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Bioactive peptides from Spanish dry-cured ham: *In vitro* and *in vivo* antioxidant effects using a *Caenorhabditis elegans* model

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ABSTRACT

Spanish dry-cured pork ham, a highly valued traditional product, is well-recognized for its intense flavor and beneficial health properties. In this study, dry-cured ham peptides were obtained after simulated gastrointestinal digestion and their antioxidant capacity was analyzed, both *in vitro* and *in vivo*. The *in vitro* antioxidant activity was evaluated by measuring the capacity of the peptide to neutralize free radicals using ABTS, DPPH, and a cellular assay with Caco-2 cells. In addition, *in vivo* tests were performed using the nematode *Caenorhabditis elegans* as the biological model. The results showed that the ham peptides exhibited higher antioxidant activity ($P < 0.05$) than the unhydrolyzed protein (control). In *C. elegans* assays, an increase in the life expectancy of peptide-fed specimens was observed under both chronic and acute oxidative stress conditions. Moreover, reactive oxygen species (ROS) levels in these nematodes were significantly lower ($P < 0.05$) than those in the control group when exposed to paraquat-induced oxidative stress. Additionally, PCR analyses suggested that this increased resistance to oxidative stress may be related to an increase in the expression of genes involved in the stress response, such as *GST-4*, *GST-10,* and *SKN*-1. Taken together, these findings support the potential of dry-cured ham peptides as functional ingredients or nutraceuticals useful to combat oxidative damage in the body.

1. Introduction

Public awareness of the health risks associated with a diet consisting of highly processed products rich in saturated fats has led to the beginning of a change in consumption habits, increasingly demanding foods that, in addition to nourishing us, provide us with additional benefits that can help improve or protect our health (Sharma et al., 2024). Therefore, in recent years, there has been growing interest in functional compounds, including bioactive peptides, which are small amino acids sequences derived from food proteins with potential health benefits (Purohit et al., 2024). Bioactive peptides derived from food, such as dry-cured ham, which is highly consumed by the population, have attracted attention because of their unique composition and their numerous biological activities (Toldrá et al., 2020). Spanish dry-cured ham undergoes a long maturation process that improves its sensory properties and releases bioactive compounds such as peptides (Benedini et al., 2012; Hernandez Correas et al., 2024; Hernández Correas et al., 2024). These peptides are generated by the action of proteolytic phenomena that occur during the ham curing process and are capable of degrading both the protein produced and those already present in the muscle tissue (Rodríguez-Nuñez et al., 1995; Toldrá et al., 2020).

Bioactive peptides have shown potential protective effects against various diseases, highlighting their antioxidant activity, which contributes to the neutralization of free radicals, thus reducing oxidative stress and the risk of developing associated pathologies (Escudero et al., 2013). Recently, novel peptides such as GPAGAP with antidiabetic activity (Dong et al., 2024); and antihypertensive peptides (T.-T. Zhang et al., 2024) further highlight the therapeutic potential of dietary peptides. Additionally, they inhibit enzymes related to hypertension (Escudero et al., 2012) and type II diabetes (Zhang et al., 2021), as well as antimicrobial (Corrêa et al., 2023), anti-inflammatory (Mudgil et al., 2019), and immunomodulatory (Pavlicevic et al., 2022) properties. In

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this sense, it has been shown that bioactive peptides can interact with enzymes through inhibition mechanisms, altering their conformation and reducing their metabolic activity (Akbarian et al., 2022). Likewise, these peptides can regulate the gene expression of abnormal signalling pathways (Cui et al., 2019) and play a key role in cholesterol metabolism (Udenigwe & Rouvinen-Watt, 2015), favouring the elimination of metabolites that lead to hyperlipidaemia.

Bioactive peptides derived from dry-cured Spanish ham have been recognized for their strong antioxidant properties (Nan et al., 2024), including their ability to scavenge free radicals and chelate metal ions (Wang et al., 2021). The enzymatic hydrolysis that occurs during the curing process generates peptides with significant antioxidant capacity, as evidenced by their ferric ion reducing power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, both of which are widely used to evaluate the antioxidant potential *in vitro* (Gallego et al., 2020; Toldrá et al., 2020). Studies have indicated that lower molecular weight peptides exhibit higher bioactivity, probably because of their better absorption (Daliri et al., 2017). These findings underscore the potential of peptides from dry-cured ham as functional ingredients that could contribute to health maintenance and the prevention of oxidative stress-related disorders (Correas et al., 2025).

The nematode *Caenorhabditis elegans* has emerged as a valuable *in vivo* multicellular model for studying the biological effects of bioactive compounds (Mudd & Liceaga, 2022), including antioxidant peptides (Mudd et al., 2022a; Urbizo-Reyes et al., 2022). Its short lifespan, well-characterized genetics, and physiological similarities to higher organisms make it an ideal system for assessing oxidative stress resistance (Moreno-Arriola et al., 2014). These multicellular models have a well-documented genome, which makes it easier to study genes related to antioxidant responses, allowing them to retain may biological pathways with mammals that then facilitates the extrapolation of results to other organisms, including humans (Miranda-Carrazco et al., 2025). Previous studies have shown that dietary peptides can increase stress resistance and activate key regulatory pathways associated with antioxidant defense mechanisms in *C. elegans* (Mudd et al., 2022a; Wu et al., 2022; Yu et al., 2020). These activities have mad bioactive peptides an object of interest in the research and development of functional foods and new therapeutic targets. Therefore, the main objective of this study was to evaluate the *in vivo* antioxidant activity of peptides derived from Spanish dry-cured ham, using *C. elegans* as an *in vivo* model. To the best of our knowledge, this is the first study to evaluate the potential of dry-cured ham peptides in this model, providing information on their role in the modulation of oxidative stress and possible health benefits.

2. Material and methods

2.1. Materials and reagents

The cured ham samples used in this study were provided by AROMAIBERICASERRANA S.L. (Balsapintada, Murcia, Spain). The hams came from pigs with a genetic line of 50 % Duroc, and 50 % White, and were subjected to the traditional curing process developed by the company, which allowed a final salt content 25 % lower than that of products made using conventional methods to be achieved. A total of 16 samples from 16 hams were used.

C. elegans, N2 wildtype strain and OP50 *E. coli* were provided by the CGC (Caenorhabditis Genetic Centre, Minneapolis, MN, USA), which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440). Primers were purchased from Integrated DNA Technologies (Coralville, IA, USA). All other chemicals in this study are reagent grade and were purchased from one of three vendors: VWR International (Murcia, Spain & Radnor, PA, USA), Sigma Aldrich (St. Louis, MO, USA), and Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Preparation of dry-cured ham-derived bioactive peptides

The peptide fraction of the ham (HPF) sample was prepared according to the method described by Hernández-Correas et al. (2025). Peptide fractions were obtained *via* chemical hydrolysis. Four samples in triplicate from four hams were used to prepare the fractions. To prepare the extracts, 8 g of the sample was weighed into an Erlenmeyer flask, and 30 mL of distilled water was added and shaken for 15–20 min using a magnetic stirrer. Next, 15 mL of 20 % trichloroacetic acid was added, and the mixture was stirred for 10 min. The Erlenmeyer flasks were filtered using a funnel and filter paper into a 50 mL volumetric flask. After filtration, the flask volume was brought to 50 mL using distilled water.

