

TESIS DOCTORAL



UCAM

UNIVERSIDAD CATÓLICA
DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

Programa de Doctorado en Ciencias de la Salud

Desarrollo de nuevas estrategias tecnológicas para la obtención de jamón curado con alto contenido en péptidos bioactivos.

Autor/a:

Noelia Hernández Correas

Directores/as:

Dr. D. Luis Tejada Portero

Dra. D^a. María Adela Abellán Guillén

Murcia, 15 Septiembre de 2025

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AUTORIZACIÓN DEL DIRECTOR DE LA TESIS PARA SU PRESENTACIÓN

El Dr. D. Luis Tejada Portero y la Dra. D^a. María Adela Abellán Guillén como Directores de la Tesis Doctoral titulada “Desarrollo de nuevas estrategias tecnológicas para la obtención de jamón curado con alto contenido en péptidos bioactivos” realizada por Dña. Noelia Hernández Correas en el Programa de Doctorado en Ciencias de la Salud, **autoriza su presentación a trámite** dado que reúne las condiciones necesarias para su defensa.

Lo que firmo, para dar cumplimiento al Real Decreto 99/2011 de 28 de enero, en Murcia a 12 de septiembre de 2025.

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This Thesis takes the form as **COMPENDIUM OF PUBLICATIONS**. It brings together concepts derived from a literature review and *in vitro* and *in vivo* trials. The compendium contains eight articles, four of which have already been published, two are currently being reviewed by journals, and two others are in development. The articles that form part of this thesis are as follows:

ARTICLE 1:

Hernández Correás, N., Liceaga, A.M., Abellán, A., Muñoz-Rosique, B., & Tejada, L. (2024). **Bioactivities derived from dry-cured ham peptides: A review.** *Antioxidants*, 14(8), 1011. <https://doi.org/10.3390/antiox1408101>

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ARTICLE 2:

Hernández Correás, N., Abellán, A., Cayuela, J. M., Bande-De León, C., & Tejada, L. (2024). **Effect of Overripening on the Physico-Chemical and Sensory Characteristics of Boneless, Salt-Reduced Iberian Dry-Cured Ham.** *Foods*, 13(10), 1588. <https://doi.org/10.3390/foods13101588>

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ARTICLE 4:

Hernández-Correas, N., Tejada, L., Molina-Valero G., Muñoz-Rosique, B., & Abellán, A. (2025). Curing strategies for Spanish dry-cured ham: Food Quality and Preference. *LWT – Food Science and Technology*

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ARTICLE 5:

Hernández-Correas, N., Rodríguez- Martínez, A., Abellán, A., Pérez-Sánchez, H., & Tejada, L. (2025). Curing strategies and bioactive peptide generation in ham: *In vitro* digestion and *in silico* evaluation. *Food Chemistry*, 484, 144360. <https://doi.org/10.1016/j.foodchem.2025.144360>

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ARTICLE 6:

Hernández-Correas, N., Abellán, A., Muñoz-Rosique B., & Tejada, L. (2025). Curing strategies for the production of a boneless dry-cured ham with up to 50% less sodium. Effect on the physicochemical profile and on sensory quality and acceptability.

Publication Date: Under Development

ARTICLE 7:

Hernández-Correas, N., Rodríguez-Martínez, A., Tejada, L., Pérez-Sánchez, H., & Abellán, A., (2025). Curing strategies for obtaining ham with 50% less sodium. Effect on the generation of bioactive peptides. *In vitro* and *in silico* evaluation.

Publication Date: Under Development

ARTICLE 8:

Hernández-Correas, N., Liceaga, A. M., Abellán, A., Noguera, C., & Tejada, L. (2024). Bioactive Peptides from Spanish Dry-Cured Ham: *In Vitro e In Vivo* Antioxidant Effects using a *Caenorhabditis elegans* model. *Meat Science*

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RESUMEN

Título: Desarrollo de nuevas estrategias tecnológicas para la obtención de jamón curado con alto contenido en péptidos bioactivos.

Introducción: El jamón curado, alimento tradicional de la dieta mediterránea altamente valorado por los consumidores, presenta un elevado contenido de sodio que se ha asociado con efectos negativos sobre la salud. La proteólisis que tiene lugar durante su proceso de curado y maduración favorece la liberación de péptidos bioactivos, que han demostrado poseer entre otras, actividad antioxidante, antihipertensiva y antidiabética. Sin embargo, la influencia de factores tecnológicos durante el procesado como pueden ser la reducción de sal, la sobremaduración, el incremento de temperatura o los cambios en las sales de curado sobre la generación y biodisponibilidad de dichos péptidos ha sido poco estudiada. Con el desarrollo de la presente tesis doctoral se pretende evaluar el efecto de dichos cambios y, al mismo tiempo, potenciar la generación de péptidos bioactivos.

Objetivos: El objetivo principal del estudio es encontrar diferentes técnicas y estrategias de procesado que permitan aumentar la concentración de péptidos bioactivos del jamón sin comprometer su calidad. Se persigue caracterizar los diferentes perfiles peptídicos generados bajo las diferentes estrategias empleadas y validar el potencial funcional de los péptidos obtenidos mediante ensayos *in vitro*, *in silico* e *in vivo*, determinando su funcionalidad en un modelo biológico.

Resultados: Los resultados de la tesis doctoral se exponen mediante una sucesión de ocho capítulos distintos que comienzan con una revisión del conocimiento existente de las bioactividades de los péptidos generados durante el proceso de curado del jamón, destacando su potencial antihipertensivo, antioxidante, antidiabético, antimicrobiano y antiinflamatorio.

En el segundo capítulo tras experimentar con técnicas de sobremaduración en jamones ibéricos, deshuesados y reducidos en sal, demostrando que el incremento en el tiempo de maduración intensificaba la proteólisis y la

acumulación de aminoácidos libres sin afectar de manera significativa ni a la textura ni a la aceptabilidad sensorial del jamón, lo que abría la puerta a evaluar el incremento de la producción de péptidos mediante esta estrategia. Estos resultados se exponen durante el tercer capítulo de la tesis que además evalúa el efecto de la temperatura revelando que los jamones sobremadurados a una temperatura controlada de 30°C obtuvieron un perfil peptídico con mayor actividad antioxidante e inhibidora de la enzima convertidora de angiotensina, mientras que a una temperatura controlada de 36°C se alcanzaba una mayor proporción de péptidos identificados pero sin desembocar en una mejora significativa de las bioactividades estudiadas.

En el capítulo cuatro se estudió el impacto de la variación durante el curado de las sales nitrificantes y la sobremaduración, mostrando que la combinación de reducción o modificación parcial del contenido de sales nitrificantes durante el proceso de salado mantenía la seguridad y calidad sensorial, a la vez que modulaba la proteólisis, la liberación de aminoácidos y los atributos de color y textura. Una vez evaluados estos parámetros y no obteniendo resultados que afectaran significativamente a la calidad del producto final o a la aceptación sensorial se continuo con el desarrollo del siguiente capítulo.

En el capítulo cinco se aplicaron técnicas *in vitro* e *in vivo* para evaluar y/o predecir la bioactividad de los péptidos identificados procedentes de los jamones elaborados con diferentes tratamientos de sales nitrificantes y distinto tiempo de maduración, observando un aumento en la concentración de péptidos con bajo peso molecular en maduraciones avanzadas, identificando en las mismas una mayor concentración de dipéptidos que mediante análisis *in silico* mostraron potencial antioxidante, inhibidor de la enzima convertidora de angiotensina e inhibidora de la dipeptidil peptidasa-4, mostrando algunos de estos dipéptidos identificados y cuantificados, similitud con fármacos ya aprobados y comercializados por la FDA (Food and Drug Administration, EEUU). Estos resultados alentaron al estudio de una reducción aún más potencial del contenido de sales nitrificantes y sodio en el jamón, evaluando la reducción de este en un 50% del contenido de sales nitrificantes y sodio, confirmando durante el capítulo seis que se podían mantener perfiles físico-químicos adecuados y una aceptación sensorial positiva de los distintos jamones evaluados sin comprometer la seguridad y calidad, lo que valida la viabilidad industrial de este enfoque. De manera

complementaria, el capítulo siete evaluó el impacto de esta reducción del 50% sobre la generación de péptidos, demostrando mediante distintas técnicas *in vitro* e *in silico* que esta estrategia no solo no compromete la bioactividad, sino que la potencia, aumentando la liberación de péptidos antioxidantes e inhibidores de la enzima convertidora de la angiotensina.

Finalmente en el capítulo ocho se evaluó la funcionalidad de los péptidos obtenidos durante el estudio anterior en un modelo *in vivo*, *Caenorhabditis elegans*, donde los péptidos derivados del jamón curado incrementaron la longevidad y la resistencia al estrés oxidativo, redujeron los niveles de ROS e indujeron la expresión de genes como *GST-4*, *GST-10* y *SKN-1*, consolidando así la evidencia de que el jamón curado mediante innovaciones tecnológicas durante su procesado puede transformarse en un alimento funcional con aplicaciones reales en la prevención de enfermedades y el cuidado de la salud.

Conclusiones: El jamón curado puede transformarse en un alimento funcional. Diversas estrategias tecnológicas, como la reducción del contenido de sal, la modificación en la composición de las sales de curado o la aplicación de procesos de sobremaduración, han demostrado favorecer la generación de péptidos bioactivos con potenciales efectos beneficiosos para la salud. Los diferentes cambios implementados durante el procesado de los jamones sientan las bases para la producción de jamones curados con mayor contenido en péptidos bioactivos, con un claro potencial para contribuir a la prevención de enfermedades crónicas relacionadas con la hipertensión y el estrés oxidativo.

Palabras Clave: Antioxidantes en los Alimentos; Nutrientes; Péptidos; Productos de Origen Animal; Propiedades de los Alimentos; Proteínas; Tecnología de los Alimentos.

ABSTRACT

Title: Development of new technological strategies for obtaining cured ham with a high content of bioactive peptides.

Introduction: Dry-cured ham, a traditional food in the Mediterranean diet that is highly valued by consumers, has a high sodium content that has been associated with negative health effects. The proteolysis that occurs during the curing and maturing process promotes the release of bioactive peptides, which have been shown to have antioxidant, antihypertensive, and antidiabetic properties, among others. However, the influence of technological factors during processing, such as salt reduction, over-maturation, increased temperature, or changes in curing salts, on the generation and bioavailability of these peptides has been little studied. The aim of this doctoral thesis is to evaluate the effect of these changes and, at the same time, to enhance the generation of bioactive peptides.

Objectives: The main objective of the study is to find different processing techniques and strategies that allow increasing the concentration of bioactive peptides in ham without compromising its quality. The aim is to characterize the different peptide profiles generated under the different strategies employed and to validate the functional potential of the peptides obtained through *in vitro*, *in silico*, and *in vivo* tests, determining their functionality in a biological model.

Results: The results of the doctoral thesis are presented in a series of eight different chapters, beginning with a review of existing knowledge on the bioactivities of peptides generated during the ham curing process, highlighting their antihypertensive, antioxidant, antidiabetic, antimicrobial, and anti-inflammatory potential.

In the second chapter, over-maturation techniques were tested on Iberian hams, deboned and reduced in salt, demonstrating that increasing the maturation time intensified proteolysis and the accumulation of free amino acids without significantly affecting the texture or sensory acceptability of the ham, which opened the door to evaluating the increase in peptide production using this

strategy. These results are presented in the third chapter of the thesis, which also evaluates the effect of temperature and revealed that hams overripened at a controlled temperature of 30°C obtained a peptide profile with greater antioxidant activity and angiotensin-converting enzyme inhibitory potency, while at a controlled temperature of 36°C a higher proportion of identified peptides were identified, but without leading to a significant improvement in the bioactivities studied.

Chapter four examined the impact of variation during the curing of nitrifying salts and overripening, showing that the combination of reduction or partial modification of the nitrifying salt content during the salting process maintained safety and sensory quality, while modulating proteolysis, amino acid release, and color and texture attributes. Once these parameters had been evaluated and no results were obtained that significantly affected the quality of the final product or sensory acceptance, the development of the next chapter continued.

In chapter five, *in vitro* and *in vivo* techniques were applied to evaluate and/or predict the bioactivity of the peptides identified from hams produced with different nitrite salt treatments and different maturation times, observing an increase in the concentration of low molecular weight peptides in advanced maturations, identifying in them a higher concentration of dipeptides that, through *in silico* analysis, showed antioxidant potential, angiotensin-converting enzyme inhibitor, and dipeptidyl peptidase-4 inhibitor. Some of these identified and quantified dipeptides showed similarity to drugs already approved and marketed by the FDA (Food and Drug Administration, USA). These results encouraged the study of an even greater potential reduction in the nitrite and sodium content of ham, evaluating a 50% reduction in nitrite and sodium content. Chapter six confirmed that adequate physicochemical profiles and positive sensory acceptance of the different hams evaluated could be maintained without compromising safety and quality, which validates the industrial viability of this approach. Complementarily, chapter seven evaluated the impact of this 50% reduction on peptide generation, demonstrating through various *in vitro* and *in silico* techniques that this strategy not only does not compromise bioactivity, but enhances it, increasing the release of antioxidant peptides and angiotensin-converting enzyme inhibitors.

Finally, chapter eight evaluated the functionality of the peptides obtained during the previous study in an *in vivo* model, *Caenorhabditis elegans*, where peptides derived from cured ham increased longevity and resistance to oxidative stress, reduced ROS levels, and induced the expression of genes such as *GST-4*, *GST-10*, and *SKN-1*, thus consolidating the evidence that cured ham, through technological innovations during its processing, can be transformed into a functional food with real applications in disease prevention and health care.

Conclusions: Dry-cured ham can be transformed into a functional food. Diverse technological strategies, such as reducing salt content, modifying the composition of curing salts, or applying overripening processes, have been shown to promote the generation of bioactive peptides with potential health benefits. The different changes implemented during ham processing lay the foundations for the production of cured hams with a higher content of bioactive peptides, with clear potential to contribute to the prevention of chronic diseases related to hypertension and oxidative stress.

Keywords: Antioxidants in Food; Nutrients; Peptides; Animal Products; Food Properties; Proteins; Food Technology.

A mis padres por apoyarme,
A mi hermana por estar siempre,
A mis abuelos por guiarme y cuidarme,
A mis amigas/os por confiar y caminar conmigo,
A mis padrinos, mis tíos, mis primos y toda mi familia,
A Ahmed por elegirme para construir un futuro de la mano,
A mi familia, sois mi vida.

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"La ciencia se compone de errores, que, a su vez, son los pasos hacia la verdad". Julio Verne (1828-1905).

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I – INTRODUCTION

I - INTRODUCTION

In recent years, growing public concern about the relationship between diet and health has led to a rethinking of the role of food in the prevention of chronic diseases. Numerous reports from international organizations, such as the World Health Organization, warn of the negative effects of excessive salt consumption, which is directly associated with hypertension, cardiovascular disease, and certain types of cancer (Grillo et al., 2019; Kwong et al., 2023; B. Wu et al., 2021). Ultra-processed products, which have a high sodium content, are one of the main sources of this mineral in the diet, contributing to the population's daily intake far exceeding the established recommendations (Monteiro et al., 2024; Popkin et al., 2024). In this context, reducing the presence of salt in processed foods while ensuring their sensory and microbiological quality is one of the major challenges facing the food industry.

At the same time, research in the field of functional foods has experienced remarkable growth (Ponte et al., 2025; Sgroi et al., 2024). These foods, in addition to providing basic nutrients, offer additional physiological benefits or help reduce the risk of chronic diseases (Mittal et al., 2024; Vignesh et al., 2024). Among the compounds of greatest interest are bioactive peptides, protein fragments that are released by enzymatic action during digestion or technological processing and that can exert antioxidant, antihypertensive, antidiabetic, anti-inflammatory, or immunomodulatory effects (Ashraf et al., 2020; Mittal et al., 2024; Purohit et al., 2024).

Cured ham, an emblematic product of the Mediterranean diet, is an example of a traditional food that can become a vehicle for functional compounds. Its prolonged curing process involves intense proteolysis of myofibrillar and sarcoplasmic proteins, resulting in a wide variety of low molecular weight peptides with bioactive potential (del Olmo et al., 2015; Escudero et al., 2013a; Gallego et al., 2020, p. 201). In fact, it has been reported that during the maturation of ham, the concentration of di- and tripeptides capable of exerting antioxidant, antihypertensive, or immunomodulatory effects increases (Heres et al., 2021, 2023; Mora, Escudero, et al., 2015).

However, the consumption of cured ham is also associated with high sodium and nitrite content, ingredients that are essential for the microbiological safety, texture, and flavor of the product, but whose excessive consumption is linked to chronic diseases. Despite growing interest, existing studies on the effect of salt reduction, variation in curing salts, or changes in processing conditions on the generation of bioactive peptides in ham are still scarce, and even less so in innovative formats such as boneless ham.

Given this lack of knowledge, competitive projects were launched, FUNPROBIOPET – CD/AG/CA2A, whose objective has been to develop healthier hams through strategies to reduce sodium and nitrifying salts, while enhancing the release of bioactive peptides. These projects were based on the hypothesis that technological modifications, such as over-maturation, temperature variation, or partial salt substitution, could increase proteolysis and promote the appearance of new bioactive peptides without deteriorating sensory quality or compromising product safety.

This doctoral thesis is developed within this framework and brings together a series of studies designed to comprehensively evaluate how changes in ham processing influence the generation, characterization, and bioactivity of peptides. Its approach responds to several gaps in knowledge and specific needs:

- Lack of information on how technological changes in processing (sodium reduction, use of alternative salts, overripening, temperature variations, nitrite reduction) affect the generation and concentration of bioactive peptides in ham.
- Lack of comprehensive studies combining *in vitro*, *in silico*, and *in vivo* approaches to demonstrate the bioactivities of ham-derived peptides. To date, most research has been limited to sequence identification, without advancing their bioavailability or functional validation in cellular or animal models.
- Lack of safe and sustainable reformulation strategies that reduce sodium and nitrites while maintaining sensory and microbiological stability and improving the health benefits of ham.

The central hypothesis of this study is that by optimizing curing conditions and using alternative salt formulations, it is possible to increase the generation of new bioactive peptides in ham, resulting in a product with greater health benefits without compromising its sensory quality. To this end, a series of studies was designed combining alternative technological strategies, advanced proteomic analyses, and bioactivity tests at different levels (*in vitro*, *in silico*, and *in vivo*).

The thesis is presented in the form of a compendium of publications, consisting of eight scientific articles that, taken together, address this issue from different perspectives. The following sections justify the studies carried out, placing their contribution in the current scientific and technological context and showing how, taken together, they respond to the overall objective of the thesis.

II – JUSTIFICATION

II - JUSTIFICATION

The purpose of this doctoral thesis is based on the need to move towards the production of healthier meat products that respond to current public health challenges and consumer demands. Cured ham, an emblematic product of the Mediterranean diet and of great socioeconomic importance in Spain, is a food with recognized sensory properties but also a high sodium and nitrite salt content, which limits its consideration as a healthy product. For this reason, reformulating ham to reduce its salt content and enhance its functional value has become a major technological, scientific, and social challenge.

The starting point for this research is found in the first chapter, which consisted of a comprehensive review of the state of the art on peptides derived from cured ham and their potential bioactivities. The decision to begin with this work responds to the need to establish a solid and up-to-date conceptual framework on the state of the art, given that, despite advances in knowledge of bioactive peptides in other food matrices—such as dairy products, soybeans, and fish—the specific literature on cured ham was very limited until a few years ago (Escudero et al., 2013a; L. Fu et al., 2021; Gallego et al., 2014).

This review systematically compiles and analyses existing findings on peptide generation mechanisms during the curing process (proteolysis by cathepsins, calpains, and exopeptidases), sequences identified in cured hams using advanced proteomics techniques, described bioactivities, highlighting the antioxidant, antihypertensive, immunomodulatory, and antidiabetic properties of different low molecular weight peptides. However, it also revealed significant gaps: the absence of comprehensive studies combining *in vitro*, *in silico*, and *in vivo* approaches; the lack of information on the influence of salt and nitrifying salt reduction on the generation of these peptides; and the virtual absence of research on boneless hams, a format of growing industrial interest. Thus, this first chapter not only compiled existing knowledge, but also clearly justified the need for the

experimental studies that make up the following chapters. Thus, this first study not only compiled existing knowledge, but also clearly justified the need for the experimental studies that make up the following studies.

The second study was conceived as a direct consequence of the conclusions of the review: if ham is a matrix rich in bioactive peptides, what technological strategies could enhance their release? One of the most interesting hypotheses was that overripening could increase proteolysis and, therefore, favor the accumulation of free amino acids and functional peptides, even in salt-reduced boneless Iberian hams. Traditionally, reducing sodium in ham has posed a risk of loss of sensory quality and microbiological stability, but to date, it has not been evaluated whether extending maturation could compensate for these limitations. In the ham industry, overripening is traditionally considered a risky process, as excessive proteolysis can lead to texture defects (softening) and bitter flavors (Cilla et al., 2005). However, it has also been observed that longer maturation times can promote the release of free amino acids and small peptides, with possible functional effects (Gallego, Mora, & Toldrá, 2018b).

Results showed that overripening intensified proteolysis without negatively altering the texture or sensory acceptability of reduced-salt boneless Iberian hams. From a sensory point of view, although slight variations in texture and flavor were observed, these did not reach levels that would cause consumers to reject the product. This evidence justified overripening as a viable strategy for combining salt reduction and deboning with the generation of bioactive peptides, opening a new avenue of research.

Once this effect had been verified, the next study took a more ambitious step by exploring the influence of temperature in combination with overripening in deboned, reduced-salt Iberian hams. If the initial review had indicated that enzymatic activity depends largely on technological factors such as time and temperature, it seemed logical to check how controlled variations could modulate the bioactive profile of the ham. The tests confirmed that processing at 30 °C enhanced antioxidant activity and angiotensin-converting enzyme inhibition, while at 36 °C a greater diversity of sequences was achieved, with more than a thousand peptides being identified using peptidomic techniques.

These findings justified that precise temperature control during over-maturation allows the generation of peptides to be directed towards specific bioactivities, providing useful tools for designing functional foods with specific applications in the prevention of hypertension and oxidative stress.

Having demonstrated in previous chapters that overripening and temperature variation could increase the release of bioactive peptides in boneless, reduced-salt Iberian hams, the next step would have been to continue working with this line of hams. However, regulatory restrictions imposed by the Protected Designation of Origin (PDO) do not allow boneless products to be marketed as “Iberian ham.” As a result, despite following a similar production process and using raw materials of comparable quality, economic profitability is significantly lower. This limitation means that it is not an economically viable product, even though it has excellent sensory characteristics. Given this situation, it was decided to focus the research on hams obtained from a more economical raw material, but with compositional characteristics similar to those of Iberian ham, especially with regard to intramuscular fat infiltration. For this reason, subsequent work was carried out with animals of the Duroc genetic line.

The next step was to address a critical aspect of ham reformulation: the presence of nitrite salts. Their technological and safety importance is indisputable, but their excessive use is increasingly being questioned by health authorities and consumers. The next study focused precisely on evaluating different formulations of nitrite curing salts and maturation times to see if it was possible to reduce nitrites and nitrates, obtaining healthier hams without compromising product quality. The results showed that it was feasible to reduce these salts and that, together with the reduction in sodium, the quality and acceptability of the ham were maintained and the generation of bioactive peptides increased, which justified that the reformulation of ham was also viable in this line of hams. This consolidates the idea that it is possible to move towards safer and healthier hams, responding to health recommendations and growing consumer demand.

At this stage, a new research question emerged: whether the peptides generated under these reformulated conditions are capable of retaining their bioactivity after digestion and demonstrating real bioavailability. To address this question, this study combined two complementary approaches: *in vitro* digestion to simulate the gastrointestinal process and evaluate the release and potential bioavailability of peptides, and *in silico* analysis using bioinformatic databases and molecular modelling to predict the possible biological activities of the identified peptides. The tests made it possible to simulate gastrointestinal digestion and predict, using bioinformatic tools, the bioactivity of the identified peptides. Di- and tripeptides with antioxidant activity, angiotensin-converting enzyme inhibitor activity, and dipeptidyl peptidase-IV inhibitor activity were observed, some of which had structural similarities to FDA-approved drugs. This chapter demonstrates that the integration of *in vitro* and *in silico* techniques is essential for validating the functionality of peptides and guiding the development of processing strategies that increase their generation.

After reviewing the results obtained in previous studies, we moved on to address a complementary challenge: we attempted to produce ham with 50% less salt, modifying both the formulation of the salt used during the rubbing and curing process and the grain size of the salt used to bury the hams. The rationale behind this was those partial reductions, although beneficial, make it difficult to achieve public health objectives. It was necessary to assess whether such a drastic reduction could maintain adequate physical-chemical parameters, texture, color, and stability, as well as positive consumer acceptance. The results showed that, under controlled conditions, it is possible to maintain the technological and sensory quality of the ham even with such drastic reductions, thus justifying the feasibility of transferring these strategies to an industrial scale.

The importance of this study lies in the fact that it is one of the first systematic approaches to the impact of reducing such a high percentage of sodium in boneless dry-cured white Spanish hams, in line with public health recommendations and consumer demands. Furthermore, it opens the possibility of combining the reduction of sodium and with other strategies, such as overripening, to enhance the generation of bioactive peptides without compromising safety or acceptability.

The results showed that reducing sodium content increased the generation of bioactive peptides, some of which had remarkable antioxidant and antihypertensive properties. Similarly, *in silico* analysis identified sequences with high potential for interaction with physiological targets related to blood pressure regulation and oxidative stress. This study demonstrated that salt reduction is not only feasible from a sensory and technological point of view but can even promote the generation of peptides with functional activities. This study complemented the previous study, offering a comprehensive view of the effects of sodium and nitrite reduction on both product quality and functional profile.

Finally, to corroborate the biological activity of the peptides, a test was performed using an *in vivo* model, *Caenorhabditis elegans*. Until then, studies had mainly used *in vitro* approaches (antioxidant assays, ACE inhibition, digestions) and *in silico* approaches (molecular modelling, prediction of biological targets). However, it was still necessary to corroborate these results in a living organism. To this end, the nematode *Caenorhabditis elegans* was used, a model widely used in biomedical research due to its genetic simplicity, its molecular homology with humans, and its suitability for studying processes related to aging, oxidative stress, and immune responses.

Results showed that the peptide extracts obtained from hams with 25% less sodium through changes in processing in the previous study significantly improved the nematodes' resistance to acute and chronic oxidative stress, prolonging their survival against pro-oxidant agents and also modulating the expression of antioxidant genes such as *GST-4*, *GST-10*, and *SKN-1*. This finding constitutes the first *in vivo* evidence of the bioactivity of peptides derived from cured ham, confirming that the results observed in *in vitro* and *in silico* analyses have real physiological relevance. The development of this study consolidated the methodological approach proposed in the thesis, which advances from technological optimization of processing to biochemical and functional characterization and, finally, *in vivo* validation.

This thesis responds to one of the main demands of society and the food industry today: the development of healthier traditional products without compromising their quality and

safety attributes. Throughout its chapters, it has been justified and demonstrated that it is possible to reformulate the cured ham production process by reducing sodium and nitrites, over-maturing and controlling temperature to increase the generation of bioactive peptides with beneficial health functions

III – OBJECTIVES

III - OBJECTIVES

The main objective of this doctoral thesis focuses on developing and evaluating new technological strategies in cured ham processing that enhance the generation of bioactive peptides in order to revalue a traditional food as a healthy product with nutraceutical potential.

In addition to the general objective, this thesis includes the following specific objectives:

- Analyze the available scientific evidence on peptides derived from cured ham, describing their most relevant biological activities and their potential application in the development of functional foods.
- Evaluate the effect of over-maturation on boneless, reduced-salt Iberian hams on their physicochemical properties, proteolysis, amino acid generation, acceptability, and sensory quality.
- Determine how over-maturation and increased temperature during the curing process influence the diversity, quantity, and bioactive potential of the peptides generated in boneless, reduced-salt Iberian hams.
- Study the impact of different nitrite salt formulations and different curing times on the physicochemical parameters, proteolysis, amino acid release, and sensory quality of cured ham.
- Identify, quantify, and predict the bioactivities associated with bioactive peptides generated under different curing strategies with different nitrite salt formulations and different processing times using different *in vitro* and *in silico* techniques.
- Analyse the physical-chemical and biochemical profile, as well as the sensory quality and acceptance, of cured hams with up to 50% less

nitrite salt content, with the aim of validating the industrial viability of their production.

