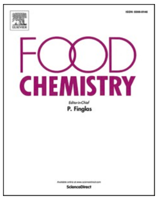
Food Chemistry 484 (2025) 144360

Contents lists available at ScienceDirect

Food Chemistry

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



Curing strategies and bioactive peptide generation in ham: In vitro digestion and in silico evaluation

Noelia Hernández Correas [a,*,1], Alejandro Rodríguez Martínez [b,1], Adela Abellán [a], Horacio Pérez Sánchez [b,*], Luis Tejada [a]

[a] *Faculty of Pharmacy and Nutrition, Universidad Católica de Murcia-UCAM, Campus de los Jerónimos, 30107 Murcia, Spain*
[b] *Structural Bioinformatics and High Performance Computing (BIO-HPC) Research Group, Universidad Católica de Murcia-UCAM, Campus de los Jerónimos, 30107 Murcia, Spain*

ARTICLE INFO

Keywords:
Bioactive peptide
Alternative salts
Antioxidant
Angiotensin-I converting enzyme (ACE)
In silico analysis, in vitro digestion

ABSTRACT

This study assessed the impact of curing salts and maturation times on peptide production and bioactivity in dry-cured hams using in vitro and in silico methods. Ninety-six hams underwent six curing treatments and two maturation stages (38 % and 42 % weight loss). Mass spectrometry identified bioactive peptides, while in silico tools predicted their bioactivities. Reduced sodium nitrifying salts (treatment IX) and 42 % weight loss showed the most significant results, enhancing low-molecular-weight peptides generation (EE, VG, VD) linked to high functionality. Antioxidant and antihypertensive activities were prominent in samples with 42 % weight loss. Peptides under 1.5 kDa were more abundant at advanced maturation stages. In silico analyses predicted ACE and DPP-IV inhibition and antioxidant effects. Dipeptides like DG, ES, and DV showed similarities to FDA-approved molecules, suggesting potential therapeutic uses. The study highlights those specific treatments boost biopeptides formation, requiring further research on their potential as functional foods or therapeutic agents.

1. Introduction

Bioactive peptides are short amino acid sequences that have the ability to exert specific physiological effects in the human body and other living organisms (*Food Protein-Derived Bioactive Peptides: Production, Processing, and Potential Health Benefits - Udenigwe - 2012 - Journal of Food Science - Wiley Online Library*, n.d.). They have several biological functions and can exert several health-promoting activities, including antioxidant activity (Escudero, Mora, Fraser, Aristoy, Arihara, & Toldrá, 2013) (Antioxidant peptides help the neutralization of free radicals in the body, which can reduce oxidative stress and the risk of chronic diseases), anti-inflammatory activity (Fu et al., 2021), antihypertensive activity (Escudero et al., 2012). Some peptides can help lower blood pressure, which is beneficial for people with hypertension or other cardiovascular risk diseases, immunomodulatory activity (Szwajkowska et al., 2011). Peptides can also modulate the immune system response, which may be useful in the treatment of autoimmune diseases and other immune disorders), antimicrobial activity (Corrêa et al., 2023), other peptides have antimicrobial properties, which means they can fight bacteria, viruses and fungi. These peptides are important for immune function, protecting the body against infections, and neuroprotective activity (Martínez Leo et al., 2022; L. Zhang et al., 2023). Certain peptides can have neuroprotective effects, protecting nerve cells from damage and helping in neurodegenerative diseases such as Alzheimer's and Parkinson's), among others.

These activities make bioactive peptides an object of interest in research and the development of functional foods and new medical therapies, which is why the main objective of this study: generate new bioactive peptides and increase their concentration in hams processed with different curing salts, corroborating their bioactivity by different in vitro and in silico analyses.

The level of peptides that exists at each stage of the ham maturation process is the result of the balance between proteolytic phenomena capable of producing peptides from muscle proteins and proteolytic phenomena capable of degrading both the peptides produced in this way, and those already existing in the muscle tissue (Abellán et al., 2018; del Olmo et al., 2015; Guo et al., 2021; Ruiz et al., 1998). The evolution of these peptides (many of them with bioactivity) is directly

---

* Corresponding authors.
E-mail addresses: nhernandez7@ucam.edu (N.H. Correas), hperez@ucam.edu (H.P. Sánchez).
[1] Co-first authorship.

https://doi.org/10.1016/j.foodchem.2025.144360
Received 21 January 2025; Received in revised form 30 March 2025; Accepted 14 April 2025
Available online 15 April 2025

related to the different phases that take place during the curing process of the ham (Gallego et al., 2019; Heres, Gallego, et al., 2022; Hernandez Correas et al., 2024). This is because the activity of proteases is affected by various factors, including changes in intrinsic or extrinsic factors that directly or indirectly affect the activity of endogenous proteases (Gallego et al., 2015; Heres et al., 2023; Toldrá & Flores, 1998). Extension of maturation, reduction of sodium chloride, the increase of other salts and the decrease of nitrite, among others, can change the activity of muscle peptidases such as aminopeptidases and carboxypeptidases, tripeptidyl peptidases (TPP) and dipeptidyl peptidases (DPP) which are able to hydrolyse tripeptides and dipeptides, respectively, from the N-terminus of protein fragments and polypeptides (Aliño et al., 2010; Andres et al., 2005; Hernandez Correas et al., 2024; Virgili et al., 2007; C.-Y. Zhou et al., 2019; G. H. Zhou & Zhao, 2007). Previous studies have related the increase in maturation time to the increase in the concentration of certain dipeptides (Heres et al., 2023).

During the study, different analyses will be carried out using in silico tools that have not been used for this purpose until now, which will allow us to predict the presence of peptides with bioactive potential in the protein sequences of ham using databases and specialised software. In addition, the gastrointestinal digestion process will be simulated to predict which peptides will be released during protein digestion. Finally, the interaction of bioactive peptides with different biomolecules and receptors in the human body will be predicted, providing information about their mechanisms of action and potential biological effects.

With the development of this study, it will be possible to corroborate the in-silico analyses by means of different in vitro techniques of identification, quantification and bioactivity analysis of the different hams processed with different salts and treatments, and thus be able to know more directly the presence and interaction of the bioactive peptides.

2. Materials and methods

For the development of this study, a total of 96 boneless cured hams from Poland with a racial percentage of 50 % Duroc and 50 % Landrace x Large White were required. Six batches of 16 fresh hams were processed under different conditions. Starting from the same genetic, physical and feeding conditions at the time of slaughter.

Fresh hams were first deboned and then salted with 6 different nitrifying salt treatments (TI, TIII, TIV, TVI, TVII and TIX) at the salting stage. These are indicated below:

TI: Standard dose of reduced nitrifying agent with more than 60 % nitrate. Salt reduced by 25 % sodium. Standard salting treatment (1 day/kg ham weight).

TIII: Double dose of reduced nitrifier with more than 60 % nitrate. Salt reduced by 25 % sodium. Standard salting treatment (1 day/kg ham weight).

TIV: Double dose of reduced nitrifier with more than 60 % nitrate. Standard salt. Standard salting treatment (1 day/kg ham weight).

TVI: Standard dose of reduced nitrifier without nitrates. Salt reduced by 25 % sodium. Standard salting treatment (1 day/kg ham weight).

TVII: Standard nitrifying salt with standard nitrification process but reduced sodium. Salt reduced by 25 % sodium but with special granulometry. Standard salting treatment (1 day/kg ham weight).

TIX: Standard nitrifying salt with standard nitrification process but reduced in sodium. Standard salt. Standard salting treatment (1 day/kg ham weight).

Each piece was salted for 24 h per kg of weight, approximately 7–8 days, in a cold room at a temperature of 3 °C at a relative humidity between 93 and 95 %.

The composition of the standard nitrifying salts is 86 % sodium chloride, 9 % sodium nitrite, 3 % sodium isoascorbate, 1.2 % potassium nitrate and 0.8 % dextrose. The dosage applied for standard salting treatment is 5 g per kilogram of fresh weight of ham.

The nitrifying salts are applied by rubbing, before starting with salting process. After salting, hams were washed with water, followed by a traditional curing process. The resting or post-salting phase started at 3 °C, rising progressively to 6 °C at a relative humidity between 70 and 80 %. This production phase ended when the hams reached 18 % loss, lasting approximately four to five months. This phase lasted for a minimum of 120 days, thus extending the post-salting phase to reduce the aw as much as possible and to try to counteract the lack of salt (Barat et al., 2013). Subsequently, the temperature was raised to 30 °C and the relative humidity decreased to 50 %. The process ended when the pieces reached a weight loss of 38 %, after approximately 14/15 months of curing.

After this last treatment, half of the hams processed under each treatment, finishing their maturation process (8 pieces per treatment), but the other half of the hams were brought to over-maturation, up to 42 % weight loss, needing to reach over-maturation for approximately 3 more months.

The percentage weight loss was determined by weighing each sample in triplicate during each processing stage. Results were expressed as percentage of weight loss, considering the fresh weight of each piece.

Finally, all samples were taken at the end of the process, on the final product (38 % weight loss for stage 4 hams and 42 % weight loss for stage 5 hams). Samples were taken using a 2 cm diameter stainless steel cylinder, in the area corresponding to the biceps femoris muscle.

This study has determined the effect of different types of nitrifying salts and the maturation time used in the deboning treatment, on the final product quality, related to peptide production and bioactivity.

2.1. Extraction of peptides - obtaining the fraction

Peptide fractions were obtained following the procedures done by Muela et al., 2024 with slight modifications. To prepare the extracts, 8 g of sample were weighed in an Erlenmeyer flask, after which 30 mL of distilled water were added, and agitated for 15–20 min in a magnetic stirrer. Then, 15 mL of 20 % trichloroacetic acid was added and shaken for 10 min. The content of the Erlenmeyer was filtered using a funnel and filter paper in a 50 mL volumetric flask. After filtration, the flask was filled with distilled water.

2.2. In vitro assays

2.2.1. Angiotensin-I-converting enzyme inhibitory activity

The ACE-I activity of the peptide fraction was conducted according to the spectrophotometric method described by Molina-Valero et al., 2024. For this purpose, 40 μL of each fraction was incubated at 37 °C with 100 μL of 5 mM HHL dissolved in 0.1 M borate buffer and 0.3 M NaCl (pH 8.3). Next, 2 mU of ECA were added to the substrate. Thirty minutes later, 150 μL of 1 M HCl was added. The formed hippuric acid was recovered with 1000 μL ethyl acetate and centrifuged 10 min at 4000*g* and the organic phase (800 μL) was collected. The ethyl acetate was removed by bringing the temperature to 95 °C. The resulting hippuric acid was resuspended in 1000 μL of distilled water and the absorbance was measured at 228 nm. ACE inhibitory activity was determined by the following equation.

ACE inhibitory activity was determined by applying the equation:

$$\text{ACE inhibitory activity }(\%) = ((\text{Acontrol} - \text{Ablank}) - (\text{Asample} - \text{Ablank}))/(\text{Acontrol} - \text{Ablank})) \times 100.$$

*Acontrol: the measure of hippuric acid produced by the action of uninhibited ACE.