2.3. Simulated gastrointestinal digestion of ham peptides

Ham samples were obtained from four pieces of dry-cured ham, produced under the same conditions and from the same source. The analysis was performed in triplicate for each sample. The Biceps femoris muscle, previously defatted to eliminate extramuscular fat, was processed by grinding and subsequent homogeneous mixing. The *in vitro* digestion was carried out following the protocol used in Hernández-Correas et al. (2025) and Minekus et al. (2014), with some modifications, adapted to this study. To simulate digestion, 5 g of the sample was subjected to physiological conditions that reproduced the oral, gastric, and intestinal phases of the human digestive process. In the oral phase, the sample was mixed with 3.5 mL of simulated salivary fluid (SSF), to which 0.5 mL of amylase (1500 U/mL, dissolved in SSF), 25 μL of $CaCl_2$ (0.3 mol/L) and 975 μL of distilled water were added. The mixture was incubated for 2.5 min at 37 °C under agitation to simulate the mechanical effect of chewing and the initial enzymatic action. During the gastric phase, simulated gastric fluid (SGF) was incorporated until a final ratio of 1:1 (*v*/v) was added to the mixture. 7.5 mL of SGF, 1.6 mL of porcine pepsin (25,000 U/mL, dissolved in SGF), 5 μL of $CaCl_2$ (0.3 mol/L), and 695 μL of water. The pH of the mixture was adjusted to 3 with HCl, and the mixture was incubated at 37 °C for 2 h with continuous agitation, reproducing the acidic conditions of the stomach and the proteolytic action of pepsin. Finally, in the intestinal phase, gastric contents (chyme) were combined with simulated intestinal fluid (SIF) in a 1:1 ratio (v/v). 11 mL of SIF, 5 mL of porcine pancreatin (800 U/mL trypsin activity), 2.5 mL of bile (160 mmol/L), 40 μL of $CaCl_2$ (0.3 mol/L), 1.31 mL of water, and the pH was adjusted to 7.0 with NaOH. The mixture was incubated for 2 h at 37 °C to allow for the joint action of pancreatic enzymes and bile salts on proteins, lipids, and carbohydrates.

During all phases of the simulated digestive process, the pH and incubation conditions were rigorously controlled, thus ensuring a reliable reproduction of the human digestive environment and allowing the evaluation of the digestibility and bioaccessibility of the compounds present in the cured ham. Samples of ham-digested peptides (HDP) were stored in a freezer at −20 °C until further use.

2.4. Antioxidant capacity

2.4.1. 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity.

The ABTS assay was performed as described by Mudd et al. (2022a, 2022b) with slight modifications. ABTS (7 mmol/L) was prepared using 2.45 mmol/L potassium persulfate and was incubated at 25 °C for 16 h. ABTS working solution was prepared fresh each day by diluting the stock solution to an absorbance of 0.700 ± 0.02 at 734 nm using distilled water. The analyses were performed based on the unified fractions obtained in Section 2.2 (HPF) and Section 2.3 (HDP). The reaction was carried out in a 96-well plate with 10 μL of HDP (1 mg/mL), 10 μL of HPF (1 mg/mL) or 10 μL of distilled water (control), and 290 μL of ABTS working solution. This mixture was incubated for 10 min at 30 °C and the absorbance was read on a Multiskan TM FC Microplate Photometer

(Thermo Fisher Scientific, Massachusetts, United States) at 734 nm. ABTS scavenging activity was reported as the Trolox Equivalent (mmol/L TE/mg sample). In addition to calculating the Trolox equivalent, the peptide concentration required for 50 % inhibition (IC_{50}) was calculated using four different concentrations (0.1, 0.25, 0.5, 1.0 mg/mL).

2.4.2. 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity

The DPPH free radical scavenging activity was determined following the method described by Hernández Correas et al. (2024). The analyses were performed based on the unified fractions obtained in Section 2.2 (HPF) and Section 2.3 (HDP). The antioxidant activity of the samples was determined using a 0.02 % (*w/v*) solution of the DPPH radical in ethanol. In Eppendorf tubes, 500 µL of ethanol, 500 µL of the sample, and 125 µL of 0.02 % (w/v) DPPH solution were added. Ethanol (500 µL), water (500 µL), and 125 µL DPPH were used for the blank. The samples were incubated for 1 h in the dark at room temperature. The samples were then centrifuged at 10,000*g* for 2 min and their absorbance was measured at 517 nm.

$$\%\text{DPPH Radical Scavenging Assay (RSA)} = (\text{Abs Blank} - \text{Abs Sample})/\text{Abs Blank x } 100.$$

To determine the IC50, the concentration of peptide (in mg/mL) required to inhibit 50 % of the DPPH radical, we applied the equation derived from the TROLOX standard.

2.4.3. Cell viability assay (MTT)

To evaluate HDP cytotoxicity, a colorimetric MTT assay, based on the ability of active mitochondria from viable cells to reduce MTT tetrazolium (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to purple formazan crystals, was performed as described previously by Martínez-Sánchez et al. (2020) with slight modifications. Cells were seeded in 96-well plates at a density of 15,000 cells per well in DMEM without antibiotics and supplemented with 10 % fetal bovine serum (FBS). Seeding was performed at a volume of 100 µL per well and incubated for 24 h at 37 °C in a 5 % CO_2 atmosphere to allow adhesion. After this time, the corresponding treatments, from the unified fractions obtained in Section 2.3 (HDP), prepared at specific concentrations according to the experimental design (0.25, 0.5, 1.0, or 2.5 mg/mL), were applied. The treatment solutions were added to the wells at an additional volume of 100 µL, thus doubling the total volume and ensuring adequate dilution of the compounds. The final concentrations were adjusted using the formula $Ci \cdot Vi = Cf \cdot Vf$, taking into account the dilution that occurs upon addition of the treatment to previously seeded cells. The plates were then incubated for another 24 h under the same conditions. After this time, the MTT solution was prepared at a concentration of 5 mg/mL in PBS. Thirty µL of the MTT/PBS mixture, calculated to reach a final concentration of 1.9 mg/mL in each well, was added to each well. The plates were protected from light by wrapping them in aluminium foil and incubating them for 4 h at 37 °C. During this time, viable cells reduced MTT to formazan, which accumulated as crystals at the bottom of the well.

After incubation, the medium was carefully removed without disturbing the attached cell monolayer, and 200 µL DMSO was added to each well to dissolve the formazan crystals. Subsequently, the plates were agitated for 30 min and protected from light, ensuring homogeneous dissolution. The absorbance was measured at 570 nm, using 620 nm as the reference wavelength, using a microplate spectrophotometer (SpectraMax I3, Molecular Devices LLC, San Jose, CA). The viability of the cells was calculated as a percentage of the absorbance of the samples against the control.

2.4.4. Cellular antioxidant activity - DCFDA

Intracellular antioxidant activity was assessed in Caco-2 cells using the ROS Detection Cell-Based Assay (DCFDA, Cayman Chemical). This assay is based on the use of 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA), a cell membrane-permeable fluorescent probe, which, when oxidized by reactive oxygen species (ROS), is converted to the highly fluorescent compound dichlorofluorescein (DCF). Caco-2 cells were cultured in 96-well plates treated for cell culture along with different concentrations of HDP, from the unified fraction obtained in Section 2.3, (0.5, 1.0 and 1.5 mg/mL) for 24 h, with the exception of control wells, which were cultured with culture medium alone. Positive (pyocyanin) and negative (*N*-acetylcysteine) controls were also included.