- Evaluate how reducing nitrifying salt content by up to 50% can influence the generation of bioactive peptides, through *in vitro* studies and *in silico* predictions of their possible functional activities.
- Corroborate the possible antioxidant effects of peptides derived from cured ham in an *in vivo* model widely used in molecular biology, *Caenorhabditis elegans*, studying the impact on longevity, resistance to oxidative stress, and the regulation of genes associated with defense against oxidative stress.

IV – COMPENDIUM OF ARTICLES

IV - COMPENDIUM OF ARTICLES

ARTICLE 1:

Hernández Correas, N., Liceaga, A.M., Abellán, A., Muñoz-Rosique, B., & Tejada, L. (2024). **Bioactivities derived from dry-cured ham peptides: A review.** *Antioxidants*, 14(8), 1011. <https://doi.org/10.3390/antiox14081011>

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Review

Bioactivities Derived from Dry-Cured Ham Peptides: A Review

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Abstract

Dry-cured ham is a traditional food in the Mediterranean diet, which, in addition to its sensory qualities, is a natural source of bioactive peptides generated during the curing process through the action of endogenous enzymes on muscle and sarcoplasmic proteins. These low-molecular-weight peptides have attracted growing interest due to their multiple bioactivities, including antihypertensive, antioxidant, antimicrobial, antidiabetic, and anti-inflammatory effects described in vitro, in vivo, and in preliminary human studies. The identification of specific sequences, such as AAPLAP, KPVAAP, and KAAAAP (ACE inhibitors), SNAAC and GKFNV (antioxidants), RHGYM (antimicrobial), and AEEEEYDPL and LGVGG (dipeptidyl peptidase-IV and α -glucosidase inhibitors), has been possible thanks to the use of peptidomics techniques, tandem mass spectrometry, and bioinformatics tools that allow their activity to be characterized, their digestive stability to be predicted, and their bioavailability to be evaluated. This review article summarizes current knowledge on the bioactivities of peptides derived from dry-cured ham, advances in their functional characterization, and challenges associated with their application in functional foods and nutraceuticals, with the aim of providing a comprehensive overview of their potential in health promotion and chronic disease prevention.

Keywords: bioactive peptides; dry-cured ham; proteolysis; functional foods; ACE-inhibitory activity; antioxidant activity; DPP-IV inhibitor



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1. Introduction

The Mediterranean culinary tradition is characterized by numerous products, particularly dry-cured ham, especially in countries such as Spain where its consumption is deeply rooted in the food culture [1]. In addition to its sensory value, derived from its characteristic texture, aroma, and flavor, dry-cured ham is a food of great interest from a nutritional and functional standpoint. During the curing process, complex biochemical transformations take place that modify the structure and composition of the muscle [2,3], generating low-molecular-weight compounds, such as free amino acids and peptides, some of which have bioactive properties with the potential to promote health and prevent disease [4].

The ham curing process involves intense and prolonged proteolysis [3,5], in which endogenous meat enzymes (calpains, cathepsins, and various peptidases) are capable of progressively degrading the myofibrillar and sarcoplasmic proteins of the muscle [6–8],

leading to the release of numerous peptides of various sizes and sequences. These peptides, in many cases, have relevant bioactivities documented by *in vitro* studies, in animal models, and, more recently, in preliminary clinical trials [4,9,10]. Among the most notable bioactivities are the antihypertensive effect, which is mediated by the inhibition of the angiotensin-converting enzyme (ACE) [11]; antioxidant activity, with the ability to neutralize free radicals and protect against oxidative damage [12]; antidiabetic action, linked to the inhibition of dipeptidyl peptidase-IV (DPP-IV) [13] and α -glucosidase [14]; antimicrobial activity against food pathogens such as *Listeria monocytogenes* [15]; and anti-inflammatory activity, related to the modulation of immune response mediators [16].

The growing interest in bioactive peptides in dry-cured ham has led to the development and application of advanced identification and characterization methodologies, such as peptidomics [17], tandem mass spectrometry (MALDI-TOF) [18], and the use of bioinformatic tools (e.g., PeptideRanker, BIOPEP-UWM, and CPPpred) [4,19] that allow us to predict, simulate, and study their bioactivity, stability, and bioavailability. These tools, together with simulated gastrointestinal digestion studies and absorption studies using cellular models, help clarify the fate and physiological impact of the peptides generated during curing. Taken together, these advances suggest that dry-cured ham can be considered not only a food of gastronomic interest, but also a matrix with functional and nutraceutical potential, whose applications could contribute to the prevention of chronic non-communicable diseases, such as high blood pressure, type-2 diabetes, and certain inflammatory processes.

This review aims to integrate and analyze current knowledge on bioactive peptides derived from dry-cured ham, with special emphasis on the biological activities described and corroborated, the mechanisms of action, and potential application in functional foods and nutraceutical products for health promotion and chronic disease prevention. The summary of the article can be seen in Figure 1.

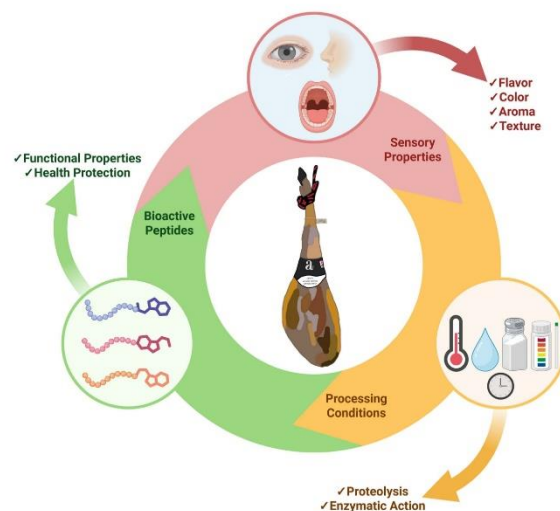


Figure 1. Properties of dry-cured ham. Processing conditions that affect palatability and bioactive profile. Created with BioRender.com (accessed on 1 July 2025).

2. Peptide Generation in Dry-Cured Ham

The generation of bioactive peptides in dry-cured ham is the result of a complex process of proteolysis that occurs during the curing process [20–22], which can last from 7 to more than 36 months (depending on the type of ham and the technological practices applied), under the combined action of a set of endogenous enzymes and, to a lesser extent, the action of microorganisms present naturally or incorporated as starter cultures in some preparations.

Calpains, calcium-dependent neutral proteases (calpaimin-1 and calpaimin-2), participate in the early stages of maturation [23–25], degrading structural proteins such as myosin, titin, desmin, and troponin T, and generating larger polypeptide fragments. Cathepsins, notably isoforms B, L, H, and D, are responsible for the proteolysis of myofibrillar and sarcoplasmic proteins under acidic pH and high-salinity conditions [25–28], acting both as endopeptidases and, in the case of cathepsin B, with a carboxypeptidase function. As the process progresses, exopeptidases (aminopeptidases, carboxypeptidases, and dipeptidyl and tripeptidyl peptidases) come to the fore, whose sequential action on the fragments generated by endopeptidases leads to the release of di- and tripeptides, as well as free amino acids that not only contribute to the development of the characteristic flavor of dry-cured ham [7,29–31], but also have significant bioactive activities in many cases [32]. This process begins after the animal is slaughtered and continues throughout all stages of curing, from salting to drying and subsequent maturation in the cellar. The peptides generated vary in size, charge, sequence, and hydrophobic/hydrophilic properties, which depend not only on the initial protein substrate and the specificity of the enzymes involved, but also on the technological conditions of the curing process. Factors that modulate the proteolysis profile, the action of enzymes, and, therefore, the generation of peptides include the curing time [33,34], which in quality dry-cured hams can exceed 30 months; the temperature and relative humidity of the maturation chambers [35]; the type and concentration of salt used; the pH of the piece; and water activity (*a_w*) [36–38]. Together, these parameters determine the residual activity of proteolytic enzymes and the rate of muscle protein degradation.

The proteolysis pattern is highly specific and results in the generation of recurrent peptide sequences, many of which have been identified as bioactive [4]. For example, peptides derived from myosin (such as FPPDVGGNVD) or creatine kinase (such as AEEEEYPDL) have been identified in dry-cured hams using tandem mass spectrometry techniques after fractionation by size exclusion chromatography (SEC) and reverse-phase high-performance liquid chromatography (RP-HPLC) [10,12,39]. Peptidomics studies have shown that the molecular weight of peptides with the highest bioactivity ranges from 400 to 2500 Da, concentrated mainly in the most polar fractions of the soluble extract [4,10,40].

In addition, the peptide profile can be used as a marker of the degree of maturation and quality of dry-cured ham, and specific peptides derived from proteins such as titin or LIM domain-binding protein-3 have been identified, which can serve as biomarkers of minimum curing time and product authenticity [41]. In short, the generation of bioactive peptides in dry-cured ham is a dynamic phenomenon that is highly dependent on processing conditions and the proteolytic metabolism of the muscle itself, with direct implications for the functional value of the food. Thus, the natural proteolysis of dry-cured ham makes this food a matrix of interest for the development of functional and nutraceutical products. In the Figure 2 it could be seen a network map summarizing the peptides identified in Spanish dry-cured ham and their associated bioactivities.

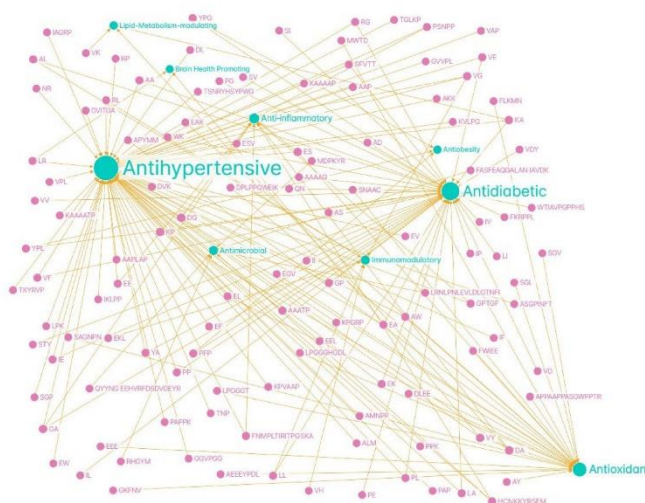


Figure 2. Network map summarizing the peptides identified in Spanish dry-cured ham (pink) and their associated bioactivities (green). Graph created with Graph Commons.

3. Bioactivity Associated with Peptides Derived from Dry-Cured Ham

The peptides that are naturally liberated during the dry-cured ham maturation process not only participate in the development of the product's sensory attributes, such as color, aroma, flavor, and texture [32], but also represent a source of bioactive compounds with potential beneficial effects for human health [4], as demonstrated by various *in vitro*, *in vivo*, and, increasingly, preliminary clinical trials [42–44].

The diversity of biological activities described for these peptides is a consequence of the variability of their sequences, sizes, and physicochemical properties, the most relevant being antihypertensive, antioxidant, antidiabetic, antimicrobial, and anti-inflammatory, among others. Among the peptides with multifunctional effects, numerous small di- and tripeptides stand out, produced by the sequential action of exopeptidases, which have the ability to act simultaneously on different physiological targets, such as the VD, WK, IE, and SI fragments, identified from proteins such as beta-enolase and alpha-enolase [32], which have demonstrated inhibitory activities against DPP-IV, ACE, and, in some cases, α -glucosidase [14]. For example, the antihypertensive activity attributed to dry-cured ham has been linked to the presence of peptides with the ability to inhibit the angiotensin-converting enzyme (ACE), such as AAPLAP, KPVAAP, and KAAAAP, which have shown relevant *in vitro* IC₅₀ values [12,45] and hypotensive effects in animal models using spontaneously hypertensive rats (SHRs), where significant decreases in systolic blood pressure have been documented after administration of dry-cured ham extracts rich in these peptides [46]. On the other hand, the antioxidant action of soluble extracts from dry-cured ham has been linked to the presence of peptides such as SNAAC, GKFNV, GLAGA, or AEEYVCL, which act through hydrogen transfer (HAT) and electron transfer (SET) mechanisms, contributing to the neutralization of free radicals and the prevention of oxidative damage in lipid systems and cellular models [47]. In the field of antidiabetic bioactivity, various peptides such as AEEYVCL, LGVGG, GGLGP, and small dipeptide fragments (VE, PP, and EA) have shown the ability to inhibit the activity of DPP-IV and α -glucosidase [48], participating in the

modulation of the postprandial glycemic response and suggesting a potential role in the control of type-2 diabetes [13].

Peptides with antimicrobial activity have also been identified, such as the pentapeptide RHGYM, which has shown efficacy against *Listeria monocytogenes* in food microbiology tests [49], representing an example of the potential use of dry-cured ham as a source of compounds with natural preservative properties. Finally, although less studied, peptides with possible anti-inflammatory activity have been reported, linked to the modulation of immune response mediators. Some in vitro studies and preliminary clinical trials have shown that regular consumption of dry-cured ham may contribute to improving the inflammatory profile and thrombogenic status in healthy subjects or those with cardiovascular risk factors [43,50].

All of this evidence positions dry-cured ham not only as a food with traditional and gastronomic value, but also as a matrix with high functional potential that can be exploited for the development of functional foods and nutraceuticals aimed at the prevention of chronic diseases.

3.1. Multifunctionality of Di- and Tripeptides Identified in Dry-Cured Ham

The di- and tripeptides generated during the maturation of dry-cured ham represent a significant fraction of the bioactive compounds present in this food. These small peptides are products of the sequential action of exopeptidases—such as dipeptidyl peptidases and tripeptidyl peptidases—and their small size gives them unique properties [51]: high solubility, greater relative resistance to gastrointestinal hydrolysis, and a higher probability of being absorbed intact through the intestinal epithelium, making them ideal candidates for exerting direct physiological effects. Several studies have identified di- and tripeptides with multifunctional activities, capable of acting on more than one biological target, such as inhibition of ACE, DPP-IV, and α -glucosidase, as well as antioxidant or glucose uptake modulating potential [32]. This multifunctional profile has been corroborated both by in vitro assays [52] and in silico studies [33,53], which highlight the frequency of these fragments in precursor proteins and their potential affinity for various enzymes and receptors. The main multifunctional di- and tripeptides identified in dry-cured ham are listed in Tables 1 and 2.

Table 1. Multifunctional dipeptides identified in dry-cured ham.

Sequence	Mass (Da)	Protein of Origin	Described Bioactivity	References
AA	204.23	Myosin7590	Antihypertensive/ Antidiabetic	[32,52,54–56]
AD	204.18	Myosin Light Chain	Antidiabetic	[14,57]
AL	188.21	Myosin	Immunomodulatory/ Antidiabetic	[10,16,18,52,58]
AS	176.08	Actin	Antidiabetic	[59,60]
AW	246.3	Myosin	Antioxidant/ Antihypertensive	[54,61]
AY	252.27	LIM Domain-Binding Protein 3	Antioxidant	[32]
DA	204.18	Actin/Myosin	Anti-inflammatory/ Antihypertensive/ Lipid-Metabolism-Modulating Activity	[18,33,61–63]
DG	190.15	Myosin	Anti-inflammatory/ Antihypertensive/ Antioxidant	[33,61,62,64]
DL	246.28	Creatine Kinase	Antihypertensive	[55]
EA	218.21	Titin/Myoglobin	Antihypertensive/ Antidiabetic	[14,52,55,62,65]
EE	276.24	Titin (fragments)	Anti-inflammatory/ Antihypertensive	[33,61,64,66]
EF	278.35	Ubiquitin-60S	Antihypertensive	[32,67]

Table 1. Cont.

Sequence	Mass (Da)	Protein of Origin	Described Bioactivity	References
EK	275.3	Myosin	Antihypertensive/ Antidiabetic	[55]
EL	244.3	Enolase	Antioxidant/ Antihypertensive	[7,32,42]
ES	234.21	Titin	Anti-inflammatory/ Antihypertensive/ Antioxidant	[33,61,62,64]
EV	277.24	Myosin	Antihypertensive	[42,58]
EW	333.34	Myosin	Antihypertensive	[14]
GA	146.15	Titin	Anti-inflammatory/ Antidiabetic/ Antihypertensive	[14,18,32,33,53,55,61–64]
GP	172.2	Titin	Antihypertensive/ Antidiabetic	[32,52,55,61,64]
IE	260.29	α -enolase	Antihypertensive/ Antidiabetic	[14,33,68]
IF	278.35	Ubiquitin-60S	Antihypertensive	[14]
II	244.33	Glyceraldehyde-3-Phosphate Dehydrogenase	Antidiabetic	[32,67,69]
IL	244.33	Enolase	Antidiabetic	[32,67,69]
IP	244.3	Troponin T	Antidiabetic	[32]
IV	230.3	Myosin	Antidiabetic	[32,67,69]
KA	217.27	Ubiquitin-60S	Antihypertensive/ Antidiabetic Lipid-Metabolism-Modulating Activity	[7,32,52,55,56,70,71]
KP	243.3	Titina	Antidiabetic/ Antihypertensive Antioxidant	[7,32,55]
LA	202.25	Creatine Kinase	Antihypertensive/ Antidiabetic	[55]
LI	244.33	Myosin	Antidiabetic	[7,32,69]
LL	244.33	Lactate Dehydrogenase	Antidiabetic/ Immunomodulatory	[7,32,67,69,72,73]
LR	287.36	Myosin Light Chain	Antihypertensive Brain Health-Promoting and Neuron-Related Activities	[7,10,32,40,56,67]
NR	289.29	Creatine Kinase	Antihypertensive	[32,67]
PE	244.25	Titin/Myosin	Antidiabetic	[14,74]
PG	172.18	Actin	Antidiabetic	[32,53,61]
PL	228.3	Creatine Kinase	Antidiabetic/ Antihypertensive	[14,52,55]
PP	212.25	Myosin	Antihypertensive/ Antidiabetic	[14,55,66,68,75]
QN	260.25	Myosin	Antidiabetic	[59,60]
RG	246.28	Troponin T LIM	Antihypertensive/ Antidiabetic	[55]
RL	287.36	Domain-Binding Protein 3	Antihypertensive/ Immunomodulatory	[7,42,72,73]
RP	271.32	Myosin	Antihypertensive	[32,55,56]
SI	246.3	α -enolasa	Antidiabetic	[14,57]
SV	232.3	Titin	Antidiabetic	[32]
VD	232.23		Antidiabetic	[14,33]
VE	246.26	Titin	Antihypertensive/ Antidiabetic	[14,55,57,66,68]
VF	262.30	Myosin Light Chain	Antihypertensive/ Antidiabetic	[14,18,58,63,64]
VG	174.20	Myosin	Anti-inflammatory/ Antihypertensive/ Immunomodulatory	[33,61,62]
VH	254.29	Troponin T	Immunomodulatory	[10,16,18,52]
VK	245.32	Myosin Light Chain LIM	Lipid-Metabolism-Modulating Activity	[7,55,70,71]
VV	216.28	Domain-Binding Protein 3; β -enolasa	Antidiabetic	[14,76]

Table 1. Cont.

Sequence	Mass (Da)	Protein of Origin	Described Bioactivity	References
VY	280.32	Enolase	Antihypertensive/ Antioxidant Brain Health-Promoting and Neuron-Related Activities	[7,10,32,40,55,56]
WK	319.4	β -enolase	Antidiabetic	[14,76]
YA	252.27	Myosin	Antihypertensive/ Antidiabetic	[59,60,77]

These di- and tripeptides represent a peptide fraction of functional interest, not only because of their abundance and ease of intestinal absorption, but also because of their multifunctional capacity. More than 40 different dipeptides have been identified in dry-cured ham, mainly derived from structural proteins. Among the dipeptides identified, we highlight those with hydrophobic or aromatic residues in their C-terminal position, such as EA, VE, GP, KA, RG, PP, VK, or DL, which have demonstrated inhibitory capacity against angiotensin-converting enzyme (ACE) in both in vitro studies and animal models [45,46,64]. The dipeptide AA has been quantified and studied because it is one of the most abundant and has the most significant inhibitory capacity [54]. The presence of basic or branched residues also seems to improve or favor the interaction of dipeptides with the active site of ACE, as has been described for EK, VF, KP, and LA [14,52,55].

Conversely, numerous dipeptides have also been identified for their antidiabetic activity, acting as inhibitors of DPP-IV and, to a lesser extent, α -glucosidase. Notable examples are EA, VE, PP, PE, IP, IL, LL, VF, SI, and SV, many of which are derived from proteins with high degradation rates during the maturation process [14,32,52,67,68]. The chemical structure and hydrophobic context of these dipeptides appear to play a crucial role in their affinity for DPP-IV, as evidenced by molecular docking models and in vitro validation [53,57]. For example, Gallego et al. (2014) identified di- and tripeptides with this activity in dry-cured hams, such as KA and GP, with $IC_{50} \approx 6-9$ mol/L [52]. In addition, during gastrointestinal digestion, active fragments such as WT, KP, PL, and IP, which are believed to be responsible for DPP-IV inhibition in the intestine, could be released [52,78].

Antioxidant activity has also been documented in dipeptides such as AW, EL, AY, KP, and VY, with the ability to donate electrons or protons through SET and HAT mechanisms. This property is particularly interesting in the context of functional foods, as it contributes to cell protection against oxidative stress, which is involved in aging processes and chronic diseases [7,61]. Some dipeptides such as GA, VG, DG, DA, and EE have been associated with anti-inflammatory effects, showing the ability to modulate pro-inflammatory signaling pathways or inhibit mediators such as TNF- α , IL-6, and cyclooxygenase-2 (COX-2), according to enzymatic and cellular studies [18,61,62,64].

The dipeptides DA, DD, EE, ES, and LL showed the ability to reduce cholesterol synthesis by inhibiting 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase). Through in vitro enzyme assays and in silico studies, Heres et al. (2021) confirmed this hypocholesterolemic capacity of dipeptides due to the in vitro enzymatic inhibition of HMG-CoA reductase (decrease in catalytic activity) and in silico models by molecular docking, demonstrating statin-like interactions with the active site of the enzyme [63].

It should be noted that some dipeptides such as EA, KP, GA, PP, and LA have clear multifunctionality, as they have been described simultaneously as antihypertensive, antidiabetic, antioxidant, and anti-inflammatory in different experimental models [14,32,52,55,61,64]. This functional convergence is particularly attractive for the design of nutraceuticals or broad-spectrum functional ingredients.

Tripeptides identified in dry-cured ham also have a high probability of intact absorption in the digestive tract and a bioactivity profile that is multifunctional in many cases

(Table 2). Their structure allows for greater diversity of interactions with biological targets, and their generation is mainly associated with the action of tripeptidyl peptidases on degraded structural proteins such as titin, myosin, and troponin.

Table 2. Multifunctional tripeptides identified in dry-cured ham.

Sequence	Mass (Da)	Protein of Origin	Described Bioactivity	References
AAP	245.27	Proteolyzed Polypeptides/Light Myosin	Antihypertensive	[40,79]
AKK	345.44	Titin (fragments)	Antihypertensive	[7,32,71]
ALM	/	/	Antihypertensive	[80]
DVK	346.39	Ubiquitin	Antihypertensive	[40,79]
EAK	346.18	Titin (fragments)	Antioxidant	[7,32]
EEE	375.29	Ubiquitin	Antioxidant	[40]
EEL	/	/	Antihypertensive	[42]
EGV	275.28	LIM Domain-Binding Protein 3	Antioxidant	[40]
EKL	388.49	Myosin Light Chain	Immunomodulatory	[7,72,73]
ESV	/	/	Antihypertensive	[42]
LPK	343.43	Myosin	Antihypertensive	[79]
PAP	297.33	Titin	Antihypertensive	[7,32,71,79]
PPF	359.42	Myosin	Antidiabetic/Antiobesity	[14]
PPK	357.42	Titin/Myosin	Antihypertensive	[79]
SGL	275.28	Creatine Kinase	Antioxidant	[40,79]
SGP	259.26	Myosin Light Chain	Antihypertensive	[7,32,71]
SGV	261.29	Creatine kinase	Antioxidant	[40,79]
STY	369.39	LIM Domain-Binding Protein 3	Antidiabetic	[32,52]
TNP	330.34	Myosin Light Chain	Antihypertensive	[7,32,71]
VAP	271.34	Myosin	Antihypertensive	[40,79]
VDY	/	/	Antidiabetic	[14]
VPL	271.34	Troponin T	Antihypertensive	[40,79]
YPG	335.35	Myosin	Antidiabetic/Antiobesity	[14]
YPL	391.46	Myosin	Antidiabetic/Antiobesity	[14]

For instance, AAP, PAP, PPK, LPK, VAP, VPL, DVK, SGP, and TNP have been identified as potent ACE inhibitors, many of them quantified directly by LC-MS/MS in dry-cured hams subjected to long maturation times [32,81]. Their efficacy is related to the presence of hydrophobic or acidic residues at the C-terminal position, as well as conformations that mimic the natural substrate of ACE [7,32,79]. Other peptide fragments have demonstrated the ability to neutralize free radicals and protect against lipid peroxidation [32,60,81–84]. These peptides, derived from creatine kinase and ubiquitin, have been shown to modulate the redox balance in cellular models and oxidized lipid systems [40]. Some *in silico* studies have also linked their activity to the density of electron-donating functional groups (–OH and –NH₂) in the structure of the tripeptide [32].

Other tripeptides such as STY have been reported to have antidiabetic activity, inhibiting enzymes such as DPP-IV, especially when they contain aromatic residues (Tyr and Trp) that can be stabilized by interactions with the active site of the enzyme [32,52]. As with dipeptides, some tripeptides show remarkable multifunctionality. For example, VAP and VPL have not only been identified as ACE inhibitors, but also as antioxidants in cellular systems [85]. Fragments such as PAP, AKK, or EKL have been described as immunomodulators, implicating them in the regulation of inflammatory responses or neuronal functions, especially when derived from proteins such as titin or light myosin [7,72,73]. Mora et al. (2020) [14] identified and characterized three peptides with antiobesity activity—PPF, YPL, and YPG—all derived from dry-cured ham. These peptides not only showed inhibition

of α -glucosidase and DPP-IV but also significantly reduced lipid accumulation in 3T3-L1 adipocytes, suggesting a potential antiadipogenic effect.

3.2. Angiotensin-Converting Enzyme (ACE) Inhibitory Peptides

Dry-cured ham is a food in which, due to its maturation process, endogenous proteolysis releases numerous peptides and free amino acids [4], several of which exhibit antihypertensive activity, mainly through the inhibition of angiotensin-converting enzyme (ACE) [45,54,61]. Table 3 presents a compilation of bioactive peptides (≥ 4 amino acids) reported in the literature for dry-cured ham with antihypertensive activity. Their sequences, molecular mass, precursor protein, type of experimental validation, antihypertensive activity (expressed as IC_{50} of ACE, % inhibition, or another equivalent measure), and the corresponding study reference are detailed.

Table 3. Peptides identified in dry-cured ham with antihypertensive activity.