**Asample: the measure of hippuric acid produced by the action of ACE with the sample.

***Ablank is the measure of unreacted HHL.

Captopril (Sigma) (1 mM) was used as a reference substance to fine-tune the technique. The results of the standard line can be consulted in the supplementary material. To calculate the IC50, (the peptide concentration required to inhibit 50 % of ACE activity), dilutions were prepared at different concentrations of the peptide fraction and the percentage inhibition was calculated for each concentration tested. Subsequently, the percentage of ACE-I activity versus the concentration of peptide fraction used (mg peptides/mL) was plotted in a spreadsheet. The equation of the line (y = ax+b) was obtained and the concentration of the peptides giving an meinhibitory activity of 50 %, i.e. IC50 = (50-b)/a, was calculated.

2.2.2. Antioxidant activity

The determination of antioxidant activity was conducted following the method Hernández-Correas et al. (2024). The antioxidant activity of the samples was then determined using a 0.02 % (*w/v*) solution of the DPPH radical in ethanol. In Eppendorf tubes, 500 uL of ethanol, 500 uL of sample, and 125 uL of 0.02 % (w/v) DPPH solution were added. For the blank, 500 uL of ethanol, 500 uL of water, and 125 uL of DPPH were used. 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.

% DPPH Radical Scavenging Assay (RSA) = (Abs Blank - Abs Sample) / 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.

2.3. Peptide identification and bioactivity analysis

Identification was determined by tandem mass spectroscopy analysis using liquid nanochromatography (nLC) from the peptide fraction obtained in Section 2.1.

Liquid nanochromatography was performed on a Dionex Ultimate 3000 nano UPLC (Thermo Scientific) with a C18 (75 μm × 50 cm) Acclaim Pepmam column (Thermo Scientific). The previously obtained fraction was loaded onto a 300 μm × 5 mm Acclaim Pepmap precolumn (Thermo Scientific) in a 2 % acetonitrile/0.05 % TFA mixture for 5 min at a flow rate of 5 μL/min. Peptide separation was performed at 40 °C for all samples. Mobile phase buffer A was composed of water and 0.1 % formic acid. Mobile phase B was composed of 20 % acetonitrile and 0.1 % formic acid. The samples were separated at 300 nL/min. Mobile phase B was increased from 4 to 45 % in 60 min; from 45 to 90 % for 1 min, followed by a 5 min wash at 90 % B-phase and a final 15 min re-equilibration at 4 % B-phase. Total chromatography time was 85 min. Identification of the peptide sequences of the peptide fractions of the different hams was performed at the Proteomics and Bioinformatics Unit of the University of Cordoba, Spain. MS2 spectra were found with SEQUEST HT against the UnitProtKB database.

The peptides of each fraction were identified and quantified using the peptide spectral matches (PSM). Quantification values were normalized, focusing on the total PSM for all peptides in the sample. Thus, the quantification of a single peptide was comparable between those of the different samples. We also performed a search for each of the identified peptides in the BIOPEP-UWM database (Minkiewicz et al., 2019). Two types of searches were performed: identification of activated biopeptides in the sample already displayed in the BIOPEP database and identification of potential biopeptides containing fragments of bioactive sequences in their primary structure already displayed in the BIOPEP database.

2.4. In silico studies

2.4.1. Digestion simulation of the identified peptides and bioactivities prediction

First, we simulated the protein digestion for all identified non digested peptides. We created a workflow to predict the digestion of 20,255 peptides using the PeptideCutter tool from Expasy website (https://web.expasy.org/peptide_cutter/, 2024, July 1). We developed a specific script that allowed us to run the different predictions for each peptide via the command line in a supercomputer.

The pepsin (EC 3.4. 23.1), trypsin (EC 3.4.21.4), and chymotrypsin (EC 3.4.21.1) enzymes were used sequentially, in that order, to simulate the digestion as realistically as possible. In this way, pepsin primarily cleaves at Phe, Tyr, Trp, and Leu in P1 or P1'. At pH 1.3, its specificity increases, favoring cleavage at Phe and Leu in P1, with minimal cleavage at other residues. This specificity is lost at pH ≥ 2 (Keil, 1992). After that, Trypsin acts and this enzyme primarily cleaves at Arg and Lys in the P1 position, with a higher preference for Arg. However, in the presence of pseudotrypsin, it can also cleave at Phe, Tyr, Trp, Met, and Cys in P1, depending on the surrounding amino acid context (Keil, 1992). Finally, Chymotrypsin primarily cleaves at Trp, Tyr, and Phe in P1 with high specificity, and to a lesser extent at Leu, Met, and His. However, cleavage is blocked when Trp in P1 is paired with Met or Pro in P1', Pro in P1' generally inhibits cleavage, Met in P1 is blocked by Tyr in P1', and His in P1 is blocked by Asp, Met, or Trp in P1 (Keil, 1992).

Following this algorithms implemented in PeptideCutter tools, we obtained the peptide fragments from the simulated digestion and we subsequently conducted an exhaustive search of these peptides in BioPep, a database that records peptide bioactivity to validate its function in the oxidative stress or hipertensive context (Minkiewicz et al., 2019).

2.4.2. Target fishing

2.4.2.1. Target protein selection.

For the in-silico analysis, a method based on target fishing was carried out using molecular docking simulations. In this way, each of the eleven dipeptides was individually confronted against two different sets of proteins, whose functions are associated with hypertensive and antioxidant bioactivities.

The proteins corresponding to each set were selected. An exhaustive search was conducted in the UniProt protein database using the application programming interface (UniProt website REST API). A cutoff of a sequence length of less than 1250 amino acids was established to ensure an adequate and varied representation of the proteins. Only those proteins with a defined active site and an identified residue at the binding site were considered.

The pertinent Gene Ontology codes (https://geneontology.org/) were also indicated to obtain the related proteins for each function. For the antihypertensive function, proteins from the filtering related to the code GO:0003073, corresponding to systemic arterial blood pressure regulation, were selected. For the antioxidant function, proteins related to the following codes were searched: GO:0072593 ("reactive oxygen species metabolic process"), GO:1903409 ("reactive oxygen species biosynthetic process"), and GO:2000379 ("positive regulation of reactive oxygen species metabolic process"). After applying this filter, 35 proteins related to the antioxidant context and 21 proteins related to the antihypertensive context were used for the target fishing calculations.

2.4.2.2. Model building.

The sequences obtained for each group of proteins were modeled through the transformer protein language model, ESM-Fold (https://github.com/facebookresearch/esm), by the command-line tool using esm-fold. All runs were launched on GPU (NVIDIA GeForce RTX 3090 24 Gb) on the cluster used by the research group.

The structures of the predicted proteins generated in pdb were converted to mol2 and pdbqt format for the subsequent docking calculations. The conversion to mol2 was done through the external scripts of Maestro Schrödinger (Schrödinger Release 2022–1: Desmond Molecular Dynamics System, D. E. Shaw Research, New York, NY, 2021. Maestro-Desmond Interoperability Tools, Schrödinger, New York, NY, 2021., n. d.), explicitly calling the applications by the command lines of Preparation Wizard and Structure Convert. Regarding conversion in pdbqt format, the prepare_receptor4.py Python script from AutoDockTools (ADT) (Morris et al., 2009) was used.

The dipeptides were modeled through the graphical interface of

Maestro, where the ligand preparation was also carried out using the LigPrep module. The generated file, in mol2, was also transformed into pdbqt format with the ligand_prep.py script in the ADT package.

2.4.2.3. Docking simulations. The docking programs used for these calculations were AutoDock Vina (Trott & Olson, 2010) and LeadFinder (Stroganov et al., 2008) via the MetaScreener suite (https://github.com/bio-hpc/metascreener). A large amount of docking simulations was carried out so that each of the peptides faced each of the proteins of both groups.

The calculations were carried out through the supercomputer of the University of Malaga, Picasso. The executions were launched on the “all_nodes” node, assigning 4 CPU cores and 4 GB RAM for each job, the same configuration for calculations with AutoDock Vina and LeadFinder. Once all the docking results were obtained, they were processed from a specific workflow. Initially, a consensus was reached regarding the results of both docking techniques, one for each protein group function. Afterward, the number of interactions in each protein residue for both techniques was calculated, and the number of interactions between the catalytic residue of each protein and the dipeptide in question was analysed. Based on this data, each dipeptide was ranked, ordering those proteins with a greater capacity for said dipeptide to bind to the key residue of the corresponding catalytic center.

2.4.3. Pharmacophoric similarity

The pharmacophore features for each dipeptide were extracted, and individual pharmacophoric models were created using LigandScout software (Wolber & Langer, 2005) via MetaScreener. These models were saved in PMZ format. Subsequently, virtual screening was performed using these models against the DrugBank library, a virtual collection of FDA-approved molecules, to identify those with high pharmacophore similarity to the dipeptides. The computations were done on the University of Malaga’s supercomputer, Picasso, with 4 CPU cores and 2 GB RAM allocated for each job. Finally, the results for each dipeptide were filtered using a Relative Pharmacophore Similarity cutoff of 0.90.

2.5. Simulated gastrointestinal digestion of ham peptides

Ham samples were prepared from three cured ham samples grinding and mixing Biceps femoris muscle after removing extramuscular fat. The methodology used was that followed by Minekus et al., 2014 (Minekus et al., 2014) with slight modifications.

For in vitro digestion, 5 g of ham were used, reproducing the physiological conditions of the oral, gastric and intestinal stages.

In the oral phase, the sample is homogenised with 3.5 mL of simulated salivary fluid (SSF), to which 0.5 mL of amylase 1500 U/m (dissolved in SSF), 25 uL of $CaCl_2$ (0.3 M) and 975 uL of H2O are added. The mixture is incubated at 37 °C for 2 min under agitation to simulate the effect of chewing and initial enzyme action.

The gastric phase is started by adding simulated gastric fluid (SGF) in an equal ratio to reach a final 50:50 (*v*/v) ratio with the sample. 7.5 mL of SGF, 1.6 mL of 25,000 U/mL porcine pepsin (dissolved in SGF), 5 uL of $CaCl_2$ (0.3 M) and 695 uL of H2O are added. The pH is adjusted to 3 by HCl. The mixture is incubated at 37 °C for 2 h, ensuring adequate mixing by constant agitation to simulate the acidic environment and enzymatic conditions of the stomach.

In the intestinal phase, the gastric contents (chyme) are combined with simulated intestinal fluid (SIF) in equal ratio (50,50 v/v). 11 mL of SIF, 5 mL of porcine pancreatin (800 U/mL trypsin activity), 2.5 mL of bile (160 mM), 40 uL of $CaCl_2$ (0.3 M), 1.31 mL of H2O and pH adjusted to 7 with NaOH are added. Incubation at 37 °C for 2 h allows the action of pancreatic enzymes and bile acids on lipids, proteins and carbohydrates.

Each stage includes precise controls of pH and incubation conditions, allowing to reproduce the human digestive process to analyse the digestibility and bioaccessibility of nutrients in a replicable way.

2.6. Quantification of dipeptides identified after in vitro digestion

Quantification of the peptides was determined from the different ham samples previously subjected to in vitro digestion. An AB Sciex (CA, USA) Eksigent Nano-LC Ultra 1D Plus liquid chromatography system coupled to a tandem mass spectrometer with an AB Sciex Instruments (MA, USA) Q/TOF TripleTOF 56,001 system with a nanoelectrospray ionisation source system was used for quantification. An Eksigent C18 trap column and nano-HPLC capillary column has been used for quantification.