Once cell adhesion was achieved, the culture medium was replaced with assay buffer and DCFDA probe in buffer was added at a final concentration between 10 µM along with 10 µL of the antioxidant control (*N*-acetylcysteine) to the wells designated as negative control, after which it was incubated for 30 min at 37 °C in the dark. After incubation, 10 µL of pyocyanin was applied to the wells designated as the positive control, followed by an additional 1 h incubation at 37 °C in the dark. Finally, the staining solution was replaced with fresh buffer, and the fluorescence reading was performed. The fluorescence generated by DCFDA oxidation was measured using a plate reader at 480 nm excitation and 510 nm emission. The fluorescence intensity reflects the intracellular ROS levels, thus, the antioxidant efficacy of the applied treatments.

2.4.5. Cellular antioxidant activity - DHE

Intracellular reactive oxygen species (ROS) was detected using the ROS Detection Cell-Based Assay (DHE) kit from Cayman Chemical. This method employs dihydroethidium (DHE), an oxidative stress-sensitive fluorescent probe that reacts primarily with superoxide anions to form fluorescent products, such as 2-hydroxyethidium (2-OH-E^+) and ethidium (E^+), whose fluorescence can be quantified.

Caco-2 cells were cultured in 96-well plates treated for cell culture along with different concentrations of HDP, from the unified fraction obtained in Section 2.3, (0.5, 1.0 and 1.5 mg/mL) for 24 h, with the exception of control wells, which were cultured with culture medium alone. Positive (pyocyanin) and negative (*N*-acetylcysteine) controls were included. Prior to the assay, the culture medium was replaced with the kit buffer, and 130 µL of DHE staining solution (5 µM in buffer) was added to each well. To the wells designated as negative controls, 10 µL of *N*-acetylcysteine was added. The plates were incubated at 37 °C for 30 min in the dark to allow the uptake and oxidation of the probe. Next, 10 µL of antimycin A was added to the wells designated as the positive control and incubated again for an additional hour under the same conditions. After incubation, the staining solution was removed and fresh buffer was added. Fluorescence was measured using a plate reader at an excitation length of 480 nm and an emission wavelength of 570 nm. The fluorescence levels obtained reflect the intracellular generation of ROS and allow evaluation of the antioxidant capacity of the applied treatments.

2.5. In vivo study using Caenorhabditis elegans

2.5.1. Maintenance and synchronization of C. elegans

N2 (wild type) specimens of *Caenorhabditis elegans* were used in this investigation. The methodology used was described by Mudd and Liceaga (2022). The worms were reared on NGM culture medium and provided with *Escherichia coli* strain OP50 as food. Throughout the process, the samples were maintained at a constant temperature of 20 °C. To obtain synchronized populations, adults with eggs were removed from the plates by washing them with M9 buffer. Subsequently, a mixture of 58 % M9 solution, 25 % sodium hypochlorite, and 17 % sodium hydroxide (NaOH) at a concentration of 5 M was added and the mixture was shaken vigorously for five minutes. The resulting suspension was centrifuged, and the liquid containing cellular debris was discarded, keeping only the sediment with the eggs. This was washed three times and then suspended in M9 solution, shaken for approximately about 8 h to allow hatching of the hatchlings and to start the

experiments with a uniform age population.

2.5.2. Survival analysis under acute oxidative stress

The acute antioxidant activity assay was performed following the methodology of Mudd and Liceaga (2022), for which previously synchronized worms in larval stage L4 were washed three times with M9 buffer solution and subsequently incubated in a solution containing HDP (from the unified fraction obtained in Section 2.3) at concentrations of 0.25, 0.5, 1.0, 1.5 and 2.0 mg/mL for 24 or 48 h at 20 °C. After incubation, the nematodes were washed with M9 buffer three times to remove any remaining peptides. Twenty to 25 nematodes per well (*ca.* 100–125/treatment) were placed in a 96-well plate by adding the same volume of a solution containing tert-butyl hydroperoxide (tBOOH) at a concentration of 5 mmol/L. Live nematodes were counted every 30 min until all worms died. Nematodes were observed under a compound microscope (Reichert- Jung Series 150 Microscope, Cambridge Instruments Inc., Buffalo, NY, USA). Worms were considered dead if they were completely straight and no longer moved when probed with a wire stick (Harrington & Harley, 1988).

2.5.3. Survival analysis under chronic oxidative stress

A chronic oxidative stress assay was performed according to the methodology described by Mudd et al. (2022a, 2022b). For this assay the nematodes were previously synchronized and grown until reaching they reach the L4 stage, after which they were washed with M9 buffer three times and then incubated for 24 or 48 h in the HDP solution (from the unified fraction obtained in Section 2.3) at concentrations of 0.25, 0.5, 1.0, 1.5 and 2.0 mg/mL. After the incubation time had elapsed, the nematodes were washed three times with M9 buffer to remove all traces of the applied treatment. Nematodes were placed on top of a 24-well transwell plate, with approximately 25–30 nematodes per well (*ca.* 100–120 nematodes per treatment) along with 200 μL of paraquat solution (25 mmol/L) and 400 μL at the bottom. The number of live and dead nematodes was counted and recorded every day (every 24 h) at the same time from the beginning until all were dead. Nematodes were observed through a compound microscope (Reichert- Jung Series 150 Microscope, Cambridge Instruments Inc., Buffalo, NY, USA).

2.5.4. Reactive oxygen species measurement

To quantify the generation of ROS, the methodology used by Zhao et al. (2018), modified by Mudd et al. (2022a, 2022b), was followed. For this assay, the nematodes were previously synchronized and reached the L4 stage before starting the assay. Once this stage was reached the nematodes were washed 3 times with M9 buffer and incubated in HPF (the unified fraction obtained in Section 2.3) at 0.25; 0.5; 1; 1.5 or 2 mg/mL for 24 or 48 h. After incubation, the worms were washed three times with M9 buffer and pre-incubated with 15 mmol/L paraquat solution for 48 h to generate ROS. After this period, worms were washed three times with M9 buffer and once more with 0.1 % tween-20 PBS solution. The solution containing the nematodes was frozen at −80 °C and sonicated for 30 min at 60 % power on ice. The nematode lysate was centrifuged for 30 min, the supernatant was collected, and the protein content of the supernatant was determined using a Pierce TM bicinchoninic acid (BCA) protein determination kit (Thermo Fisher, Waltham, MA). 50 μL of each sample lysate (previously adjusted to the lowest concentration) were placed in a black-walled 96-well plate along with 50 μL of 2,7-dichlorofluorescein (DCFH-DA) to a final concentration of 50 μM. The samples were incubated for 90 min, at 37 °C and the fluorescence level was measured with a fluorometer (Fluoroskan Ascent FL Microplate Fluorometer and Luminometer, Thermo Fisher Scientific, Waltham, MA, USA) at 485 nm excitation and 535 nm emission.

