment with the specific peptides and we quantified the content of Nrf2 as a readout of activation of the Keap1/Nrf2 pathway. Among the

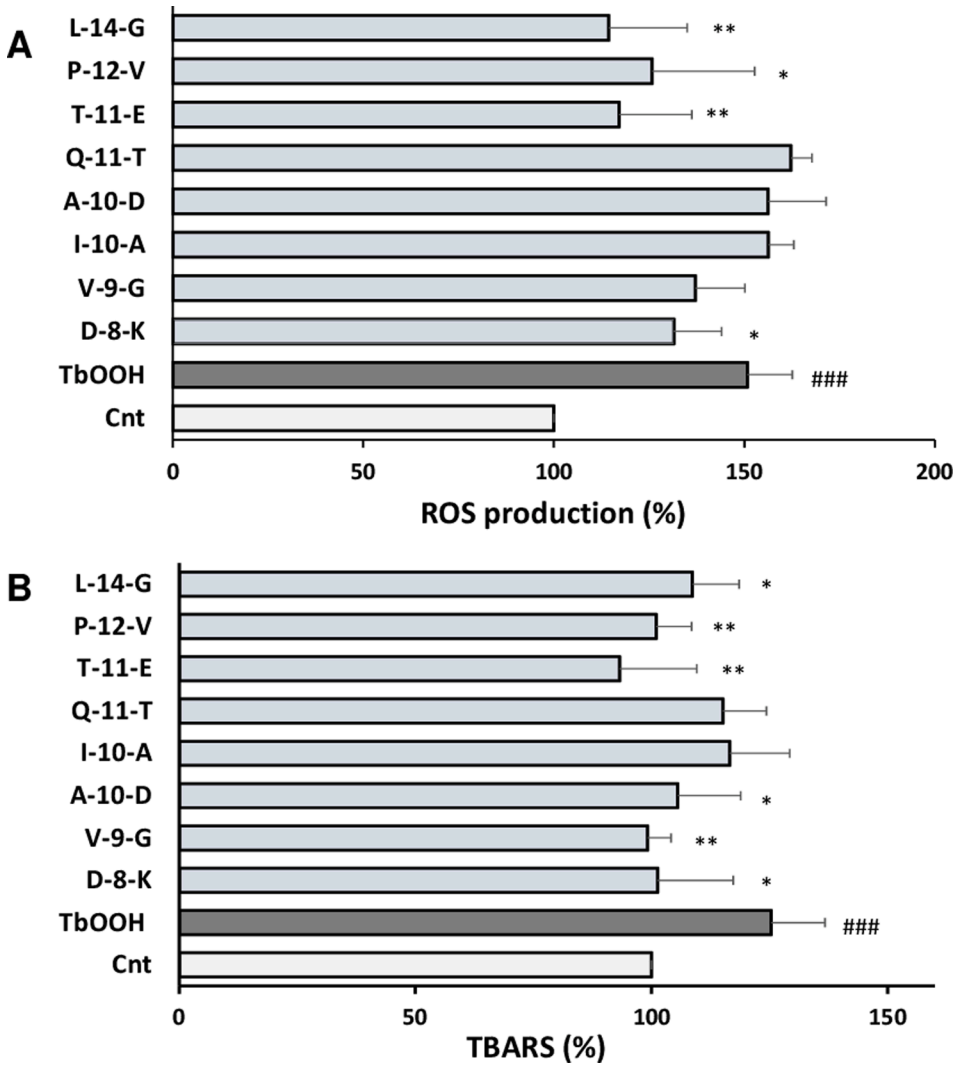


Fig. 3. Caco-2 cells treated with PEF peptides showed protection against oxidative stress. (A) ROS production was evaluated in Caco-2 cells pre-treated with the indicated peptides. In brief, cells were treated with peptides (0.05 mg/mL) for 24 h, then loaded with 20 μM CMH_2DCFDA. To induce oxidative stress, cells were subjected to TbOOH (250 μM) during the ROS detection (90 min). Data were expressed as a percentage of the values observed in nontreated group. (B) Lipid peroxidation evaluated by the production of thiobarbituric acid-reactive substances (TBARS). Caco-2 cells were pre-treated with peptides (0.05 mg/mL) for 24 h and oxidative stress was induced with 250 μM TbOOH. TBARS production was estimated fluorometrically and the data were expressed as a percentage of the values observed in nontreated group. ### $p < 0.001$ vs. nontreated group (Cnt). Means of at least 3 experiments were compared. ** $p < 0.01$, * $p < 0.05$ vs. treated group (TbOOH).