Previously lyophilised digested peptide extracts were resuspended in 100 μL of 0.1 % (*v*/v) TFA. 10 μL of each sample was cleaned and concentrated using standard bed format Zip-Tip C18 tip cartridges from Millipore Corporation. 4 μL of the supernatant was concentrated using the C18 trap column at a flow rate of 3 mL/min for 5 min and using 0.1 % TFA as the mobile phase. The trap column is automatically switched online to the capillary nano-HPLC column.

The mobile phases are 0.1 % (v/v) FA in H2O as solvent A, and 0.1 % (v/v) FA in ACN as solvent B. HPLC conditions are a linear gradient of 5 % to 35 % solvent B for 90 min, and 10 min from 35 % to 65 % solvent B, at a flow rate of 0.3 μL/min at 30 °C.

Finally, the column output was coupled to the electrospray ionisation source and the tandem mass spectrometer was set to operate in positive polarity and information-dependent acquisition mode, with a 250 ms TOF MS sweep from 300 to 1250 *m/z*, followed by 50 ms product ion sweeps from 100 to 1500 m/z on the 50 most intense 1–5 charged ions.

Quantification of the peptide sequences of the previously digested peptide fractions from the different hams was performed at Metabolomics facilities of the Central Service of Research Support (SCAI), University of Cordoba (Spain).

2.7. Statistical analysis

All analyses of our samples were performed in triplicate. Statistical analysis of our samples was performed using SPSS software (version 21.0, IBM Corporation, Armonk, NY, USA). To evaluate how the different treatments affected the analyses carried out, a one-way ANOVA was performed between cured hams with different treatments. When the treatment effect was significant ($p < 0.05$), the results were compared using a Fisher’s LSD test.

3. Results and discussion

3.1. In vitro assays

3.1.1. Evaluation of antihypertensive activity (ACE—I) in hams with different curing losses and salt treatments

The results of the angiotensin-converting enzyme (ACE—I) inhibitory activity in ham samples processed with different curing salts and maturation times are presented in Table 1. The IC50 values, expressed in mg/mL of peptides, indicate the concentration of peptides from the peptide fraction, necessary to inhibit 50 % of ACE activity. A general trend towards improved antihypertensive activity (decreased IC50) was observed at later stages of maturation (Stage 5) compared to Stage 4. At Stage 4, treatment IV showed the lowest average IC50 value (0.108 mg/mL), suggesting high efficacy as an ACE inhibitor, while treatment IX had the highest value (0.187 mg/mL). In Step 5, IC50 values decreased markedly in most treatments. For example, treatment III reduced its IC50 from 0.153 mg/mL to 0.099 mg/mL, and treatment I improved from 0.139 mg/mL to 0.107 mg/mL. Supplementary material includes Fig. SI showing the evolution of the angiotensin I-converting enzyme inhibitory activity of the peptide fraction of the different processed cured ham samples as a function of the concentration of peptides present in the samples.

Table 1
Effect of processing conditions and salt treatment on IECA (IC50). Results are expressed in mg/ml of peptides as mean values ± SEM.

		IECA (IC50)	
		Processing Stage	
	Salt Treatment	4	5
	I	0.139 ± 0.03^{abc}	0.107 ± 0.036^{bc}
	III	0.153 ± 0.020^{ac}	0.099 ± 0.047^{b}
	IV	0.108 ± 0.013^{bc}	0.148 ± 0.007^{abc}
	VI	0.166 ± 0.048^{ac}	0.126 ± 0.039^{bc}
	VII	0.162 ± 0.009^{ac}	0.126 ± 0.009^{bc}
	IX	0.187 ± 0.095^{a}	0.153 ± 0.027^{ac}
	Interaction	0.234	
	Salt Treatment	0.221	
p-value	Processing Stage	**0.043**	

SEM: standard error of mean.
p-value Processing Conditions: One-way ANOVA between different processing conditions (*p*-value significant at p ≤ 0.05).

Results suggest a significant influence of ripening time on ACE-I activity ($p = 0.043$), while no significant differences were found between the different salt treatments ($p = 0.221$) or in the interaction between both factors ($p = 0.234$). The improvement in antihypertensive activity with curing time may be attributed to an increased release of bioactive peptides due to the action of endogenous peptidases (Escudero et al., 2012; Escudero, Mora, Fraser, Aristoy, & Toldrá, 2013; Sentandreu & Toldrá, 2001; Sentandreu & Toldrá, 2007). Furthermore, this increase in antihypertensive activity in hams with prolonged maturation verifies the stability of these peptides, which has already been demonstrated in other studies (Escudero et al., 2014). Similar results have been reported in other studies where prolonged ripening increased antihypertensive bioactivity (Hernandez Correas et al., 2024). The absence of significant differences between treatments suggests that the composition of the curing salts has less impact than the curing time on the generation of ACE-I peptides.

3.1.2. *Antioxidant activity*

The antioxidant activity of the ham extracts was evaluated by the DPPH RSA, the results of which are presented in Table 2. The IC50 values, also expressed in mg/mL, allow the evaluation of the antioxidant capacity of the different treatments and stages. In Stage 4, treatments IV and VII showed the lowest IC50 values (0.682 mg/mL), indicating a high antioxidant capacity. In contrast, treatment IX had the highest value (1.05 mg/mL). In stage 5, results showed more marked variations, treatment IX improved significantly from 1.05 mg/mL to 0.692 mg/mL, while treatment VI worsened, increasing from 0.861 mg/mL to 1.103 mg/mL. Supplementary material includes Fig. SII showing the evolution of the in vitro antioxidant capacity of the peptide fraction of the different processed cured ham samples, depending on the concentration of peptides present. Antioxidant peptides in Spanish cured hams have shown excellent stability against the presence of up to 8 % salt content despite processing conditions with higher temperatures (Gallego et al., 2018; Hernandez Correas et al., 2024), which explains the results obtained in which we observed that treatments IV and IX, despite having higher salt content, showed greater antioxidant activity. In other types of ham, such as Jinhua ham, the peptides showed less stability to fluctuations and salt percentages higher than 8 % (Zhu et al., 2014). The results of the study of Nan et al. (2024) showed that peptides (<3 KDa) extracted from low-salt cured ham had higher antioxidant activity, resulting in peptides with strong free radical scavenging activity.

Table 2
Effect of processing conditions and salt treatment on DPPH (IC50). Results are expressed in mg/ml as mean values ± SEM.

		DPPH (IC50)	
	Salt Treatment	Processing Stage	
		4	5
	I	1.079 ± 0.076^{ad}	0.927 ± $172{,}09^{adefg}$
	III	0.906 ± 0.109^{defg}	0.879 ± 0.047^{befg}
	IV	0.682 ± 0.114^{c}	0.976 ± 0.036^{adef}
	VI	0.861 ± 0.046^{bcdefg}	1.103 ± 0.226^{a}
	VII	0.682 ± 0.1^{bcg}	0.795 ± 0.049^{bcfg}
	IX	1.05 ± 0.25^{ade}	0.692 ± 0.048^{bc}
p-value	Interaction	**0.000**	
	Salt Treatment	**0.026**	
	Processing Stage	0.684	

SEM: standard error of mean.
p-value Processing Conditions: One-way ANOVA between different processing conditions (*p*-value significant at p ≤ 0.05).

Significant differences were found between treatments ($p = 0.026$) and a significant interaction between treatment and stage ($p = 0.000$), indicating that both factors affect antioxidant activity jointly. Treatments with alternative salts and special granulometry, such as VII, showed promising results. These findings are consistent with previous research highlighting the importance of processing conditions in preserving antioxidant activity (Hernandez Correas et al., 2024), such as the study by Wang et al., 2021 where the results showed that mutton ham, Jinhua ham and Xuanwei ham with the same maturation time have different proteolytic reactions, displaying different antioxidant capacities. This study also reaffirms that published by Mora et al., 2016 which also showed that the protein in dry-cured ham is affected by the raw material and processing parameters like salt content, temperature, humidity and maturation time. Other studies have suggested that peptides with higher antioxidant potential are produced the longer the processing time, (Xing et al., 2016) showing the ham at the end of maturation that the chelating activity and free radical inhibitory activity was potentially superior (Li et al., 2022), coinciding with the results obtained.

3.2. *Bioactive peptide identification*

3.2.1. *Identification of peptides present in hams with a weight < 1.5 kDa*

Table 3 shows the total number of peptides identified in the different cured ham samples processed under different treatments and conditions. In addition, the number of peptides identified with a mass lower than 1.5 kDa is specified. Peptides with bioactive potential were identified in all samples, with a higher proportion of peptides <1.5 kDa in treatments VII (991 in stage 4 and 1367 in stage 5) and IV (874 in stage 4 and 1392 in stage 5), observing for almost all samples how an increase in maturation time leads to an increase in the number of peptides present and in the amount of peptides with a size smaller than 1.5 kDa. These results verify the activity of the proteolytic enzymes with increasing maturation time (Heres et al., 2023; Li et al., 2022). Peptides <1.5 kDa exhibit higher bioavailability and functional activity, underlining the importance of treatments that favour their generation. The results obtained by Li et al., 2022 showed that the oxidation of peptides could be related to their molecular weight, so that a greater number of fractions with a small molecular weight showed higher antioxidant activity. Other studies reported that the molecular size and amino acid composition play important roles in the antioxidant activities of protein hydrolysates (Harnedy et al., 2017). Heres et al. showed an increase in dipeptide concentration with the duration of processing, obtaining values that suggest an intense action of muscle dipeptidyl peptidase enzymes during processing.

Table 3
Identification of peptides present in hams processed under different treatments and conditions.

		TI	**TIII**	**TIV**	**TVI**	**TVII**	**TIX**
F4	**Total**	1648	2160	2238	2439	2487	2097
	<1.5 kDa	632	824	673	874	991	785
F5	**Total**	1367	2331	2660	2598	2808	2482
	<1.5 kDa	679	1237	1293	1392	1367	1096

Summary tables (SI – SII – SIII- SIV- SV- SVI) containing some of the sequences identified in the peptide fraction of each sample have been added in the supplementary material. Peptides with a mass lower than 1.5kDA containing within their sequence the dipeptides quantified a posteriori, after in vitro and in silico digestion have been selected. These results corroborate the presence of peptides that after digestion can give rise to smaller peptides (dipeptides) that can exert bioactivity.

3.3. Bioactivity analysis based on amino acid composition

3.3.1. Comparative study of peptides identified in each peptide fraction from hams produced with different salts with putative activity

To contextualise the bioactive profile of the samples analysed, the data were normalized using *Z*-score, which allowed the variations between samples to be graphically represented in relation to the average of the different bioactivities, using a heatmap. Fig. I shows these results, where the intensity of the red color reflects an over-representation of a specific bioactivity with respect to the average of the three samples. An exhaustive search for peptide precursors potentially containing bio-peptides in their sequence, susceptible to activation following protein cleavage during digestion, was carried out. This strategy is especially valuable for detecting sequences that cannot be identified by conventional proteomics techniques, such as peptide chains smaller than seven amino acids.