2.5.5. C. elegans gene expression using real time qPCR

L4 nematodes, that were previously synchronized, were exposed to the HDP fraction for 24 h. The nematodes were then washed with M9 buffer and lysed using TRIzol reagent. RNA extraction was carried out following the protocols described by Mudd et al. (2022a). The extracted RNA was reverse-transcribed into cDNA using the Superscript™ Reverse Transcriptase kit, following the manufacturer's guidelines (Thermo-Fisher Scientific, Waltham, MA, USA). Quantitative Real-Time PCR was performed using a StepOnePlus™ Real-Time PCR system (Applied Biosystems Inc., Waltham, MA, USA) and SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) as the detection method. The primer sequences used for real-time PCR were as follows:

ACT-1	F: 5′ – TCCAAGAGAGGTATCCTTAC −3′, R: 5′ - CGGTTAGCCTTTGGATTGAG - 3′
DAF-16	F: 5′ – CTTCAAGCCAATGC- CACTACC −3′, R: 5′ - GGAGATGAGTTGGATGTTGATAGC −3′
SOD- 3	F: 5′ – CCAACCAGCGCTGAAATTCAATGG −3′; R: 5′ - GGAACC-GAAGTCGCGCTTAATAGT −3′
SKN-1	F: 5′ – GACGTCAATTTATG- GAGTGTCG −3′, R: 5′ - GAAGATGTTTTGTCGTGATCCG −3;
GST-4	F: 5′ – TGGAGACTCATTGACTTGGG −3′, R: 5′ - TCCTTTCTTGTTGCCACG −3′
GST-10	F: 5′ – CGTGCCACAACTTTACTACTTC −3′, R: 5′ - CAACT-GACCAAGGAGCATTC −3′

2.6. Statistical analysis

All analyses were performed in triplicate. Statistical analysis of the samples was performed using SPSS software (version 21.0, IBM Corporation, Armonk, NY, USA). To evaluate how the different treatments affected the analyses, one-way ANOVA was performed between the different treatments. When the treatment effect was significant ($P < 0.05$), the results were compared using the Fisher's LSD test. For *C. elegans* survival, the data were analyzed using the Kaplan-Meyer method and a log-rank test using the JMP® statistical software (Cary, NC, USA).

3. Results and discussion

3.1. In vitro antioxidant capacity of peptides after simulated gastrointestinal digestion

The antioxidant activity of the samples was determined by measuring the DPPH and ABTS radical scavenging activities. These assays are usually classified as electron transfer-based methods, but they can also be used in hydrogen atom transfer reactions (Prior et al., 2005). The results obtained are presented in Tables 1 and 2, respectively. ABTS assay revealed that peptides from digested dry-cured ham (HDP) had greater antioxidant activity than undigested peptides (PF). This is evidenced by a significantly ($P < 0.05$) lower IC_{50} for HDP (0.279 ± 0.019 mg/mL) than for PF (0.362 ± 0.088 mg/mL). The increase in the reducing power of hydrolyzed digests may be due to several factors: with an increase in the degree of hydrolysis, polar or charged groups became

Table 1
Results of the *in vitro* antioxidant activity of dry-cured ham peptides determined by ABTS, measured as IC_{50} (mg/mL) values ± SEM*.

Sample code	ABTS IC_{50} (mg/mL)
PF	0.362^{a} ± 0.088
HDP	0.279^{b} ± 0.019
Control	–

PF: Peptide Fractionzz.
HDP: Ham Digested Peptides.
Control: Distilled water. «–»: not detected / not applicable.
Samples that do not share the same letter (a, b) are significantly different (P < 0.05).
* SEM: standard error of mean.

Table 2
Results of the *in vitro* antioxidant activity of ham peptides determined by DPPH, measured as IC50 (mg/mL) values ± SEM*.

Sample code[1]	DPPH
	IC_{50} (mg/mL)
PF	$0.873^{a} \pm 0.027$
HDP	$0.431^{b} \pm 0.092$
Control	–

PF: Peptide Fraction.
HDP: Ham Digested Peptides.
Control: Distilled water. «–»: not detected / not applicable.
Samples that do not share the same letter (a, b) are significantly different ($P < 0.05$).
* SEM: standard error of mean.

more exposed. This phenomenon has been observed in protein hydrolysates, where reduction in molecular weight leads to exposure of polar and ionizable groups, enhancing solubility and redox interactions (Honrado et al., 2024). Also, Cui et al., 2021 reported that enzymatic hydrolysis releases more polar hydrophilic functional groups, which may further support the enhanced reducing (electron-donating) capacity of the hydrolysate. In addition, peptide cleavage and the greater availability of free amino acids (FAA) during digestion may provide an additional source of protons and electrons to maintain a high redox potential (Zhu et al., 2008). These physicochemical changes may also explain the greater radical-scavenging capacity of HDP. This result suggests that the simulated digestion process releases smaller peptides or peptides with more active chemical structures capable of efficiently donating electrons or hydrogen (Gallego et al., 2017). Similar results were obtained in other studies, where the peptide fraction also showed lower ABTS activity compared to the peptide fraction that had undergone digestion (Gallego et al., 2020; Mudd et al., 2022b).

Similar results were obtained in the DPPH assay, where the IC_{50} value of HDP (0.431 ± 0.092 mg/mL) was significantly lower than that of PF (0.873 ± 0.027 mg/mL) ($P < 0.05$). This confirms that the peptides generated after simulated digestion have a greater capacity to eliminate DPPH-type free radicals, reaffirming their superior antioxidant potential, most likely towing to the presence of hydrophobic peptides (Liao et al., 2016). In other studies conducted with corn protein hydrolysates, the DPPH radical scavenging activity of digested peptide hydrolysates significantly increased their reducing power by up to 50 % (Ren et al., 2018).

The different patterns of DPPH and ABTS radical scavenging are probably related to the structure of the peptides produced at different stages (Hernández-Ledesma et al., 2007; Zhu et al., 2008), and differences in reactive species, mechanisms, reaction conditions, reaction kinetics, and quantification methods make comparisons between both difficult (Gallego et al., 2020).

3.1.1. Caco-2 cytotoxicity (MTT)

The Caco-2 monolayer cell system is widely used as a model to study the cytotoxicity of bioactive peptides (Hu et al., 2024). The results of the MTT assay in Caco-2 cells shown in Fig. 1 indicate that HDP peptides do not exhibit significant cytotoxicity up to a concentration of 2.5 mg/mL HDP, where there was an increase in absorbance due to the formation of formazan, indicating that cell viability was decreased compared to the control. A slight increase in cell viability was observed at low concentrations (0.5–1.0 mg/mL), suggesting a possible cell-protective effect. Similar results were observed when cells were exposed to other peptide hydrolysates from different matrices, which also showed no cytotoxicity (Liang et al., 2018; Mudd et al., 2022a, 2022b; Urbizo-Reyes et al., 2022; Zhang et al., 2019). Recently other authors observed a decrease in cell viability when the concentration of peptides from Xuanwei ham

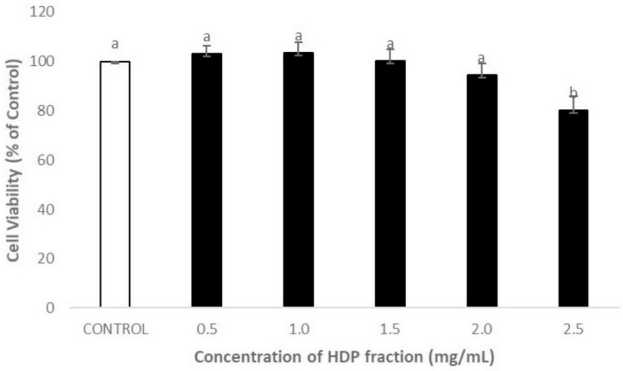

Fig. 1. Effect of simulated gastrointestinal ham digested peptides (HDP) on the viability of Caco-2 cells, reported as Cell Viability % using the MTT assay. Control contained a simulated digestion with water instead of ham peptides. Values represent mean observations of three replicates ± SEM. Trials that do not share the same letter (a, b) are significantly different ($P < 0.05$).

exceeded 0.8 mg/mL in HaCat cells (Xie et al., 2024). These findings are fundamental for their application as nutraceuticals as they validate their safety in intestinal cell models.