peptides examined, **D-8-K, T-11-E** and **P-12-V** facilitated the translocation of Nrf2 into the nucleus (Fig. 4, panel A). As expected, the peptide **A-10-D**, which was predicted to lack interaction with Keap1, induced no accumulation of Nrf2 in the nuclear fraction. After Nrf2 translocation into the nucleus, it interacts with the Antioxidant Response Elements (ARE) sequences, which promote the expression of phase II and antioxidant enzymes (Baird & Dinkova-Kostova, 2011). To verify if PEF peptides act through this pathway, we evaluate by RT-PCR the expression of ARE-regulated enzymes in Caco-2 cells treated with the 5 antioxidant peptides plus the negative control, for 24 h. As depicted in Fig. 4 (panels B-E), the peptides **V-9-G**, **P-12-V**, and **L-14-G** demonstrated the ability to enhance HMOX1 expression through the activation of the Nrf2 pathway (panel B). Among them, only **P-12-V** induced NQO1 expression (panel C). Both **V-9-G** and **P-12-V** were found to induce TXNRD1 (panel D). However, it should be noted that the impact on SOD1 expression by Nrf2 activation through the peptides was lower (panel E), as SOD1 is not the primary target gene of Nrf2 activation (Tonelli et al., 2018; Tonolo, Moretto, et al., 2020).

3.5. PEF bioactive peptides inhibit the expression of pro-inflammatory cytokines and the activation of NF-κB pathway

The anti-inflammatory role of Nrf2 is well-established (Wardyn et al., 2015). Recently, we have confirmed the crosstalk between Nrf2 and NF-κB, using milk and soy-derived bioactive peptides that showed, among antioxidant properties, anti-inflammatory effects (Tonolo et al., 2022). Therefore, we treated Caco-2 cells with peptides for 24 h, either alone or in the presence of TNF-α for the last 2 h. TNF-α is a potent inducer of pro-inflammatory cytokines and chemokines, such as IL-6, TNF-α, and IL-8. After treatment, cells were harvested, and mRNA was extracted and retrotranscribed into cDNA. The cDNA was then amplified using qRT-PCR with primers specific to the mentioned cytokines. As shown in Fig. 5 (panels A–C), some peptides affected the cytokine-induced upregulation of these genes in the presence of TNF-α. Among the six peptides tested, **P-12-V** demonstrated the most significant anti-inflammatory activity. In this regard, considering that Nrf2 inhibits the activation of the transcription factor NF-κB by preventing its translocation into the nucleus, and NF-κB induces the expression of various pro-inflammatory