Sequence	Mass (Da)	Protein of Origin	Validation Methodology	Antihypertensive Activity	Reference
AAATP	429.5	Allantoicase (metabolic enzyme)	In vitro (ACE inhibition), in vivo (SHR* rat)	$IC_{50} = 100 \mu M$ PAS* decrease = -25.6 mmHg (8 h)	[45]
AAPLAP	538.6	Heavy chain of myosin XV	In vitro (ACE inhibition)	$IC_{50} = 14.38 \mu M$	[86]
AMNPP	528.6	Myosin 3 (heavy chain)	In vitro (ACE inhibition)	$IC_{50} = 304.5 \mu M$	[86]
ASGPINFT	805.9	Myosin regulatory light chain 2	In vitro (ACE inhibition)	$IC_{50} = 975 \mu M$	[45]
DVITGA	574.6	Light chain of myosin	In vitro (ACE inhibition)	$IC_{50} = 900 \mu M$	[45]
FNMPLTIRITPGSKA	1646.0	Fragment of myofibrillar protein	In vitro (ACE inhibition)	$>70\%$ inhibition at $170 \mu M$	[55]
GGVPGG	442.5	Elastin	In vitro (ACE inhibition)	79.9% inhibition at 1 mol/L	[55]
GVVPL	483.6	Heavy chain of myosin	In vitro (ACE inhibition)	$IC_{50} = 956 \mu M$	[80]
HCNKKYRSEM	1295.5	Heavy chain of dynein 3	In vitro (ACE inhibition)	$>70\%$ inhibition at $170 \mu M$	[55]
IAGRP	512.6	Titin (myofibrillar protein)	In vitro (ACE inhibition)	$IC_{50} = 25.94 \mu M$	[86]
IKLPP	566.7	Myosin IXb (heavy chain)	In vitro (ACE inhibition)	$IC_{50} = 193.9 \mu M$	[86]
KAAAAAP	527.6	Light chain 3 of myosin	In vitro (ACE inhibition)	$IC_{50} = 19.79 \mu M$	[86]
KAAAAATP	628.7	PR-domain zinc finger protein 2	In vitro (ACE inhibition)	$IC_{50} = 25.64 \mu M$	[86]
KPGRP	553.7	Titin	In vitro (ACE inhibition)	$IC_{50} = 67.08 \mu M$	[86]
KPVAAP	581.7	Myosin	In vitro (ACE inhibition)	$IC_{50} = 12.37 \mu M$	[86]
KVLPG	512.7	Phosphoglycerate kinase 1	In vitro (ACE inhibition)	$IC_{50} = 265.4 \mu M$	[86]
PAPPK	508.6	Light chain 1/3 of myosin	In vitro (ACE inhibition)	$IC_{50} = 199.6 \mu M$	[86]
PSNPP	510.5	Titin	In vitro (ACE inhibition)	$IC_{50} = 192.8 \mu M$	[86]
SFVTT	553.6	Glyceraldehyde-3-phosphate dehydrogenase	In vitro (ACE inhibition)	$IC_{50} = 395 \mu M$	[80]

Table 3. Cont.

Sequence	Mass (Da)	Protein of Origin	Validation Methodology	Antihypertensive Activity	Reference
TGLKP	514.6	Aspartate aminotransferase	In vitro (ACE inhibition)	IC ₅₀ = 51.57 µM	[86]
TKYRVP	762.9	Titin	In vitro (ACE inhibition)	>70% inhibition at 170 µM	[55]
TSNRYHSYPWG	1367.4	Protein kinase	In vitro (ACE inhibition)	>70% inhibition at 170 µM	[55]

* PAS = systolic blood pressure; SHRs = spontaneously hypertensive rats. In the activity column, the inhibition percentages correspond to the concentration indicated when no IC₅₀ was reported.

As can be seen in Table 3, most of the antihypertensive peptides identified in dry-cured ham are 5–7 amino acids, with molecular masses of ~500–800 Da. Several peptides are derived from myofibrillar (muscle) proteins, such as myosin (AAPLAP and KPVAAP), titin (IAGRP, KPGRP, PSNPP, and TKYRVP), and myosin light chains, whereas others are derived from soluble muscle proteins (aspartate aminotransferase in TGLKP) and from connective tissue proteins such as elastin (GGVPGG) [9]. Peptides from less conventional proteins such as dinein (HCNKKYRSEM) [87] or metabolic enzymes such as allantoicase (AAATP) [9] have also been detected. These studies reflect the extensive proteolysis taking place during curing, which releases fragments of multiple muscle proteins [4,45].

The main mechanism of action of these peptides in lowering blood pressure is through the inhibition of the angiotensin-converting enzyme (ACE) [9]. ACE is a zinc-dependent metalloprotease that catalyzes the conversion of angiotensin I to angiotensin II, a potent vasoconstrictor; it also degrades bradykinin (vasodilator). Therefore, ACE inhibition produces a vasodilator effect and a decrease in blood pressure [88]. Many of the peptides identified act as competitive ACE inhibitors, binding to the active site of the enzyme and preventing substrate binding. ACE inhibitor peptides usually have hydrophobic, cyclic, or even aromatic amino acids at their C-terminal end, which interact strongly with the enzyme. In fact, it has been observed that the peptides with the best inhibitory potential in dry-cured ham contain proline (P) in the C-terminal (e.g., KPVAAP, AAPLAP, KAAAAAP, and IAGRP), which is consistent with the preferences of ACE inhibitors. Many known inhibitors, such as the tripeptides IPP and VPP in milk, have a C-terminal proline [57]. In addition, several peptides contain a basic residue (Lys or Arg) in a position close to the C-terminus (e.g., Lys in the penultimate position in TGLKP or Arg in IAGRP). These positively charged amino acids could favor electrostatic interactions with the active site of ACE, which has negatively charged residues and zinc ions. The combination of an internal basic residue and a proline at the C-terminus appears to contribute to the high inhibitory affinity reported for bioactive peptides.

The number of amino acids contained in the peptide is also a factor to consider. Although it has been suggested that longer peptides may interact with more sites in ACE, this does not always translate into greater potency because they may be less stable and more prone to degradation [33,35]. In the case of dry-cured ham, peptides with 4–7 amino acids tend to show the lowest IC₅₀ (i.e., the highest activity). For example, KPVAAP exhibits the lowest IC₅₀ (~12 µM), followed by AAPLAP (6 aa, ~14 µM), whereas longer peptides such as TSNRYHSYPWG or FNMPLTIRITPGSKA showed significant inhibitory activity only at relatively high concentrations (170 µM for >70% inhibition). This suggests that long peptides may act after fragmentation into shorter active peptides during gastrointestinal digestion [86]. In this sense, many of the listed peptides are susceptible to degradation by digestive peptidases, generating di- and tripeptides that may also act as ACE inhibitors. In addition to ACE inhibition, some peptides may modulate other relevant targets in

hypertensive pathophysiology. Other study evaluated the ability of peptide fractions from dry-cured ham bones to inhibit the endothelin-converting enzyme-1 (ECE-1) and platelet-activating factor-associated phospholipase A₂ (PAF-AH) [55]. Some inhibitory activity was observed on ACE-1 (although it decreased after *in vitro* digestion), as well as marked inhibition of PAF-AH after simulated gastrointestinal digestion. ACE-1 generates endothelin, a potent vasoconstrictor; its inhibition by peptides could complement the effect of ACE inhibition. PAF-AH is involved in vascular inflammation; its inhibition could confer additional vasoprotective effects. These findings suggest that dry-cured ham peptides could have multiple cardioprotective effects, acting on several pathways.

In animal models, the impact is more evident. In a study conducted by Escudero et al. 2013, the synthesized pentapeptide AAATP (dose 30 mg/kg) was administered by gastric tube to SHR (spontaneously hypertensive rats), and a marked reduction of ~25.6 mmHg in systolic pressure was observed after 8 h [45]. Similarly, the peptides Ala-Ala (AA) and Ala-Trp (AW) present in dry-cured ham were recently evaluated in rats. Both achieved acute reductions in systolic pressure (~20 mmHg) after oral administration [54,61]. However, peptides were rapidly metabolized; thus, their effect was transient. In contrast, 4–6 amino-acid-long peptides may have a slightly longer half-life, releasing active dipeptides gradually during gastrointestinal digestion [89].

A relevant question is whether dry-cured ham consumption has antihypertensive effects in humans. Traditionally, its consumption has been discouraged in hypertensive individuals due to its relatively high salt content; however, recent research suggests that the bioactive peptides present may partially counteract the hypertensive effect of sodium. A recent study with more than 13,000 Spanish adults followed for a period of 4.5 years found that high consumption of dry-cured ham (≥ 50 g at least 5 times/week) was not associated with a higher incidence of hypertension compared to low consumption (< 1 time/week). These results indicated that, in a real dietary context, dry-cured ham did not have the expected harmful effect of contributing to high blood pressure. The authors suggested that the ACE-inhibiting peptides present in dry-cured ham could explain the impact on neutralization of the salt [90]. Controlled clinical trials have also been reported in the literature. Two studies carried out by Montoro-García et al., 2017, and Montoro-García et al., 2022, showed a trend towards reduced systolic and diastolic blood pressure [43,50], with the latter study showing, after 8 weeks of consumption, a significant decrease of ~2–3 mmHg in both systolic and diastolic pressure 24 h after ingestion of dry-cured ham, compared to the boiled ham control group. In addition, the latter study also observed a reduction in total blood cholesterol in the dry-cured ham group [43].

3.3. Antioxidant Peptides

During dry-cured ham maturation, endogenous proteolysis releases peptides that can act as antioxidants [39]. Table 4 shows the antioxidant peptides (> 3 amino acids) identified in dry-cured ham. These peptides have been obtained either directly by protein hydrolysis during curing or by specific extraction and purification techniques from dry-cured hams. Their antioxidant activities have been validated mainly *in vitro* by free radical scavenging assays (DPPH and ABTS), ferric reducing power (FRAP), or oxygen radical absorbance capacity (ORAC), and even in cellular models of oxidative stress.

Table 4. Peptides with antioxidant activity identified in dry-cured ham.

Sequence	Mass (Da)	Protein of Origin	Validation Methodology	Antioxidant Activity	Reference
AEEEYPDL	1109	Creatine kinase (muscle)	In vitro (ABTS capture, ORAC)	ABTS: 1474 nmol TEAC/mg; ORAC: 960 nmol TE/mg (high antioxidant activity)	[39]
APPAAPPASGWPPTR	1743	/	Cell model (HaCaT + UVA)	Protected HaCaT cells from oxidative damage caused by UVA, increasing their survival and antioxidant response	[91,92]
APYMM	611.76	/	In vitro (ABTS)	ABTS: 0.12 mg/mL	[93]
DLEE	576	/	In vitro (ABTS, ORAC); cell model (Caco-2)	ABTS: 148 µmol TE/g; ORAC: 1032 µmol TE/g (similar to glutathione); reduced intracellular ROS and activated the Nrf2-Keap1 pathway	[94]
DPLPPGWEIK	1331	E3 ubiquitin ligase	Cell model (HaCaT keratinocytes + UVA)	Increased the viability of UVA-damaged HaCaT cells (~10–15% vs. control), activating the Nrf2-Keap1 antioxidant pathway	[91,92]
FLKMN	652	Myosin light chain	In vitro (DPPH)	DPPH: 65% inhibition at 1 mg/mL; OH [•] : 60% at 1 mg/mL	[95]
FWIEE	706.84	/	In vitro (ABTS)	ABTS: 0.23 mg/mL	[93]
GKFNV	564	/	In vitro (DPPH)	DPPH: 92.7% inhibition at 1 mg/mL	[47,95]
LPGGGHGD	822	/	In vitro (O [•])	OH [•] : 85% at 1 mg/mL	[95]
LPGGGT	501	/	In vitro (DPPH)	DPPH: 65% inhibition at 1 mg/mL; OH [•] : 60% at 1 mg/mL	[95]
MWTD	551.61	/	In vitro (ABTS)	ABTS: 0.4 mg/mL	[93]
SAGNPN	640	Integrin α-3 (muscle)	In vitro (DPPH)	DPPH: 50% inhibition at 1.5 mg/mL (moderate antioxidant activity)	[12]
SNAAC	555	Heavy myosin chain	In vitro (DPPH, reducing power)	DPPH: 95.7% at 3 mg/mL; FRAP: Abs ₇₀₀ = 1.7 at 1 mg/mL (potent antioxidant)	[96,97]

TE = Trolox Equivalent; TEAC = antioxidant capacity equivalent to Trolox. DPPH: % radical inhibition; FRAP: absorbance at 700 nm (reducing power).

The results in the table show that multiple endogenous peptides in dry-cured ham exhibit strong antioxidant activity. AEEEYPDL showed high antioxidant power, even higher than that of reduced glutathione [98]. This peptide was quantified at 0.148 fg/g of cured ham using MRM mass spectrometry. The levels of peptides present in cured ham depend largely on the type of peptide and the technological processing conditions [39]. Similarly, the peptide SNAAC achieved almost 96% DPPH radical scavenging activity, positioning itself as one of the most potent antioxidant peptides described in the literature. However, in vitro digestion studies reveal that these long peptides can be degraded in the gastrointestinal tract, losing much of their original activity [9].

In Chinese dry-cured hams (e.g., Xuanwei), novel antioxidant peptides have been discovered using combined extraction and in silico screening strategies. In particular, the peptides DPLPPGWEIK and APPAAPPASGWPPTR showed the ability to protect skin cells (HaCaT) from UV-induced oxidative stress, increasing their cell viability and activating the cytoprotective Nrf2/Keap1 pathway [91,92], constituting the first evidence of dry-

cured ham peptides capable of protecting human cells from oxidative damage caused by radiation. It should be noted that many of the most active antioxidant peptides share common structural motifs: they are small to moderate in size, often enriched in amino acids such as proline (P) and glycine (G) [91], and frequently contain residues with side chains capable of donating hydrogen or electrons or acid groups for chelating metals. These characteristics facilitate the neutralization of free radicals and the interruption of oxidative cascades [82,99].

3.4. Dipeptidyl Peptidase-IV Inhibitory Peptides

Several peptides generated in dry-cured ham have been shown to inhibit the enzyme dipeptidyl peptidase-IV (DPP-IV). This enzyme is responsible for the rapid degradation of incretin hormones such as glucose-dependent insulintropic peptide (GIP) and glucagon-like peptide type-1 (GLP-1), both of which are involved in stimulating postprandial insulin secretion. Inhibition of DPP-IV prolongs the half-life of these hormones, facilitating better blood glucose control, which justifies its consideration as a therapeutic target in the treatment of type-2 diabetes mellitus [100].

Given that the most effective peptides against DPP-IV tend to be very short, dipeptides or tripeptides with proline or alanine in the penultimate residue have not yet been identified or corroborated in dry-cured ham. In this context, several peptides generated during the curing process of dry-cured ham have shown inhibitory potential against this enzyme. Specifically, it has been reported that the sequences AAAAG and AAATP, extracted from Spanish dry-cured ham, exhibit DPP-IV inhibitory activity, with IC_{50} values between 6.3 and 9.7 mol/L, indicating a moderate affinity for the enzyme [52]. Similarly, in a 2021 study, two peptides (WTIAVPGPPHS and FKRPPPL) were identified after hydrolysis of dry-cured pork loin with pepsin and pancreatin. These two peptides were the most promising DPP-IV inhibitors. Using *in silico* analysis, the authors detected sequences with abundant motifs recognized by BIOPEP as DPP-IV inhibitors [78].

3.5. Anti-Inflammatory Peptides

Research on anti-inflammatory peptides in dry-cured ham is still in early stages, but recent advances indicate great potential. Unlike antioxidant and DPP-IV inhibitor peptides, in the anti-inflammatory field, much of the evidence comes from the effect of peptide mixtures from dry-cured ham in biological models. For instance, a peptide extract from Xuanwei dry-cured ham was shown to significantly reduce the production of inflammatory mediators (*in vitro* and *in vivo*). In LPS-stimulated RAW264.7 macrophages, it inhibited the secretion of nitric oxide (NO) and pro-inflammatory cytokines (TNF- α and IL-6) [94,101], whereas in a mouse model of colitis, it attenuated weight loss, inflammatory infiltration in the colon, and the expression of COX-2, among other markers [94]. These observations suggest that the peptides generated in dry-cured ham could exert systemic anti-inflammatory effects. In another study, Gallego et al. (2019) [55] evaluated the anti-inflammatory activity of peptides isolated from Spanish dry-cured ham by inhibiting platelet-activating factor acetylhydrolase (PAF-AH), autotaxin (ATX), and lipoxygenase (LOX). PAF-AH activity was inhibited by up to 26.06% with a fraction containing 19 peptides, with FNMPLTIRITPGSKA being identified as the most active peptide. ATX was inhibited by up to 57.49% with a fraction containing 13 peptides, with the strongest inhibition being that of the PSNPP pentapeptide, and LOX was inhibited by up to 23.33% with a fraction of 5 peptides, with HCNKKYRSEM showing the strongest inhibitory activity.

A recent study identified GPTGF, along with other peptides derived from the hydrolysis of collagen from ham bones, as potential anti-inflammatory agents. *In vitro* tests showed that bone peptide hydrolysate (in which GPTGF is one of the main components)

suppresses the release of IL-6 and TNF- α in activated macrophages, suggesting that GPTGF and similar peptides could reduce the pro-inflammatory polarization of macrophages and cytokine production [101].

3.6. Other Bioactivities Associated with Peptides Identified in Dry-Cured Ham

Besides the antioxidant, antihypertensive, anti-inflammatory, and antidiabetic properties commonly reported in bioactive peptides derived from dry-cured ham, recent research has identified a number of complementary bioactivities of functional interest.

The fermentation process of dry-cured ham can cause protein degradation through the combined action of endogenous enzymes and microbial activity, resulting in higher concentrations of bioactive peptides [91,102]. Several peptides generated during the proteolysis of dry-cured ham have demonstrated potent activity against some food pathogens. In particular, the pentapeptide RHGYM was isolated from Spanish dry-cured ham and demonstrated a great ability to inhibit the growth of *Listeria monocytogenes* (MIC = 6.25 mol/L), being the most effective of those identified by Castellano et al. 2016 [49]. Other peptides, such as HCNKKYRSEM and MDPKYR, which contain the central KYR sequence with recognized antimicrobial activity, also showed efficacy, although at higher concentrations (MIC $\approx$ 50 mol/L). Similar results were obtained when three peptide fractions were extracted, namely QYYNG EEHVRFDSDVGEYR, LRNLPNLEVLDLGTNFI, and FASFEAQGALAN IAVDK from Chinese Xuanwei dry-cured ham, which produced a significant inhibition of the growth of *Escherichia coli* O157:H7 [7].

4. Use of Bioactive Peptides Derived from Dry-Cured Ham as Potential Nutraceuticals

The current trend toward developing foods with beneficial properties has opened up the possibility of using bioactive peptides present in dry-cured ham as nutraceutical ingredients or in the design of functional foods. Various studies have shown that these peptides can have beneficial effects on human health [43,50,90,103], suggesting its potential preventive or adjuvant application towards chronic diseases. For the development of nutraceuticals, concentrated sources of active peptides are necessary. In the case of dry-cured ham, methods for extracting the soluble peptide fractions after maturation have been tested, followed by separation techniques such as ultrafiltration or chromatography, to isolate the peptides of interest [46,104]. These fractions constitute the fundamental raw material for the development of nutraceuticals and enriched functional foods. A crucial step is demonstrating that peptides retain their bioactivity when administered in biological models. Numerous peptides isolated from dry-cured ham have shown activity using in vitro systems and animal models [95,98]. Peptide extracts from dry-cured ham have also exhibited anti-inflammatory effects, attenuating the production of pro-inflammatory cytokines [94,101]. This evidence is supported by clinical trials that have been conducted, which have achieved a significant decrease in blood pressure [43].

Bioactive peptides in dry-cured ham could be incorporated into different formats containing a standardized dose of peptides with defined bioactivity. Another possibility could be to fortify foods with these peptides [46,102]. Similarly, fat-free soups or broths made from dry-cured ham, concentrated in peptides, could be developed to act as functional broths, providing physiological benefits [105,106]. An important technological challenge is to ensure the stability of the peptides during product processing and storage, protecting them from thermal or pH degradation.

In this regard, techniques such as microencapsulation in polymer or lipid matrices have been successful in protecting bioactive peptides from degradation and releasing them in a controlled manner in the gastrointestinal tract. Recent studies describe the

nanoencapsulation of peptides in liposomes and polyelectrolyte nanoparticles, which improves their gastric stability and intestinal absorption [107–109]. In the Figure 3 it could be seen the different techniques used for the encapsulation of bioactive peptides.

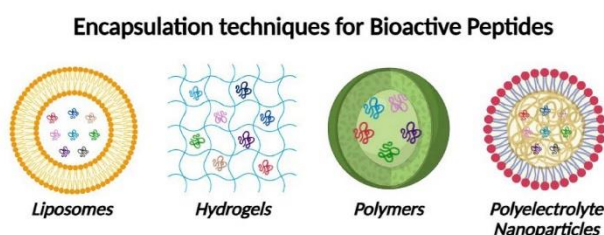


Figure 3. Techniques used for the encapsulation of bioactive peptides. Created with [BioRender.com](#) (accessed on 10 August 2025).

Such approaches could be applied to dry-cured ham peptides to develop more effective nutraceuticals. As with any nutraceutical ingredient, it is essential to ensure the safety of dry-cured ham peptides, although, as they are derived from a traditional food, these peptides have no known intrinsic toxicity; in fact, regular consumption of dry-cured ham in moderate amounts has been associated with neutral or beneficial effects on blood pressure and lipids in humans [90]. However, concentrating these peptides in a supplement may require further evaluation. Fortunately, there are already precedents for food peptides approved in nutraceuticals [110–112].

The nutraceutical application of peptides derived from dry-cured ham is a promising field. Different peptides with well-defined activities could be incorporated into supplements or functional foods aimed at the general population or at-risk groups. Before commercialization, it will be essential to demonstrate their efficacy in clinical trials and optimize formulations to ensure the release and bioavailability of these peptides in the body. With advances in research and technological innovation, it is not inconceivable that dry-cured ham—or its peptide extracts—will also become an important element in nutraceutical strategies for health promotion in the near future.

5. Conclusions and Future Directions

The bioactive peptides identified in dry-cured ham represent an interesting and rapidly evolving field of study. Current evidence suggests that dry-cured ham, beyond its culinary value, provides molecules with functional potential that could help prevent or mitigate risk factors for various chronic diseases. In particular, peptides have been characterized to have the ability to modulate cardiovascular function (by inhibiting the angiotensin-converting enzyme, blocking the endothelin axis, or inhibiting the PAF-AH factor), improve cellular oxidative balance (by scavenging free radicals and increasing the bioavailability of nitric oxide), and regulate glycemic metabolism (by inhibiting enzymes such as DPP-IV and α -glucosidase). Recent studies also highlight the multifunctionality of many of these peptides, where short sequences such as AA, VE, or KP simultaneously exhibit antihypertensive, antioxidant, and antidiabetic activities. This multifunctional profile is particularly interesting, as it suggests a synergistic or comprehensive effect on several physiological pathways, which could be exploited to address complex conditions such as metabolic syndrome through dietary interventions.

While some human studies have shown encouraging results, additional controlled clinical trials are needed. These studies should more directly evaluate the impact of dry-

cured ham or its isolated peptides on clinical markers such as blood pressure, lipid profile, glycemic control, inflammatory status, etc. Long-term interventions in specific populations are of particular interest to confirm whether the incorporation of dry-cured ham into a balanced diet can have a protective or therapeutic effect. Recent clinical trials with dry-cured ham indicated a slight decrease of ~2–3 mmHg in blood pressure after several weeks of consumption, a clinically relevant result at the population level. Therefore, confirming and expanding these findings is a priority.

The conditions under which dry-cured ham is produced also influence the amount and type of peptides generated. Recent studies have shown that strategies such as prolonging maturation or slightly increasing the temperature in reduced-salt dry-cured hams can increase proteolysis and thus the release of bioactive peptides. These future lines of research point to the possibility of designing healthier versions of dry-cured hams by modulating the technological process. However, these changes must be balanced with the sensory quality of the dry-cured hams. Proteomics applied to different processing conditions is a valuable tool for identifying which practices optimize the generation of specific peptides without compromising safety or flavor. In the future, the use of proteolytic microbial starters or technological adjuvants that enhance the formation of certain functional peptides during curing could also be explored.

A promising direction is the valorization of dry-cured ham by-products as an additional source of peptides. For example, collagen peptides with notable anti-inflammatory activity, such as the pentapeptide GPTGE, have been isolated from dry-cured ham bones. The extraction of these peptides from by-products could lead to new functional ingredients, contributing to the sustainability of the industry. At the same time, the formulation of nutraceuticals based on dry-cured ham peptides is a field ready to be explored. In the coming years, we are likely to see dietary supplements with bioactive peptides aimed at controlling hypertension or improving metabolic health.

The mechanisms of action of many of these peptides *in vivo* are not yet known in detail. Further investigation into how they interact with their molecular targets will help understand their real therapeutic scope. Research into these interactions will open up new potential applications such as neurological health and appetite control. It is also important to evaluate the matrix effect within the ham itself, as the absorption of peptides may be affected by other components (e.g., fats and salt). Simulated gastrointestinal digestion and absorption studies in cell cultures will continue to be useful in determining what fraction of dry-cured ham peptides reaches the blood circulation in intact form, thus optimizing the consumption patterns.

Recently, technological advances have significantly expanded the set of tools available for discovering and characterizing bioactive peptides. *In silico* screening platforms, which integrate protein sequence databases (UniProt and BIOPEP-UWM) with predictive algorithms such as PeptideRanker, have become an indispensable tool for rapidly identifying candidate peptides with bioactive potential before conducting more costly *in vitro* or *in vivo* assays [58]. These bioinformatics tools can also simulate gastrointestinal digestion and predict the release of active fragments, guiding the selection of enzymes for hydrolysis [113].

Another new approach that has recently been used is the integration of peptidomics with molecular docking and molecular dynamics simulations to predict the interaction of peptides with specific molecular targets, such as ACE, DPP-IV, or α -glucosidase. Recent studies have shown that combining LC-MS/MS-based peptidomics with *in silico* docking not only allows for highly reliable identification of peptide sequences, but also provides mechanistic information on binding modes, stability, and potential synergistic effects [114]. This computational experimental process can accelerate the elucidation of multifunctional

peptides from complex matrices such as cured ham, supporting their use in functional foods and nutraceutical formulations [113,115].

Finally, the convergence of advanced analytical chemistry, big data, and simulation is expected to transform future research in this field. Future studies should integrate omics datasets to better understand the matrix effects and systemic impact of cured ham peptides, combining these approaches with well-designed clinical trials to validate their functional potential. This integrative strategy will be essential to translate the growing mechanistic evidence into practical dietary recommendations, functional product development, and nutraceutical formulations. In this context, dry-cured ham has gone from being considered only a traditional product to being recognized as a food with functional potential thanks to the peptides liberated during its maturation. In the coming decades, there is likely to be an increase in research aimed at applying this knowledge in innovative ways, from the development of healthier meat products to the creation of specific dietary supplements, allowing cured ham, backed by a solid scientific basis, to consolidate its position not only as part of gastronomic culture, but also as a valuable ally in promoting health.

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Article

Effect of Overripening on the Physico-Chemical and Sensory Characteristics of Boneless, Salt-Reduced Iberian Dry-Cured Ham

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Abstract: The aim of this study was to evaluate the effect of extended maturation and temperature increase on the physico-chemical, biochemical, instrumental color and texture, sensory, and acceptability parameters of cured and boneless Iberian hams. Given the limited knowledge in this area, our objective was to develop a ham with enhanced proteolysis, potentially leading to increased bioactive peptide generation and superior sensory characteristics compared to salt-reduced counterparts. To achieve this, a batch of hams cured up to 38% loss at 30 °C and two batches cured up to 42% loss at 30 °C and 36 °C were evaluated. Results showed that the increase in processing time and temperature significantly enhanced ($p < 0.05$) ham proteolysis and amino acid content without adversely affecting its texture. No significant differences were observed in instrumental texture parameters or sensory attributes as evaluated by consumers. These processing conditions also increased the content of free amino acids, improving the product quality. Overall, these processing modifications resulted in hams with excellent sensory acceptability and enhanced bioactive potential despite the salt reduction.

Keywords: ham; processing technology; temperature increase; overripeness



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1. Introduction

Iberian ham is a high-quality product that, due to its nutritional profile and sensory characteristics, is highly appreciated by consumers [1,2]. It is one of the leading products of the Spanish meat industry, and this can be generalized to any country in the Mediterranean basin since the demand for this product is increasing [3].

The production process of cured ham is carried out first by salting the ham, then post-salting, drying, and finally, a long maturation period [4,5].

The quality of the cured ham that is finally obtained will depend on how proteolysis has evolved during the production process in response to changes in the different factors that occur during production, such as temperature and relative humidity, excluding considerations regarding the quality of the meat utilized, which also constitutes a pertinent factor for consideration.