3.1.2. Cellular antioxidant study

Cells present in the human intestine, including Caco-2 cells, play an essential role as epithelial protective barriers. However, this function can be compromised as a result of oxidation, which could generate additional negative effects on the body (Aguilar-Toalá & Liceaga, 2021). In this sense, the peptides present in cured ham could be of great help in protecting cells from oxidative damage, since both DHE (Fig. 2) and DCFDA (Fig. 3) assays revealed a significant decrease in intracellular ROS levels in Caco-2 cells treated with HDP compared to positive controls. In both assays, peptide (HDP) concentrations of 1.0 and 1.5 mg/mL were the most effective, significantly reducing ROS-associated fluorescence ($P < 0.05$). This indicates that HDP not only acts directly on free radicals, but also modulates intracellular antioxidant defense mechanisms. Hu et al., 2016 showed that peptides extracted from Jinhua hams were capable of reducing intracellular ROS generation by 25 % and 36 % in cells pretreated with 200 and 400 μg/mL of peptides, respectively.

This data aligns with other that have found similar results when examining peptides from other peptide matrices. The finding suggest

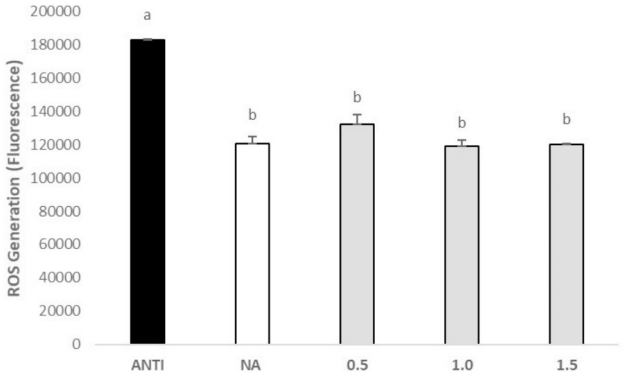

Fig. 2. Cellular antioxidant activity in Caco-2 cells, reported as ROS generation in DHE assay, for dry-cured ham peptides after simulated gastrointestinal digestion (HDP) at different concentrations (mg/mL). Antimycin (ANTI) was used as a positive control in ROS generation and *N*-acetylcysteine (NA) as a negative control. Values represent mean observations of three replicates ± SEM. Trials that do not share the same letter (a, b) are significantly different ($P < 0.05$).

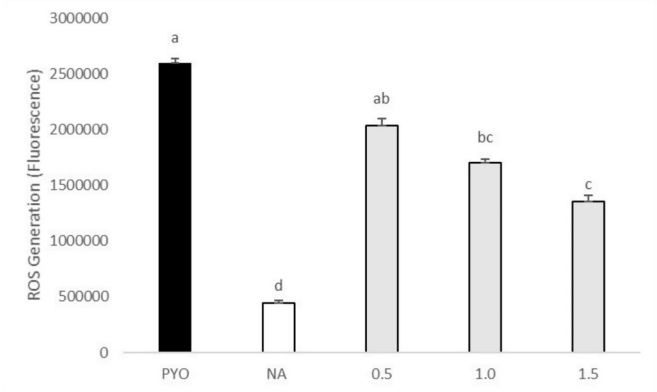

Fig. 3. Cellular antioxidant activity in Caco-2 cells, reported as ROS generation in DCFDA assay, for dry-cured ham peptides at different concentrations (mg/mL) after simulated gastrointestinal digestion (HDP). Pyocyanin (PYO) was used as a positive control in ROS generation and *N*-acetylcysteine (NA) as a negative control. Values represent mean observations of three replicates ± SEM. Trials that do not share the same letter (a, b) are significantly different ($P < 0.05$).

that a fraction of peptides from canary seeds provided homogeneous and gradually increased cellular antioxidant protection, offering cellular antioxidant activity (CAA) of up to 80 % (Urbizo-Reyes et al., 2022).

Xing et al. (2018) reported the ability of DLEE (a peptide isolated from ham) to prevent H_2O_2-induced ROS generation in Caco-2 cells. The relative antioxidant capacity of DLEE during the cell culture test was similar to that of glutathione, a natural cellular antioxidant. Therefore, DLEE could effectively function as an exogenous antioxidant to prevent the excessive intracellular accumulation of ROS. Other studies conducted on HhepG2 cells damaged by H_20_2 showed that antioxidant peptides derived from Huanwei ham after simulated gastrointestinal digestion upregulated the activity of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), and glutathione reductase (GR)(Hu et al., 2023). This upregulation of antioxidant enzymes suggests that these peptides may play a crucial role in mitigating oxidative stress and protecting cells from damage caused by reactive oxygen species.

3.2. *In vivo* antioxidant capacity

3.2.1. *Lifespan analysis of C. elegans under acute oxidative stress*

Tetrazolium hydroperoxide (t-BOOH) is an organic hydroperoxide commonly used to induce oxidative stress in various cell types (Sestili et al., 1998). Its toxicity is due to the generation of free radicals such as butoxyl radicals through the Fenton reaction, which depletes intracellular thiols and glutathione reserves, thereby weakening antioxidant systems and causing cell death (Baker & He, 1991). In this experiment, tBOOH was used to generate acute oxidative stress in L4-stage *C. elegans* nematodes, that had been previously treated with dry-cured ham digestion peptides (HDP) for 24 or 48 h.

Exposure of *C. elegans* to acute stress by t-BOOH demonstrated that pre-incubation with HDP significantly increased ($P < 0.05$) the survival rate as a function of time and dose (Fig. 4A and B) than in those not