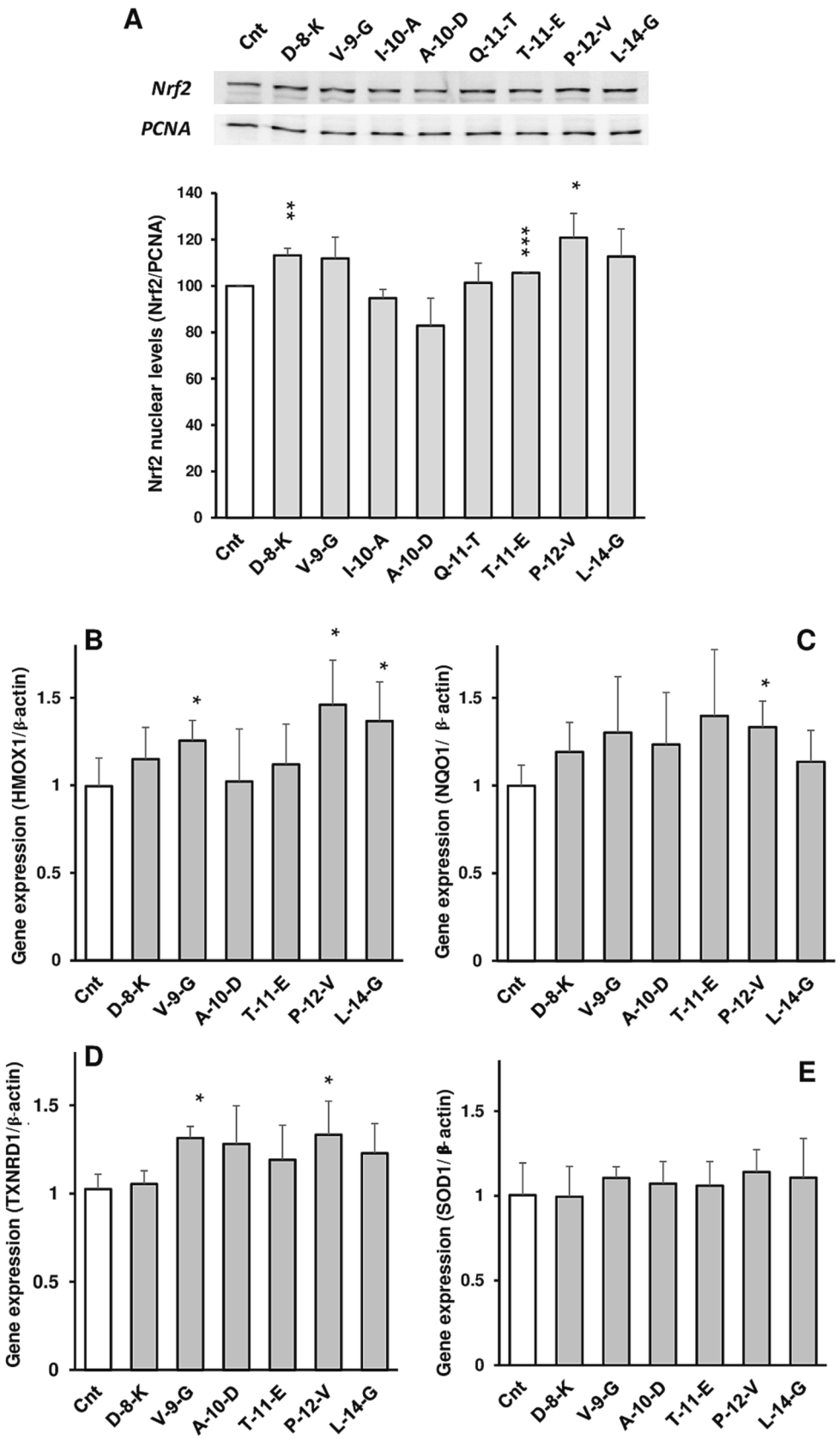


Fig. 4. Activation of Keap1/Nrf2 pathway in Caco-2 cells treated with the indicated peptides. (A) Nrf2 levels were estimated by Western blot detection in isolated nuclear fractions as indicated in Materials and methods. The densitometric analysis of three different experiments using PCNA as a loading control was reported. (B–E) The expression of antioxidant enzymes in Caco-2 cells treated with the indicated peptides was carried out by qRT-PCR. ARE-regulated enzymes: (B) HMOX1, (C) NQO1, (D) TXNRD1, and (E) SOD1 were reported and the data were normalized to an endogenous reference gene (β-actin). Means of at least 3 experiments were compared. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