Throughout these stages, complex proteolysis and lipolysis reactions occur that contribute to developing the sensory profile that our ham will finally have [6]. It is mainly during maturation that proteins and lipids undergo intense processes of proteolysis and lipolysis, affecting the flavor and texture of the cured ham. Final flavor formation has to develop over a long maturation time at high temperatures [7,8]. These changes depend mainly on the duration of the ripening process, and this has been corroborated in different cured products [9–12]. Increased maturation time or overripening will lead to increased proteolysis and, consequently, to an increase in the amount of potentially bioactive peptides with beneficial properties for the consumer's health [13].

Salt is the main ingredient, after meat, in the preparation of cured ham, which is involved in proteolysis, water holding capacity (WHC), and organoleptic characteristics. In addition, it decreases water activity (aw), thus contributing to the stability of the final product [5,14].

Throughout the processing stages, salting and drying procedures yield varying profiles of salt and water content, which undergo fluctuations over time. It is crucial to quantify and evaluate how these two variables vary in order to see their effects on the biochemical evolution and sensory characteristics that develop during ham processing; control of each stage is also necessary to obtain a tasty and safe product [5].

Today's concern about sodium intake and its relationship with certain diseases [15] has led to new initiatives to reduce the consumption of salty foods. However, in products such as traditional dry sausages this is not an easy task, since salt plays an essential role in controlling the chemical and microbial processes in ham.

In a study by Zhao et al. [16], it was observed that the salting process inhibited the potential activity of the proteolytic enzymes cathepsin B and L to only 9.31% and 13.66%, respectively, of their original potential activity, thus confirming the inhibitory role of salt in proteolysis.

Similar results were described by Harkouss et al. [17], who corroborated the promoting role of temperature and the inhibitory role of salt and drying in increasing proteolytic intensity [18].

Water activity generally affects microbial growth and the action of certain enzymes involved in proteolytic reactions, which are responsible for the development of the flavor, aroma, and texture of ham [11,19,20].

To deal with the undesirable effects of salt reduction (reduction of flavor and aroma, undesirable effects on texture, and problems with stability and color formation [21]), several options have been proposed, one of which shows that prolonging the maturation phase results in better flavor and aroma properties in hams and also reinforces muscle firmness due to muscle endopeptidases and exopeptidases that generate a large amount of free amino acids and peptides [22–25]. It has also been described that a reduction in moisture by dehydration is linked to a more persistent aroma and other sensory features [10,25,26]. One of these studies also showed that the acceptability of ham decreased with increasing curing time. High pastiness and stickiness values seemed to be more correlated with decreased acceptability despite a significant increase in aroma. These textural changes were the result of excessive proteolysis [22].

Even so, knowledge in this area is very scarce, which is why in this work we pretend to evaluate the effect of the extension of maturation on ham characteristics, with the aim of developing a ham with high proteolysis (probably more significant biopeptide generation) and with better sensory characteristics than a ham reduced in salt due to an increase in maturation time (overripening).

Knowledge in this area is relatively scarce for boneless Iberian hams reduced in salt since it has never been done. Therefore, it is unknown what side effects would be produced if the temperature or maturation time were increased.

2. Materials and Methods

2.1. Preparation and Collection of Cured Iberian Ham Samples

A total of 48 Iberian hams were needed for the development of this study. Three batches of 16 Iberian hams each were processed.

Pork legs were first deboned and then salted with sea salts and nitrifying agents at a rate of 0.8 days/kg in a cold storage chamber at a temperature of 3 °C.

After salting, the pork legs were washed with water and, after this, a traditional curing process was carried out. The resting or post-salting stage began at 3 °C, gradually increasing to 6 °C. This processing phase ended when the Iberian hams reached 18% shrinkage.

Subsequently, the temperature was raised to 30 °C. The process ended when the pieces reached a weight loss of 38%. After this last processing, the first batch had finished

its maturation process, but the two remaining batches of Iberian hams were taken to overripening, with up to 42% weight loss in each but in different processing conditions: The second batch was processed at a temperature of 30 °C and the third batch at 36 °C.

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

Finally, 8 cured Iberian hams were selected from each batch (48 hams). Samples were taken at the following stages: I: raw muscle; II: beginning of post-salting; III: end of post-salting; IV: drying phase (33% weight loss); V: final product (38% weight loss for lot I and 42% for lots II and III).

2.2. Physicochemical Analyses

The analysis of the physicochemical parameters of the samples was carried out in triplicate for each type of dry-cured Iberian ham.

Moisture content analysis were determined following the gravimetric procedure described in ISO1442 [27].

The determination of total nitrogen (TN) and non-protein nitrogen (NPN) was carried out by the Kjeldahl method [28]. For the preparation of NPN extract, the method followed by Abellán et al. [29] was used. The crude protein value was obtained by multiplying the TN value by 6.25. The proteolysis index was determined as the percentage of the ratio of non-protein nitrogen to total nitrogen [30].

For salt content analysis, the Volhard methodology of ISO1841-1 [31] was used and slightly modified. The Folch method [32] was used to measure the intramuscular fat content of Iberian hams.

The evaluation of water activity was carried out by gravimetry using the AOAC 920.153 method [33].

Free amino acid (FAA) content was determined following the method described by Abellán et al. [34].

2.3. Determination of Instrumental Color and Texture Profile Analysis (TPA)

Instrumental color assessment utilized colorimetry with a colorimeter (HunterLab, Colorflex) following the CIELab system. The outcomes were represented by the L*, a*, and b* coordinates, denoting luminosity, red–green index, and yellow index, respectively. Additionally, the saturation parameter (chroma) and hue angle (h*) values were obtained. The results were averaged from three measurements [35].

For instrumental texture analysis, a QTS-25 texturometer (Brookfield CNS Farnell, Borehamwood, Hertfordshire, England) equipped with a 25 kg load cell and a 10 mm-diameter probe was employed. Texture Pro v. 2.1 software facilitated data interpretation [36].

The biceps femoris muscle was dissected into 10 × 10 × 10 mm parallelepipeds, with three parallelepipeds per sample. Testing was conducted at a controlled room temperature of 20 °C.

The procedure involved subjecting the sample to two consecutive cycles at a constant speed of 30 mm/s, with 50% compression perpendicular to the muscle fibers.

Texture profile analysis (TPA) was conducted to assess the hardness, deformation concerning hardness, adhesiveness, cohesiveness, recoverable deformation, springiness, gumminess, and chewiness.

2.4. Sensory Analysis

For the sensory analysis of the dry-cured Iberian ham, a consumer test was performed with an untrained panel. The analysis was carried out following the method described by Muñoz-Rosique et al. [35] with slight modifications. For this analysis, a total of 200 anonymous participants attended on a voluntarily basis and without having received any type of prior information or training. The study was conducted in a single session where the participants evaluated the three batches of hams. The age range chosen was 18 to 55 years.

For the study, participants evaluated the samples using a questionnaire that included the hedonic assessment of appearance, color, odor, texture, salty taste, overall taste, and overall acceptability. Each attribute was scored by assigning a numerical value using a hedonic scale between 1 (I dislike it very much) to 5 (I like it very much).

The samples used for the consumer sensory test were complete slices of the cross-section of the piece, following the method established in the UNE-ISO standard 6658:2019 [37] and UNE-ISO 4121:2006 [38], which were coded with a random three-digit number.

The results were obtained by calculating the average score given to each product attribute evaluated by the consumer. To determine the preference between the three samples, the percentage values of choice of the consumer panel were obtained in each type of dry-cured ham studied.

2.5. Statistical Analysis

All analyses of our samples were performed in triplicate. Statistical analysis of our samples was performed using SPSS software package (version 21.0, IBM Corporation, Armonk, NY, USA). All results were expressed with the mean and standard error. The results shown in the tables are expressed with two decimal places.

To evaluate the effect of overripening, a one-way ANOVA was performed between batches I and II. The effect of temperature was also evaluated using a one-way ANOVA between hams from lots II and III.

When the effect of overripeness or temperature was significant ($p < 0.05$), the results were compared using Tukey's HSD test. Differences were considered statistically significant when p -values were equal to or below 0.05. Relationships among the studied factors are presented using appropriate tables and figures.

3. Results and Discussion

3.1. Analysis of the Physical–Chemical Parameters of Iberian Ham during Processing

Figure 1 shows the changes in the physicochemical parameters of Iberian dry-cured ham throughout the maturation process.



Figure 1. Changes in the physicochemical parameters of Iberian dry-cured ham throughout the maturation process.

Figure 1 shows the evolution of the physicochemical parameters and salt content of boneless cured Iberian hams reduced in salt during the different stages of processing. The different processing stages affected all the physicochemical parameters. Moisture content in the cured ham decreased significantly ($p < 0.001$) during all stages but mainly between post-salting (stage III) and the final stage (stage V). In contrast, intramuscular fat

content increased ($p < 0.05$), as did protein values. This parameter increased progressively throughout all the stages, with significant differences being observed between the raw muscle (stage I) and the final product (stage V) but not between the final stages of processing (stage IV and stage V). The same can be observed in the total nitrogen (TN) or non-protein nitrogen (NPN) content, where the evolution throughout processing was gradual, with significantly higher values ($p < 0.05$) being observed mainly between the end of post-salting (stage III) and the end of processing (stage V).

Consequently, PI increased significantly during the different processing stages ($p < 0.05$). The increase in temperature that occurs during the final stages of processing (stage IV and stage V) was directly related to the increase in temperature that occurs during these stages of ham processing, resulting in an increase in the activity of proteolytic enzymes [23,25,29].

The physicochemical modifications that took place during processing coincide with those observed during the processing of other cured hams [26,35].

NaCl concentration increased throughout processing but mainly at the beginning of the post-salting stage (stage II). This significant increase was due to the incorporation of curing salts associated with the manufacturing process ($p < 0.05$). An increase in NaCl values was observed between stages I and II, as well as between the end of post-salting and the last two stages (stages IV and V), which was due to the decrease in moisture content, which takes place during last stages of ham processing. The final salt content indicates a correct diffusion of salt, which is important not only for the texture and flavor of the ham but also to ensure the microbiological quality of the ham [14,19,35]. The ash content remained stable throughout processing, and significant differences were only observed between stage I (raw muscle) and stage II (beginning of post-salting).

3.2. Effect of Change in Processing Conditions in the Storage Phase on the Physicochemical Parameters of Iberian Ham

Table 1 shows the effect of the overmaturation along with the increase in the temperature on the physicochemical parameters obtained from Iberian ham.

Table 1. Effect of different processing conditions on the physicochemical parameters of Iberian ham. Results are expressed as means values $\pm$ SEM.

	Processing Conditions			<i>p</i> -Value	
	I	II	III	Overmaturation	Temperature
Moisture	35.39 $\pm$ 1.16 ^a	29.81 $\pm$ 0.57 ^b	32.13 $\pm$ 1.63 ^{ab}	0.00	0.21
%NaCl	3 $\pm$ 0.16 ^a	3.1 $\pm$ 0.18 ^a	3.3 $\pm$ 0.18 ^a	0.69	0.45
Fat	40.95 $\pm$ 1.64 ^a	48.66 $\pm$ 0.11 ^b	47.6 $\pm$ 0.68 ^b	0.00	0.15
Ash	0.88 $\pm$ 0.01 ^a	0.84 $\pm$ 0.01 ^b	0.84 $\pm$ 0.01 ^b	0.01	0.88
Protein	26.42 $\pm$ 0.22 ^a	25.66 $\pm$ 0.33 ^b	25.51 $\pm$ 0.11 ^b	0.07	0.67

I: Traditional processing up to 38% weight loss at 30 °C. II: Traditional processing up to 42% weight loss at 30 °C. III: Traditional processing up to 42% weight loss with temperature increase up to 36 °C in the storage phase. SEM: standard error of mean. The *p*-value processing conditions: one-way ANOVA^{a,b} between conditions I, II, and III. Values within a row with different superscripts differ significantly at $p < 0.05$.

Table 1 shows the results obtained for the different physicochemical parameters with the different processing conditions. In batch I, the final percentage of weight loss or loss was 38% at 30 °C, and for batches II and III the final percentage of weight loss was 42% in both batches, at 30 °C for batch II and at 36 °C for batch III.

Overripening had a significant effect ($p < 0.05$) on moisture content, with the lowest moisture content being obtained in the hams of lot II as a result of the increase in ripening time, which is consistent with the progressive weight loss experienced by hams with continued ripening [9,12,24].

The salt content of the hams was not significantly affected by the different processing conditions; a slight increase in salt content was observed between hams from lots I and II. A

more marked increase in salt content was observed between hams from lot I and lot III. The increase in salt content was due to the increase in the maturing time of hams from lots II and III. An increase in curing time causes the water content to decrease and, consequently, the salt concentration increases [18,39]. Proteolysis in hams can be affected by humidity and salt content. A high salt content could slow down the activity of proteases, but this was not the case with our hams, which were reduced in salt [40,41].

Overripening also significantly affected the intramuscular fat content of the ham, with significantly higher values being obtained in lots II and III compared to lot I. Other studies carried out on Jinhua hams related this increase in processing time and temperature increase to the acceleration of lipid oxidation and a higher concentration of volatile components directly related to flavor development [7].

Ash content increased significantly in lots II and III with respect to lot I due to overripening, which decreases the moisture content of the ham [42].

3.3. Effect of Processing Conditions on the Biochemical Parameters of Boneless Iberian Ham during the Final Storage Stage

Figure 2 shows the effect of changing the processing conditions of Iberian ham on biochemical parameters.

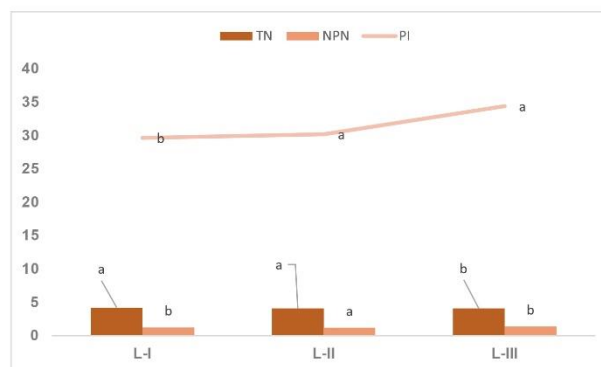


Figure 2. Changes in biochemical parameters with different processing conditions of Iberian dry-cured ham. Values within a row with different superscripts differ significantly at $p < 0.05$.

Figure 2 shows the biochemical content of boneless cured Iberian hams reduced in salt in the final stages of processing of the different batches. Total nitrogen and protein content did not vary significantly despite the changes in processing time and temperature applied to batches II and III. Similar values were obtained for the three batches in both parameters studied.

The proteolysis index was significantly affected ($p < 0.05$) by the processing changes in the ham. Hams from lots I and II obtained values similar to each other and significantly lower than those of hams from lot III. This was due to the increase in temperature in the final phase of the Iberian hams from lot III, which may have resulted in the significant variations that took place during proprotein degradation, which induces protease activity [5]. Similar results were observed in other studies, where increasing the temperature and drying level significantly ($p < 0.05$) increased the NPN content and the IP [43–45]. The hams from batch II were not affected by the increase in processing time for the hams from batch I, and this may have been because there comes a moment in which, despite continuing with the processing, proteolysis remains stagnant and is not favored by the increase in processing time.

Non-protein nitrogen content was not significantly affected by the different processing conditions; higher values were observed in the hams of lot III, which had up to 42% weight loss or loss at 36 °C. This may have been due to the increase in the temperature of the cellar in the final phase, since increasing the temperature increased proteolysis and, with it, the activity of cathepsins B and L, which contribute to the production of smaller peptides and free amino acids, which also contribute to the development and quality of the cured ham [6]. Similar results have been obtained for Jinhua hams [46].

The non-significant results for these fractions (TN and NPN) and the similar results for all batches can be explained by the fact that we know that over-translation and temperature increase protein degradation. Thus, the salt content in all hams was reduced, which also increases cathepsin activity [16,46].

Results for the effect of different processing conditions on Iberian dry-cured ham on free amino acid (FAA) content in the final product are shown in Table 2.

Table 2. Effect of processing conditions on free amino acid content (FAA) of Iberian dry-cured ham. Results are expressed in g/kg of dry matter as mean values $\pm$ SEM.

FAA	Processing Conditions			p-Value	
	I	II	III	Overmaturation	Temperature
Asp	-	-	-	-	-
Glu	2.57 $\pm$ 0.29 ^a	3.99 $\pm$ 0.04 ^b	3.95 $\pm$ 0.33 ^b	0.00	0.89
Ser	1.04 $\pm$ 0.18 ^a	0.98 $\pm$ 0.07 ^a	1 $\pm$ 0.01 ^a	0.86	0.85
His	0.81 $\pm$ 0.07 ^a	1.12 $\pm$ 0.01 ^b	1.25 $\pm$ 0.03 ^b	0.03	0.01
Gly	2.4 $\pm$ 0.18 ^a	3.17 $\pm$ 0.02 ^b	3.51 $\pm$ 0.04 ^b	0.03	0.00
Thr	0.33 $\pm$ 0.02 ^b	0.39 $\pm$ 0.03 ^b	1.32 $\pm$ 0.15 ^a	0.25	0.00
Arg	0.64 $\pm$ 0.16 ^a	0.17 $\pm$ 0.02 ^{ab}	0.13 $\pm$ 0.00 ^b	0.13	0.07
Ala	6.65 $\pm$ 0.56 ^a	8.54 $\pm$ 0.11 ^b	9.92 $\pm$ 0.11 ^b	0.00	0.00
Tyr	0.47 $\pm$ 0.05 ^b	0.7 $\pm$ 0.05 ^a	0.49 $\pm$ 0.02 ^{ab}	0.05	0.04
Cys	-	-	-	-	-
Val	2.15 $\pm$ 0.16 ^a	2.85 $\pm$ 0.02 ^b	3.13 $\pm$ 0.07 ^b	0.03	0.04
Met	0.69 $\pm$ 0.06 ^a	0.63 $\pm$ 0.02 ^a	0.63 $\pm$ 0.08 ^a	0.55	0.97
Trp	0.23 $\pm$ 0.02 ^b	0.31 $\pm$ 0.01 ^a	0.3 $\pm$ 0.01 ^{ab}	0.05	0.72
Phe	1.24 $\pm$ 0.09 ^b	1.5 $\pm$ 0.01 ^{ab}	1.93 $\pm$ 0.18 ^a	0.15	0.13
Ile	1.6 $\pm$ 0.12 ^a	1.94 $\pm$ 0.02 ^a	2.11 $\pm$ 0.04 ^b	0.14	0.04
Leu	2.51 $\pm$ 0.18 ^b	2.94 $\pm$ 0.02 ^{ab}	3.39 $\pm$ 0.105 ^a	0.23	0.02
Lys	3.18 $\pm$ 0.35 ^a	4.05 $\pm$ 0.32 ^a	4.39 $\pm$ 0.06 ^a	0.25	0.50
Pro	1.17 $\pm$ 0.11 ^b	1.51 $\pm$ 0.00 ^{ab}	1.62 $\pm$ 0.01 ^a	0.00	0.12
Total FAA	26.67	34.35	39.03	0.00	0.00

I: Traditional processing up to 38% weight loss at 30 °C. II: Traditional processing up to 42% weight loss at 30 °C. III: Traditional processing up to 42% weight loss with temperature increase up to 36 °C in the storage phase. SEM: standard error of mean. The p-value processing conditions: one-way ANOVA^{a,b} between conditions I, II, and III. Values within a row with different superscripts differ significantly at $p < 0.05$.

Changes in the concentration of free amino acids also indicate changes in ham proteins during storage [47]. The different processing conditions of the salt-reduced boneless cured Iberian ham significantly affected the composition of free amino acids. No values were detected for the amino acids aspartate (Asp) and cysteine (Cys), coinciding with the results obtained in a study on lamb ham, where an increase in maturation time resulted in undetectable values of this amino acid [47]. Serine (Ser), methionine (Met), arginine (Arg), phenylalanine (Phe), and lysine (Lys) were not significantly affected by the different processing conditions (overripeness and temperature) to which the different batches were subjected. Similar but slightly higher values were observed in almost all the hams from

batch I, except for phenylalanine and lysine, where the highest values were obtained from batches II and III. These results coincide with those found in another study, where increased storage time increased the concentration of these amino acids [47]. For the rest of the free amino acids, temperature and over-maturing led to significant changes in the free amino acid content. For histidine (His), glycine (Gly), alanine (Ala), tyrosine (Tyr), and valine (Val) overripening and temperature produced significant changes ($p < 0.05$) in the concentration of these amino acids. Glutamic acid (Glu), tryptophan (Tro), and proline (Pro) were also significantly affected ($p < 0.05$) only by overripening, without the increase in temperature, producing significant changes in their concentration. In the case of threonine (Thr), isoleucine (Ile), and leucine (Leu) it was temperature that produced significant differences ($p < 0.05$) in their concentration. For all free amino acids, these processing changes favored a significant increase in their concentration, with the hams of batch III being in most cases the ones that showed the highest concentrations. Therefore, peptidase activity was significantly affected by the increase in temperature during the final phase and overripening [4], as was the total free amino acid (FAA) content, which was significantly higher in batches II and III than in batch I, with batch III having the highest values.

The results obtained for batch I are similar to those obtained in the study by Muñoz-Rosique et al. [35] for traditionally cured hams. In the first place, the reduction in salt did not cause any decrease in the amino acid content, as corroborated by the study by Cittadini et al. [48].

FAAs are also a type of taste substance. Glutamic acid is the amino acid associated with the umami taste of Jinhua ham [7]. In addition, alanine is associated with sweet taste and leucine with bitter taste. A decrease in their concentration during ham maturation can diminish these flavors [47], something that did not occur in our hams since overripening and temperature had the opposite effect of increasing the concentrations of these amino acids. Lysine is related to the flavor of ripening [7,47], coinciding with the results obtained for our hams, where the lysine values for lots II and III were the highest.

In addition, it was observed that, with respect to the amino acid content of the hams from batch I, there was an increase in the concentration of almost all amino acids, reflecting the fact that the temperature and overripening favored the activity of the peptidases. Similar results were observed in another study, where processing time increased the total free amino acid content in Jinhua hams with increased maturation time and processing temperature [7].

A higher content of free amino acids is directly related to an increase in the substrate of the Strecker degradation reaction, which is responsible for the formation of aromatic molecules that directly contribute to the flavor of cured ham [8,49].

3.4. Instrumental Color and Texture Profile

Table 3 shows the effect of different processing on the instrumental color obtained from Iberian ham.

Table 3. Effect of processing conditions on instrumental color. Results are expressed as means values $\pm$ SEM.

	Processing Conditions			p-Value	
	I	II	III	Overmaturation	Temperature
L	49.2 $\pm$ 1.03 ^b	46.03 $\pm$ 0.44 ^a	47.54 $\pm$ 1.44 ^b	0.01	0.33
a	28.62 $\pm$ 0.99 ^a	30.22 $\pm$ 0.25 ^a	28.82 $\pm$ 0.77 ^a	0.14	0.11
b	34.75 $\pm$ 0.63 ^a	34.47 $\pm$ 0.57 ^a	31.93 $\pm$ 1.42 ^a	0.74	0.12
C	45.06 $\pm$ 1.01 ^a	45.85 $\pm$ 0.52 ^a	43.15 $\pm$ 0.98 ^b	0.5	0.03
h	50.59 $\pm$ 0.74 ^b	48.73 $\pm$ 0.44 ^a	47.75 $\pm$ 1.7 ^a	0.05	0.59

I: Traditional processing up to 38% weight loss at 30 °C. II: Traditional processing up to 42% weight loss at 30 °C. III: Traditional processing up to 42% weight loss with temperature increase up to 36 °C in the storage phase. SEM: standard error of mean. The p-value processing conditions: one-way ANOVA^{a,b} between conditions I, II, and III. Values within a row with different superscripts differ significantly at $p < 0.05$.

Instrumental color parameters were not affected by the temperature or overripeness of the cured Iberian ham ($p > 0.05$ in all cases), coinciding with the data obtained in similar studies [22]. The brightness value (L) was slightly higher in the hams from lot I, as well as in the h parameter. A decrease in L* values was directly related to the decrease in moisture or, in other words, the dehydration of the cured ham, since a decrease in water increases the concentration of pigments such as nitrosyl-myoglobin. As a consequence, there is a reduction in luminosity (L*) [50]. The range of values for parameters a, b, and c was similar for all the batches of hams, so they were not significantly affected by overripeness or increased temperature.

Table 4 shows the effect of different processing conditions on the instrumental texture of Iberian dry-cured ham.

Table 4. Effect of different processing conditions on instrumental texture of Iberian dry-cured ham. Results are expressed as means values $\pm$ SEM.

	Processing Conditions			p-Value	
	I	II	III	Overmaturation	Temperature
C1 hardness (N)	15.31 $\pm$ 2.05 ^a	17.87 $\pm$ 2.39 ^a	16.9 $\pm$ 1.45 ^a	0.43	0.73
C1 hardness deformation (mm)	2.97 $\pm$ 0.00 ^a	2.98 $\pm$ 0.00 ^a	2.98 $\pm$ 0.00 ^a	0.64	0.57
C1 adhesiveness (mJ)	0.03 $\pm$ 0.02 ^b	0.24 $\pm$ 0.09 ^a	0.36 $\pm$ 0.10 ^a	0.04	0.38
C2 hardness (N)	14.63 $\pm$ 1.95 ^a	16.66 $\pm$ 2.23 ^a	15.67 $\pm$ 1.33 ^a	0.50	0.71
C2	0.62 $\pm$ 0.04 ^a	0.67 $\pm$ 0.03 ^a	0.63 $\pm$ 0.03 ^a	0.39	0.41
C2 recoverable deformation (mm)	1.43 $\pm$ 0.13 ^a	1.50 $\pm$ 0.11 ^a	1.45 $\pm$ 0.14 ^a	0.65	0.78
C2 springiness (mm)	2.1 $\pm$ 0.15 ^a	2.31 $\pm$ 0.13 ^a	2.19 $\pm$ 0.13 ^a	0.31	0.53
C2 gumminess (N)	9.84 $\pm$ 1.77 ^a	12.31 $\pm$ 2.01 ^a	10.94 $\pm$ 1.39 ^a	0.37	0.58
C2 chewiness (mJ)	22.2 $\pm$ 5.15 ^a	29.82 $\pm$ 5.65 ^a	25.39 $\pm$ 4.31 ^a	0.34	0.54

I: Traditional processing up to 38% weight loss at 30 °C. II: Traditional processing up to 42% weight loss at 30 °C. III: Traditional processing up to 42% weight loss with temperature increase up to 36 °C in the storage phase. SEM: standard error of mean. The p-value processing conditions: one-way ANOVA^{a,b} between conditions I, II, and III. Values within a row with different superscripts differ significantly at $p < 0.05$.

Overripening and the increase in processing temperature in the final phase did not have a significant effect on the different parameters defined for the instrumental texture. Only the adhesiveness was significantly higher in hams from lots II and III with respect to lot I, with lot III obtaining the highest values. Although there were no significant differences, for the rest of the parameters, slightly higher values were observed for hams from lots II and III with respect to lot I. Higher hardness values have been attributed to water content, since texture is positively related to moisture loss in ham [10]. As a general rule, the lower the moisture content, the better the consistency [51]. Despite showing greater proteolysis, the texture parameters analyzed did not indicate a softer texture. This could be due to the positive effect of temperature and overripening on hardness, since for both batches (II and III) the hardness was higher than for batch I, with these results also coinciding with those obtained in other studies [9,43].

Table 5 shows the effect of different processing conditions on the sensory analysis profile of Iberian dry-cured ham.

Table 5 shows the acceptability scores for dry-cured hams under different processing conditions given by the consumer panel. Despite the different processing conditions, the consumer panel scored the three types of ham very similarly, finding significant differences only for color, with overripeness significantly affecting these parameters. Therefore, consumer acceptability of sensory traits was similar for all the types of cured hams studied. Similar results were described by Cilla et al. [22]. Therefore, we can say that neither overripening nor the increase in temperature in the final processing phase affected the organoleptic characteristics of the salt-reduced boneless cured Iberian ham. Prolonged

ripening or overripening could increase lipid oxidation, the Maillard reaction, or Strecker amino acid degradation, leading to the development of compounds responsible for the flavor of the ham [49].