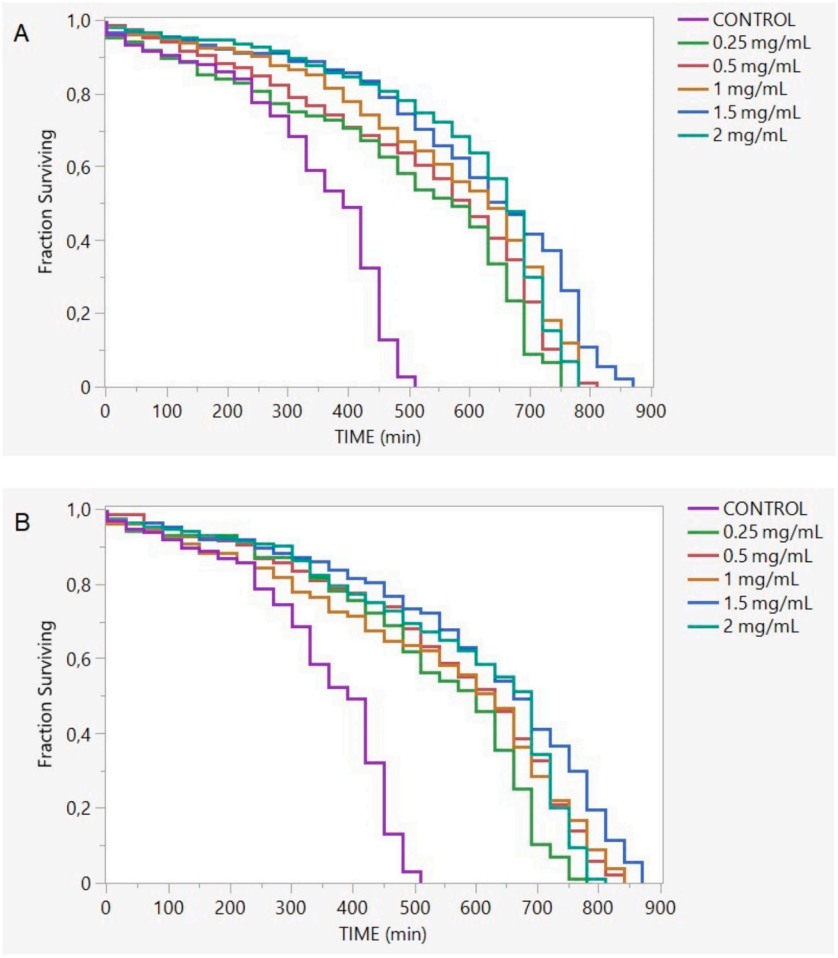

Fig. 4. Survival rate (in minutes) of *C. elegans* exposed to acute oxidative stress in the presence of t-BOOH. Survival rate after being exposed to different concentrations (ranging from 0.25 to 2.0 mg/mL) of dry-cured ham digested peptides for (A) 24 and (B) 48 h.

exposed to HDP (control). Concentrations of 1.0 and 1.5 mg/mL were the most effective after 24 and 48 h of pretreatment. The mean survival rate of nematodes exposed to a concentration of 1.0 mg/mL was approximately 630 min, while in the control group it was 390 min, which the survival rate was less than 50 %. Similarly, nematodes treated with a concentration of 1.0 mg/mL HDP for 48 h showed a mean survival of 600 min, compared to 390 min in the control treatment. This antioxidant effect was enhanced by increasing the HDP concentration to 1.5 mg/mL. These findings demonstrate that *C. elegans* is a useful model for demonstrating that peptides derived from dry-cured ham can offer protection against acute oxidative stress in a living system. Similar results were obtained for açai (*Euterpe oleracea* Mart.) extracts, and nematodes treated with the extract survived significantly longer under tBOOH-induced oxidative stress (Bonomo et al., 2014).

This suggests that HDP induces a state of greater resistance to oxidative stress, probably by activating the endogenous antioxidant pathways. Several studies using digested peptides from different food matrices also showed similar results, indicating that incubation of *C. elegans* with digested peptides increased their survival rate due to the peptides' antioxidant capacity (Mudd et al., 2022b; Urbizo-Reyes et al., 2022). In another study, the survival rate of *C. elegans* treated with 1 mg/mL of three synthetic peptides identified in C-phycocyanin from L*imnospira platensis* was much higher than that of the control group (no peptide incubation), and the mean survival time was significantly prolonged, with an increase in maximum lifespan of >15.8 % (Zhao et al., 2025). Other studies investigated the effect of treatment with a trypsin inhibitor isolated from tamarind seeds (TTI) on the survival of *C. elegans* under bacterial infection conditions and.

exposure to the stressor t-BOOH, with increased survival ($P < 0.05$) (Matias, 2024). After simulated digestion, *Porphyra haitanensis* proteins decreased oxidative stress in *C. elegans* and increased its survival rate by increasing the activity of antioxidant enzymes and reducing the levels of malondialdehyde, lipofuscin, and reactive oxygen species (Ye et al., 2025).

3.2.2. Lifespan analysis of C. elegans under chronic oxidative stress

Paraquat (methyl viologen) is a non-selective contact herbicide and known mitochondrial toxicant (Balaban et al., 2005; Bora et al., 2021). In this study, we used *C. elegans* as a model to investigate the effect of chronic exposure to paraquat on the survival rate of nematodes previously exposed to different concentrations of digested peptides from dry-cured ham (HDP). Nematodes pretreated (24 or 48 h) with HDP showed greater longevity than the control (Fig. 5A and B). Again, the best results were observed for those nematodes exposed to the intermediate HDP concentrations (1.0–1.5 mg/mL). These data reinforce the hypothesis that dry-cured ham peptides impart a sustained protective effect.

The average survival rate of nematodes exposed to a concentration of 1.0 mg/mL was approximately 4.5 days, while in the control group, it was 3 days, after which the survival rate was less than 50 %. Similarly, nematodes treated with a concentration of 1.0 mg/mL HDP for 48 h showed an average survival of 5 days, compared to 3 days for the control. This antioxidant effect was enhanced by increasing HDP concentration to 1.5 mg/mL. No significant increase ($P > 0.05$) in half-life was observed at higher HDP concentrations in the 24- and 48-h assays.

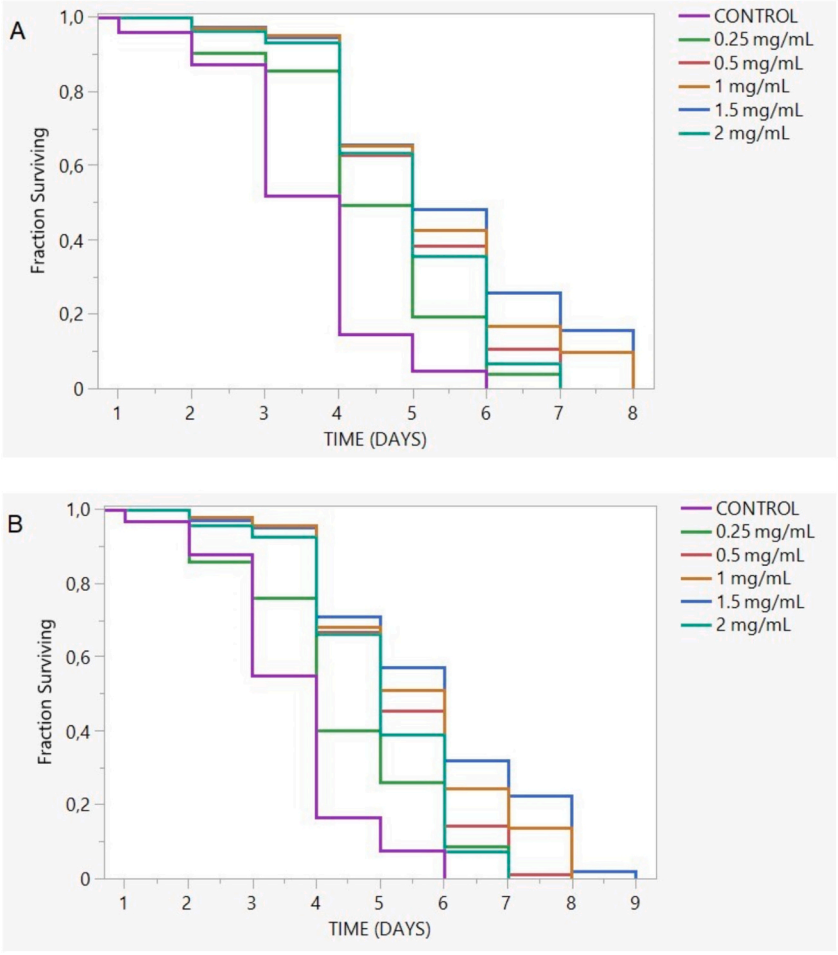

Fig. 5. Survival rate (in days) of *C. elegans* exposed to chronic oxidative stress, paraquat. Survival rate after being exposed to different concentrations (ranging from 0.25 to 2.0 mg/mL) of ham digested peptides for (A) 24 h and (B) 48 h.