The heatmap organises the bioactivities according to their frequency of occurrence in the different samples, arranging the rows and columns in such a way as to minimise the intersection of the dendrogram lines. The light blue lines represent the values of the coefficients. In addition, in the supplementary material, in Fig. SIII, the bioactivities highlighted in each sample are presented individually, associating the different groups of bioactivities to the colours indicated on the left edge of the heatmap (Fig. 1).

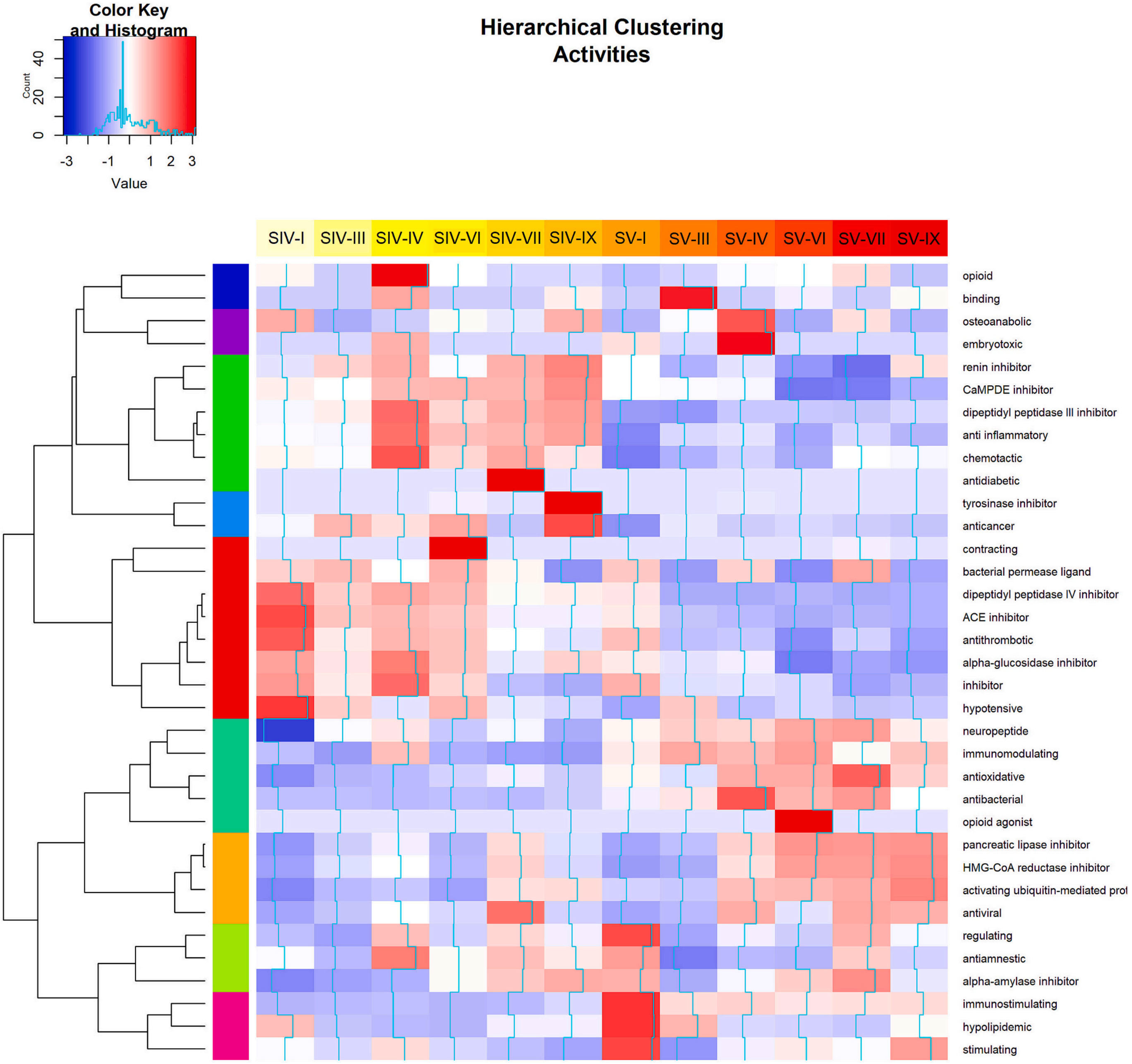


Fig. 1. Heatmap and dendrogram of bioactivities of the different ham samples studied. Quantification of bioactivity is regarding the mean. The grouping relationship between the groups of activities is defined.

Regarding the bioactivities evaluated in vitro, specifically the antihypertensive and antioxidant activities, it is highlighted that the antihypertensive activity is predominant in the samples processed with up to 38 % weight loss (SIV), as evidenced by the more intensely red areas in those columns. Particularly, the SIV-VI sample stands out for its hypotensive potential, as well as for its renin and angiotensin-converting enzyme (ACE) inhibitory capacity. These bioactivities have been extensively studied in cured ham (Heres, Mora, & Toldrá, 2021; Toldrá et al., 2023). On the other hand, the SV-treated samples show a higher antioxidant potential, suggesting a higher bioactivity in these samples. This strong antioxidant potential is consistent with the results previously obtained by in vitro studies (Table 2), other studies also showed increased antioxidant potential with increasing processing time (Harnedy et al., 2017; Li et al., 2022). This antioxidant property is influenced by their hydrophobic amino acid content. Zheng et al., 2016 (Zheng et al., 2016) demonstrated that hydrophobic amino acids are capable of neutralizing various free radicals, while Asp and Glu exhibit reduced properties. These observations provide insight into the high DPPH free radical-scavenging activity observed in our experiments. Additionally, hydrophobic amino acids contributed to enhancing the antioxidant activity of ham, thereby improving the quality of the dry-cured ham throughout the maturation process.

It should be noted that for many of these bioactivities there are no previous studies that specifically characterise them in cured ham, let alone in hams produced under controlled conditions. However, previous research has suggested that cured ham is a promising source of dipeptidyl peptidase IV (DPP-IV) inhibitors, peptides that could be adjuvants in the treatment of type II diabetes (Bailey et al., 2023; Montoro-García et al., 2017, 2022). In this regard, samples from the SIV group show a remarkable predominance of this bioactivity. Additionally, HMG-CoA reductase inhibitors play a crucial role in the prevention of premature cardiovascular risk diseases. Previous studies have identified numerous dipeptides in ham as responsible for this inhibition (Heres, Yokoyama, et al., 2021; Heres, Yokoyama, et al., 2022; Nie et al., 2020). A marked potential for this bioactivity is observed in phase V samples, probably due to the increase of small peptides, such as dipeptides, during prolonged maturation processes.

3.4. *In silico bioactivity*

3.4.1. *In silico digestion simulation of the identified peptides and prediction of bioactivities*

First, the completed list of peptides without digestion and the results obtained by this in silico digestion simulation (20,255 peptides in total, file provided in the Supplemental Data) were analysed for potential bioactivity. After a final bibliographic revision of the results obtained, seven peptides exhibited bioactive properties, results are presented in Table 4. Two of these peptides were identified as Dipeptidyl Peptidase IV inhibitors, which improve glucose control in type 2 diabetes (Makrilakis, 2019). Their immunomodulating function is also under investigation (T. Zhang et al., 2021). Other peptides with similar registered functions include DNIQGITKPAIR and FLGFPTTKTYFPHF, which exhibit immunomodulating and stimulating activities. The peptides IVGRPRHQG and VVYPWTQRF demonstrated anti-hypertensive bioactivity, likely due to their role as ACE inhibitors (Cutrell et al., 2023). Furthermore, antioxidative activity was detected in the peptide LEQQVDDLEGSLEQEKK.

Table 4
Bioactivities predicted for complete peptides.

Peptides	Bioactivity
DNIQGITKPAIR	Immunomodulating
FLGFPTTKTYFPHF	Stimulating
FN	Dipeptidyl peptidase IV inhibitor
IVGRPRHQG	ACE inhibitor
LEQQVDDLEGSLEQEKK	Antioxidative
LVVYPWTQRF	Dipeptidyl peptidase IV inhibitor
VVYPWTQRF	ACE inhibitor

3.4.2. *Prediction and quantification of bioactivities associated with peptides obtained after in silico digestion*

The use of in silico tools allows the prediction of bioactivities prior to experimental validations, optimizing the identification of functional peptides. Table 5 presents the predicted bioactivities associated with the dipeptides obtained after digestion, the dipeptide DA is identified as an inhibitor of ACE and the enzymes dipeptidyl peptidase III and glutamate carboxypeptidase II. The dipeptide DG is mainly associated with antioxidant and antihypertensive activities. G shows a dual bioactive profile as an ACE and DPP-IV inhibitor. The dipeptides ES and EE stand out for their immunomodulatory and stimulatory activities. Finally, PA and VD are associated with inhibitory effects on DPP-IV, suggesting their possible application in glycemic regulation.

Following this, an in-silico digestion was performed to predict the number of peptides identified that would give rise to the dipeptides studied. Fig. 2 represents the quantification of the number of peptides that after simulation would lead to the formation of these dipeptides.

3.4.3. *Target prediction associated with bioactivities of dipeptides identified after digestion*

Target fishing results performed for antioxidant (Fig. 3) and antihypertensive (Fig. 4) functions in characterized peptides show some possible target proteins related to these bioactivities which can explain the function of some of the peptides. Raw data obtained from the docking results is available in Supplementary Data as xlsx files.

Regarding the antioxidant activity, most of the characterized dipeptides interact with the main residue in the active sites of AKR1C1 and AKR1C3. Block action of AKR1C1 and AKR1C3 could be beneficial in reducing oxidative stress and enhancing the effectiveness of cancer treatments (Khayami et al., 2020; Matsunaga et al., 2013; Zuo et al., 2022). Another target with strong interaction found is IMMP2L, specially for EE and ES dipeptides. This protein has been studied as possible oxidant agent (Lawther et al., 2023). There is another with interaction in most of the peptides with three or more contacts with the key residue of active site, ACOX1, a generator of Reactive Oxygen Species (ROS) (Zeng et al., 2017).

Some dipeptides have demonstrated potential antihypertensive bioactivity exhibiting significant binding interactions to target proteins. EE and DV dipeptides interacted with CPA3, a carboxypeptidase implicated in cardiovascular diseases with indirect effects on hypertension management (Lewicki et al., 2019). Additionally, Hsd11b2, a protein critical for mineralocorticoid receptor activation, contributes to hypertension through sodium retention and increased blood pressure. In silico analyses revealed that the EE dipeptide binds strongly to Hsd11b2, with multiple interactions (M. A. Bailey et al., 2022). Furthermore, KLK1 and NDST3, both of which play significant roles in blood pressure regulation, exhibited strong binding interactions with dipeptides such as DG, DV,

Table 5
Prediction of bioactivities associated with peptides obtained after in silico digestion.