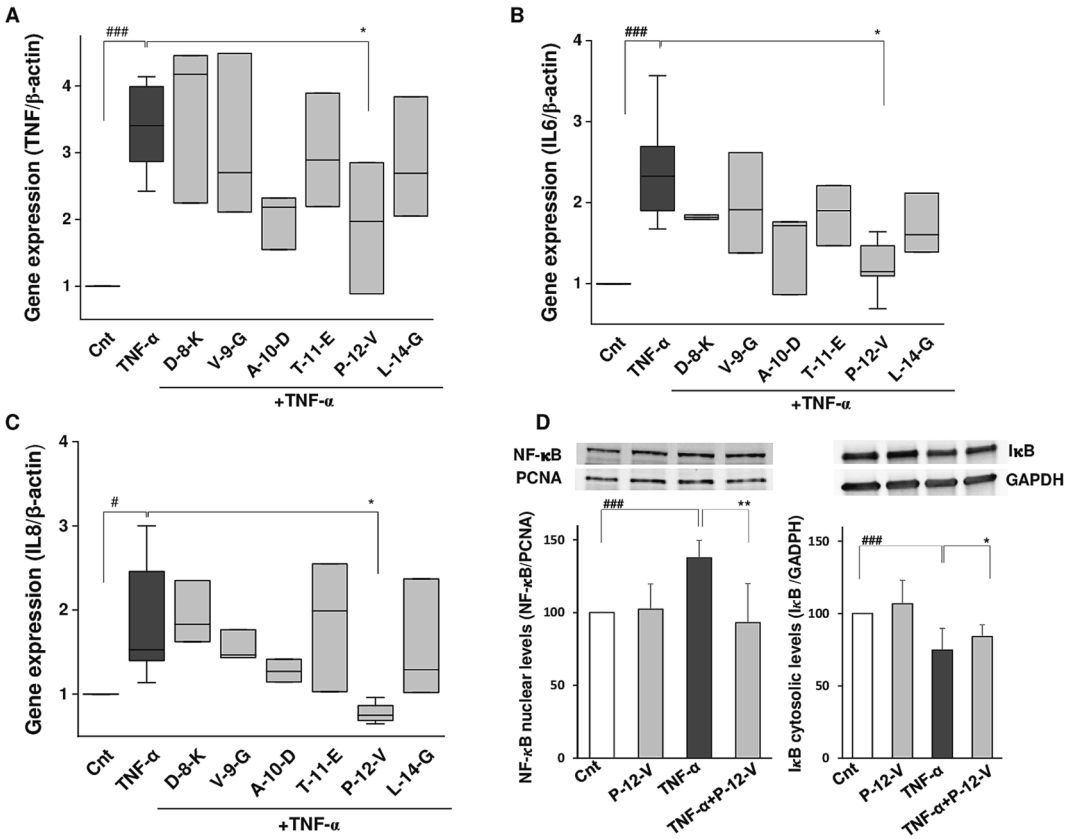


Fig. 5. Anti-inflammatory effects of bioactive peptides. (A-C) mRNA expression of IL-6, IL-8 and TNF-α in Caco-2 cells stimulated with bioactive peptides with or without 2 ng/mL TNF-α challenge. mRNA expression was determined by qRT-PCR and data were normalized to an endogenous reference gene (β-actin). Expression levels of treated cells are relative to values of untreated cells set as 1. Data are shown as mean ± SEM of 5 independent experiments. Significance was determined by the one-way analysis of variance (ANOVA) test *$p < 0.05$; **$p < 0.01$. (D) NF-κB and IκB levels: Caco-2 cells were pre-incubated with 0.05 mg/mL of each peptide for 24 h, before the exposure to 2 ng/mL TNF-α for 2 h. Western blot detection of the abundance of NF-κB in nuclear fractions and IκB in the cytosolic compartment was performed. Densitometric analysis was carried out using PCNA or GAPDH as the loading control for the nuclear and cytosolic fractions, respectively. Means of at least 3 experiments were compared. ### $p < 0.001$ and # $p < 0.05$ vs. nontreated group (Cnt); ** $p < 0.01$ and *$p < 0.05$ vs. only TNF-α-treated group.

genes, including those encoding cytokines and chemokines, we evaluated the translocation of the mentioned transcription factor into the nucleus in Caco-2 cells treated with **P-12-V**, before the exposure to TNF-α. As depicted in Fig. 5D, **P-12-V** reduced the levels of NF-κB in the nucleus (panel D), while increasing the cytosolic amount of the NF-κB inhibitor (IκB), indicating a reduction in the activation of the pro-inflammatory pathway.

3.6. *PEF bioactive peptides modulates oxidative metabolism*

The activity of the mitochondrial electron transport chain couples O_2 consumption to ATP production (Nolfi-Donegan et al., 2020; Vercellino & Sazanov, 2022). As side products of O_2 reduction, physiological levels of ROS are produced (Nolfi-Donegan et al., 2020). In healthy functional mitochondria, a decrease in O_2 consumption also leads to a reduction in ROS generation (Hulbert et al., 2007). Therefore, we aimed to determine whether the sunflower PEF-derived bioactive peptides had any effect on mitochondrial activity, which, in turn, could impact mitochondrial ROS (mROS) generation. To do this, we measured the oxygen consumption rate (OCR) in Caco-2 cells after 2 h of treatment with PEF-derived bioactive peptides. Among the peptides, **P-12-V**, **D-8-K**, and **V-G-9** reduced the basal and the ATP-linked OCR (Fig. 6 panel A and C). **P-12-V** significantly reduced also maximal OCR. Additional peptides, including **I-10-A**, **Q-11-T** and **T-11-E** had a significant effect on the maximal OCR (Fig. 6 panel B). Remarkably, **A-10-D** also impacted on the latter, although it proved ineffective in altering both basal and ATP-linked OCR. In all cases, the OCR trend was maintained, even if slightly reduced, as indicated in Fig. S4, suggesting that mitochondria remained functional after treatment with PEF-derived peptides. These data suggest that, in addition to the activation of the Keap1/Nrf2 pathway, which leads to the expression of antioxidant enzymes, a reduction in mitochondrial activity may contribute to the antioxidant effect of selected peptides.

4. Conclusions

In this study, we have introduced an innovative method for producing a sunflower seed protein isolate (SPI) designed for food applications. Moving from the protein isolate, we obtained a sunflower peptide-enriched fraction (PEF). We commenced by assessing the impact of this matrix on the well-being of a model animal, with the goal of identifying the responsible peptides and elucidating their molecular mechanisms. After obtaining evidence of the fraction's positive impact on stressed animals, attributable to its antioxidant and anti-inflammatory properties, our focus shifted to the exploration of antioxidant peptides within PEF. We concentrated primarily on the Keap1/Nrf2 pathway, which plays a pivotal role in cellular antioxidant capacity. Our results revealed that several peptides, having the highest *in silico* interaction scores with Keap1, indeed exhibited significant antioxidant capabilities. Remarkably, out of the seven tested peptides, five were able to significantly reduce oxidative stress in a cellular model. Furthermore, for three of these peptides, we observed an impact on mitochondrial metabolism. In the case of one peptide, PADVTPEEKPEV, we successfully demonstrated both its antioxidant and anti-inflammatory