Table 5. Effect of different processing conditions on the sensory analysis profile of Iberian dry-cured ham. Results are expressed as means values $\pm$ SEM.

	Processing Conditions			p-Value	
	I	II	III	Overmaturation	Temperature
Appearance	4.15 $\pm$ 0.07 ^a	4 $\pm$ 0.07 ^a	4.14 $\pm$ 0.07 ^a	0.11	0.14
Color	4.28 $\pm$ 0.06 ^b	4.1 $\pm$ 0.07 ^a	4.23 $\pm$ 0.07 ^a	0.04	0.15
Odor	3.87 $\pm$ 0.07 ^a	3.94 $\pm$ 0.07 ^a	3.86 $\pm$ 0.08 ^a	0.53	0.47
Texture	3.95 $\pm$ 0.07 ^a	3.87 $\pm$ 0.07 ^a	3.9 $\pm$ 0.08 ^a	0.48	0.83
Salty taste	3.82 $\pm$ 0.08 ^a	3.78 $\pm$ 0.08 ^a	3.66 $\pm$ 0.08 ^a	0.68	0.28
Global flavor	4.01 $\pm$ 0.07 ^a	3.94 $\pm$ 0.07 ^a	3.91 $\pm$ 0.07 ^a	0.50	0.78
General acceptance	4.05 $\pm$ 0.07 ^a	3.95 $\pm$ 0.06 ^a	3.94 $\pm$ 0.07 ^a	0.33	0.86

I: Traditional processing up to 38% weight loss at 30 °C. II: Traditional processing up to 42% weight loss at 30 °C. III: Traditional processing up to 42% weight loss with temperature increase up to 36 °C in the storage phase. SEM: standard error of mean. The p-value processing conditions: one-way ANOVA^{a,b} between conditions I, II, and III. Values within a row with different superscripts differ significantly at $p < 0.05$.

4. Conclusions

Increasing processing time and temperature significantly increased proteolysis, but this did not result in undesirable changes in texture. No significant differences were observed in any of the analyzed parameters defining instrumental texture. The consumer panel did not detect worse texture in these hams; the texture was not significantly affected.

Furthermore, these changes in processing conditions increased the content of free amino acids, benefiting the consumer, as well as being responsible for aromas and attributes that contribute to the development of positive effects in cured ham.

These changes did not modify the sensory attributes evaluated by the participants, as no significant differences were found between the different batches—only for the instrumental color, which scored significantly lower in the overripe ham without temperature increase.

In general, despite the reduction in salt, these new processing conditions benefited the hams, with all of them obtaining very good scores in the sensory panel, with texture not being affected by increased proteolysis and with a higher content of free amino acids. In addition, increased proteolysis and NPN will lead to a higher production of peptides with possible bioactivity.

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Overripening and increased temperature: Alternative strategies to enhance peptide production and bioactivity in salt-reduced boneless cured Iberian hams

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ABSTRACT

Iberian ham is renowned for its unique flavour and artisanal tradition, but its potential as a source of bioactive compounds remains underexplored. This study presents an innovative view of how overripening and controlled temperature increase can significantly improve the production and diversity of bioactive peptides in salt-reduced cured Iberian hams. Forty-eight hams were divided into three groups: traditionally cured, overripened with 42 % weight loss at 30 °C, and overripened with 42 % weight loss at 36 °C. Comparing them, our results reveal significant advances in proteolysis, generating peptides with potent antioxidant and angiotensin converting enzyme-I (ACE-I) inhibitory activities. In particular, hams cured at 30 °C showed superior bioactive profiles, reaching the lowest IC50 values for ACE-I activity, while those processed at 36 °C exhibited the highest diversity, identifying 1,313 unique peptides by advanced nano-liquid chromatography-mass spectrometry. In terms of antioxidant activity, overripeness significantly affected ($p \leq 0.05$) antioxidant activity, with both overripened hams showing the highest antioxidant activity. These findings redefine the role of Iberian ham as a functional food with potential applications in cardiovascular and metabolic health management, while maintaining the authenticity and quality of traditional Iberian ham.

1. Introduction

Bioactive peptides are short chains of amino acids that have specific biological effects in the human body. These peptides can have a variety of functions in the body, and some of them have been shown to have bioactive properties beneficial to health (Zaky et al., 2022). Bioactive peptides can be found in a variety of foods, such as meat, fish, dairy products and legumes. In addition, bioactive peptides are also being researched and developed as functional ingredients in the food and supplement industry, with the aim of improving the health and well-being of the population (Peighambari et al., 2021). The search for functional foods that offer health benefits beyond basic nutrition has prompted studies on animal products, where fermentation and maturation processes promote the release of these bioactive compounds.

Iberian ham is a quality product that, due to the fermentation and

maturation process, has the capacity to generate bioactive peptides (Martín et al., 1998; Rosell & Toldrá, 1998). During the production of ham, the meat undergoes a maturation and fermentation process involving enzymes and bacteria (Alía et al., 2019; Bidlas & Lambert, 2008). This process can lead to the formation of bioactive peptides from the proteins present in the meat.

Maturation is a process in which endogenous enzymes and microorganisms present in the meat work together to influence the generation of bioactive peptides (Ruiz et al., 1998). During maturation that the muscle proteins present in meat undergo enzymatic and proteolytic degradation processes due to the presence of cathepsins and peptidases, which convert proteins into smaller peptide fragments with bioactive potential. Overripening provides more time for these enzymes to act, which can result in increased protein breakdown and thus release of bioactive peptides.

Abbreviations: AA, (aminoacid); AAs, (aminoacids); ACE-I, (angiotensin-I converting enzyme).

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In addition, the peptides that are initially formed during maturation may undergo additional changes as the process progresses. These changes may include post-translational modifications that affect the biological activity of the peptides (Heres et al., 2023; Hortós, 1996; Li et al., 2022a; Ruiz et al., 1998).

These peptides may have beneficial properties for health, and some studies have suggested that certain bioactive peptides derived from ham may have antioxidant, antihypertensive and anti-inflammatory effects (Bersuder et al., 1998; Escudero, Aristoy et al., 2012, 2012; Saiga et al., 2003). Some bioactive peptides found in ham have shown antioxidant activity, which means they can help neutralise free radicals in the body. Free radicals can contribute to oxidative stress, which is linked to ageing and various diseases (Escudero et al., 2013; Gallego et al., 2020; Saiga et al., 2003).

It has been suggested that certain bioactive peptides derived from ham may have antihypertensive properties, meaning that they may help lower blood pressure. These peptides may act as inhibitors of angiotensin-converting enzyme, which is involved in blood pressure regulation (Escudero, Aristoy et al., 2012; Heres, Yokoyama et al., 2021; Mora, Escudero, Arihara et al., 2015).

Some studies have indicated that certain bioactive peptides in ham may have anti-inflammatory properties (Martínez-Sánchez et al., 2017; Xing et al., 2022).

The ability to reduce inflammation could be beneficial to health, as chronic inflammation is associated with various diseases (Uetake et al., 2015).

In general, is supposed that overripening may be associated with a higher concentration of bioactive peptides in ham, as it provides more opportunities for the formation and accumulation of these compounds. Some studies related that proteolysis mostly breaks down proteins into relatively large polypeptides and peptides, also generating some peptides smaller than 3 kDa, the value of which increases with prolonged postmortem time (Fu et al., 2017).

It is important to note that the effect of overripening on the formation of bioactive peptides may vary depending on several factors, such as specific production conditions, maturation temperature, meat composition, and curing practices used. Furthermore, although some studies suggest benefits associated with bioactive peptides, research on bioactive peptides in foods such as ham is still a developing area, and more studies are needed to fully understand their effects and mechanisms of action.

Previous studies on cured hams have shown that overripening and increases in processing temperature can increase proteolysis, which generates a greater amount of low molecular weight peptides with these bioactive properties. However, although increased temperature accelerates proteolysis, it can negatively influence the stability of certain peptides, which could compromise their activity.

Despite these advances, the study of overripeness and its influence on salt-reduced Iberian hams is still limited. There is evidence that overripening at controlled temperatures can maximise the generation of bioactive peptides, but how increased temperature and overripening specifically affect the formation of antioxidant and antihypertensive peptides in this type of product has not been comprehensively evaluated.

For all these reasons, the present study aims to evaluate how overripening and increased temperature influence the generation of bioactive peptides in boneless Iberian hams reduced in salt. It is hypothesised that a longer overripening time and a moderate temperature will improve the release of peptides with bioactive properties.

2. Material and methods

2.1. Preparation and collection of cured Iberian ham samples

Total of 48 boneless salt-reduced Iberian hams were needed for the development of this study. Three batches of 16 Iberian hams were processed under the same conditions. Starting from the same genetic,

physical and feeding conditions at the time of slaughter.

Fresh hams were first deboned and then salted with sea salts and nitrifying agents at a rate of 0.8 days/kg, (with the aim of obtaining a cured ham reduced by 25% salt each piece was salted for 19.2 h for each kg of weight, approximately 7–8 days) in a cold storage chamber at a temperature of 3 °C. The composition of the nitrifying salts is 86 % sodium chloride, 9 % sodium nitrite, 3 % sodium isascorbate, 1.2 % potassium nitrate and 0.8 % dextrose. The dose applied is 5 g per kilogram of fresh weight of ham. The nitrifying salts are applied by rubbing just before the salting process begins.

After salting, Iberian hams were washed with water and, after this, a traditional curing process was carried out. The resting or post-salting stage began at 3 °C, gradually rising to 6 °C. This processing phase ended when the Iberian hams reached 18% shrinkage, lasting approximately four to five months. This phase was extended for a minimum of 120 days, thus prolonging the post-salting phase to reduce as much as possible and try to counteract the lack of salt (Barat et al., 2013).

Subsequently, the temperature was raised to 30 °C. The process ended when the pieces reached a weight loss of 38%, after approximately 14/15 months of curing. After this last processing, the first batch finished its maturation process, but the two remaining batches of Iberian hams were taken to overripening, both up to 42% weight loss, but in different processing conditions, the second batch was processed at a temperature of 30 °C and the third batch at 36 °C, needing to reach overripening for approximately 3 more months. The percentage weight loss was determined by weighing each sample in triplicate during each of the processing stages. The results were expressed as percent 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 lot I “CON” and 42% for lots II “RIB4” and III “RIB5”). Samples were taken using a 2 cm diameter stainless steel cylinder, in the area corresponding to the biceps femoris muscle.

2.2. Non-protein nitrogenous compounds (NPN)

For preparing this fraction, two grams of the sample were weighed and added to an Erlenmeyer flask containing 30 mL of distilled water and shaken for 15–20 min. 15 mL of 20% trichloroacetic acid was then added and shaken for 10 min. The entire contents were filtered using a funnel and filter paper; after filtration, the 50 mL volumetric flask was made up to the mark with distilled water (Abellán et al., 2018). After obtaining the fraction, the total non-protein nitrogen content was determined using the Kjeldahl method (Official Methods of Analysis, 22nd Edition (2023), n.d.).

2.3. Antioxidant activity

For the determination of antioxidant activity, a standard was made with the 98 % TROLOX (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) reagent, which is an analog of vitamin E with antioxidant capacity, used to react with the DPPH radical (2,2-diphenyl-1-picrylhydrazyl). The stock solution of TROLOX (2 Mm) was prepared by weighing 12.5 mg of TROLOX and diluting it in 25 mL of ethanol. 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 µL of ethanol, 500 µL of sample, and 125 µL of 0.02% (w/v) DPPH solution were added. For the blank, 500 µL of ethanol, 500 µL of water, and 125 µL 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 (Bersuder et al., 1998).

To calculate the concentration of our sample that inhibits 50% of the DPPH radical (IC50), we use the equation of the TROLOX standard we performed previously.

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2.4. Angiotensin-I-converting enzyme inhibitory activity

The ACE-I activity of the NPN fraction was carried out using the spectrophotometric used in a previous study (Munoz-Rosique et al., 2023). The method is based on the spectrophotometric measurement at 228 nm of absorbance of the hippuric acid released in the reaction. The ACE inhibitory activity was determined applying Eq. (1):

$$\text{ACEinhibitoryactivity}(\%) = ((A_{\text{control}} - A_{\text{blank}}) - (A_{\text{sample}} - A_{\text{blank}})) / (A_{\text{control}} - A_{\text{blank}}) \times 100 \quad (1)$$

A_{control} being the measurement of hippuric acid produced by the action of non-inhibited ACE, A_{sample} the measurement of the hippuric acid produced by the action of ACE with the sample, and A_{blank} the measurement of non-reacting HHL. The IC50 value was obtained in triplicate.

2.5. Peptide identification and bioactivity analysis

Identification was determined by tandem mass spectroscopy analysis using liquid nanochromatography (nLC) from the NPN fraction obtained in Section 2.1 (Bueno-Gavilá et al., 2019).

Liquid nanochromatography was performed on a Dionex Ultimate 3000 nano UPLC (Thermo Scientific) with a C18 (75 μm x 50 cm) Acclaim Pepmap column (Thermo Scientific). The previously obtained fraction was loaded onto a 300 μm x 5 mm Acclaim Pepmap precolumn (Thermo Scientific) in a 2% acetonitrile/0.05% TFA mixture for 5 min at a flow rate of 5 $\mu\text{L}/\text{min}$. Peptide separation was performed at 40 $^{\circ}\text{C}$ 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 NPN fractions of the different hams was performed at the Proteomics and Bioinformatics Unit of the University of Córdoba, Spain. MS2 spectra were found with SEQUEST HT against the UniProtKB database.

The peptides of each hydrolysate 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., 2022). 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.

2.6. Statistical analysis

All analyses were performed in triplicate. Statistical analysis of our samples was considered significant if $p < 0.05$. Statistical analysis of our samples was performed with SPSS software (version 21.0, IBM Corporation, Armonk, NY, USA).

Most of the data obtained were analyzed using a one-way ANOVA or factorial ANOVA test. Biostatistical analysis of the identified peptide data was performed with R (version 3.4.1; <https://www.r-project.org>).

3. Results and discussion

In order to explain the results, it is necessary to know the protein and non-protein nitrogen content and the proteolysis index of the samples analysed, where the 42% overripe Iberian ham at 36 $^{\circ}\text{C}$ stands out. These results can be seen in a previous study carried out by the group and recently published (Hernández Correas et al., 2024).

3.1. Evaluation of antioxidant activity in hams produced under different processing conditions

The assessment of antioxidant activity by studying the DPPH radical is a technique widely used in many studies for this purpose (Marinova & Batchvarov, 2011).

Several studies have demonstrated the antioxidant capacity of the peptides present in cured ham (Escudero, Aristoy et al., 2012; Escudero et al., 2013; Gallego, Mora, Reig et al., 2018). Despite this, there are no studies that evaluate whether the antioxidant capacity of the peptides increases with overripening or the temperature increase. The antioxidant capacity of the peptides present in cured ham is directly related to hydrophobic amino acids, so the presence of this activity will also imply the presence of hydrophobic amino acids associated with greater bioactivity (Wang et al., 2021).

Fig. 1 shows the evolution of the in vitro antioxidant capacity of the non-protein nitrogen fraction of the different processed Iberian ham samples according to the concentration of peptides present in them. All the samples analysed showed a higher antioxidant capacity the higher the concentration of peptides in the sample.

The sample with the highest antioxidant capacity was the Iberian ham overripened to 42% weight loss at 30 $^{\circ}\text{C}$ (RIB4) reaching an inhibition of >70%. The maturation time is a crucial factor in the development of proteolysis, which in turn plays a crucial role in the development of bioactive peptides, as proteolysis begins with the hydrolysis of myofibrillar proteins by endopeptidases, leading to the accumulation of polypeptides. These polypeptides are broken down into small peptides and amino acids by the action of exopeptidases. Over-ripening results in increased proteolysis, which can lead to an increase in the concentration of peptides with bioactivity, including an increase in the concentration of peptides with antioxidant activity (Mora, Escudero, Aristoy et al., 2015; Schivazappa & Virgili, 2020; Wang et al., 2021). It is possible that at 30 $^{\circ}\text{C}$, the enzymes functioned more efficiently, allowing a controlled proteolysis that favoured the release of specific antioxidant peptides. Antioxidant activity in cured ham peptides is strongly related to the presence of hydrophobic amino acids such as leucine, isoleucine, valine, and alanine. In the case of RIB4 ham, a high concentration of these amino acids was observed in the peptides identified, which could have contributed significantly to their ability to neutralise free radicals. Studies indicate that peptides containing these hydrophobic amino acids in key positions tend to exhibit higher antioxidant activity. In the rest of the samples, an increase in antioxidant capacity is also observed as the concentration of peptides increases, this capacity being greater in RIB5, so that the ham with the lowest antioxidant capacity was the control ham, which was not subjected to any treatment and followed the traditional curing process (CON).

The table below (Table 1) shows the in vitro antioxidant activity of Iberian ham processed under different conditions, with increasing the temperature or overripening. The concentration of non-protein nitrogen (mg/mL) required to inhibit 50% of the antioxidant activity (IC50) was evaluated. Both traditionally processed ham (CON) and overripe ham without (RIB4) or with increased temperature (RIB5) showed

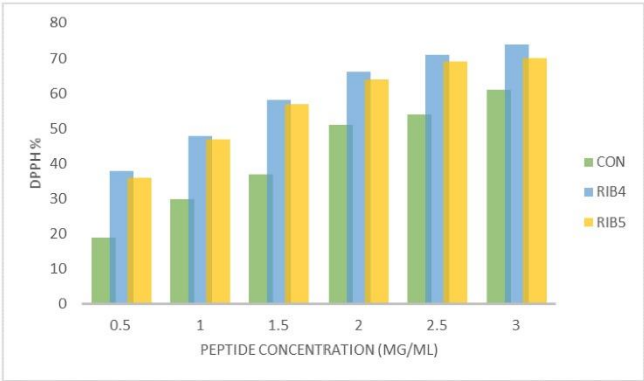


Fig. 1. Evolution of antioxidant activity as a function of peptide concentration in hams with different processing conditions.

Table 1
Effect of processing conditions on DPPH during the final stage. Results are expressed in mg/mL as means values $\pm$ SEM.

Processing Conditions	p value				
Formulation	CON	RIB4	RIB5	Processed	Temperature
DPPH(IC50)	1.76 $\pm 0.241^a$	1.144 $\pm 0.194^b$	1.172 $\pm 0.075^b$	0.037	0.426

CON: DRYING 33% loss (30 °C); STORAGE UP TO 38% decrease (30 °C)
RIB4: DRYING 33% loss (30 °C); STORAGE UP TO 42% decrease (30 °C)
RIB5: DRYING 33% loss (30 °C); STORAGE UP TO 42% decrease (36 °C)
SEM: standard error of mean.
p-value Processing Conditions: One-way ANOVA between different processing conditions (p-value significant at $p \leq 0.05$).

antioxidant activity.
Processing significantly affected ($p \leq 0.05$) the uptake of the DPPH radical in the samples. In contrast, temperature increase did not

significantly influence the antioxidant activity of the samples, although the highest antioxidant capacity was shown by RIB4 overripe without temperature increase. These hams showed the highest antioxidant capacity as they reached the IC50 with the lowest concentration of peptides (1.144 ± 0.194 mg/mL), probably because overripeness affected the activity of the enzymes responsible for proteolysis, although the processing temperature did not affect significantly ($p \leq 0.05$), obtaining very similar data between samples, and it may be possible that the temperature neither favours nor harms the activity of the enzymes responsible for protein degradation. There are no studies that help us to corroborate these hypotheses, as this is the first time this type of test has been carried out.

For all hams, the concentration necessary to inhibit 50% has been lower than 2 mg/mL, obtaining an IC50 around a concentration lower than 1.2 mg/mL in overripe Iberian hams, obtaining in other studies the IC50 at concentrations higher than 2.5 mg/mL or even higher than 4.5 mg/mL, (Hu et al., 2023; Li et al., 2022b; Xing et al., 2018) thus highlighting the quality of the meat from Iberian pigs. A previous study

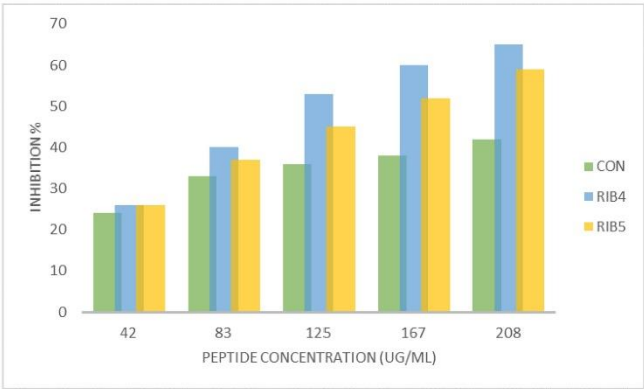


Fig. 2. Evolution of ACE-I activity as a function of peptide concentration in hams with different processing conditions.

showed that the antioxidant activity was significantly higher in hams from Iberian pigs than in hams from white pigs (Muñoz-Rosique et al., 2023).

3.2. Evaluation of antihypertensive activity (ACE-I) in hams processed under different processing conditions

One of the key enzymes in the control and regulation of blood pressure is angiotensin I-converting enzyme, due to its involvement in the renin angiotensin-aldosterone system (RAAS) (Ji et al., 2020). The following figure (Fig. 2) shows the evolution of the inhibition of this enzyme during a test as a function of peptide concentration in Iberian hams with different curing times and processed at different temperatures, where RIB4 showed the highest ACE-I activity probably due to a combination of moderate temperature and prolonged over-ripening, which allowed efficient generation of inhibitory peptides. RIB5 hams (36 °C), showed lower ACE-I activity, probably due to enzymatic denaturation or excessive degradation of peptides caused by the high temperature. CON hams had the lowest ACE-I activity, due to limited proteolysis, which prevented the release of a significant amount of ACE inhibitory peptides (Alía et al., 2019; del Olmo et al., 2015; Martín et al., 1998). Table 2 shows the effect of processing (percentage of loss) and curing temperature on the ACE inhibitory activity, represented as the concentration of peptides required (mg/mL) to inhibit 50% of the activity of this enzyme (IC50). All the samples studied showed ACE inhibitory activity, which increased with increasing peptide concentration. This result could be a consequence of the presence of peptides smaller than 3 kDa (Noorani & Nazeer, 2020; Sahingil et al., 2022). The ham with the highest ACE-I activity (IC50) was RIB4, showing the greatest potential to help control cardiovascular risk diseases, affirming the conclusions of other studies that report a benefit of ham consumption on blood pressure and cardiometabolic markers in individuals at risk of cardiovascular disease (Montoro-García et al., 2022). In addition, a study conducted at the University of Navarra showed that after an average of 11 years and a high consumption of ham (equal to or more than five times a week) middle-aged patients did not report an increased incidence of hypertension cases despite the high salt content (Rico-Campà et al., 2020). The IC50 of the final product was lower, which means higher activity, in the Iberian ham overripened up to 42% weight loss and processed at 30 °C (RIB4), with significant differences ($p \leq 0.05$) in processing observed between the CON ham, processed up to 38% weight loss. This may probably be due to the longer curing time to which the RIB 4 hams were subjected, these results are in agreement with other studies that saw an increase of >40% in the concentration of a dipeptide (AA) when the curing time was increased (Heres, Yokoyama et al., 2021). In this study, the hams had up to 42% loss of weight. This could imply that with maturation up to this percentage of weight loss, proteolysis increases, producing a higher concentration of bioactive peptides, which would lead to an increase in bioactivity in the samples, which coincides with other results observed in various studies, in which it has

Table 2
Effect of processing conditions on ACE-I during the final stage. Results are expressed in mg/mL as means values $\pm$ SEM.

Processing Conditions				p-value	
Formulation	CON	RIB4	RIB5	Processed	Temperature
ACE-I (IC50)	0.172 ±0,037 ^a	0.131 ±0,025 ^c	0.158 ±0,003 ^b	0.037	0.042

CON: DRYING 33% loss (30 °C); STORAGE UP TO 38% decrease (30 °C)
RIB4: DRYING 33% loss (30 °C); STORAGE UP TO 42% decrease (30 °C)
RIB5: DRYING 33% loss (30 °C); STORAGE UP TO 42% decrease (36 °C)
SEM: standard error of mean.
p-value Processing Conditions: One-way ANOVA between different processing conditions (p-value significant at $p \leq 0.05$).

been seen that this activity increases significantly in the last phase of curing in Serrano and Panxian hams (Montoro-García et al., 2017; Schivazappa & Virgili, 2020). Other authors also observed an increase in the activity of certain dipeptides (Escudero, Mora et al., 2012).

Curing temperature also significantly ($p \leq 0.05$) affected ACE-I (comparing RIB4 hams with RIB5 hams), with higher activity being observed in those hams in which the processing temperature was not increased (RIB4). This could be due to the fact that a temperature of 36 °C was not within the appropriate temperature range for the optimal activity of proteolytic enzymes, especially endopeptidases, which are highly sensitive to temperature changes, and although a slight increase in temperature can accelerate enzymatic reactions, too high temperatures can denature these enzymes or reduce their efficiency, with this increase in processing temperature being a possible inhibitory factor for certain enzymes involved in peptide formation. Despite this observation, it is interesting to note that previous studies on other types of ham, such as Jinhua ham, have shown that maturation at high temperatures, similar to 36 °C, promotes proteolysis by stimulating chemical and biochemical reactions essential for protein degradation. Zhang et al. (2011) and Luo et al. (2021) confirmed that the use of high temperatures during curing of Jinhua ham promoted the generation of proteolytic compounds and the development of bioactive peptides. However, the differences observed between the results of Jinhua ham and Iberian ham may be due to variability in meat types, pig genetics, and the specific enzymes and microorganisms involved in each case.

It is possible that Iberian pork, which is rich in intramuscular fat and has unique characteristics due to its traditional curing process, is more sensitive to temperature variations compared to other types of ham. Therefore, while in products such as Jinhua ham high temperatures may be beneficial, in the case of Iberian ham there may be a more limited temperature window to ensure optimal proteolytic enzyme activity, which maximises the production of bioactive peptides.

In addition, it is important to consider that peptides generated at higher temperatures could be different in structure or size, which could influence their functionality.

During a previous study it was observed that the consumption of cured ham with a high percentage of bioactive peptides had a positive influence on the regulation of glycaemia and cholesterol in healthy patients, so instead of restricting its consumption, it was seen that regular consumption of this product had positive effects on the regulation of the development of cardiovascular risk diseases (Montoro-García et al., 2017).

The presence of ACE-I activity in the hams studied could help to counteract the harmful effects of sodium in the body, helping to reduce or reducing the risk of suffering or developing cardiovascular risk diseases (Moorthy et al., 2021; Udenigwe & Aluko, 2012).

3.3. Bioactive peptide identification

Peptides present in the analysed ham samples (CON, RIB4, RIB5) were identified by nLC-MS/MS analysis. The figure below (Fig. 3) shows the number of peptides identified per sample. The ham with the highest number of peptides identified was RIB5 (1313), followed by RIB4 (954) and finally the traditionally processed ham, CON, where 411 peptide sequences were identified. These values are in agreement with the bioactivity measured in vitro obtained so far, where hams not subjected to changes during processing (CON) showed the lowest activity for the

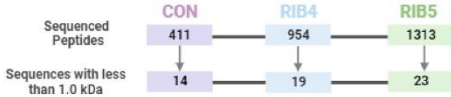


Fig. 3. Number of peptides identified per sample. Number of peptides identified with <1.0 kDa.

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bioactivities studied.

These results corroborate the increase in proteolysis that occurs with overripening and with it a higher activity of endo- and exo-peptidases, which in turn leads to a higher number of identified sequences, and smaller peptides can be obtained thanks to a longer proteolysis time.

In the peptides obtained in this proteomic study, no peptides already registered in the BIOPEP database were found, but a huge number of sequences were found that presented fragments of potential biopeptides containing in their primary structure fragments of bioactive sequences already shown in the BIOPEP database.

Other studies did find unique sequences in cured ham already identified, with origin in different types of myosin protein, being identified as peptides with more ACE-I activity (Le et al., 2020; Martini et al., 2019; Mora et al., 2017; Xing et al., 2021). Other studies showed that the AAATP peptide had the highest ACE-I activity, but also exerted a multifunctional effect because it also exerted inhibitory activity on DPPIV (Escudero, Mora et al., 2012). Other sequences with antihypertensive capacity were also identified, such as ASGPINFT and DVITGA, both being myosin as their protein of origin (Escudero et al. (2013)).