DIPEPTIDE	FUNCTION
DA - 1	ACE inhibitor', 'dipeptidyl peptidase III inhibitor', 'glutamate carboxypeptidase II inhibitor
DG - 2	ACE inhibitor
EE - 3	Stimulating', 'glutamate carboxypeptidase inhibitor', 'glutamate carboxypeptidase II inhibitor
ES - 4	Dipeptidyl peptidase IV inhibitor
GA - 5	ACE inhibitor', 'dipeptidyl peptidase IV inhibitor
VG - 6	ACE inhibitor', 'dipeptidyl peptidase IV inhibitor
VD - 7	Dipeptidyl peptidase IV inhibitor
PA - 8	Dipeptidyl peptidase IV inhibitor
IE - 9	ACE inhibitor', 'glutamate carboxypeptidase inhibitor

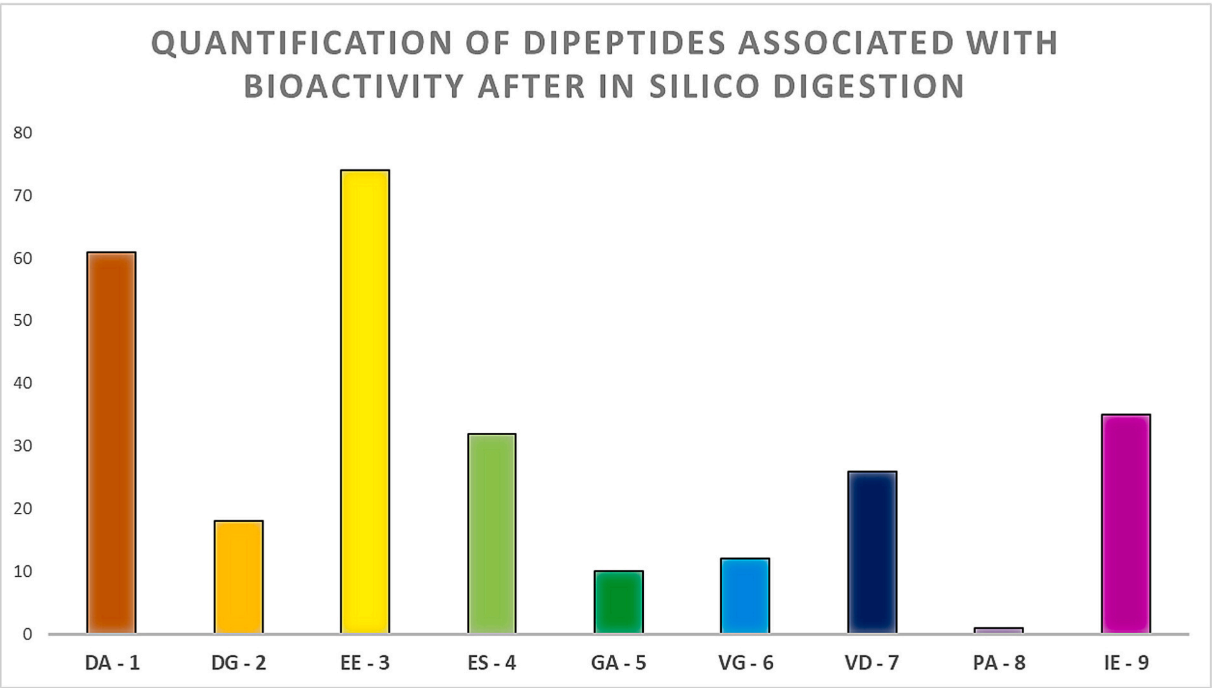


Fig. 2. Quantification of dipeptides associated with bioactivity after in silico digestion.

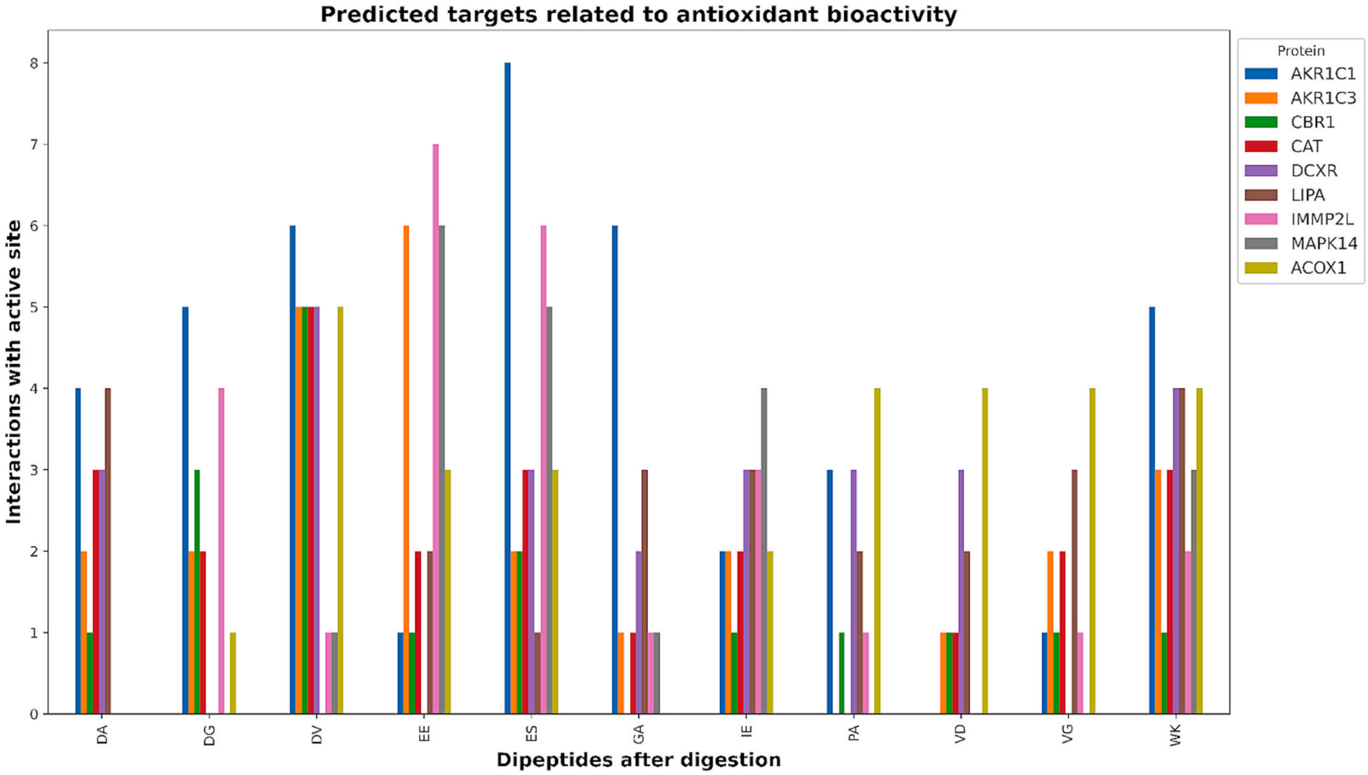


Fig. 3. Predicted targets related to antioxidant bioactivity.

EE, IE, and WK (Oppelaar et al., 2024; Sotiropoulou et al., 2009).

3.4.4. *Pharmacological similarity of the chemical structure of dipeptides identified after in silico digestion*

The potential of bioactive peptides to play a preventive role or support therapeutic treatments has garnered significant research interest, as it could help lower the prevalence of risk-related diseases and reduce healthcare expenses. Table 6 details the pharmacological similarity of the chemical structure of the dipeptides identified after in silico digestion. The dipeptides were compared with approved molecules in the DrugBank using pharmacophoric similarity scores, with those with values above 0.90 standing out.

The results reveal that dipeptides such as DG and ES obtained scores of 0.959, showing significant similarities with anti-inflammatory and

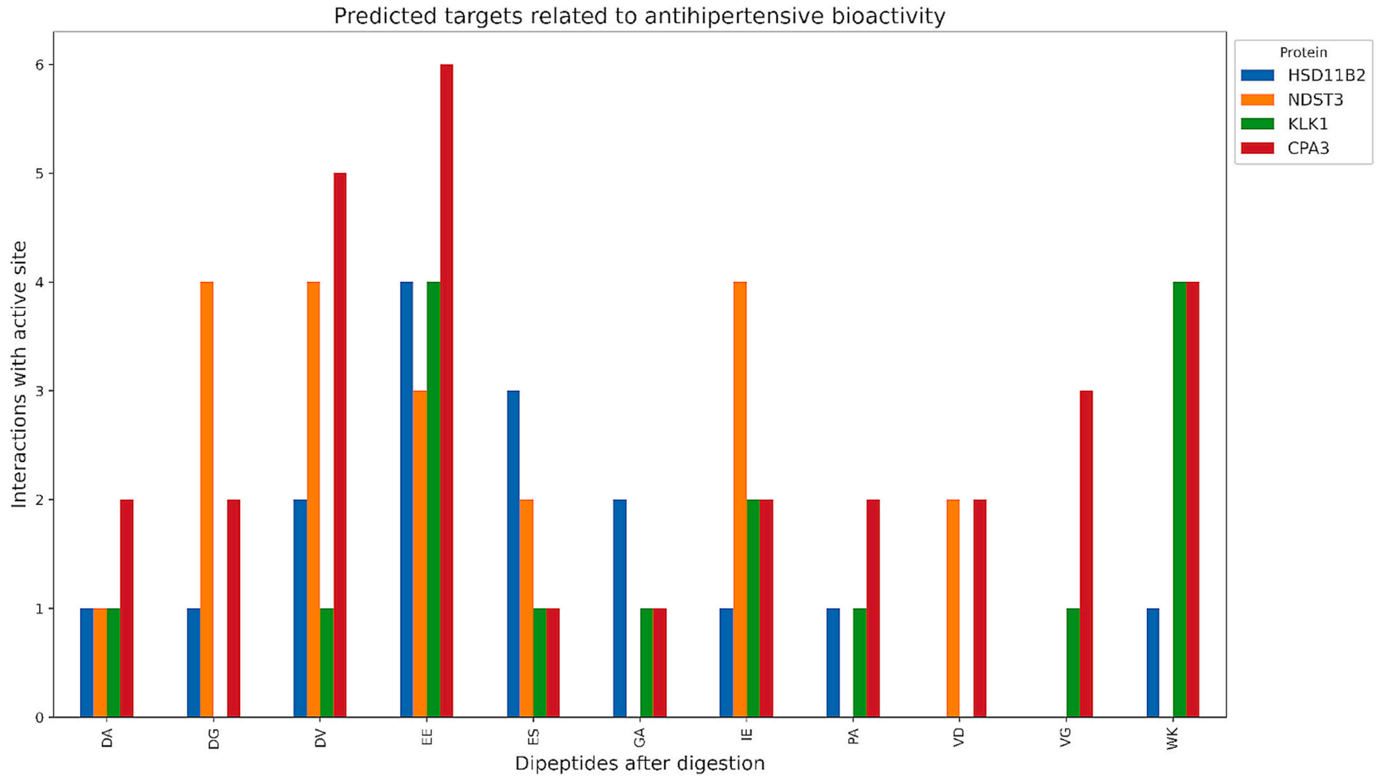


Fig. 4. Predicted targets related to antihypertensive bioactivity.

Table 6
Pharmacological similarity of the chemical structure of dipeptides identified after in silico digestion.