Several studies have shown that certain peptides can prolong the lifespan of *C. elegans* under paraquat-induced oxidative stress. For example, peptides from sea cucumbers (Wu et al., 2022), ginseng (Wang, Huang, et al., 2016), edible crickets (Mudd et al., 2022b), canary seeds (Urbizo-Reyes et al., 2022), and purple sea urchin (Zhao et al., 2018) and Asian hard clams (Jia et al., 2018) showed positive and protective effects towards nematode survival rates. In another study using antioxidant peptides from a hydrolysate of *Sepia esculenta*, superoxide dismutase (SOD) activity increased significantly when worms were co-treated with paraquat and peptides, suggesting that antioxidant peptides modulate intracellular antioxidant defense (Yu et al., 2020). Specific amino acid residues of peptides, such as hydrophobic residues, are considered to play a key role in enhancing the activity of antioxidant enzymes. In the future, the effect of these amino acid residues could be confirmed by substitution to better understand their role in antioxidant activity.

As paraquat causes oxidative stress through increased ROS generation (Blanco-Ayala et al., 2014), ROS levels were measured to corroborate these results.

3.2.3. Reactive oxygen species (ROS)

Oxidative stress results from an imbalance between the production of reactive oxygen species (ROS) and the body's ability to neutralize them, leading to cell damage and development of chronic diseases (Sharifi-Rad et al., 2020). Although the human body produces endogenous antioxidants, dietary antioxidants play a crucial role in mitigating oxidative damage (Frei, 2004). Bioactive peptides have been identified as potential antioxidant agents due to their ability to modulate enzymatic activity (Qiao et al., 2021), regulate the molecular mechanisms of the Keap1-Nrf2-ARE, NF-κB, MAPK, and PI3K/AKT/mTOR pathways, and act on multiple pathways simultaneously to exert their antioxidant-mediated functional activities (Lv et al., 2022).

The results obtained from the acute and chronic oxidative stress analyses were in agreement with those observed in the quantification of ROS levels (Fig. 6). HDP treatment significantly reduced the ROS levels in nematodes exposed to paraquat. Concentrations of 0.5, 1.0 and 1.5 mg/mL were particularly effective ($P < 0.05$), showing levels of ROS comparable to those of the negative control without paraquat (C) (Fig. 6). Excessive generation of intracellular ROS damages cell structure and function, leading to aging and disease (Rodriguez et al., 2013); suggesting that digested ham peptides decrease endogenous ROS accumulation and protect against oxidative damage.

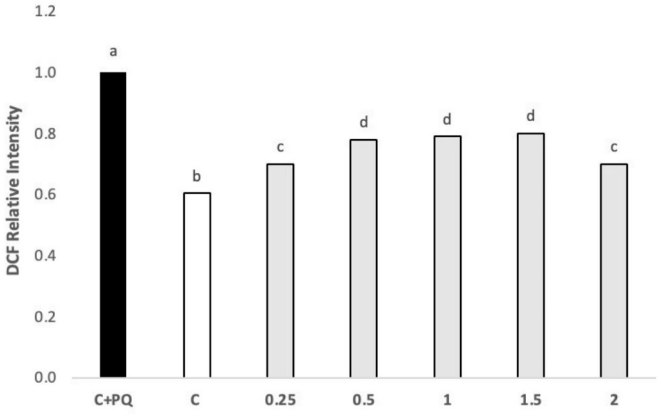

Fig. 6. Reactive oxygen species (ROS) levels in *C. elegans* pretreated with different concentrations of dry-cured ham digested peptides (at different concentrations in mg/mL) and then exposed to paraquat (PQ). The DCF (dichlorofluorescin) intensity was determined using a fluorometer at excitation 485 nm and emission 535 nm. C + PQ = control (with paraquat, no peptides), C = control (without paraquat and without peptides). The results represent the adjusted fluorescence values from three independent assays repeated thrice, those that do not share the same letter (a, b, c, d) are significantly different ($P < 0.05$).

Similar results were observed in other studies, where intracellular ROS levels decreased when paraquat was applied after pre-treatment with peptides (Han et al., 2018; Jia et al., 2018; Mudd et al., 2022a; Tsai et al., 2020; Urbizo-Reyes et al., 2022; Wu et al., 2022; Yu et al., 2020). A reduction in ROS levels was also observed in nematodes when pre-incubated with 2.0 and 4.0 mg/mL peptides from *Angelica sinensis* protein hydrolysate and exposed to paraquat (2 mmol/L) (Wang, Huang, et al., 2016).

The main pathways through which these antioxidants exert their protective effects involve reduction of oxidative stress and enhancement of the body's antioxidant defense systems. Accordingly, our findings indicate that the antioxidant peptides identified in this study, together with those described in previous studies, exhibit antioxidant activity by enhancing endogenous defense mechanisms and decreasing the generation of harmful ROS. The antioxidant potential of peptides produced through enzymatic breakdown of meat proteins, particularly those from dry-cured ham, has been widely investigated *in vitro*. Nevertheless, to our knowledge, this is the first study to evaluate the antioxidant activity of peptides resulting from the simulated gastrointestinal digestion of Spanish dry-cure using *C. elegans* as an *in vivo* model. Based on our findings, we confirmed that the antioxidant effect of HDPs involves the neutralization of free radicals, which is one of the key mechanisms by which they mitigate both acute and chronic oxidative stress conditions at the cellular level (Caco-2 cells) and *in vivo* (*C. elegans*).