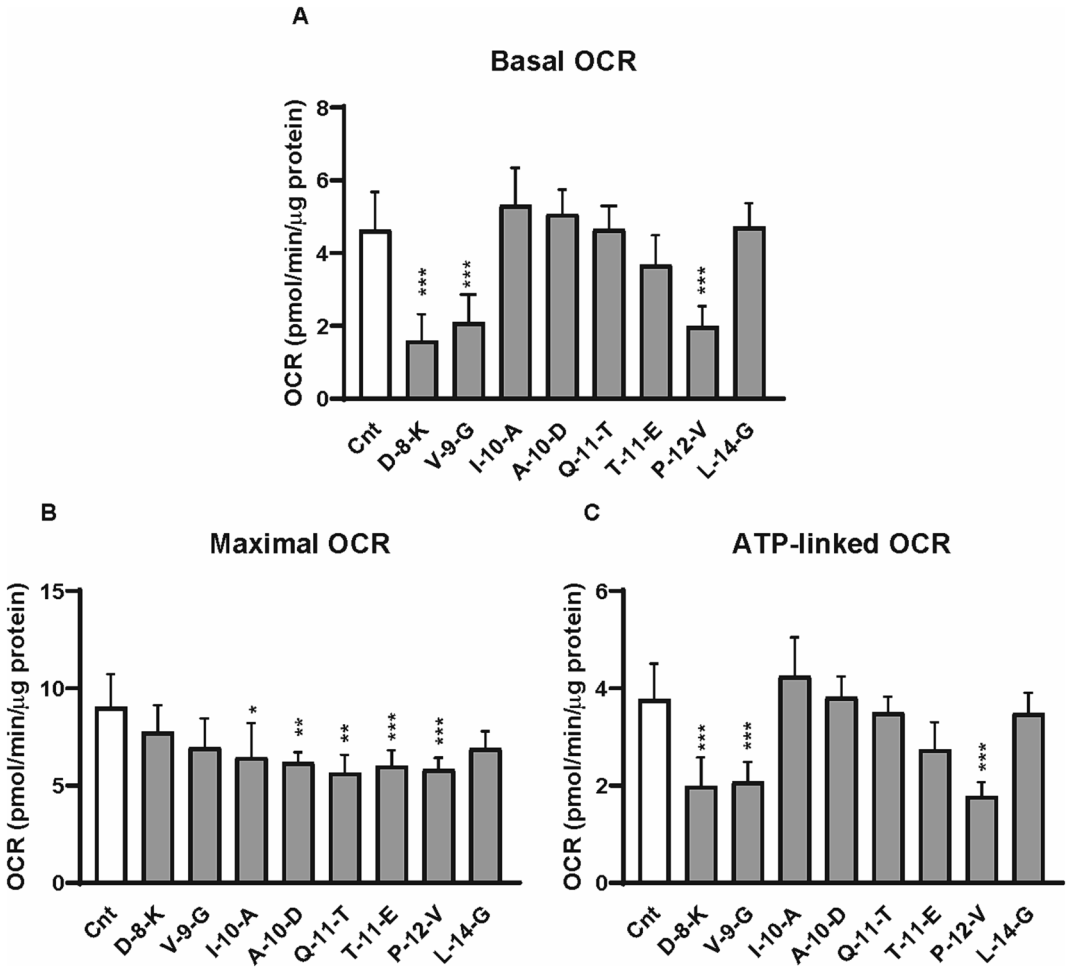


Fig. 6. Mitochondrial metabolism modulations by PEF-derived bioactive peptides. OCR measurement in Caco-2 cells after 2 h of treatment with 0.05 mg/mL of the PEF-derived bioactive peptides. (A) basal respiration; (B) maximal respiration; (C) ATP-linked respiration. To calculate basal and maximal respiration, non-mitochondrial O_2 consumption was subtracted from absolute values. ATP-linked respiration was calculated as the difference between basal and oligomycin-insensitive O_2 consumption. Data are normalized by protein content and are presented as mean ± SD. Means of at least 3 experiments were compared. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

activities. Considering the link between inflammation, oxidative stress, and the pathogenesis of numerous chronic diseases, understanding how bioactive peptides, obtained from residues, can influence metabolic pathways is crucial for the development of functional foods with beneficial effects on human health and on environment sustainability.

Funding

This research was funded by POR-FESR project: "L'Agricoltura del Futuro e gli Alimenti Funzionali: una sfida per la ricerca e il rilancio del territorio veneto (F.A.&A.F.)".