Dipeptide	DrugBank Code	Score	Function
DG	DB00138	0.959	Anti-inflammatory
ES	DB02321	0.959	Antioxidant Vasodilation/Blood pressure reduction
DV	DB02677	0.955	Vasodilatation/blood pressure reduction
DA	DB01835	0.954	Anti-inflammatory
DG	DB02499	0.954	Antioxidant Vasodilation/Blood pressure reduction
DG	DB01833	0.952	Antioxidant Vasodilation/Blood pressure reduction
PA	DB12783	0.951	Parkinson’s disease
SA	DB12783	0.951	Parkinson’s disease
DG	DB04700	0.947	Antioxidant Vasodilation/Blood pressure reduction
DG	DB02381	0.945	Antioxidant Vasodilation/Blood pressure reduction
DG	DB00143	0.945	Antioxidant Vasodilation/Blood pressure reduction
DG	DB03408	0.945	Antioxidant Vasodilation/Blood pressure reduction
DG	DB02999	0.943	Antioxidant
SA	DB01835	0.938	Anti-inflammatory
SA	DB12967	0.936	Antioxidant
PA	DB12967	0.936	Antioxidant
SA	DB04083	0.935	Vasodilatation/blood pressure reduction
PA	DB04083	0.935	Vasodilatation/blood pressure reduction
VG	DB01686	0.931	Antioxidant Vasodilation/Blood pressure reduction
VG	DB05779	0.93	Immunomodulator
SA	DB12237	0.928	Antioxidant Vasodilation/Blood pressure reduction
PA	DB12237	0.928	Antioxidant Vasodilation/Blood pressure reduction
PA	DB06762	0.922	Vasodilatation/blood pressure reduction

antioxidant and vasodilator compounds. The main constituent amino acids of porcine myofibrillar proteins are E, D and K (15 and 9.5 respectively). These amino acids can interact with metal ions through their charged residues and inactivate the prooxidant activity of metal ions (Saiga et al., 2003), which may explain the significant similarity with antioxidant and vasodilator compounds obtained in these in silico studies. Other dipeptides, such as DV (0.955) and DA (0.954), were associated with vasodilatory and anti-inflammatory properties, respectively. Several studies mentioned above have shown that peptides derived from dry-cured ham have an antihypertensive potential. In this line, the dipeptide DA has already been quantified in other studies in dry-cured ham produced with less salt, showing in vitro inhibitory activity on several proinflammatory enzymes (Heres, Gallego et al., 2022), which corroborates the results obtained in silico. This similarity suggests that these compounds could act as natural alternatives to commercial drugs, with additional benefits derived from their food origin. In silico analysis shows the importance of dipeptides in the bioactivity of dry-cured hams, showing the multifunctionality of dipeptides (Gallego et al., 2019), and further assays are needed to clarify the idea of possible degradation of small peptides during gastrointestinal digestion and intestinal absorption.

3.5. *Quantification of dipeptides identified after in vitro digestion*

Table 7 presents the quantification of bioactive dipeptides after in vitro digestion of hams treated with different salts and processed in two stages of maturation (38 % and 42 % weight loss). The quantified dipeptides studied were: EE, IE, GA, VD, DA, DG, VG, PA, DV, and ES. Also, several dipeptides appear to show multiple functionalities, probably determined by their structure, sequence and amino acid composition. This underlines their possible role in both sensory properties and health benefits associated with ham, particularly in its sodium-reduced variants (Gallego et al., 2019). There was a significant increase in the concentration of dipeptides such as EE, GA, VD, and VG in prolonged treatments such as IX and VI at stage 5 ($p < 0.01$). In other studies,

Table 7
Quantification of dipeptides identified after in vitro digestion.

Salt Treatment	Processing Stage	EE mg/mL	IE mg/mL	GA mg/mL	VD mg/mL	DA mg/mL	DG mg/mL	VG mg/mL	PA mg/mL	DV mg/mL	ES mg/mL
IX	4	4.646 ± 0.61	3.026 ± 0.193	3.277 ± 0.522	3.41 ± 0.433	1.67 ± 0.299	0.763 ± 0.069	2.611 ± 0.336	0.687 ± 0.09	3.25 ± 0.419	1.4 ± 0.052
IX	5	12.239 ± 3.11	7.74 ± 2.585	6.782 ± 1.682	8.54 ± 2.762	3.05 ± 0.675	0.919 ± 0.18	8.94 ± 3.434	1.884 ± 0.694	6.21 ± 1.366	1.546 ± 0.088
VII	4	5.259 ± 0.62	4.327 ± 1.213	3.947 ± 0.363	3.447 ± 0.807	1.616 ± 0.192	0.911 ± 0.07	4.328 ± 0.959	1.034 ± 0.195	3.263 ± 0.59	1.409 ± 0.092
VII	5	5.94 ± 0.099	2.888 ± 0.035	5.046 ± 0.076	3.573 ± 0.044	2.356 ± 0.011	0.964 ± 0.008	2.773 ± 0.015	1.13 ± 0.014	4.319 ± 0.09	1.645 ± 0.000
VI	4	5.254 ± 0.392	2.725 ± 0.155	4.536 ± 0.202	3.484 ± 0.037	1.727 ± 0.08	0.877 ± 0.016	3.9 ± 0.411	1.084 ± 0.17	3.224 ± 0.055	2.063 ± 0.104
VI	5	7.648 ± 0.36	4.272 ± 0.707	5.726 ± 0.108	4.621 ± 1.067	2.433 ± 0.37	0.761 ± 0.088	4.559 ± 0.975	0.972 ± 0.127	4.787 ± 1.048	2.052 ± 0.060
IV	4	3.8 ± 0.507	3.534 ± 0.509	2.857 ± 0.197	4.362 ± 0.37	1.281 ± 0.205	0.748 ± 0.041	3.948 ± 0.542	1.283 ± 0.035	3.379 ± 0.442	1.515 ± 0.018
IV	5	6.91 ± 0.102	5.231 ± 0.602	4.89 ± 0.098	5.782 ± 0.331	3.079 ± 0.174	0.883 ± 0.027	6.044 ± 0.211	1.974 ± 0.078	5.018 ± 0.377	2.107 ± 0.104
III	4	3.7 ± 0.317	2.51 ± 0.132	2.757 ± 0.776	2.959 ± 0.202	1.09 ± 0.005	0.65 ± 0.072	2.637 ± 0.339	0.934 ± 0.156	2.38 ± 0.004	1.898 ± 0.044
III	5	8.16 ± 1.146	2.436 ± 0.43	4.36 ± 0.389	4.685 ± 0.924	2.055 ± 0.211	0.655 ± 0.074	4.445 ± 0.975	1.287 ± 0.036	4.448 ± 0.493	1.996 ± 0.015
I	4	3.85 ± 0.228	2.515 ± 0.158	3.174 ± 0.03	3.074 ± 0.07	1.085 ± 0.026	0.686 ± 0.004	2.449 ± 0.104	0.862 ± 0.022	2.383 ± 0.051	1.756 ± 0.01
I	5	5.246 ± 0.536	1.584 ± 0.324	2.9 ± 0.189	2.19 ± 0.409	1.343 ± 0.185	0.426 ± 0.047	1.299 ± 0.278	0.544 ± 0.043	1.998 ± 0.305	1.519 ± 0.129
	Salt Treatment	0.095	0.052	**0.028**	0.113	**0.016**	**0.000**	0.150	0.421	**0.040**	**0.000**
p-value	Processing Stage	**0.000**	0.328	**0.002**	0.077	**0.000**	0.659	0.204	0.296	**0.003**	0.352
	Both	**0.000**	**0.006**	**0.001**	**0.012**	**0.000**	**0.001**	**0.011**	0.092	**0.001**	**0.000**

dipeptides DA, EE and ES were identified as major inhibitors of HMG-CoA reductase (HMG-CoAR), and in silico studies suggested interactions comparable to statins (Heres et al., 2021). These results show that for most of the dipeptides quantified, gastrointestinal digestion and intestinal absorption as well as interactions with the food matrix have not reduced their bioavailability. Treatment IX in hams processed up to 42 % weight loss (stage 5) presented the highest concentrations of EE (12.239 ± 3.11 mg/mL) and VG (8.94 ± 3.434 mg/mL), reflecting an optimal balance between endogenous peptidase activity and saline medium conditions, which favoured protein hydrolysis. DA and DG dipeptides, although less prevalent, showed a significant increase in advanced treatments such as IV and VII. Prolonged maturation (stage 5) significantly ($p < 0.01$) increased the concentration of most dipeptides. Other studies also suggest higher protein digestibility at longer processing times, resulting in higher generation of amino acids and di- and tri-peptides. (Paolella et al., 2015).

These results are consistent with previous studies showing that prolonged maturation enhances the generation of small peptides with high bioactivity, such as ACE inhibitors and antioxidants (Heres et al., 2023; Hernández-Ledesma et al., 2011; Toldrá et al., 2023). Prolonged maturation could also allow for greater breakdown of residual proteins, enhancing the availability of substrates for the formation of specific dipeptides, increasing the concentration of dipeptides as maturation time rises (Gallego et al., 2015, 2022; Heres et al., 2023). Some of this dipeptides have been identified in low-salt ham. It is noteworthy that some showed anti-inflammatory activity and cardiovascular potential (Heres et al., 2022). Likewise, dipeptides such as VD and PA, associated with DPP-IV inhibition, stand out for their potential as adjuvants in glycemic control, reinforcing their relevance in functional applications for cardiovascular and metabolic health. The quantification of the peptides helps us to corroborate the in-silico analyses carried out and is of vital importance to know the concentration of these peptides and to be able to corroborate the predicted in silico bioactivities that would highlight the use of these bioactive compounds as possible adjuvants or drugs for the treatment of various pathologies.

4. Conclusions

This study has shown that curing salts and prolonged maturation times significantly enhance the production of bioactive peptides in dry-cured hams. Among the various conditions evaluated, the treatment using standard nitrifying salts with reduced sodium (treatment IX) and a prolonged maturation time of 42 % weight loss (stage 5) proved to be the most effective. These conditions resulted in the highest concentrations of low-molecular-weight peptides (<1.5 kDa) such as EE, VG, and VD, known for their high bioavailability and robust bioactive potential. Antioxidant and antihypertensive activities were most prominent in samples with a 42 % weight loss, closely linked to the increased generation of low-molecular-weight peptides (<1.5 kDa).

In silico analyses predicted specific bioactivities for these peptides, including ACE inhibition, DPP-IV inhibition, and antioxidant properties. Notably, dipeptides like DG, ES, and DV exhibited strong structural similarity to FDA-approved molecules in DrugBank, underscoring their potential as natural alternatives or complements to commercial drugs. These findings were corroborated by in vitro evaluations, identifying and quantifying the presence of these dipeptides, which revealed a direct correlation between extended curing times and improved bioactive potential. The digestions did not significantly affect their concentration or availability.

The results highlight the importance of optimizing curing conditions and processing times to maximize the production of bioactive peptides, which could be leveraged for applications in cardiovascular and metabolic health. Furthermore, these peptides may serve as functional food ingredients or therapeutic agents capable of reducing the incidence of chronic diseases, thereby contributing to the development of innovative health strategies and reducing healthcare costs.