Another of the most studied bioactivities is antioxidant, peptides with this bioactivity have been identified in ham, such as SAGNPN, SNAAC and AEEYYPDL, which have the capacity to donate electrons, helping to neutralise oxidative capacity (Escudero et al., 2013; Gallego, Mora & Toldrá, 2018; Mora et al., 2014).

A variety of peptides with antihypertensive capacity have also been identified in Iberian ham, with greater potential than those present in white ham. The most frequently repeated sequences that coincide with those identified in BIOPEP are PPK, PAP and AAP (Heres, Yokoyama et al., 2021). Numerous dipeptides found in Iberian ham, such as EA, PP and VE, have also been identified, showing multifunctional bioactivities (Escudero et al., 2013). Despite the progress made, no studies have been found that identify the peptides present in Iberian hams subjected to different processing conditions, where the alterations that occur in the peptides when the ham is subjected to different factors during the curing process can be observed. In a previous study carried out with hams of different genetic lines and reduced in salt, numerous peptide precursors were identified that could give rise to sequences identified as bioactive in the BIOPEP database. The dipeptide DV was identified among the peptide precursors present in all types of hams studied (Muñoz-Rosique et al., 2023).

3.3.1. Study of peptide identified with putative activity

In order to contextualise the type of bioactivity possessed by the samples analysed, a Zscore was carried out to plot the variation between the samples with respect to the mean of the different bioactivities (heatmap). The results obtained can be seen in Fig. 4, knowing that the higher the intensity of the red colour, the more this bioactivity will be over-represented with respect to the average of the three samples.

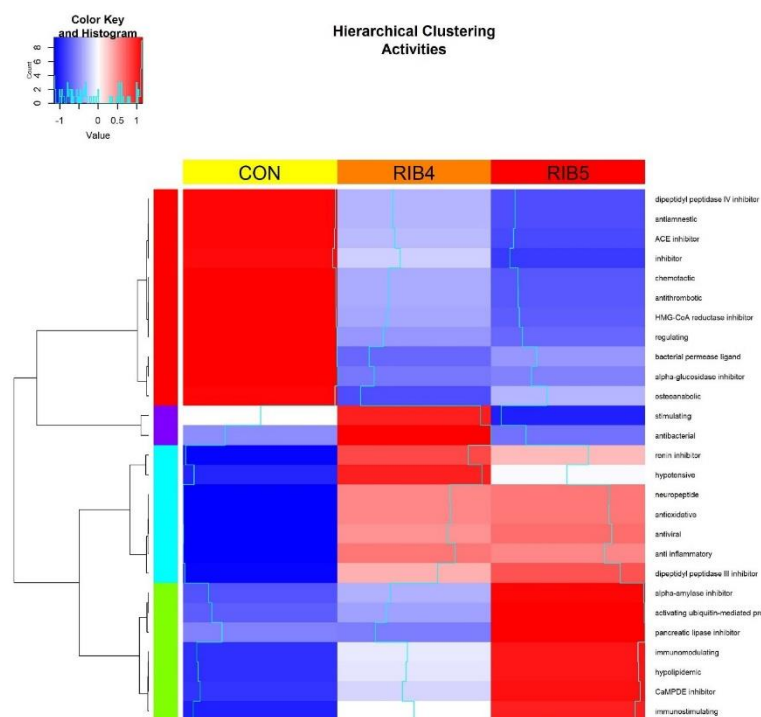
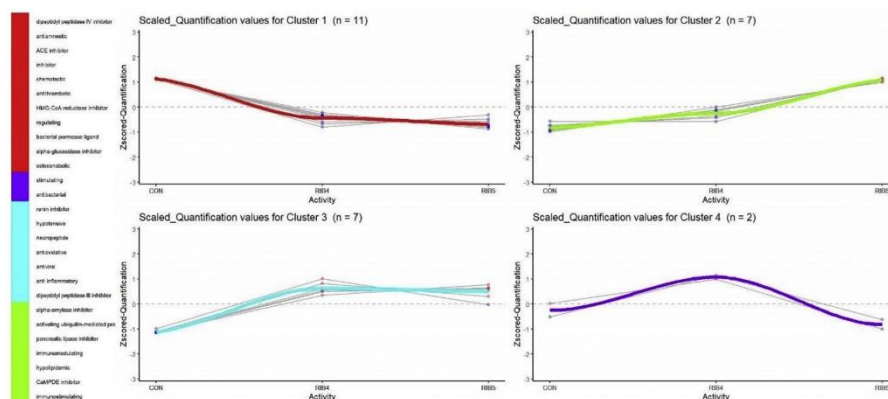


Fig. 4. Heatmap and dendrogram of bioactivities of the different ham samples studied. Quantification of bioactivity is regarding the mean. The grouping relationship between the groups of activities is defined.



An exhaustive search for peptide precursors that may contain biopeptides in their sequence and that could be activated after cleavage of the proteins after digestion has been carried out. This technique is very useful for sequences that are impossible to detect by proteomics techniques (chains of less than seven amino acids).

The bioactivities represented in the heatmap are associated based on the degree of occurrence in each sample. The order of the rows and columns is designed to avoid intersection of the lines of the dendrogram. The lines that can be seen in light blue represent the value of the coefficient. In addition, in Fig. 5 we have shown individually in which sample each group of activities is prominent, associating the groups of bioactivities to the colours represented on the left side of the heatmap (Fig. 4).

In the case of CON ham, the main activities in which it stands out are DPPIV inhibitor, antihemostatic, ACE inhibitor, inhibitory, chemotactic, antithrombotic, HMG-CoA reductase (hydroxymethylglutaryl-CoA reductase) inhibitor, regulatory, bacterial permease ligand, Alpha-glucosidase inhibitor and osteoanabolic. For most of these bioactivities there are no previous studies that define or identify them in cured ham and even less in hams produced under specific conditions. There are studies that claim that ham would be a good source of DPPIV and that these peptides could be an adjunct in the treatment of type II diabetes (Li et al., 2022; Montoro-García et al., 2017).

HMG-CoA reductase inhibitors carry out a very important role in the development and appearance of cardiovascular risk diseases in a premature way, there are studies that have identified numerous dipeptides in ham that have been identified as responsible for the inhibition of this coenzyme (Heres, Mora et al., 2021; Nie et al., 2020). In vitro studies showed the antihypertensive capacity of the sample as the concentration of peptides in the sample increased and although it is not the one with the highest inhibitory potential this could be due to the variability within the same sample and the representativeness of the sample.

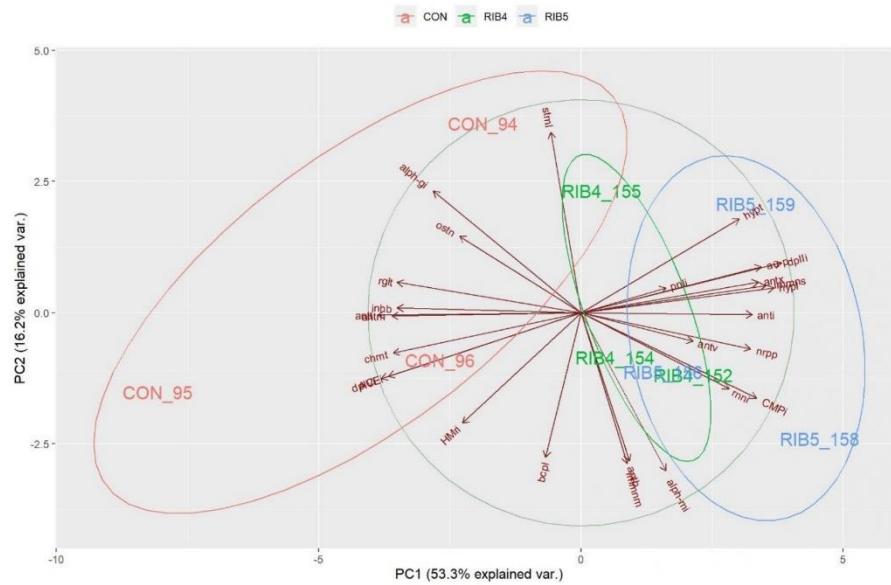
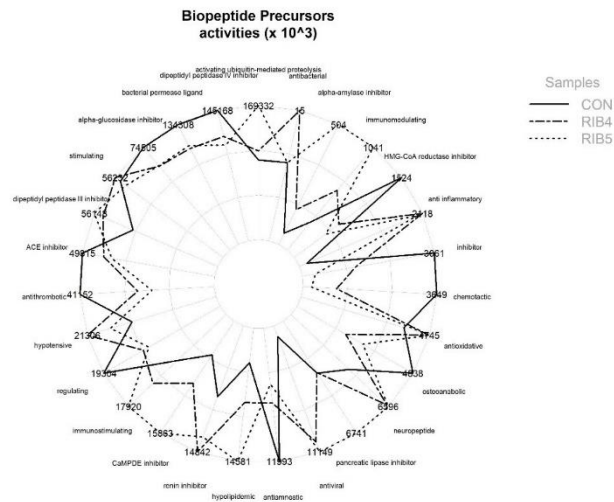
RIB4 sample stands out for its stimulating, antibacterial, renin inhibitor, hypotensive, neuropeptide, antioxidant, antiviral, anti-inflammatory and dipeptidyl peptidase III inhibitor properties. As in the case of the previous sample there are many bioactivities that have not been previously studied, there is no literature describing, identifying or defining these bioactivities in foods such as ham. The strong antioxidant potential observed in this sample coincides with the *in vitro* study carried out previously (Table 2). In addition, its hypotensive and renin inhibitory potency is noteworthy. These bioactivities have been

extensively studied in ham (Heres, Yokoyama et al., 2021; Toldrá et al., 2023). Other studies have also identified peptides with anti-inflammatory activity, reducing the symptoms of inflammatory bowel disease in previous studies in mice and proposing these peptides as a functional drug in patients suffering from inflammatory bowel disease (Xing et al., 2023).

RI B5 hams were noted for their neuropeptide, antioxidative, antiviral, anti-inflammatory, DPPIII inhibitor, Alpha-amylase inhibitor, activating ubiquitin-mediated protein, pancreatic lipase inhibitor, immunomodulating, hypolipidemic, CaMPDE inhibitor and Immunostimulating bioactivities, most of which have not yet been studied.

As mentioned, each group of bioactivities was represented by a colour and in Fig. 5 we can observe four clusters, one for each group of bioactivities, defined by a specific colour designated in the heatmap (Fig. 4), where we can quantify the values of that group of bioactivities for the three samples studied. The bioactivities represented by the red group (angiotensin converting enzyme inhibitors, DPPIV inhibitors, antithrombotics and regulators, among others) show a decreasing trend with increasing temperature. This indicates that higher temperatures (RIB5, 36 °C) inhibit the formation of these peptides, with traditional curing (CON) being the most effective method to preserve these bioactivities. The immunomodulatory and immunostimulatory activities of the green cluster increase with temperature, reaching a maximum at RIB5 (36 °C), suggesting that overcuring at high temperatures favours the generation of these peptides. The bioactivities of the blue cluster (renin inhibitors, hypotensives and antioxidants) reach their maximum at 30 °C (RIB4), but decrease when the temperature rises to 36 °C. The purple cluster, related to antimicrobial activity, also has an optimal performance at RIB4 (30 °C), with a significant reduction at RIB5, indicating that higher temperatures do not favour the activities of this cluster, with over-ripening at 30 °C being an ideal equilibrium for almost all the bioactivities represented.

In the samples analysed we found fragments of bioactive sequences, which is why in Fig. 6 we observe a spider web graph with the normalised quantification of all the peptide precursors found in the three types of hams studied. This distribution makes it possible to differentiate the hams according to their bioactivities. The potential bioactivity of the peptides identified in each of the samples is reflected by using the same scale and amplitude of the axis, thus allowing comparison between samples, with RIB4 samples containing the most sequence fragments with antioxidant activity (4,745,000) followed by RIB5 samples. These



results are consistent with the determination of antioxidant activity against DPPH *in vitro* in this study. As for ACE inhibitory activity, the samples with the highest proportion of sequence fragments with this bioactivity were those from hams processed under traditional conditions (CON), results that do not coincide with the ACE-I activity measured *in vitro* but could be explained given the variability between the samples analysed.

3.3.2. Multivariate analysis

In order to reduce the dimensionality of the data and to detect the most important causes of variability, PCA was applied to the different bioactivities detected in the Iberian hams produced under different processing conditions. For this analysis, the three most similar and representative samples of each type of processed ham obtained from the bioactivity analyses were chosen, using for the analysis the bioactivities shown by the peptides identified as biopeptide precursors. The 9 samples and 27 variables yielded two principal components that explained 69.5% of the variation in the data set. In Fig. 7, the bioactivities are represented as a function of the first and second principal components.

The first principal component (PC1) explained 53.3% of the total variability, and was mainly defined by the bioactivities: DPPIII inhibitor, Hypolipidemic, Immunostimulating, Activating ubiquitin-mediated protein, Antioxidative, CaMPDE inhibitor, Anti-inflammatory, Neuropeptide, Hypotensive, Renin-inhibitor, Antiviral, Pancreatic lipase inhibitor, Alpha-amylase inhibitor, Antibacterial, Immunomodulating. These bioactivities were the most prominent in overripe hams with increasing temperature and were located close to each other on the positive side of the horizontal axis. PC1 could be associated with the effect of as all hams that underwent overripening up to 42% weight loss (RIB4 and RIB5) were located on the positive side of PC1, while the samples of hams processed in the traditional way (CON) were located on the negative side of PC1 (Fig. 7).

Thus, the traditionally processed hams, located on the negative side of PC1, showed less presence of the bioactivities DPPIII inhibitor, Hypolipidemic, Immunostimulating, Activating ubiquitin-mediated protein, Antioxidative, CaMPDE inhibitor, Anti-inflammatory, Neuropeptide, Hypotensive, Renin-inhibitor, Antiviral, Pancreatic lipase inhibitor, Alpha-amylase inhibitor, Antibacterial and Immunomodulating

Table 3
Loading of the two first principal components.

Activity	Loadings PC1	Loadings PC2
Antithrombotic	-0.252	-0.005
DPPIII inhibitor	0.248	0.112
DPPIV inhibitor	-0.247	-0.148
Hypolipidemic	0.239	0.055
ACE inhibitor	-0.239	-0.143
Antimnesic	-0.234	-0.007
Chemotactic	-0.232	-0.090
Immunostimulating	0.229	0.061
Regulating	-0.228	0.068
Inhibitor	-0.228	0.010
AI-MP	0.223	0.104
Antioxidative	0.220	0.069
CaMPDE inhibitor	0.217	-0.192
Anti-inflammatory	0.212	-0.004
Neuropeptide	0.210	-0.081
Hypotensive	0.196	0.210
Renin inhibitor	0.183	-0.171
Alpha-G inhibitor	-0.183	0.273
Osteoanabolic	-0.150	0.171
HMG-CoA inhibitor	-0.147	-0.248
Antiviral	0.139	-0.064
Pancreatic lipase inhibitor	0.105	0.056
Alpha-amylase inhibitor	0.105	-0.355
Antibacterial	0.060	-0.333
Immunomodulating	0.057	-0.339
Bacterial permease ligand	-0.044	-0.325
Stimulating	-0.038	0.405

than the samples on the positive side.

The second principal component (PC2) explained 16.2% of the total variability, and was mainly defined by the bioactivities Stimulating and Alpha-glucosidase inhibitor (positive values), Alpha-amylase inhibitor and Immunomodulating (negative values), whose factor loadings were 0.405, 0.273, -0.355 and -0.339, respectively (Table 3).

PC2 was positively with the bioactivities Stimulating, Alpha-G inhibitor, Hypotensive, Osteoanabolic, DPPIII inhibitor, Activating ubiquitin-mediated, Antioxidative, Regulating, Immunostimulating, Pancreatic lipase inhibitor, Hypolipidemic and Inhibitory negatively with the rest of the bioactivities analysed (Fig. 2), which were little affected by the different maturation conditions. The different bioactivities were related to overripening; this resulted in a higher proteolytic activity leading to a higher concentration of peptides with possible bioactivity (Cilla et al., 2005; Prevotnik et al., 2014; Rosell & Toldra, 1996).

3.4. Bioactivity analysis based on amino acid composition of the peptides identified

Identification of the peptides present was carried out by liquid chromatography coupled to mass spectrometry. The spectra obtained were analysed by comparison with reference proteome databases of the species of origin of the protein. A relative quantification of the peptides of each sample (PSMs) has also been obtained. For the analysis of the results, the quantification values have been normalised to make them comparable between samples.

The identification of the peptides present on the samples was carried out on the three types of hams studied, the control batch matured to 38% of curing under traditional processing conditions (CON), the ham overripened to 42% of weight loss under normal production conditions (RIB4) and finally the ham overripened to 42% of curing in which the processing temperature was increased to 36 °C (RIBV). In bioactivity studies characteristics and structural properties of the sequences are very important aspects to take into account (Zhu et al., 2013).

Most of the peptides that were identified in the ham samples had a molecular weight of <3 kDa (Fig. 3) and sequential chains of between 6 and 20 amino acids. Size, composition or even hydrophobicity are some of the characteristics that can influence peptide bioavailability or stability. Approximately 15 to 20 sequences were selected from those identified in each ham, taking into account that they had a size of <1 kDa and a maximum of 10AA in their peptide chain. Other authors reported that these peptides produced a higher antioxidant activity (Qian et al., 2008) and inhibitory of the ACE enzyme as they are more efficiently accommodated to its active centre (Natesh et al., 2003). It was additionally required that >50% of the AA contained in the peptide chain had to be hydrophobic, as this contributes to the development or increase of certain bioactivities (Shazly et al., 2017; Tang et al., 2010). According to several studies, an increase in processing time can lead to a decrease in the size of peptides and thus to an increase in their antioxidant activity (Li et al., 2022b; Mora et al., 2014; Rosell & Toldra, 1998; Ruiz et al., 1998), as short AA sequences are more likely to be bioactive (Heres et al., 2022; Huang et al., 2017; Tang et al., 2010).

The amino acid sequences of the peptides identified from traditionally processed Iberian hams (CON) are shown in Table 4.

The most important sequences found were DGIIDKED and DEDETAL, which contain 75% of hydrophobic AA, with >50% of AA conferring ACE-I activity in both cases. This bioactivity would be even more present in those sequences containing hydrophobic AA residues in the three C-terminal positions (Tejada et al., 2022). Also, the AGPSIVHR sequence contains the SI dipeptide of the protein -enolase in its chain, which has been described as DPP4 inhibitory peptides (Lan et al., 2015). These dipeptides could be responsible for the high DPPIV inhibitory activity observed in this study for the CON sample (Fig. 5).

The APAPAPKE sequence identified in these hams contains three hydrophobic AAs at the C-terminus that confer ACE-I activity. This

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Table 4
Identification of Peptides Present in CON Liams with a weight <1 kDa.

N	Peptide Seq	Exp. Mass	Protein Source	Protein Accession
1	FDFIHL	791,409	tRNA methyltransferase	I3LIG35
2	AGPSIVHR	836,474	Actin, alpha skeletal muscle	P68137
3	FAGDDAPR	848,390	Actin, alpha skeletal muscle	Q6QAAQ1; P68137; I3LVD5
4	KPTDKHK	853,489	Creatine kinase M type	Q5XLD3
5	APAPAPPKE	877,478	Uncharacterized protein	A0A286ZVM3
6	KRDTFKE	878,410	Uncharacterized protein	A0A287BG25; A0A287AGJ6
7	SDEVEFH	886,342	Troponin T fast skeletal muscle type	A0A287AFK2; A0A287A421; Q75NI2
8	DSGDGVTHN	901,365	Actin, alpha skeletal muscle	P68137
9	ADAEAGGGKK	903,453	Myosin-2	F18S65
10	DGIIDKED	904,426	Myosin regulatory light chain 2	Q5XLD2
11	ADGGGDISVK	918,453	Fast skeletal muscle troponin C	A1XQV5
12	DEDETTAL	935,384	Actin, alpha skeletal muscle	P68137
13	LEETKAK	947,504	Myosin-4	Q9TV62
14	LGTDADKEH	985,459	L-lactate dehydrogenase	A0A286ZXT7

peptide comprises the Pro-Ala-Pro sequence, one of the most repeated sequences among the bioactive peptides described in the literature (Mora, Escudero, Arihara et al., 2015, 2015; Muñoz-Rosique et al., 2023), which would confer good antioxidant activity to the sample (Wattanasiritham et al., 2016). Recently, some dipeptides have also been identified in salt-reduced cured hams that are closely linked to anti-inflammatory activity, which could help prevent cardiovascular risk pathologies (Heres et al., 2022, 2023). These dipeptides are PA, GA, DA and DG and could be derived from sequences found in CON (AGPSIVHR, FAGDDAPR, APAPAPPKE, DSGDGVTHN, ADAEAGGGKK, DGIIDKED, ADGGGDISVK, LGTDADKEH); whether these peptides confer ACE-I activity and in what quantity they are present would need to be studied.

Amino acid sequences of the peptides identified from Iberian hams overripe up to 42% weight loss without temperature increase (RIB4) are shown in Table 5.

The bioactivity of peptides is linked to their amino acid composition and therefore to their sequence. The presence of amino acids such as leucine (L), valine (V), alanine (A), proline (P), and glycine (G) in the sequences enhances antioxidant activity (Luna-Vital et al., 2015). Likewise, the presence of these amino acids together with others such as isoleucine (I), lysine (K) or phenylalanine (F) in the C- or N-terminal position increases the radical scavenging capacity of the bioactive peptides. Of the 20 sequences selected, 16 had some of these amino acids at the C- or N-terminal position. Moreover, in the sequences identified, these hydrophobic residues were mostly found at the carbon-terminal position, which is considered to enhance a greater antioxidant effect than when they are in the N-terminal position (Tejada et al., 2022).

In RIB4 samples, the sequences AKEWGYAD and YETDAIQR were found to have bulky hydrophobic amino acids with electronic affinity to the hydrogen bond at the third position next to the terminal carbon, which is identified with a high antioxidant potential (Li & Li, 2013). Also the presence of other amino acids such as glutamic acid (E) and aspartic

Table 5
Identification of peptides present in RIB4 hams with a weight <1 kDa.

N	Peptide Seq	Exp. Mass	Protein Source	Protein Accession
1	DSGDGVTH	787,322	Actin, alpha skeletal muscle / Actin, cytoplasmic 1 / Actin gamma 1	Q6QAAQ1; P68137; I3LVD5
2	SPEKPPE	825,399	SAM and SH3 domain containing 1	A0A286ZVI6
3	GAGPALEAF	832,420	URB1 ribosome biogenesis 1 homolog	A0A286ZYB9
4	VGDEAQS	833,400	Actin, alpha skeletal muscle / Actin, cytoplasmic 1 / Actin gamma 1	Q6QAAQ1; P68137; I3LVD5
5	DGGGDISVK	847,416	Fast skeletal muscle troponin C	A1XQV5
6	AGDDAPRAV	871,427	Actin, alpha skeletal muscle / Actin, cytoplasmic 1 / Actin gamma 1	Q6QAAQ1; P68137; I3LVD5
7	GTADADKEH	872,374	L-lactate dehydrogenase	A0A286ZXT7
8	MDAIIKKK	875,502	Uncharacterized protein / Troponin alpha 1 chain	A0A286ZRF1; A0A287AN33
9	IDSGDGVTH	900,406	Actin, alpha skeletal muscle	P68137
10	DSGDGVTHN	901,365	Actin, alpha skeletal muscle	P68137
11	DEDETTAL	935,384	Actin, alpha skeletal muscle	P68137
12	NGSGLVKAGI	949,510	Actin, alpha skeletal muscle	P68137
13	ISQIISGGK	959,516	Calcium binding protein 2	I3L711
14	AGFAGDDAPR	976,448	Actin, alpha skeletal muscle / Actin, cytoplasmic 1 / Actin gamma 1	Q6QAAQ1; P68137; I3LVD5
15	DGGGDISVKE	976,458	Fast skeletal muscle troponin C	A1XQV5
16	AKEWGYAD	981,431	Carbonic anhydrase 3	Q5S184
17	SEDKLRDK	990,521	Troponin T fast skeletal muscle	A0A287AFK2; A0A287A421; Q75NI2
18	YETDAIQR	995,479	Myosin 2 / Myosin 4 / Myosin 7	Q9TV62; F18S64; F18S65; A0A287AGU3
19	VLDSDGVTH	999,474	Actin, alpha skeletal muscle	P68137

acid (D) may be responsible for the antioxidant properties (Saiga et al., 2003). These amino acids are present in >90% of the sequences studied in all the samples analysed. The AGDDAPRAV peptide caught our attention since firstly 66.67% of the amino acids it contains are hydrophobic in character, with alanine and valine located at the N- and C-terminus respectively, and also including aspartic acid in its sequence. The identification of these unique and particular sequences in our samples may be responsible for the high antioxidant activity of our sample. These results are in agreement with the DPPH activity measured in vitro.

The amino acid sequences of the peptides identified from Iberian hams overripened up to 42% weight loss with temperature increase up to 36 °C (RIB5) are shown in Table 6.

Recently, in other studies, meat protein hydrolysates have been reported to contain cryptides which are little peptide sequences encrypted within longer peptides that need further processing, such as gastrointestinal digestion to release their bioactivity (Gathercole et al., 2023).

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Table 6
Identification of peptides present in RIB5 hams with a weight <1 kDa.

N	Peptide Seq	Exp. Mass	Protein Source	Protein Accession
1	DSGDGVTH	787,322	Actin, alpha skeletal muscle / Actin, cytoplasmic 1	Q6QAAQ1; P68137
2	MEAAHSK	815,372	Calcium-transporting ATPase	A0A287APK5
3	VGDEAQS	833,400	Actin, alpha skeletal muscle / Actin, cytoplasmic 1	Q6QAAQ1; P68137
4	VGGASLKPE	857,473	Triosephosphate isomerase	A0A286ZRV2
5	VIISAPSAD	872,472	Glyceraldehyde-3-phosphate dehydrogenase	A0A287BG23
6	MDAIKKK	875,502	Uncharacterized protein / Troponin alpha-1 chain	A0A286ZRE1; A0A287AN33
7	DSGDGVTH	901,365	Actin, alpha skeletal muscle	P68137
8	NKDLKLE	901,499	Retinitis pigmentosa GTPase regulator interacting protein 1	F1S8J2
9	DLEEATLQ	918,441	Myosin 4 / Myosin 2 / Myosin heavy chain 8 / Myosin 7	F1S862; Q9TV62; F1S865; A0A287AGU3
10	GMFASGIHE	930,399	Actin, alpha skeletal muscle	P68137
11	FDDTYDR	931,379	Carbonic anhydrase 3	Q5S1S4
12	LEEETKAK	947,504	Myosin-4 / Myosin heavy chain 8	F1S862; Q9TV62
13	ISAPSADAPM	959,450	Glyceraldehyde-3-phosphate dehydrogenase	A0A287BG23
14	LSEAGSIKK	974,552	CAP-Gly domain containing linker protein 1	F1REW5
15	ADQLTEEQ	975,427	Calmodulin 2	F1S145
16	AGFAGDDAPR	976,448	Actin, alpha skeletal muscle / Actin, cytoplasmic 1	Q6QAAQ1; P68137
17	LGTDADKEH	985,459	L-lactate dehydrogenase	A0A286ZXT7
18	NNLGLINT	987,511	Triosephosphate isomerase	A0A286ZRV2
19	LYEQHLGK	987,526	Myosin-4 / Myosin-2	Q9TV62; F1S865
20	RQQLGELSR	989,476	L-lactate dehydrogenase / L-lactate dehydrogenase	F1S805; A0A286ZXT7
21	YETDAIQR	995,479	Myosin-4 / Myosin-2 / Myosin heavy chain 8 / Myosin 7	F1S862; Q9TV62; F1S865; A0A287AGU3
22	YVGDEAQS	996,463	Actin, alpha skeletal muscle / Actin, cytoplasmic 1	Q6QAAQ1; P68137
23	AEVYHHLK	996,526	Beta-enolase	A0A287AZR0

The end products of proteolysis are tri- and di- and dipeptides and free amino acids that accumulate in large quantities in the final stages of processing (Mora et al., 2013). It has been observed that in the later stages of Jinhua ham, due to the higher temperatures used, a large generation of di-peptides, such as VE, PL, AH and AR and tri-peptides, such as LPK, SGL, AAP, SGV, and LHA, occurs (Zhu et al., 2017). These di- and tri-peptides could be derived from sequences identified in RIB5 (MEAAHSK, VGGASLKPE). Among the identified sequences we can also find some of those that could be di-peptides such as DA, PA and VG, although some studies identify a higher proportion of these di-peptides

in traditionally cured hams (Heres et al., 2022). Even so, most of the sequences identified (DSGDGVTH, VGDEAQS, VGGASLKPE, VIISAPSAD, VIISAPSAD, MDAIKKK, DSGDGVTHN, ISAPSADAPM, ADQLTEEQ, AGFAGDDAPR, LGTDADKEH, YETDAIQR, YVGDEAQS) could derive these dipeptides, contributing to their anti-inflammatory and antihypertensive activity.