CRediT authorship contribution statement

Federica Tonolo: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Sara Coletta:** Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – review & editing. **Federico Fiorese:** Methodology, Investigation. **Alessandro Grinzato:** Software, Validation, Formal analysis, Writing – review & editing. **Marica Albanesi:** Methodology, Software, Formal analysis, Investigation, Data curation (zebrafish larvae), Writing – review & editing. **Alessandra Folda:** Methodology, Investigation. **Stefania Ferro:** Methodology, Investigation. **Agnese De Mario:** Data curation, Writing – review & editing. **Ilaria Piazza:** Investigation. **Cristina Mammucari:** Formal analysis, Writing – review & editing. **Giorgio Arrigoni:** Software. **Oriano Marin:** Software, Writing – review & editing. **Giulia Cestonaro:** Investigation, Resources. **Luigi Nataloni:** Resources. **Enrico Costanzo:** Resources, Writing – review & editing, Funding acquisition. **Claudia Lodovichi:** Conceptualization, Writing – review & editing, Funding acquisition. **Maria Pia Rigobello:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition. **Marina de Bernard:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Enrico Costanzo, Luigi Nataloni and Giulia Cestonaro (Cereal Docks S.p.A.) has patent # IT102022000016812 pending to Assignee.

Data availability

No data was used for the research described in the article.

Acknowledgments

Graphical abstract created with BioRender.com. Federica Tonolo thanks "iNEST- Interconnected Nord-Est Innovation ECS00000043".

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.138124.

References

Aebersold, R., & Mann, M. (2003). Mass spectrometry-based proteomics. *Nature, 422* (6928), 198–207. https://doi.org/10.1038/nature01511

Akbarian, M., Khani, A., Eghbalpour, S., & Uversky, V. N. (2022). Bioactive peptides: Synthesis, sources, applications, and proposed mechanisms of action. *International Journal of Molecular Sciences, 23*(3), Article 1445. https://doi.org/10.3390/ijms23031445

Bai, Y., Liu, H., Huang, B., Wagle, M., & Guo, S. (2016). Identification of environmental stressors and validation of light preference as a measure of anxiety in larval zebrafish. *BMC Neuroscience, 17*(1), 63. https://doi.org/10.1186/s12868-016-0298-z

Baird, L., & Dinkova-Kostova, A. T. (2011). The cytoprotective role of the Keap1-Nrf2 pathway. *Archives of Toxicology, 85*, 241–272. https://doi.org/10.1007/s00204-011-0674-5

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., Bohn, T., Bourlieu-Lacanal, C., Boutrou, R., Carrière, F., Clemente, A., Corredig, M., Dupont, D., Dufour, C., Edwards, C., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., … Recio, I. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols, 14*(4), 991–1014. https://doi.org/10.1038/s41596-018-0119-1

Chou, M.-Y., Hsiao, C.-D., Chen, S.-C., Chen, I.-W., Liu, S.-T., & Hwang, P.-P. (2008). Effects of hypothermia on gene expression in zebrafish gills: Upregulation in differentiation and function of ionocytes as compensatory responses. *Journal of Experimental Biology, 211*(19), 3077–3084. https://doi.org/10.1242/jeb.019950

D'Opazo, V., Calpe, J., Escrivá, L., Melo Nazareth, T., Meca, G., & Luz, C. (2023). Identification and evaluation of antioxidant activity of bioactive casein-derived peptides during in vitro digestion and transepithelial transport in Caco-2 cells. *Food Bioscience, 53*, Article 102763. https://doi.org/10.1016/j.fbio.2023.102763

De'Nobili, M. D., Bernhardt, D. C., Basanta, M. F., & Rojas, A. M. (2021). Sunflower (*Helianthus annuus* L.) seed hull waste: Composition, antioxidant activity, and filler performance in pectin-based film composites. *Frontiers in Nutrition*, *8*. doi: 10.3389/fnut.2021.777214.

Dominguez, C., Boelens, R., & Bonvin, A. M. J. J. (2003). HADDOCK: A protein–protein docking approach based on biochemical or biophysical information. *Journal of the American Chemical Society, 125*(7), 1731–1737. https://doi.org/10.1021/ja026939x

Donaldson, M. R., Cooke, S. J., Patterson, D. A., & Macdonald, J. S. (2008). Cold shock and fish. *Journal of Fish Biology, 73*(7), 1491–1530. https://doi.org/10.1111/j.1095-8649.2008.02061.x

Duffuler, P., Bhullar, K. S., de Campos Zani, S. C., & Wu, J. (2022). Bioactive peptides: From basic research to clinical trials and commercialization. *Journal of Agricultural and Food Chemistry, 70*(12), 3585–3595. https://doi.org/10.1021/acs.jafc.1c06289