3.2.4. Gene expression levels in C. elegans

To explore the molecular pathways associated with the antioxidant properties of these peptides, the expression levels of various antioxidant-related genes (*DAF-16*, *SOD-3*, *SKN-1*, *GST-4* and *GST-10*) were analyzed using real-time quantitative PCR (RT-qPCR) after a 24 h exposure to different concentrations of HDP. Gene expression analysis (Fig. 7) revealed a significant increase ($P < 0.05$) in the upregulation of genes (*GST-4*, *GST-10*, *SKN-1*, and *SOD-3*) associated with the oxidative stress response after treatment with HDP. In *C. elegans*, the transcriptional regulator *SKN-1* is important for resistance to oxidative stress and acts on multiple longevity pathways. Notably, the *SKN-1* gene, analogous to the Nrf2 transcription factor in mammals, showed significant overexpression, suggesting activation of endogenous cellular defense pathways (An & Blackwell, 2003). Our results indicate that *SKN-1* mediates the longevity of the nematodes through stress resistance of the p38 MAPK pathway (Inoue et al., 2005). Since *SKN-1* appears to be involved in damage control and stress resistance (Oliveira et al., 2009), endogenous antioxidant systems regulated by activated skn-1 may explain the increased resistance to oxidative stressors and increased survival. Similar results were reported in a study in which peptides from sesame cake prolonged the lifespan of *C. elegans* by upregulating *SKN-1* (Wang, Ma, et al., 2016). In a study conducted by Cai et al. (2022), a rice bran peptide (KF-8) prolonged the lifespan and improved the health of *C. elegans via* upregulation of *SKN-1* and *DAF-16*. Activation of the *DAF-16* gene, which is involved in longevity and stress, also supports this hypothesis. It is known that *DAF-16* is upregulated by different antioxidant defense pathways, including insulin-like growth factor (IGF) (Chávez et al., 2007, p. 16), the immune pathway (independent of p38 MAPK) mediated in the epidermis in response to fungal infections or in situations of oxidative stress Afridi & Tu, 2025), and some parallel pathways such as JNK-1 which can be activated through alternative phosphorylation other than AKT. There may also be interactions between *SKN-1* and *HSF-1* (Back et al., 2010). In contrast, exposure of nematodes to HDP did not result in overexpression of this gene, suggesting that the observed upregulation of *SOD-3* (a canonical *DAF-16* target) at 1.5 mg/mL may be either the result of indirect crosstalk or partially mediated by *SKN-1* or *HSF* under these conditions. Similar results were obtained in a study by Li et al. (2024), in which glycerophosphocholine (GPC) was used to test its effect on longevity and stress resistance in *C. elegans*, the upregulation of the *SOD-3* gene did not imply

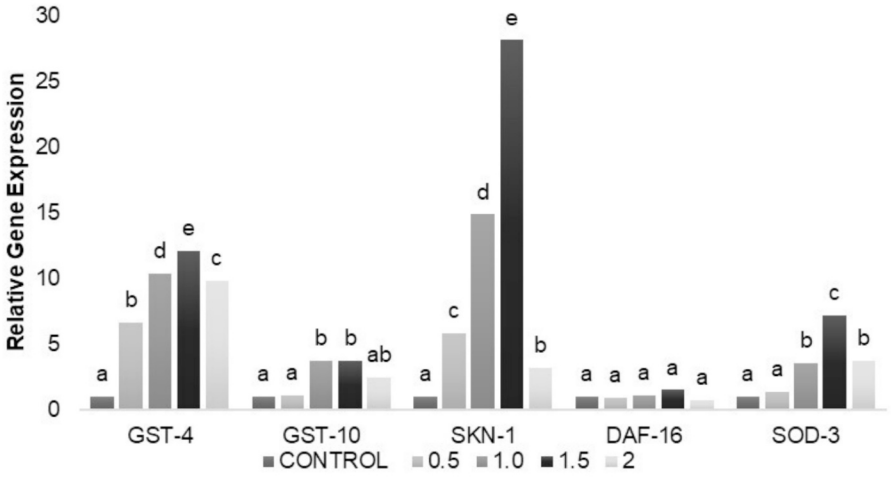

Fig. 7. Oxidative stress-related genes analysis. Effect of simulated gastrointestinal dry-cured ham digested peptides (HDP) on genes related to *C. elegans* oxidative stress response. Trials for each gene that do not share the same letter (a, b, c, d, e) are significantly different ($P < 0.05$).

the upregulation of *DAF-16*, whereas *SKN-1* was overexpressed. Their results indicate that this molecule could play an important role in resistance to external stress by activating the insulin/IGF-1 signalling pathway. In agreement with these observations, a very recent study reported that the antioxidant dipeptide Leu-Tyr improved longevity and health span in *C. elegans* by modulating the IIS, MAPK and HSF pathways (Fatah et al., 2025). This further supports the role of bioactive peptides as regulators of stress resistance and lifespan through interconnected molecular signalling cascades.

In our study, *GST-4* showed significant ($P < 0.05$) upregulation. This gene is overexpressed by two different antioxidant pathways (Raj, 2021): the insulin-like growth factor (IGF) and epidermal growth factor (EGF) pathways. *GST-4* signalling in the insulin-like growth factor pathway depends on the overexpression of skn-1 (Zhang et al., 2022), indicating that the antioxidant effect of HDP is most likely largely mediated through the activation *of SKN-1*, rather than a direct response through the overexpression of *DAF-16*. Additionally, *GST-10* expression is only moderately affected, showing no significant changes beyond the 0.5 and 1.0 mg/mL peptide doses.

Our findings point to positive regulation of antioxidant molecular mechanisms by peptides from digested dry-cured ham, demonstrating their ability to overexpress genes involved in different pathways.

4. Conclusions

This study provides solid evidence that bioactive peptides derived from Spanish dry-cured ham, after undergoing a simulated gastrointestinal digestion process, show striking antioxidant activity both *in vitro* and *in vivo*. Through chemical and cellular assays and using the *Caenorhabditis elegans* biological model, we have demonstrated that these peptides not only have the ability to neutralize free radicals but also significantly decrease intracellular levels of ROS without generating cytotoxic effects.

Furthermore, it should be noted that in the *C. elegans* model, digested peptides (HDP) significantly improved the survival rate of these organisms exposed to conditions of acute and chronic oxidative stress. This protective effect was correlated with a decrease in ROS and overexpression of key genes involved in antioxidant defense, such as *SKN-1, GST-4, GST-10*, and *SOD-3*, suggesting that peptides from Spanish dry-cured ham can not only act directly as radical neutralizing agents but also stimulate endogenous cell protection mechanisms. Activation of the *SKN-1*-regulated pathway, analogous to the Nrf2 transcription factor in mammals, highlights the potential of these peptides to modulate genetic pathways associated with longevity, stress resistance, and prevention of cellular oxidative damage, which are promising candidates for the development of functional ingredients or nutraceuticals.

Together, these findings expand our knowledge of the potential of traditional foods, such as Spanish dry-cured ham, not only as a staple food part of our diet but also as a promising vehicle for bioactive peptides with protective properties against degenerative processes related to oxidative stress.

Although *C. elegans* provides a robust *in vivo* model sharing conserved antioxidant and longevity pathways (IIS, MAPK, SKN-1/NRF2) with mammals, they are not identical, with some differences existing when compared to the latter. Therefore, caution should be exercised when extrapolating these findings, and future studies should validate the antioxidant and health-promoting effects of ham-derived peptides in mammalian models, as well as validating their efficacy in human clinical trials. Nevertheless, the presence of bioactive peptides in functional food matrices such as from Spanish dry-cured ham represents a promising avenue for preventive nutritional strategies based on scientific evidence.

Consent form

The authors declare that they have been informed during the course of the entire work and authorize the submission of the manuscript.

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CRediT authorship contribution statement

Noelia Hernández Correas: Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis. **Andrea**

M. Liceaga: Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization. **Adela Abellán:** Writing – review & editing, Resources, Methodology, Conceptualization. **Clara Noguera:** Writing – original draft, Resources, Investigation. **Silvia Montoro:** Visualization, Software, Investigation, Data curation. **Luis Tejada:** Supervision, Project administration, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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