3.5. Comparative study of the peptides identified in the three types of ham studied

The comparative study of the peptides identified between the different samples has been carried out by representing in intersection graphs the peptides that are common and not common to each of the hydrolysates.

The intersection graphs presented indicate, in the horizontal bar chart on the left-hand side of the graph, the number of peptides identified in each sample. On the other hand, the vertical bar chart at the top of the graph indicates the number of common peptides between the samples, which in turn are represented as points at the intersection of the two diagrams.

Fig. 8 shows the intersection plot of peptides with potential activity (which include bioactive sequences in their structure) of the NPN extracts of the studied hams. As can be seen in the graph, the samples where a greater number of putative bioactive peptides have been identified are those corresponding to the fractions from the extracts of hams overripe up to 42% weight loss at 30 °C (RIB 4) and the extracts of hams overripe up to 42% weight loss at 36 °C, the latter being the one in which the highest number of peptides with potential activity have been identified and, in addition, the one in which the highest number of peptides with potential activity not common to the rest of the samples have been identified (391).

4. Conclusions

The present study has shown that increased maturation time and processing temperature in overripe Iberian hams (RIB4 and RIB5) favours the generation of bioactive peptides. In particular, hams over-ripened at 30 °C (RIB4) showed the highest antioxidant activity and the highest angiotensin-converting enzyme (ACE-I) inhibitory activity, suggesting that this temperature is optimal for maintaining the stability and functionality of bioactive peptides. The results indicate that the temperature of 30 °C optimises the action of proteases responsible for the release of peptides with these activities.

Increasing the temperature to 36 °C (RIB5) increased the total amount of peptides identified, but did not result in a significant improvement in bioactive activities, suggesting that higher temperatures promote more aggressive proteolysis that may degrade important peptides. This finding has direct implications for the industry, as it highlights the importance of carefully controlling the over-ripening temperature to maximise the bioactive yield of hams without compromising the functionality of the peptides generated. Peptide precursors found in the overripe hams showed, in general terms, greater hypolipidemic, anti-inflammatory, immunomodulatory and immunostimulatory activity. In the case of the traditionally produced Iberian hams, the inhibitory activity of DPPIV, regulatory, HMG-CoA reductase inhibitory and osteoanabolic activity were highlighted. These bioactivities have yet to be extensively studied.

In all types of hams studied, the peptide precursors identified were able to give rise to sequences identified as bioactive in the literature.

In terms of practical applications, the results suggest that overripe hams, especially at moderate temperatures, could be considered

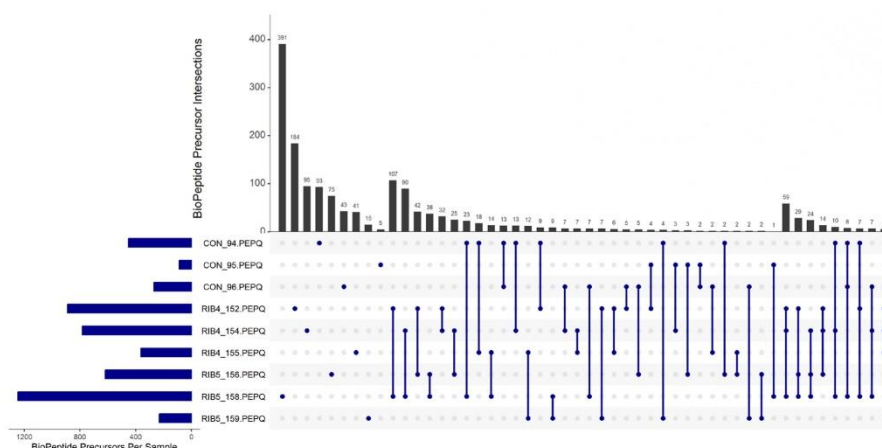


Fig. 8. Graph of intersections of peptides with fragments of sequences with bioactivity identified in the different extracts of the hams studied.

products with a higher functional potential, providing health benefits such as blood pressure reduction and antioxidant effect, which could positively impact the development of healthier meat products.

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Ethical responsibilities

Protection of humans and animals. The authors declare that no experiments on humans or animals have been carried out for this research.

- Confidentiality of data. The authors declare that no patient data appear in this article. Furthermore, the authors have acknowledged and followed the recommendations according to the SAGER guidelines depending on the type and nature of the study.
- Right to privacy and informed consent. The authors declare that no patient data appear in this article.
- Use of artificial intelligence to generate texts. The authors declare that they have not used any type of generative artificial intelligence in the writing of this manuscript or for the creation of figures, graphs, tables or their corresponding captions or legends

CRediT authorship contribution statement

Noelia Hernández-Correas: Methodology, Validation, Formal analysis, Investigation, Writing – review & editing, Visualization. **Adela Abellán:** Investigation, Resources, Writing – original draft. **Beatriz Muñoz-Rosique:** Methodology, Formal analysis, Investigation, Writing – review & editing, Visualization. **Cindy Bande-De León:** Investigation, Visualization, Data curation, Software. **Rafael Gómez:** Resources, Investigation. **Luis Tejada:** 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.

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ARTICLE 4:

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1 **Title:** Curing strategies for Spanish dry-cured ham: Food Quality and Preference

2

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1 **ABSTRACT**

2 This study evaluated, for the first time, the effect of different nitrite salt formulations
3 and curing times on physicochemical, biochemical, sensory, and instrumental
4 parameters in Spanish cured ham. For this purpose, a total of 96 boneless hams were
5 pro-cessed with six different salt treatments (variations in nitrate/nitrite content, particle
6 size, and sodium reduction) applied during salting, and two curing times (standard
7 ripening and overripening). Parameters such as composition, water activity, proteolysis,
8 free amino acids, color, texture, and sensory acceptability were analysed. The results
9 showed that the concentration of nitrifying salts and the prolongation of maturation
10 significantly affected proteolysis and amino acid release, directly influencing col-or,
11 texture, and perceived sensory profile. Partial sodium reduction and adjustment of salt
12 composition maintained the overall quality of the product without compromising the
13 safety or acceptability of the cured ham. These findings are relevant for developing
14 curing strategies with less impact on consumer health, while ensuring the traditional
15 properties of cured ham.

16

17 **Keywords:** nitrifying salts; dry-cured ham; curing process; physicochemical
18 quality; proteolysis; sensory analysis.

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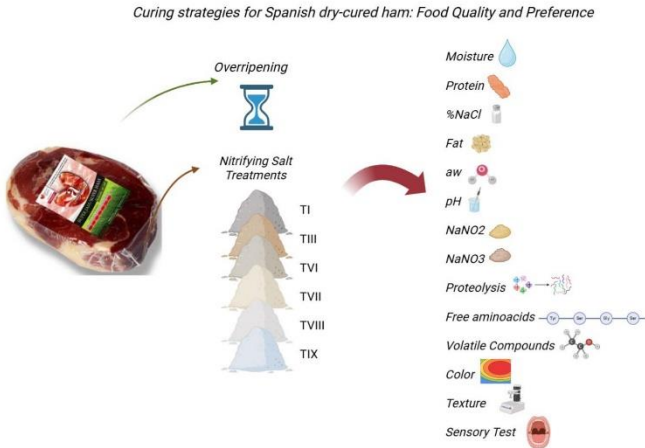
1 INTRODUCTION

2 Nowadays, high salt intake, mainly in the form of sodium chloride (NaCl), is a global
3 public health problem. The average intake recommended by the World Health
4 Organization (WHO) is being exceeded in most countries, which advises a maximum of
5 5g of salt per day [1], equivalent to about 2g of sodium [2]. Many specialists have
6 linked a daily diet high in sodium to high blood pressure and, as a result, to a high risk
7 of cardiovascular disease, which is one of the leading causes of death worldwide [2,3].
8 In the case of nitrites, the WHO has not established a maximum consumption value for
9 nitrites, but the European Food Safety Authority (EFSA) has set the Acceptable Daily
10 Intake (ADI) for nitrites at 0.07mg/kg of body weight per day.

11 Most processed foods are high in sodium, and cured meat products are one of the main
12 sources of sodium, as NaCl is used for its many technological and organoleptic
13 functions: microbiological preservation thanks to the reduction in water activity caused
14 by the salting characteristic of cured products, improvement in texture thanks to the
15 solubilization of myofibrillar proteins, and the salty taste typical of these foods [4–7]. In
16 particular, traditional Spanish cured ham contains high concentrations of salt and, in
17 addition, nitrifying salts (nitrates and nitrites) are added during the salting process,
18 which are essential to ensure safety against *Clostridium botulinum* [8,9], stabilize the
19 characteristic pink color, and help develop the typical aroma [10,11]

20 The use of nitrite salts (nitrates and nitrites) as curing agents in meat products has a long
21 history [12]. Their role as microorganism inhibitors and color developers has been
22 extensively studied. However, excessive consumption of nitrites poses risks, such as the
23 formation of nitrosamines, compounds with carcinogenic potential [13]. Because of this,
24 the European Union strictly regulates the maximum permitted levels, Directive

1 2006/52/EC [14], and current research focuses on reducing the amount added while
2 maintaining the safety and optimal sensory quality of the product [11,15].
3 With this objective in mind, various strategies have been implemented to reduce sodium
4 and nitrite salts in cured meat products, such as the partial replacement of NaCl with
5 KCl, CaCl₂, or MgCl₂ [16–18], the addition of lactate or diacetate as additional
6 preservatives [7], and the use of emerging technologies such as high pressure or
7 ultrasound to ensure microbiological stability in low-salt products [19–22].
8 In a product as beloved and appreciated as Spanish cured ham, there are numerous
9 studies on NaCl reduction [23], substitution with other salts or changes during
10 processing linked to salt reduction [24]. Although sodium reduction strategies have
11 been studied extensively, the specific role of nitrite/nitrate modulation in dry-cured ham
12 remains poorly understood. Until now, no studies have been conducted on how changes
13 in the concentration of nitrite salts would influence the physicochemical, biochemical,
14 instrumental, and sensory parameters of the final product. That is why the objective of
15 this study was to comprehensively evaluate the effect of different formulations of
16 nitrifying salts during salting and different maturation times on boneless cured ham,
17 seeing how these changes affected different physicochemical, biochemical,
18 instrumental, and sensory parameters (Figure 1). The information obtained in this study
19 aims to serve as a basis for the development of healthier and safer cured hams, without
20 compromising consumer acceptance. This will be the first time that the effect of
21 nitrite/nitrate concentration in combination with over-maturation in boneless cured
22 white hams will be studied.



1
2 *Figure 1. Summary of the article's objective. Created with BioRender.com (accessed on*
3 *8 September 2025).*

1 MATERIAL AND METHODS

2 2.1 Preparation and Collection of Dry-Cured Ham Samples

3 A total of 96 deboned cured hams were required for the development of this study. Six
4 batches of 16 fresh hams were processed under different conditions. Starting from the
5 same genetic, physical and feeding conditions at the time of slaughter.

6 Fresh hams were first deboned and then salted with 6 different nitrifying salt treatments
7 (I, III, VI, VII, VIII and IX) at the salting stage. These are indicated in the table below:

8
9 **Table 1.** Nitrifying salt treatments used in the different batches produced.

Treatment	Nitrifying salts – Amount and Composition	Burying Salt Composition
I	Standard concentration + 60%: • 1.12g/kg: 24.4% sodium nitrite, 32.5% sodium isoascorbate, 36.5% potassium nitrate, 6.5% sugar. • 6.88g/kg reduced sodium salt.	50% normal coarse salt – 50% reduced fine salt
III	Double concentration (10 g instead of 5 g): • 1.4g/kg: 24.4% sodium nitrite, 32.5% sodium isoascorbate, 36.5% potassium nitrate, 6.5% sugar. • 8.6g/kg reduced sodium salt.	50% normal coarse salt – 50% reduced fine salt
VI	Standard concentration without nitrates: • 0.7g/kg: 75% sodium nitrite, 25% sodium isoascorbate. • 4.3g/kg reduced sodium salt	50% normal coarse salt – 50% reduced fine salt
VII	Standard concentration (higher nitrite proportion): • 0.7g/kg: 64.3% sodium nitrite, 21.4% sodium isoascorbate, 8.6% potassium nitrate, 5.7% dextrose. • 4.3g/kg reduced sodium salt	Reduced sodium salt, intermediate grain size
VIII	Double concentration: • 1.4g/kg: 24.4% sodium nitrite, 32.5% sodium isoascorbate, 36.5% potassium nitrate, 6.5% sugar. • 8.6g/kg reduced sodium salt.	Standard Coarse Salt
IX	Standard concentration (higher nitrite proportion): • 0.7g/kg: 64.3% sodium nitrite, 21.4% sodium isoascorbate, 8.6% potassium nitrate, 5.7% dextrose. • 4.3g/kg reduced sodium salt	Standard Coarse Salt

10
11 Each piece was salted for 0.8 days per kg of weight, approximately 7-8 days, in a cold
12 room at a temperature of 3°C.

13 Normally, the dosage applied for standard nitrifying treatment is 5 g per kilogram of
14 fresh weight of ham and the composition of the standard nitrifying salts is 64.3% %
15 sodium nitrite, 21.4% % sodium isoascorbate, 8.6 % potassium nitrate and 5.7 %

1 dextrose. The dosage applied for standard salting treatment is 700 mg per kilogram of
2 fresh weight of ham. The remainder up to 5 g/kg is standard salt (NaCl) without any
3 modification.

4 The nitrifying salts are applied by rubbing just before the start of the salting pro-cess.
5 After salting, hams were washed with water, followed by a traditional curing process.
6 The resting or post-salting phase started at 3°C, rising progressively to 6°C. This
7 production phase ended when hams reached 18% loss, lasting approximately four to
8 five months. This phase lasted for a minimum of 120 days, thus extending the post-
9 salting phase to reduce the aw as much as possible and to try to counteract the lack of
10 salt [25]. Subsequently, the temperature was raised to 30°C. The process ended when
11 the pieces reached a weight loss of 38%, after approximately 14/15 months of curing.

12 After this last treatment, half of the hams processed under each treatment finished their
13 maturation process (8 pieces per treatment – Processing Stage 4), but the other half of
14 the hams were brought to overripening, up to 42% weight loss (Processing Stage 5),
15 needing to reach overripening for approximately 3 more months.

16 The percentage weight loss was determined by weighing each sample in triplicate
17 during each of the processing stages. The results were expressed as percentage weight
18 loss, considering the fresh weight of each piece.

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

23 This study has determined the effect of different types of nitrifying salts and the
24 maturation time used in deboning treatment on the quality of the final product in
25 relation to its physicochemical and biochemical profile and sensory acceptance.

2.2 Physicochemical, biochemical analyses. Determination of volatile compounds.

The analysis of the physicochemical parameters of the samples was carried out in triplicate for each type of dry-cured ham. Moisture content analysis was determined following the gravimetric procedure described in ISO1442 [26]. The crude protein value was obtained by multiplying the TN value by 6.25. For salt content analysis, the Volhard methodology of ISO1841-1 [27] was used and slightly modified. The Folch method [28] was used to measure the intramuscular fat content of dry-cured hams. The evaluation of water activity was carried out by gravimetry using the AOAC 920.153 method [29]. Nitrate and nitrite contents were determined at SIPA using a validated method based on the Griess reaction, following nitrate reduction to nitrite, in accordance with ISO 14673-1/AOAC for meat products. Results were expressed as mg/kg of sample.

The determination of total nitrogen (TN) and non-protein nitrogen (NPN) was carried out by the Kjeldahl method [30]. For the preparation of NPN extract, the method followed by Muñoz-Rosique et al. [31] was used. The proteolysis index was determined as the percentage of the ratio of non-protein nitrogen to total nitrogen [24]. Free amino acid (FAA) content was determined following the method described by Abellán et al. [32]. Volatile compound analyses were performed at SIPA (University of Extremadura, Cáceres) using gas chromatography–mass spectrometry (GC-MS).

2.3 Determination of Instrumental Color and Texture Profile Analysis (TPA)

Instrumental color assessment utilized colorimetry with a colorimeter (HunterLab, Colorflex) following the CIELab system. The outcomes were represented by the L*, a*, and b* coordinates, denoting luminosity, red–green index, and yellow index,

1 respectively. Additionally, the saturation parameter (chroma) and hue angle (h^*) values
2 were obtained. The results were averaged from three measurements [23].
3 For instrumental texture analysis, a QTS-25 texturometer (Brookfield CNS Farnell,
4 Borehamwood, Hertfordshire, England) equipped with a 25 kg load cell and a 10 mm-
5 diameter probe was employed. Texture Pro v. 2.1 software facilitated data interpretation
6 [33].
7 The biceps femoris muscle was dissected into $10 \times 10 \times 10$ mm parallelepipeds, with
8 three parallelepipeds per sample. Testing was conducted at a controlled room
9 temperature of 20 °C.
10 The procedure involved subjecting the sample to two consecutive cycles at a constant
11 speed of 30 mm/s, with 50% compression perpendicular to the muscle fibers.
12 Texture profile analysis (TPA) was conducted to assess the hardness, deformation
13 concerning hardness, adhesiveness, cohesiveness, recoverable deformation, springi-
14 ness, gumminess, and chewiness.

15

16 **2.4 Sensory Analysis**

17 For the sensory analysis of the dry-cured ham, a consumer test was performed with an
18 untrained panel. The analysis was carried out following the method described by
19 Muñoz-Rosique et al. [23]. For this analysis, a total of 200 anonymous participants
20 attended on a voluntary basis and without having received any type of prior information
21 or training. The study was conducted in a single session where the participants
22 evaluated the hams cured with the different salt treatments. The age range chosen was
23 18 to 55 years.
24 For the study, participants evaluated the samples using a questionnaire that included
25 the hedonic assessment of appearance, color, odor, texture, salty taste, overall taste, and

1 overall acceptability. Each attribute was scored by assigning a numerical value using a
2 hedonic scale between 1 (I dislike it very much) to 5 (I like it very much).
3 The samples used for the consumer sensory test were complete slices of the cross-
4 section of the piece, following the method established in the UNE-ISO standard
5 6658:2019 [33] and UNE-ISO 4121:2006 [34], which were coded with a random three-
6 digit number.
7 The results were obtained by calculating the average score given to each product
8 attribute evaluated by the consumer. To determine the preference between the three
9 samples, the percentage values of choice of the consumer panel were obtained in each
10 type of dry-cured ham studied.

11

12 **2.5 Statistical analysis**

13 All analyses of our samples were performed in triplicate. Results were expressed as
14 means values $\pm$ standard error of the mean (SEM).

15 The effect of the different salt treatments and curing stages (standard maturation and
16 overripening) was evaluated using a two-way analysis of variance (ANOVA),
17 considering “nitrifying salts treatment” and “processing stage” as fixed factors. When
18 significant effects ($p \leq 0.05$) were observed, Fisher’s Least Significant Difference
19 (LSD) test was applied to identify differences among means.

20 For the sensory evaluation data, a one-way ANOVA was performed to assess
21 differences among treatments, followed by LSD post-hoc comparisons when
22 appropriate. Statistical analyses were performed using the SPSS software package
23 (version 21.0, IBM Corporation, Armonk, NY, USA).

24

25

26

1 RESULTS AND DISCUSSION

2 3.1. Effect of Change in Processing Conditions on the Physicochemical and 3 Biochemical Parameters of Spanish Dry-Cured Ham

4 The results of the physical-chemical analyses are presented in Table 2. The different salt
5 treatments significantly affected moisture content, NaCl percentage, water activity, and
6 pH. The processing stage (overripening) significantly affected moisture content, NaCl
7 percentage, fat content, water activity, and pH.

8 **Table 2.** Effect of different salt treatments and the curing process on the physicochemical parameters
9 obtained from the samples of Spanish Dry-Cured Hams. Results are expressed as means values $\pm$ SEM*.

Salt Treatment	Processing Stage	% Moisture	% Protein	% NaCl	% Fat	Water Activity	pH
I	4	46.69 $\pm$ 1.94 ^c	31.01 $\pm$ 0.11 ^{abc}	2.7 $\pm$ 0.29 ^c	26.77 $\pm$ 1.18 ^{ab}	0.92 $\pm$ 0.01 ^{df}	5.82 $\pm$ 0.02 ^{def}
I	5	34.37 $\pm$ 0.38 ^a	35.4 $\pm$ 0.87 ^a	2.4 $\pm$ 0.1 ^{cd}	32.94 $\pm$ 1.45 ^a	0.84 $\pm$ 0.01 ^c	5.88 $\pm$ 0.02 ^{cdef}
III	4	47.52 $\pm$ 1 ^e	28.53 $\pm$ 0.48 ^c	2.72 $\pm$ 0.12 ^c	27.93 $\pm$ 1.52 ^{ab}	0.92 $\pm$ 0 ^f	5.93 $\pm$ 0.05 ^{cde}
III	5	37.25 $\pm$ 1.52 ^{b^c}	31.14 $\pm$ 0.92 ^{abc}	2.92 $\pm$ 0.19 ^c	29.21 $\pm$ 2.86 ^{ab}	0.88 $\pm$ 0.01 ^e	5.8 $\pm$ 0.04 ^{ef}
VI	4	46.48 $\pm$ 1.51 ^c	32.80 $\pm$ 0.73 ^{abc}	2.43 $\pm$ 0.09 ^{cd}	26.37 $\pm$ 2.57 ^{ab}	0.91 $\pm$ 0 ^{df}	5.81 $\pm$ 0.02 ^{def}
VI	5	36.05 $\pm$ 1.2 ^b	33.29 $\pm$ 2.62 ^{abc}	2.57 $\pm$ 0.03 ^{cd}	32.74 $\pm$ 2.07 ^a	0.86 $\pm$ 0.01 ^{bc}	5.72 $\pm$ 0.08 ^f
VII	4	50.13 $\pm$ 0.78 ^f	28.08 $\pm$ 2.26 ^{bc}	2.6 $\pm$ 0.1 ^{cd}	22.34 $\pm$ 3.66 ^b	0.92 $\pm$ 0 ^f	6.03 $\pm$ 0.03 ^{bc}
VII	5	40.65 $\pm$ 2.18 ^{cd}	28.91 $\pm$ 5.57 ^{bc}	2.57 $\pm$ 0.09 ^{cd}	24.34 $\pm$ 5.01 ^{ab}	0.88 $\pm$ 0 ^e	5.79 $\pm$ 0.02 ^{ef}
VIII	4	50.1 $\pm$ 0.88 ^f	29.24 $\pm$ 1.4 ^{bc}	4.17 $\pm$ 0.33 ^a	23.04 $\pm$ 2.31 ^b	0.90 $\pm$ 0 ^{ad}	6.57 $\pm$ 0.17 ^a
VIII	5	42.93 $\pm$ 0.82 ^d	33.82 $\pm$ 1.29 ^{ab}	4.03 $\pm$ 0.28 ^a	24.7 $\pm$ 2.32 ^{ab}	0.89 $\pm$ 0 ^{ae}	5.84 $\pm$ 0.03 ^{def}
IX	4	47.62 $\pm$ 1.47 ^e	27.73 $\pm$ 1.1 ^c	3.5 $\pm$ 0.1 ^b	25.17 $\pm$ 2.56 ^{ab}	0.91 $\pm$ 0 ^{df}	6.18 $\pm$ 0.05 ^b
IX	5	39.27 $\pm$ 1.48 ^{cd}	32.67 $\pm$ 3.07 ^{abc}	3.5 $\pm$ 0.1 ^b	30.91 $\pm$ 4.02 ^{ab}	0.88 $\pm$ 0.01 ^{be}	5.99 $\pm$ 0.04 ^{cd}
<i>p-value</i>							
Salt Treatment		0.006	0.149	0.000	0.113	0.025	0.000
Processing Stage		0.000	0.014	0.452	0.035	0.000	0.000

10 *Two-way ANOVA. a, b, c, d Values within a column with different superscripts differ significantly at $p \leq 0.05$ (Fisher*
11 *LSD Test).*

12 SEM: standard error of mean.

13

14 Moisture varied between 34.37% (treatment I, stage 5) and 50.13% (treatment VII, stage
15 4). This decrease in moisture content with advanced maturation reflects the loss of water
16 through dehydration during post-salting and maturation. A significant re-duction in
17 moisture was observed in all treatments when moving from stage 4 to 5, reflecting water

1 loss during overripening. NaCl ranged from 2.0% (I, 5) to 4.17% (IX, 4), showing that
2 treatment IX achieved the highest salt content. Fat content varied from 22.34% (VII, 4)
3 to 32.94% (I, 5). Water activity (a_w) ranged from 0.84 (I, 5) to 0.92 (several treatments
4 in stage 4), clearly decreasing during overripening. The final pH of the hams showed
5 differences linked to the salt treatment; in all cases, the pH tended to rise slightly with
6 the increase in nitrifying salts. The range observed was from 5.72 (VI, 5) to 6.57 (IX,
7 4). These results show that the reduction in moisture and a_w over the curing period was
8 a determining factor in microbiological stability, even in treatments with lower salt
9 concentrations, which is with what has been described by other authors [3,18], the
10 reduction in water content and, consequently, the decrease in water activity during the
11 curing process guarantees the inhibition of microbial growth even when less NaCl is
12 used [18]. Several studies describe that all pathogens stop growing below 0.87 [35],
13 although most bacteria don't grow below a water activity of less than 0.91, I feel that
14 the minimum growth limit for *Clostridium Botulinum* is approximately 0.93 and, under
15 certain conditions, even 0.96, according to the technical guidance of the Food and Drug
16 Administration (FDA). [36]. Likewise, the higher pH in treatment IX with a higher
17 concentration of salt and nitrifying agents may be related to the greater water retention
18 capacity and lower enzymatic activity under these conditions [16]. In contrast,
19 treatments with reduced nitrifying salts content and prolonged maturation showed lower
20 pH values, probably associated with greater proteolytic activity and the release of
21 compounds during curing.

22 Overall, these results indicate that a moderate decrease in NaCl together with
23 adjustments in the formulation of nitrite salts affected water retention and the
24 composition of the hams, but the overripening process up to 42% weight loss stabilized
25 many of these values, ensuring low and safe water activity in all treatments. This is

consistent with previous studies that showed that reduced-salt hams need a little more time to reach lower levels of this parameter but can achieve this without compromising microbial stability [18].

Table 3 shows the residual concentrations of nitrite (NaNO_2) and nitrate (NaNO_3) (in mg/kg) in the hams after the two maturation stages (4 and 5) for each treatment. These data allow us to evaluate the extent to which the different curing treatments contributed nitrifiers to the final product and how they evolved during the process. Nitrates added during the salting process can be partially reduced to nitrites by the action of nitrate-reducing bacteria, mainly staphylococci. This conversion, which depends on the initial concentration and maturation time, allows a residual level of nitrite to be maintained in the early stages and contributes to the development of color and oxidative stability of the product, as has been described in other studies [37,38].

Table 3. Effect of different salt treatments and the curing process on volatile compounds content obtained from the samples of boneless Spanish Dry-Cured Hams. Results are expressed in mg/Kg of dry matter as means values $\pm$ SEM*.