Guo, S., Ge, Y., & Na Jom, K. (2017). A review of phytochemistry, metabolite changes, and medicinal uses of the common sunflower seed and sprouts (*Helianthus annuus* L.). *Chemistry Central Journal, 11*(1), 95. https://doi.org/10.1186/s13065-017-0328-7

Hadidi, M., Aghababaei, F., & McClements, D. J. (2024). Sunflower meal/cake as a sustainable protein source for global food demand: Towards a zero-hunger world. *Food Hydrocolloids, 147*, Article 109329. https://doi.org/10.1016/j.foodhyd.2023.109329

Han, X., Aslanian, A., & Yates, J. R. (2008). Mass spectrometry for proteomics. *Current Opinion in Chemical Biology, 12*(5), 483–490. https://doi.org/10.1016/j.cbpa.2008.07.024

Heck, A. J., & Krijgsveld, J. (2004). Mass spectrometry-based quantitative proteomics. *Expert Review of Proteomics, 1*(3), 317–326. https://doi.org/10.1586/14789450.1.3.317

Hulbert, A. J., Pamplona, R., Buffenstein, R., & Buttemer, W. A. (2007). Life and death: Metabolic rate, membrane composition, and life span of animals. *Physiological Reviews, 87*(4), 1175–1213. https://doi.org/10.1152/physrev.00047.2006

Karamać, M., Kosińska, A., Estrella, I., Hernández, T., & Dueñas, M. (2012). Antioxidant activity of phenolic compounds identified in sunflower seeds. *European Food Research and Technology, 235*(2), 221–230. https://doi.org/10.1007/s00217-012-1751-6

Kurcinski, M., Jamroz, M., Blaszczyk, M., Kolinski, A., & Kmiecik, S. (2015). CABS-dock web server for the flexible docking of peptides to proteins without prior knowledge of the binding site. *Nucleic Acids Research, 43*(W1), W419–W424. https://doi.org/10.1093/nar/gkv456

Kyprianou, T.-D., Pörtner, H. O., Anestis, A., Kostoglou, B., Feidantsis, K., & Michaelidis, B. (2010). Metabolic and molecular stress responses of gilthead seam bream *Sparus aurata* during exposure to low ambient temperature: An analysis of mechanisms underlying the winter syndrome. *Journal of Comparative Physiology B, 180*(7), 1005–1018. https://doi.org/10.1007/s00360-010-0481-y

Lee, H., Heo, L., Lee, M. S., & Seok, C. (2015). GalaxyPepDock: A protein–peptide docking tool based on interaction similarity and energy optimization. *Nucleic Acids Research, 43*(W1), W431–W435. https://doi.org/10.1093/nar/gkv495

Leeming, J. (2021). Meet the food pioneer whose meat replacements are rocking the gravy boat. *Nature, 590*(7844), 176. https://doi.org/10.1038/d41586-021-00264-z

Lo, S.-C., Li, X., Henzl, M. T., Beamer, L. J., & Hannink, M. (2006). Structure of the Keap1:Nrf2 interface provides mechanistic insight into Nrf2 signaling. *The EMBO Journal, 25*(15), 3605–3617. https://doi.org/10.1038/sj.emboj.7601243

Lu, D.-L., Ma, Q., Sun, S.-X., Zhang, H., Chen, L.-Q., Zhang, M.-L., & Du, Z.-Y. (2019). Reduced oxidative stress increases acute cold stress tolerance in zebrafish. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 235*, 166–173. https://doi.org/10.1016/j.cbpa.2019.06.009

Manzoor, M., Singh, J., & Gani, A. (2022). Exploration of bioactive peptides from various origin as promising nutraceutical treasures: In vitro, in silico and in vivo studies. *Food Chemistry, 373*, Article 131395. https://doi.org/10.1016/j.foodchem.2021.131395

Nolfi-Donegan, D., Braganza, A., & Shiva, S. (2020). Mitochondrial electron transport chain: Oxidative phosphorylation, oxidant production, and methods of measurement. *Redox Biology, 37*, Article 101674. https://doi.org/10.1016/j.redox.2020.101674

Reid, C. H., Patrick, P. H., Rytwinski, T., Taylor, J. J., Willmore, W. G., Reesor, B., & Cooke, S. J. (2022). An updated review of cold shock and cold stress in fish. *Journal of Fish Biology, 100*(5), 1102–1137. https://doi.org/10.1111/jfb.15037

Rivero-Pino, F. (2023). Bioactive food-derived peptides for functional nutrition: Effect of fortification, processing and storage on peptide stability and bioactivity within food matrices. *Food Chemistry, 406*, Article 135046. https://doi.org/10.1016/j.foodchem.2022.135046

Schiermeier, Q. (2019). Eat less meat: UN climate-change report calls for change to human diet. *Nature, 572*(7769), 291–292. https://doi.org/10.1038/d41586-019-02409-7