Continued research is essential to validate these findings through additional experimental assays and clinical studies. Such efforts would confirm the bioactivities of these compounds and assess their viability as therapeutic agents or adjuvants derived from functional foods. This study underscores the transformative potential of bioactive peptides in bridging the gap between nutrition and medicine, paving the way for their integration into both fields.

CRediT authorship contribution statement

Noelia Hernández Correas: Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis. **Alejandro Rodríguez Martínez:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Adela Abellán:** Writing – original draft, Resources, Investigation. **Horacio Pérez Sánchez:** Visualization, Software, Investigation, Data curation. **Luis Tejada:** Supervision, Project administration, Funding acquisition, Conceptualization.

Funding

This study is part of the project "Desarrollo de alimentos funcionales a partir de nuevas fuentes de proteína y biopéptidos (FUNPROBIOPET) - CD/AG/CA2A", created within the Complementary Agri-Food Plan AGROALNEXT in the framework of the Recovery, Transformation and Resilience Plan and funded by the European Union - NextGenerationEU, the Ministry of Science, Innovation and Universities and by the Autonomous Community of the Region of Murcia.

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.

Acknowledgments

The authors thank all the colleagues who support our work on a daily basis.

Supercomputing resources in this work have been supported by the Plataforma Andaluza de Bioinformática of the University of Malaga and by the supercomputing infrastructure of the NLHPC (ECM-02, Powered@NLHPC).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2025.144360.

Data availability

Data will be made available on request.

References

Abellán, A., Salazar, E., Vázquez, J., Cayuela, J. M., & Tejada, L. (2018). Changes in proteolysis during the dry-cured processing of refrigerated and frozen loin. *LWT, 96*, 507–512. https://doi.org/10.1016/j.lwt.2018.06.002

Aliño, M., Grau, R., Toldrá, F., & Barat, J. M. (2010). Physicochemical changes in dry-cured hams salted with potassium, calcium and magnesium chloride as a partial replacement for sodium chloride. *Meat Science, 86*(2), 331–336. https://doi.org/10.1016/j.meatsci.2010.05.003

Andres, A. I., Ventanas, S., Ventanas, J., Cava, R., & Ruiz, J. (2005). Physicochemical changes throughout the ripening of dry cured hams with different salt content and processing conditions. *European Food Research and Technology, 221*(1), 30–35. https://doi.org/10.1007/s00217-004-1115-y

Bailey, C. J., Flatt, P. R., & Conlon, J. M. (2023). An update on peptide-based therapies for type 2 diabetes and obesity. *Peptides, 161*, Article 170939. https://doi.org/10.1016/j.peptides.2023.170939

Bailey, M. A., Krilis, G., Stewart, K., & Grenier, C. (2022). Mice with Brain-Specific Deletion of Hsd11b2 have an impaired renal, vascular and tubular response to high salt intake. *The FASEB Journal, 36*(S1). https://doi.org/10.1096/fasebj.2022.36.S1.R5176

Corrêa, J. A. F., de Melo Nazareth, T., da Rocha, G. F., & Luciano, F. B. (2023). Bioactive Antimicrobial Peptides from Food Proteins: Perspectives and Challenges for Controlling Foodborne Pathogens. *Pathogens, 12*(3), 477. https://doi.org/10.3390/pathogens12030477

Cutrell, S., Alhomoud, I. S., Mehta, A., Talasaz, A. H., Van Tassell, B., & Dixon, D. L. (2023). ACE-Inhibitors in Hypertension: A Historical Perspective and Current Insights. *Current Hypertension Reports, 25*(9), 243–250. https://doi.org/10.1007/s11906-023-01248-2

Escudero, E., Aristoy, M.-C., Nishimura, H., Arihara, K., & Toldrá, F. (2012). Antihypertensive effect and antioxidant activity of peptide fractions extracted from Spanish dry-cured ham. *Meat Science, 91*(3), 306–311. https://doi.org/10.1016/j.meatsci.2012.02.008

Escudero, E., Mora, L., Fraser, P. D., Aristoy, M.-C., Arihara, K., & Toldrá, F. (2013). Purification and Identification of antihypertensive peptides in Spanish dry-cured ham. *Journal of Proteomics, 78*, 499–507. https://doi.org/10.1016/j.jprot.2012.10.019

Escudero, E., Mora, L., Fraser, P. D., Aristoy, M.-C., & Toldrá, F. (2013). Identification of novel antioxidant peptides generated in Spanish dry-cured ham. *Food Chemistry, 138*(2–3), 1282–1288. https://doi.org/10.1016/j.foodchem.2012.10.133

Escudero, E., Mora, L., & Toldrá, F. (2014). Stability of ACE inhibitory ham peptides against heat treatment and in vitro digestion. *Food Chemistry, 161*, 305–311. https://doi.org/10.1016/j.foodchem.2014.03.117

Fu, L., Xing, L., Hao, Y., Yang, Z., Teng, S., Wei, L., & Zhang, W. (2021). The anti-inflammatory effects of dry-cured ham derived peptides in RAW264.7 macrophage cells. *Journal of Functional Foods, 87*, Article 104827. https://doi.org/10.1016/j.jff.2021.104827

Gallego, M., Mora, L., Aristoy, M. C., & Toldrá, F. (2015). Titin-derived peptides as processing time markers in dry-cured ham. *Food Chemistry, 167*, 326–339.

Gallego, M., Mora, L., Reig, M., & Toldrá, F. (2018). Stability of the potent antioxidant peptide SNAAC identified from Spanish dry-cured ham. *Food Research International, 105*, 873–879. https://doi.org/10.1016/j.foodres.2017.12.006

Gallego, M., Mora, L., & Toldrá, F. (2019). The relevance of dipeptides and tripeptides in the bioactivity and taste of dry-cured ham. *Food Production, Processing and Nutrition, 1*(1), 2. https://doi.org/10.1186/s43014-019-0002-7

Gallego, M., Toldrá, F., & Mora, L. (2022). Quantification and in silico analysis of taste dipeptides generated during dry-cured ham processing. *Food Chemistry, 370*, Article 130977. https://doi.org/10.1016/j.foodchem.2021.130977

Guo, X., Wang, Y., Lu, S., Wang, J., Fu, H., Gu, B., Lyu, B., & Wang, Q. (2021). Changes in proteolysis, protein oxidation, flavor, color and texture of dry-cured mutton ham during storage. *LWT, 149*, Article 111860. https://doi.org/10.1016/j.lwt.2021.111860

Harnedy, P. A., O'Keeffe, M. B., & FitzGerald, R. J. (2017). Fractionation and identification of antioxidant peptides from an enzymatically hydrolysed Palmaria palmata protein isolate. *Food Research International (Ottawa, Ont.), 100*(Pt 1), 416–422. https://doi.org/10.1016/j.foodres.2017.07.037

Heres, A., Gallego, M., Mora, L., & Toldrá, F. (2022). Identification and Quantitation of Bioactive and Taste-Related Dipeptides in Low-Salt Dry-Cured Ham. *International Journal of Molecular Sciences, 23*(5). https://doi.org/10.3390/ijms23052507. Article 5.

Heres, A., Mora, L., & Toldrá, F. (2021). Inhibition of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase enzyme by dipeptides identified in dry-cured ham. *Food Production, Processing and Nutrition, 3*(1), 18. https://doi.org/10.1186/s43014-021-00058-w

Heres, A., Mora, L., & Toldrá, F. (2023). Bioactive and Sensory Di- and Tripeptides Generated during Dry-Curing of Pork Meat. *International Journal of Molecular Sciences, 24*(2), Article 2. https://doi.org/10.3390/ijms24021574

Heres, A., Yokoyama, I., Gallego, M., Toldrá, F., Arihara, K., & Mora, L. (2021). Antihypertensive potential of sweet Ala-Ala dipeptide and its quantitation in dry-cured ham at different processing conditions. *Journal of Functional Foods, 87*, Article 104818. https://doi.org/10.1016/j.jff.2021.104818

Heres, A., Yokoyama, I., Gallego, M., Toldrá, F., Arihara, K., & Mora, L. (2022). Impact of oxidation on the cardioprotective properties of the bioactive dipeptide AW in dry-cured ham. *Food Research International, 162*, Article 112128. https://doi.org/10.1016/j.foodres.2022.112128

Hernandez Correas, N., Abellán Guillén, A., Muñoz Rosique, B., Bande De León, C. M., Gómez, R., & Tejada, L. (2024). Overripening and increased temperature: Alternative strategies to enhance peptide production and bioactivity in salt-reduced boneless cured Iberian hams. *Applied Food Research, 4*, Article 100639. https://doi.org/10.1016/j.afres.2024.100639

Hernández-Ledesma, B., del Mar Contreras, M., & Recio, I. (2011). Antihypertensive peptides: Production, bioavailability and incorporation into foods. *Advances in Colloid and Interface Science, 165*(1), 23–35. https://doi.org/10.1016/j.cis.2010.11.001

Keil, B. (1992). Specificity of proteolysis. *Springer-Verlag*. https://doi.org/10.1007/978-3-642-48380-6

Khayami, R., Hashemi, S. R., & Kerachian, M. A. (2020). Role of aldo-keto reductase family 1 member B1 (AKR1B1) in the cancer process and its therapeutic potential. *Journal of Cellular and Molecular Medicine, 24*(16), 8890–8902. https://doi.org/10.1111/jcmm.15581

Lawther, A. J., Zieba, J., Fang, Z., Furlong, T. M., Conn, I., Govindaraju, H., … Walker, A. K. (2023). Antioxidant Behavioural Phenotype in the Immp2l Gene Knock-Out Mouse. *Genes, 14*(9). https://doi.org/10.3390/genes14091717. Article 9.

Lewicki, Ł., Siebert, J., Koliński, T., Piekarska, K., Reiwer-Gostomska, M., Targoński, R., Trzonkowski, P., & Marek-Trzonkowska, N. (2019). Mast cell derived carboxypeptidase A3 is decreased among patients with advanced coronary artery disease. *Cardiology Journal, 26*(6), 680–686. https://doi.org/10.5603/CJ.a2018.0018

Li, P., Xu, F., Zhou, H., Gao, Y., Zhu, H., Nie, W., Wang, Z., Wang, Y., Deng, J., Zhou, K., & Xu, B. (2022). Evolution of antioxidant peptides and their proteomic homology during processing of Jinhua ham. *LWT, 166*, Article 113771. https://doi.org/10.1016/j.lwt.2022.113771

Makrilakis, K. (2019). The Role of DPP-4 Inhibitors in the Treatment Algorithm of Type 2 Diabetes Mellitus: When to Select, What to Expect. *International Journal of*

Environmental Research and Public Health, 16(15), 2720. https://doi.org/10.3390/ijerph16152720

Martínez Leo, E. E., Rojas Herrera, R. A., & Segura Campos, M. R. (2022). Biopeptides with Neuroprotective Effect in the Treatment of Neuroinflammation Induced by Adiposity-based Chronic Disease. *Food Reviews International, 38*(5), 1017–1032. https://doi.org/10.1080/87559129.2020.1762639

Matsunaga, T., Hojo, A., Yamane, Y., Endo, S., El-Kabbani, O., & Hara, A. (2013). Pathophysiological roles of aldo–keto reductases (AKR1C1 and AKR1C3) in development of cisplatin resistance in human colon cancers. *Chemico-Biological Interactions, 202*(1), 234–242. https://doi.org/10.1016/j.cbi.2012.09.024

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., … Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food – an international consensus. *Food & Function, 5*(6), 1113–1124. https://doi.org/10.1039/C3FO60702J

Minkiewicz, P., Iwaniak, A., & Darewicz, M. (2019). BIOPEP-UWM Database of Bioactive Peptides: Current Opportunities. *International Journal of Molecular Sciences, 20*(23). https://doi.org/10.3390/ijms20235978. Article 23.