Tonelli, C., Chio, I. I. C., & Tuveson, D. A. (2018). Transcriptional regulation by Nrf2. *Antioxidants & Redox Signaling, 29*(17), 1727–1745. https://doi.org/10.1089/ars.2017.7342

Tonolo, F., Fiorese, F., Moretto, L., Folda, A., Scalcon, V., Grinzato, A., Ferro, S., Arrigoni, G., Bindoli, A., Feller, E., Bellamio, M., Marin, O., & Rigobello, M. P. (2020). Identification of new peptides from fermented milk showing antioxidant properties: Mechanism of action. *Antioxidants, 9*(2), Article 117. https://doi.org/10.3390/antiox9020117

Tonolo, F., Folda, A., Cesaro, L., Scalcon, V., Marin, O., Ferro, S., Bindoli, A., & Rigobello, M. P. (2020). Milk-derived bioactive peptides exhibit antioxidant activity through the Keap1-Nrf2 signaling pathway. *Journal of Functional Foods, 64*, Article 103696. https://doi.org/10.1016/j.jff.2019.103696

Tonolo, F., Folda, A., Scalcon, V., Marin, O., Bindoli, A., & Rigobello, M. P. (2022). Nrf2-activating bioactive peptides exert anti-inflammatory activity through inhibition of the NF-κB pathway. *International Journal of Molecular Sciences, 23*(8), Article 4382. https://doi.org/10.3390/ijms23084382

Tonolo, F., Grinzato, A., Bindoli, A., & Rigobello, M. P. (2023). From in silico to a cellular model: molecular docking approach to evaluate antioxidant bioactive peptides. *Antioxidants, 12*(3), Article 665. https://doi.org/10.3390/antiox12030665

Tonolo, F., Moretto, L., Grinzato, A., Fiorese, F., Folda, A., Scalcon, V., Ferro, S., Arrigoni, G., Bellamio, M., Feller, E., Bindoli, A., Marin, O., & Rigobello, M. P. (2020). Fermented soy-derived bioactive peptides selected by a molecular docking approach show antioxidant properties involving the Keap1/Nrf2 pathway. *Antioxidants, 9*(12), Article 1306. https://doi.org/10.3390/antiox9121306

Tonolo, F., Sandre, M., Ferro, S., Folda, A., Scalcon, V., Scutari, G., Feller, E., Marin, O., Bindoli, A., & Rigobello, M. P. (2018). Milk-derived bioactive peptides protect against oxidative stress in a Caco-2 cell model. *Food and Function, 9*, 1245–1253. https://doi.org/10.1039/c7fo01646h

Tseng, Y.-C., Chen, R.-D., Lucassen, M., Schmidt, M. M., Dringen, R., Abele, D., & Hwang, P.-P. (2011). Exploring uncoupling proteins and antioxidant mechanisms under acute cold exposure in brains of fish. *PLoS ONE, 6*(3), Article e18180. https://doi.org/10.1371/journal.pone.0018180

van Meerloo, J., Kaspers, G. J. L., & Cloos, J. (2011). *Cell sensitivity assays: The MTT assay* (pp. 237–245). doi: 10.1007/978-1-61779-080-5_20.

Vercellino, I., & Sazanov, L. A. (2022). The assembly, regulation and function of the mitochondrial respiratory chain. *Nature Reviews. Molecular Cell Biology, 23*(2), 141–161. https://doi.org/10.1038/s41580-021-00415-0

Virtanen, P., Gommers, R., Oliphant, T. E., Haberland, M., Reddy, T., Cournapeau, D., et al. (2020). SciPy 1.0: fundamental algorithms for scientific computing in Python. *Nature Methods, 17*(3), 261–272. https://doi.org/10.1038/s41592-019-0686-2

Wardyn, J. D., Ponsford, A. H., & Sanderson, C. M. (2015). Dissecting molecular cross-talk between Nrf2 and NF-κB response pathways. *Biochemical Society Transactions, 43* (4), 621–626. https://doi.org/10.1042/BST20150014

Wu, S. M., Liu, J.-H., Shu, L.-H., & Chen, C. H. (2015). Anti-oxidative responses of zebrafish (*Danio rerio*) gill, liver and brain tissues upon acute cold shock. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 187*, 202–213. https://doi.org/10.1016/j.cbpa.2015.05.016

Zaky, A. A., Simal-Gandara, J., Eun, J.-B., Shim, J.-H., & Abd El-Aty, A. M. (2022). Bioactivities, applications, safety, and health benefits of bioactive peptides from food and by-products: A review. *Frontiers in Nutrition, 8*. https://doi.org/10.3389/fnut.2021.815640