Molina-Valero, G., Buendía-Moreno, L., Bande-De León, C., Bueno-Gavilá, E., & Tejada, L. (2024). Production of Protein Hydrolysates Teff (Eragrostis tef) Flour with Antioxidant and Angiotensin-I-Converting Enzyme (ACE-I) Inhibitory Activity Using Pepsin and Cynara cardunculus L. *Extract. Current Issues in Molecular Biology, 46*(10). https://doi.org/10.3390/cimb46100672. Article 10.

Montoro-García, S., Velasco-Soria, Á., Mora, L., Carazo-Díaz, C., Prieto-Merino, D., Avellaneda, A., Miranzo, D., Casas-Pina, T., Toldrá, F., & Abellán-Alemán, J. (2022). Beneficial Impact of Pork Dry-Cured Ham Consumption on Blood Pressure and Cardiometabolic Markers in Individuals with Cardiovascular Risk. *Nutrients, 14*(2), Article 2. https://doi.org/10.3390/nu14020298

Montoro-García, S., Zafrilla-Rentero, M. P., Celdrán-de Haro, F. M., Piñero-de Armas, J. J., Toldrá, F., Tejada-Portero, L., & Abellán-Alemán, J. (2017). Effects of dry-cured ham rich in bioactive peptides on cardiovascular health: A randomized controlled trial. *Journal of Functional Foods, 38*, 160–167. https://doi.org/10.1016/j.jff.2017.09.012

Mora, L., Calvo, L., Escudero, E., & Toldrá, F. (2016). Differences in pig genotypes influence the generation of peptides in dry-cured ham processing. *Food Research International, 86*, 74–82. https://doi.org/10.1016/j.foodres.2016.04.023

Morris, G. M., Huey, R., Lindstrom, W., Sanner, M. F., Belew, R. K., Goodsell, D. S., & Olson, A. J. (2009). AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *Journal of Computational Chemistry, 30*(16), 2785–2791. https://doi.org/10.1002/jcc.21256

Muela, T., Abellán, A., Bande-De León, C., Gómez, P., & Gil, M. D. (2024). Effect of Macro and Microalgae Addition on Nutritional, Physicochemical, Sensorial, and Functional Properties of a Vegetable Cream. *Foods, 13*(11), Article 11. https://doi.org/10.3390/foods13111651

Nan, Y., Mu, B., Ge, C., Chen, S., Cui, M., Li, H., Zhao, C., Wang, J., Piao, C., & Li, G. (2024). Exploring the novel antioxidant peptides in low-salt dry-cured ham: Preparation, purification, identification and molecular docking. *Food Chemistry, 446*, Article 138697. https://doi.org/10.1016/j.foodchem.2024.138697

Nie, W., Zhou, K., Wang, Y., Wang, Z.-M., Xie, Y., Zhou, H., & Xu, B.-C. (2020). Isolation and identification of bioactive peptides from Xuanwei ham that rescue oxidative stress damage induced by alcohol in HHL-5 hepatocytes. *Food & Function, 11*(11), 9710–9720. https://doi.org/10.1039/D0FO02329A

del Olmo, A., Calzada, J., Gaya, P., & Nuñez, M. (2015). Proteolysis and Flavor Characteristics of Serrano Ham Processed under Different Ripening Temperature Conditions. *Journal of Food Science, 80*(11), C2404–C2412. https://doi.org/10.1111/1750-3841.13078

Oppelaar, J. J., Ferwerda, B., Romman, M. A., Sahebdin, G. N., Zwinderman, A. H., Galenkamp, H., … Vogt, L. (2024). Genetic Variance in Heparan Sulfation Is Associated With Salt Sensitivity. *Hypertension, 81*(10), 2101–2112. https://doi.org/10.1161/HYPERTENSIONAHA.124.23421

Paolella, S., Falavigna, C., Faccini, A., Virgili, R., Sforza, S., & Dall'Asta, C., Dossena, A., & Galaverna, G.. (2015). Effect of dry-cured ham maturation time on simulated gastrointestinal digestion: Characterization of the released peptide fraction. *Food Research International, 67*, 136–144. https://doi.org/10.1016/j.foodres.2014.10.026

Ruiz, J., López, R. C., Rojas, M. T. A., Tejeda, J. L., Córdoba, J. J., & Martín, L. M. (1998). Influencia de las condiciones de elaboración sobre la proteolisis durante la maduración del jamón ibérico. *Food science and technology international = Ciencia y tecnología de alimentos internacional, 4*(1), 17–22.

Saiga, A., Tanabe, S., & Nishimura, T. (2003). Antioxidant activity of peptides obtained from porcine myofibrillar proteins by protease treatment. *Journal of Agricultural and Food Chemistry, 51*(12), 3661–3667. https://doi.org/10.1021/jf021156g

Sentandreu, M., & Toldrá, F. (2001). Dipeptidyl peptidase activities along the processing of Serrano dry-cured ham. *European Food Research and Technology, 213*(2), 83–87. https://doi.org/10.1007/s002170100355

Sentandreu, M. A., & Toldrá, F. (2007). Evaluation of ACE inhibitory activity of dipeptides generated by the action of porcine muscle dipeptidyl peptidases. *Food Chemistry, 102*(2), 511–515. https://doi.org/10.1016/j.foodchem.2006.04.018

Sotiropoulou, G., Pampalakis, G., & Diamandis, E. P. (2009). Functional Roles of Human Kallikrein-related Peptidases*. *Journal of Biological Chemistry, 284*(48), 32989–32994. https://doi.org/10.1074/jbc.R109.027946

Stroganov, O. V., Novikov, F. N., Stroylov, V. S., Kulkov, V., & Chilov, G. G. (2008). Lead Finder: An Approach To Improve Accuracy of Protein−Ligand Docking, Binding Energy Estimation, and Virtual Screening. *Journal of Chemical Information and Modeling, 48*(12), 2371–2385. https://doi.org/10.1021/ci800166p

Szwajkowska, M., Teter, A., Barłowska, J., Król, J., & Litwinczuk, Z. (2011). Bovine milk proteins as the source of bioactive peptides influencing the consumers' immune system—A review. *Animal Science Papers and Reports, 29*, 269–280.

Toldrá, F., & Flores, M. (1998). The Role of Muscle Proteases and Lipases in Flavor Development During the Processing of Dry-Cured Ham. *Critical Reviews in Food Science and Nutrition, 38*(4), 331–352. https://doi.org/10.1080/10408699891274237

Toldrá, F., Reig, M., Gallego, M., & Mora, L. (2023). Bioactive Peptides in Meat and Meat Products. *Meat and Muscle Biology, 7*(3). Article 3. https://doi.org/10.22175/mmb.16243.

Trott, O., & Olson, A. J. (2010). AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of Computational Chemistry, 31*(2), 455–461. https://doi.org/10.1002/jcc.21334

Virgili, R., Saccani, G., Gabba, L., Tanzi, E., & Soresi Bordini, C. (2007). Changes of free amino acids and biogenic amines during extended ageing of Italian dry-cured ham. *LWT - Food Science and Technology, 40*(5), 871–878. https://doi.org/10.1016/j.lwt.2006.03.024

Wang, J., Guo, M., Wang, Q., Dong, J., Lu, S., Lyu, B., & Ma, X. (2021). Antioxidant activities of peptides derived from mutton ham, Xuanwei ham and Jinhua ham. *Food Research International, 142*, Article 110195. https://doi.org/10.1016/j.foodres.2021.110195

Wolber, G., & Langer, T. (2005). LigandScout: 3-D pharmacophores derived from protein-bound ligands and their use as virtual screening filters. *Journal of Chemical Information and Modeling, 45*(1), 160–169. https://doi.org/10.1021/ci049885e

Xing, L., Hu, Y., Hu, H., Ge, Q., Zhou, G., & Zhang, W. (2016). Purification and identification of antioxidative peptides from dry-cured Xuanwei ham. *Food Chemistry, 194*, 951–958. https://doi.org/10.1016/j.foodchem.2015.08.101

Zeng, J., Deng, S., Wang, Y., Li, P., Tang, L., & Pang, Y. (2017). Specific Inhibition of Acyl-CoA Oxidase-1 by an Acetylenic Acid Improves Hepatic Lipid and Reactive Oxygen Species (ROS) Metabolism in Rats Fed a High Fat Diet. *The Journal of Biological Chemistry, 292*(9), 3800–3809. https://doi.org/10.1074/jbc.M116.763532

Zhang, L., Bai, Y.-Y., Hong, Z.-S., Xie, J., & Tian, Y. (2023). Isolation, Identification, Activity Evaluation, and Mechanism of Action of Neuroprotective Peptides from Walnuts: A Review. *Nutrients, 15*(18), 4085. https://doi.org/10.3390/nu15184085

Zhang, T., Tong, X., Zhang, S., Wang, D., Wang, L., Wang, Q., & Fan, H. (2021). The Roles of Dipeptidyl Peptidase 4 (DPP4) and DPP4 Inhibitors in Different Lung Diseases: New Evidence. *Frontiers in Pharmacology, 12*. https://doi.org/10.3389/fphar.2021.731453

Zheng, L., Zhao, Y., Dong, H., Su, G., & Zhao, M. (2016). Structure–activity relationship of antioxidant dipeptides: Dominant role of Tyr, Trp, Cys and Met residues. *Journal of Functional Foods, 21*, 485–496. https://doi.org/10.1016/j.jff.2015.12.003

Zhou, C.-Y., Pan, D.-D., Bai, Y., Li, C.-B., Xu, X.-L., Zhou, G.-H., & Cao, J.-X. (2019). Evaluating endogenous protease of salting exudates during the salting process of Jinhua ham. *LWT, 101*, 76–82. https://doi.org/10.1016/j.lwt.2018.11.026

Zhou, G. H., & Zhao, G. M. (2007). Biochemical changes during processing of traditional Jinhua ham. *Meat Science, 77*(1), 114–120. https://doi.org/10.1016/j.meatsci.2007.03.028

Zhu, C.-Z., Zhang, W.-G., Kang, Z.-L., Zhou, G.-H., & Xu, X.-L. (2014). Stability of an antioxidant peptide extracted from Jinhua ham. *Meat Science, 96*(2, Part A), 783–789. https://doi.org/10.1016/j.meatsci.2013.09.004

Zuo, X., Zeng, H., Wang, B., Yang, X., He, D., Wang, L., Ouyang, H., & Yuan, J. (2022). AKR1C1 Protects Corneal Epithelial Cells Against Oxidative Stress-Mediated Ferroptosis in Dry Eye. *Investigative ophthalmology & visual science, 63*(10), 3. https://doi.org/10.1167/iovs.63.10.3