Salt Treatment	Processing Stage	NaNO ₂ (mg/kg)	NaNO ₃ (mg/kg)
I	4	<2.5	213 $\pm$ 13 ^{ab}
I	5	<2.5	227 $\pm$ 14 ^a
III	4	<2.5	114.55 $\pm$ 22.45 ^c
III	5	<2.5	118 $\pm$ 10 ^c
VI	4	<2.5	111.35 $\pm$ 11.65 ^e
VI	5	<2.5	103.15 $\pm$ 15.85 ^e
VII	4	<2.5	79.8 $\pm$ 0.2 ^c
VII	5	<2.5	114 $\pm$ 5 ^c
VIII	4	<2.5	125 $\pm$ 5 ^{dc}
VIII	5	<2.5	164.5 $\pm$ 35.5 ^{cd}
IX	4	<2.5	105 $\pm$ 1 ^e
IX	5	<2.5	143 $\pm$ 1 ^d
<i>p-value</i>			
Salt Treatment		-	0.000
Processing Stage		-	0.009

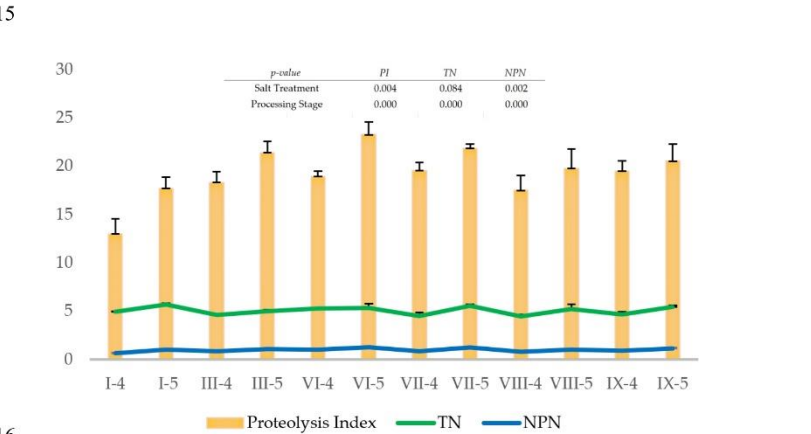
Two-way ANOVA. a, c, d Values within a row with different superscripts differ significantly at $p \leq 0.05$ (Fisher LSD Test).

SEM: standard error of mean.

1 Residual nitrite was virtually undetectable in all cases. The table indicates “<2.5” mg/kg
2 for NaNO₂, suggesting that the level was below the minimum quantifiable limit (<2.5
3 mg/kg) in both phases 4 and 5 for all treatments. In all cases, the nitrite added during
4 salting was consumed or disappeared almost completely before the final stage. This may
5 be because nitrite is highly reactive, participates in the formation of the pink color
6 (nitrosomyoglobin), reacts with oxidizing compounds and microorganisms, and can be
7 oxidized to nitrate or volatilized [39]. The results obtained confirm that, regardless of
8 the initial dose, nitrite does not persist in the final product. Previous studies have
9 observed that at the end of the curing process, residual nitrite is usually in the order of 2
10 ppm inside the ham, even with different salt formulations, thus amply complying with
11 regulatory food safety limits [40].

12 As for NaNO₃, the situation is different. Nitrate is much more stable than nitrite and can
13 act as a reservoir for nitrite during maturation. Table 2 shows that nitrate did remain in
14 the hams, with significant differences depending on the curing treatment and processing
15 time. In general, treatments formulated with a higher initial nitrate proportion yielded
16 higher residual concentrations. Treatment I had 213 mg/kg of NaNO₃ in phase 4 and
17 227 mg/kg in phase 5, remaining one of the highest. Similarly, treatment IX retained
18 105 mg/kg in phase 4, rising to 143 mg/kg in phase 5. In contrast, in treatment VI
19 around 100–111 mg/kg of nitrate was still detected. This may be because, although no
20 nitrate was added to the salt mixture, part of the nitrite initially dosed was transformed
21 into nitrate during the process [12,40,41]. Although the main process of interest is the
22 reduction of nitrate to nitrite, under certain oxidation conditions the reverse reaction can
23 also occur, with the conversion of residual nitrite to nitrate. This phenomenon has been
24 described in raw-cured products during the final stages of maturation and may explain
25 occasional increases in nitrate during prolonged maturation phases [38]. Treatment VII

1 (standard sodium-reduced nitrifier, with special grain size) showed the lowest nitrate
2 values: 80 mg/kg in phase 4 and 114 mg/kg in phase 5, possibly due to slower
3 penetration or different conversion due to the grain size of the salt. Treatment III
4 (double dose of reduced nitrifying agent, >60% nitrate) had moderate residual nitrate
5 levels (114–118 mg/kg), lower than those of treatment I de spite its higher dose,
6 suggesting that a larger portion of nitrate may have been con-verted to nitrite (and then
7 consumed) or dispersed in these hams.
8 In all cases, prolonged maturation (phase 5) tended to maintain or even slightly increase
9 the nitrate measured compared to phase 4. This small increase could be attributed to the
10 continuous conversion of nitrite to nitrate over time and/or to the concentration of
11 nitrate as the ham loses water.
12 The results of the nitrogenous fractions parameters evaluated are presented in Figure 2:
13 total nitrogen (TN), protein, non-protein nitrogen (NPN), and proteolysis index
14 (NPN/TN).



16
17 Figure 2. Effect of different salt treatments and the curing process on the biochemical parameters ob-tained from the
18 samples of Spanish Dry-Cured Hams.

1 The different salt treatments significantly affected non-protein nitrogen and the
2 proteolysis index (Sup. Table 1). The processing phase (overripening) significantly
3 affected all the biochemical parameters evaluated. Total nitrogen (TN) ranged from 4.44
4 (VIII, 4) to 5.66 (I, 5). Protein ranged from 27.73% (VIII, 4) to 35.40% (I, 5). NPN
5 varied from 0.64 (I, 4) to 1.23 (VI, 5). The proteolysis index reached a minimum of
6 12.99% (I, 4) and a maximum of 23.25% (VI, 5). The increases in stage 5 indicate
7 greater proteolysis, especially in VI and VII, suggesting that a reduction in nitrifying
8 salts enhances protease activity [42]. In fact, a marked increase in NNP content and
9 proteolysis index was observed when moving from phase IV to phase V in all
10 treatments, but this was particularly noticeable in those with lower nitrifying agent
11 content. These data are consistent with the idea that reducing nitrite/nitrate levels
12 together with reducing NaCl could decrease the inhibition of proteolytic enzymes,
13 intensifying the endogenous proteolysis of ham. Previous studies have reported similar
14 increases in proteolysis when the NaCl concentration in cured products is reduced,
15 attributing this to the increased activity of cathepsins and peptidases [23,43,44]. These
16 data are consistent with those described by Gratacós-Cubarsi et al, [45]. Additional
17 results for this figure can be found in Sup. Table 1.

18

19 The results of the free amino acids concentration ($\mu\text{mol/g}$ of dry matter) present in the
20 different hams are shown in Table 4, The total amino acid content was significantly
21 affected by both the different nitrifying salt treatments used and the processing stage. In
22 particular, the following amino acids were significantly affected by the different salt
23 treatments used: Alanine (Ala), Glutamine (Gln), Glycine (Gly), Histidine (Hys),
24 Isoleucine (Ile), Lysine (Lys), Methionine (Met), Proline (Pro), Serine (Ser), and Valine
25 (Val). On the other hand, overripening up to 42% weight loss significantly affected the

1 following amino acids: Alanine (Ala), Arginine (Arg), Aspartic acid (Asp), Proline
2 (Pro), and Serine (Ser).

3 A clear increase was observed in stage 5 compared to 4, indicating greater FAA release
4 during overripening. This is consistent with the intensification of proteolysis described
5 above: as more proteins are broken down in phase 5, more amino acids are released into
6 the muscle tissue. In addition, salt treatments with lower nitrite/nitrate levels tended to
7 show higher FAA contents. In fact, the highest total FAA levels were detected in
8 treatments VI and VII, precisely those with reduced doses of nitrifying salts. In contrast,
9 treatments with the “standard” nitrifying formulation appeared to generate somewhat
10 lower amounts of free amino acids, possibly due to slightly less pronounced proteolysis.
11 These results reinforce the idea that reducing nitrifying salts in-creases proteolysis and
12 the accumulation of FAA, direct precursors of flavor [11,23,24,46].

13 If we analyse individual amino acids, we can see that several of them were significantly
14 affected by salt treatments or maturation. In general, formulations with lower
15 nitrite/nitrate levels tended to accumulate more of certain sweet amino acids and bitter
16 amino acids, which could affect the flavor profile of the ham [47]. The treatments with
17 lower nitrifying agent content, particularly VI and VII (Table 2), had higher
18 concentrations of sweet amino acids such as alanine and serine (21–27 $\mu\text{mol/g}$ and 11–
19 13 $\mu\text{mol/g}$, respectively) and bitter amino acids such as valine and isoleucine (15–17
20 $\mu\text{mol/g}$ and 6–7 $\mu\text{mol/g}$). These profiles suggest a more intense and complex flavor,
21 where sweet and bitter notes could reinforce the overall perception, although their
22 sensory evaluation was intermediate (overall taste 3.21–3.33; overall acceptance 3.3–
23 3.5).

24

25

1 **Table 4.** Effect of different salt treatments and the curing process on free amino acid content (FAA) obtained from the samples of Spanish Dry-Cured Hams. Results are
 2 expressed in $\mu\text{mol/g}$ of dry matter as means values $\pm$ SEM^a.

Salt Treatment	I		III		VI		VII		VIII		IX		<i>p-value</i>	
Processing Stage	4	5	4	5	4	5	4	5	4	5	4	5	Salt Treatment	Processing Stage
Ala	13.9 $\pm$ 0.35 ^a	13.3 $\pm$ 0.2 ^a	16.95 $\pm$ 2.55 ^{ac}	15.6 $\pm$ 2.95 ^{ac}	27.95 $\pm$ 3.3 ^{ab}	21.3 $\pm$ 3.5 ^{abc}	30.51 $\pm$ 0.9 ^a	24.8 $\pm$ 0.8 ^{abcd}	20.41 $\pm$ 1.4 ^{ac}	18.8 $\pm$ 0.6 ^{ac}	26.1 $\pm$ 4.0 ^{bc}	20.3 $\pm$ 1.5 ^{abcd}	0.000	0.018
Arg	5.51 $\pm$ 0 ^b	10.9 $\pm$ 2.6 ^{ab}	11.6 $\pm$ 0 ^a	9.9 $\pm$ 2.4 ^{ab}	4.4 $\pm$ 2.36 ^a	9.23 $\pm$ 4.7 ^{ab}	1.321 ^{0^b}	10.71 $\pm$ 0.1 ^b	8.1 $\pm$ 6.19 ^{ab}	16.25 $\pm$ 1.95 ^a	nd	5.8 $\pm$ 1.16 ^b	0.471	0.025
Asn	4.4 $\pm$ 0.13 ^a	4.5 $\pm$ 0.1 ^{ab}	5.64 $\pm$ 0.01 ^c	5.2 $\pm$ 0.09 ^{abc}	5.77 $\pm$ 0.34 ^c	5.38 $\pm$ 0.78 ^{bc}	5.23 $\pm$ 0.55 ^{bc}	5.6 $\pm$ 0.57 ^{bc}	5.59 $\pm$ 0.04 ^{bc}	5.52 $\pm$ 0.14 ^{bc}	5.56 $\pm$ 0.19 ^{bc}	5.49 $\pm$ 0.05 ^{bc}	0.056	0.689
Asp	21.4 $\pm$ 1.8 ^{abcd}	29.5 $\pm$ 0.1 ^a	24.8 $\pm$ 1.45 ^{abcd}	28.9 $\pm$ 1.85 ^{ab}	19.95 $\pm$ 6.65 ^{abcd}	28.3 $\pm$ 1.8 ^{abc}	19.1 $\pm$ 1.5 ^{cd}	19.7 $\pm$ 3.5 ^{abcd}	16.2 $\pm$ 3.9 ^d	22.65 $\pm$ 2.9 ^{abcd}	21.1 $\pm$ 0.1 ^{abcd}	18.45 $\pm$ 3.05 ^d	0.102	0.037
Cys	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	-	-
Glu	20.5 $\pm$ 1.3 ^a	18.9 $\pm$ 0.15 ^a	27.6 $\pm$ 2 ^a	20.5 $\pm$ 1.1 ^a	31.65 $\pm$ 0.65 ^a	26.15 $\pm$ 3.75 ^a	25.34 $\pm$ 3.99 ^a	33.95 $\pm$ 1.65 ^a	28.32 $\pm$ 0 ^a	30.2 $\pm$ 1.7 ^a	24.15 $\pm$ 12.50 ^a	27.85 $\pm$ 3.15 ^a	0.578	0.939
Gln	46.45 $\pm$ 4.55 ^a	54.6 $\pm$ 3.4 ^{ab}	69.1 $\pm$ 3.85 ^{abcd}	62.3 $\pm$ 0.80 ^{abcd}	75.25 $\pm$ 6.95 ^{abc}	81.75 $\pm$ 13.95 ^{ac}	82.05 $\pm$ 1.25 ^a	87.25 $\pm$ 1.25 ^a	66.9 $\pm$ 1.05 ^{abcd}	78.4 $\pm$ 12.6 ^{cd}	65.2 $\pm$ 8.05 ^{abcd}	63.7 $\pm$ 14.45 ^{abcd}	0.013	0.407
Gly	6.14 $\pm$ 0.23 ^a	6.29 $\pm$ 0.09 ^{ab}	8.36 $\pm$ 0.13 ^{ab}	7.86 $\pm$ 0.27 ^a	9.78 $\pm$ 0.04 ^{cd}	8.79 $\pm$ 0.8 ^{ab}	10.88 $\pm$ 0.28 ^a	10.64 $\pm$ 0.67 ^a	9.39 $\pm$ 0.81 ^{ab}	8.56 $\pm$ 0.23 ^{ab}	9.27 $\pm$ 1.13 ^{abcd}	8.58 $\pm$ 0.58 ^{ab}	0.000	0.127
His	5.67 $\pm$ 0.2 ^a	5.81 $\pm$ 0.06 ^a	8.04 $\pm$ 0.33 ^{abcd}	6.81 $\pm$ 0.12 ^{cd}	8.31 $\pm$ 0.57 ^{bcd}	8.41 $\pm$ 3.4 ^{cd}	8.84 $\pm$ 0.28 ^{ab}	8.64 $\pm$ 0.23 ^{abcd}	7.08 $\pm$ 0.02 ^{abcd}	8.1 $\pm$ 0.75 ^{abc}	8.88 $\pm$ 1.33 ^a	6.86 $\pm$ 0.57 ^{abcd}	0.009	0.347
Ile	5.28 $\pm$ 0.04 ^a	5.49 $\pm$ 0.01 ^a	6.69 $\pm$ 0.21 ^{bc}	6.18 $\pm$ 0.11 ^c	6.9 $\pm$ 0.04 ^a	6.65 $\pm$ 0.31 ^{bcd}	6.24 $\pm$ 0.14 ^{cd}	6.81 $\pm$ 0.15 ^a	6.46 $\pm$ 0.3 ^{bcd}	6.67 $\pm$ 0.08 ^{cd}	6.32 $\pm$ 0.02 ^{cd}	6.56 $\pm$ 0.12 ^{bcd}	0.000	0.411
Lys	46.45 $\pm$ 4.55 ^a	54.6 $\pm$ 3.4 ^{ab}	69.1 $\pm$ 3.85 ^{abcd}	62.3 $\pm$ 0.80 ^{abcd}	75.25 $\pm$ 6.95 ^{abc}	81.75 $\pm$ 13.95 ^{ac}	82.05 $\pm$ 1.25 ^a	87.25 $\pm$ 1.25 ^a	66.9 $\pm$ 1.05 ^{abcd}	78.4 $\pm$ 12.6 ^{cd}	65.2 $\pm$ 8.05 ^{abcd}	63.65 $\pm$ 14.45 ^{abcd}	0.013	0.407
Met	1.7 $\pm$ 0.04 ^{ab}	1.55 $\pm$ 0.01 ^a	2.16 $\pm$ 0.01 ^{abcd}	1.99 $\pm$ 0 ^{cd}	2.4 $\pm$ 0.02 ^{bc}	2.16 $\pm$ 0.02 ^{cd}	2.06 $\pm$ 0.26 ^{bcd}	2.46 $\pm$ 0.02 ^d	1.84 $\pm$ 0.09 ^{abc}	2.31 $\pm$ 0.18 ^{cd}	2.14 $\pm$ 0.22 ^{abcd}	2.33 $\pm$ 0.14 ^{cd}	0.001	0.220
Phe	nd	nd	1.28 $\pm$ 0 ^a	1.32 $\pm$ 0 ^a	1.13 $\pm$ 0.06 ^a	1.15 $\pm$ 0.03 ^a	1.05 $\pm$ 0.05 ^a	1.16 $\pm$ 0.12 ^a	1.13 $\pm$ 0 ^a	1.21 $\pm$ 0 ^a	1.09 $\pm$ 0.03 ^a	1.21 $\pm$ 0 ^a	0.838	0.998
Pro	8.9 $\pm$ 0.3 ^{abcd}	8.7 $\pm$ 0.07 ^{cd}	10.3 $\pm$ 0.3 ^{abcd}	9.23 $\pm$ 0.42 ^{abcd}	12.2 $\pm$ 0.25 ^d	11 $\pm$ 0.9 ^{ab}	6.83 $\pm$ 1.7 ^{cd}	11.9 $\pm$ 0 ^d	4.4 $\pm$ 0.29 ^a	10.2 $\pm$ 0.29 ^{abcd}	7.37 $\pm$ 1.24 ^{bc}	10.44 $\pm$ 0.46 ^{abcd}	0.002	0.001
Ser	8.4 $\pm$ 1 ^{ac}	9.58 $\pm$ 0.04 ^{ac}	11.7 $\pm$ 0.2 ^{bc}	11.1 $\pm$ 0.29 ^{ab}	13.1 $\pm$ 0 ^a	12.9 $\pm$ 1 ^{bc}	9.03 $\pm$ 0.43 ^{cd}	13.2 $\pm$ 0.3 ^d	7.6 $\pm$ 0.09 ^a	12.51 ^{1^{cd}}	8.29 $\pm$ 0.12 ^{ac}	7.15 $\pm$ 1.09 ^a	0.000	0.002
Thr	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	-	-
Trp	1.44 ^{0^a}	1.51 $\pm$ 0.13 ^a	1.38 $\pm$ 0.33 ^a	1.24 $\pm$ 0 ^a	1.5 $\pm$ 0 ^a	1.42 $\pm$ 0.18 ^a	nd	1.42 $\pm$ 0 ^a	1.57 $\pm$ 0.15 ^a	nd	nd	1.61 $\pm$ 0.02 ^a	0.458	0.337
Tyr	3.3 $\pm$ 0.17 ^{bc}	3.35 $\pm$ 0.08 ^{bc}	4.32 $\pm$ 0.23 ^{abc}	4 $\pm$ 0.16 ^{abc}	4.78 $\pm$ 0.15 ^a	4.05 $\pm$ 0.32 ^{abc}	3.99 $\pm$ 0.11 ^{bc}	4.44 $\pm$ 0.02 ^{ab}	4.08 $\pm$ 0.67 ^{abc}	4.63 $\pm$ 0.06 ^a	3.21 $\pm$ 0.79 ^a	4.24 $\pm$ 0.14 ^{abc}	0.108	0.463
Val	11.3 $\pm$ 0.1 ^c	11.5 $\pm$ 0.15 ^d	14.65 $\pm$ 0.55 ^{abcd}	12.84 $\pm$ 0.1 ^{cd}	17.2 $\pm$ 1 ^a	15.44 $\pm$ 1.5 ^{abc}	15.15 $\pm$ 0.05 ^{abc}	16.54 $\pm$ 0.55 ^{ab}	13.34 $\pm$ 0.1 ^{cd}	14.7 $\pm$ 0.7 ^{bcd}	13.15 $\pm$ 0.75 ^{bcd}	14.35 $\pm$ 0.45 ^{bcd}	0.000	0.863
TOTAL FAA	210.7	239.9	293.51	267.1	249	319.1	317.5	325.7	289.5	346.3	256.9	268.4	0.000	0.000

3 Two-way ANOVA. ^{a, b, c, d} Values within a row with different superscripts differ significantly at $p \leq 0.05$ (Fisher LSD Test).

4 SEM: standard error of mean.

1 In contrast, treatment I, with a higher concentration of residual nitrate (213–227 mg/kg),
2 showed lower levels of FAA (alanine ~13–14 $\mu\text{mol/g}$; valine ~11–12 $\mu\text{mol/g}$) but
3 achieved the best scores for color (4.34), overall taste (3.84), and overall acceptance
4 (3.92) (Sup. Table 2). This apparent discrepancy indicates that moderation in
5 proteolysis and FAA may be perceived as a more balanced and acceptable sensory
6 profile. On the other hand, overripening (phase V) had a significant impact on amino
7 acids such as alanine, arginine, aspartic acid, proline, and serine, increasing their levels
8 compared to phase IV. This suggests that some amino acids increase disproportionately
9 with maturation time [48].

10 The total free amino acid content was higher in hams with reduced nitrifying salts and
11 prolonged curing, reinforcing the evidence that reducing nitrifying agents in-creases
12 proteolysis and the release of flavor precursors [49]. This finding is important from a
13 sensory point of view, as FAAs contribute to basic flavors and are substrates for curing
14 flavor reactions (such as Maillard reactions) [50]. These results are consistent with
15 previous studies that indicate a greater accumulation of FAAs in reduced-salt meat
16 products, attributed to the lower inhibitory effect of NaCl on proteolytic enzymes
17 [23,24,51].

18

19 Table 5 shows the content of volatile compounds produced in hams. expressed as a
20 percentage of the total chromatographic area (% relative abundance) for each group of
21 identified compounds. Various key compounds derived from lipid and protein
22 oxidation, microbial metabolism, and amino acid degradation reactions were evaluated.

23

24

1 **Table 5.** Effect of different salt treatments and the curing process on volatile compounds content obtained from the samples of boneless Spanish Dry-Cured Hams. The relative
2 abundance of each compound was calculated as the percentage of its chromatographic peak area relative to the total chromatographic area as means values $\pm$ SEM^a.

Salt Treatment	I		III		VI		VII		VIII		IX		μ -value	
Processing Stage	4	5	4	5	4	5	4	5	4	5	4	5	Salt Treatment	Processing Stage
Acetic acid	2.174 $\pm$ 0.43 ^{bc}	0.682 $\pm$ 0 ^d	2.271 $\pm$ 0.27 ^{cd}	2.253 $\pm$ 0.77 ^{cd}	4.3 $\pm$ 1.53 ^{cd}	3.582 $\pm$ 0.48 ^{cd}	5.17 $\pm$ 0.4 ^d	3.005 $\pm$ 0.25 ^{cd}	4.3 $\pm$ 0.68 ^{cd}	2.683 $\pm$ 0.34 ^{cd}	2.3 $\pm$ 0.01 ^{cd}	2.543 $\pm$ 1.14 ^{cd}	0.011	0.014
Ethyl Acetate	1.231 $\pm$ 0.241 ^{ac}	1.93 $\pm$ 1.31 ^{abc}	2.033 $\pm$ 1.42 ^{abc}	3.777 $\pm$ 0 ^a	0.942 $\pm$ 0 ^d	0.502 $\pm$ 0.089 ^d	0.917 $\pm$ 0.41 ^{bc}	0.366 $\pm$ 0.09 ^d	0.79 $\pm$ 0.46 ^{bc}	2.875 $\pm$ 1.15 ^{ac}	0.86 $\pm$ 0.06 ^{bc}	1.286 $\pm$ 0 ^{bc}	0.049	0.118
Butanal, 3-methyl-	0.649 $\pm$ 0.03 ^c	1.74 $\pm$ 0.23 ^{cd}	0.784 $\pm$ 0.103 ^d	2.652 $\pm$ 1.264 ^a	0.896 $\pm$ 0.102 ^d	1.773 $\pm$ 0.191 ^{cd}	0.878 $\pm$ 0.024 ^d	1.844 $\pm$ 0.343 ^{ab}	0.687 $\pm$ 0.09 ^b	1.399 $\pm$ 0.012 ^{cd}	0.734 $\pm$ 0.13 ^d	1.8 $\pm$ 0.54 ^{bc}	0.715	0.001
Butanal, 2-methyl-	0.277 $\pm$ 0.027 ^b	0.523 $\pm$ 0.1 ^b	0.301 $\pm$ 0.054 ^b	0.427 $\pm$ 0.005 ^d	0.362 $\pm$ 0.039 ^b	0.778 $\pm$ 0.041 ^d	0.332 $\pm$ 0.01 ^b	0.756 $\pm$ 0.12 ^c	0.311 $\pm$ 0.04 ^d	0.695 $\pm$ 0.03 ^d	0.291 $\pm$ 0.02 ^d	0.674 $\pm$ 0.13 ^d	0.023	0.000
Cyclohexane	16.882 $\pm$ 0.71 ^b	18.18 $\pm$ 1.69 ^a	13.844 $\pm$ 1.31 ^b	9.31 $\pm$ 8.4 ^b	14.087 $\pm$ 2.23 ^b	33.076 $\pm$ 0.3 ^a	14.319 $\pm$ 1.09 ^b	17.538 $\pm$ 3.52 ^b	10.46 $\pm$ 1.96 ^b	14.95 $\pm$ 2.12 ^b	12.95 $\pm$ 0.3 ^d	21.953 $\pm$ 5.3 ^{ab}	0.157	0.051
Perlimol	0.940 $\pm$ 2 ^{bc}	0.640 $\pm$ 0 ^{cd}	0.924 $\pm$ 0.2 ^{cd}	0.702 $\pm$ 0.33 ^{cd}	0.484 $\pm$ 0.123 ^d	0.8054 ^{cd}	0.7540 $\pm$ 0.39 ^{cd}	1.2440 $\pm$ 1.5 ^d	1.265 $\pm$ 0.15 ^d	1.20840 $\pm$ 1.8 ^{cd}	0.8 $\pm$ 0.33 ^{cd}	0.8740 $\pm$ 3 ^{cd}	0.074	0.857
1-Pentanol	0.84 $\pm$ 0.21 ^{cd}	0.51 $\pm$ 0.016 ^d	0.787 $\pm$ 0.015 ^{ab}	0.554 $\pm$ 0.211 ^d	0.67 $\pm$ 0.024 ^{cd}	0.52 $\pm$ 0.01 ^b	0.854 $\pm$ 0.032 ^{cd}	1.011 $\pm$ 0.206 ^{cd}	0.808 $\pm$ 1.31 ^{cd}	1.111 $\pm$ 0.031 ^d	0.66 $\pm$ 0.12 ^{cd}	0.992 $\pm$ 0.41 ^{cd}	0.195	0.980
Toluene	-	0.61 $\pm$ 0.281 ^a	0.97 $\pm$ 0.56 ^{ab}	1.18 $\pm$ 0.45 ^a	1.46 $\pm$ 0.67 ^a	1.62 $\pm$ 0.56 ^a	0.839 $\pm$ 0 ^a	0.739 $\pm$ 0.17 ^a	0.98 $\pm$ 0 ^{cd}	0.96 $\pm$ 0.54 ^{cd}	0.74 $\pm$ 0.29 ^a	1.043 $\pm$ 0.06 ^{ab}	0.123	0.393
Hexanal	37.541 $\pm$ 6 ^{cd}	17 $\pm$ 3.02 ^{cd}	22.244 $\pm$ 8 ^{cd}	20.1 $\pm$ 8.75 ^{cd}	20.3 $\pm$ 3.82 ^{cd}	14.42 $\pm$ 4.8 ^d	25.740 $\pm$ 55 ^{cd}	32.36 $\pm$ 1.11 ^b	29.740 $\pm$ 4 ^a	32.442 $\pm$ 52 ^a	24.4 $\pm$ 1.9 ^{cd}	26.9 $\pm$ 3.6 ^{cd}	0.037	0.475
Benzoic acid, 3-methyl	-	0.274 $\pm$ 0.07 ^{cd}	0.343 $\pm$ 0 ^d	1.540 $\pm$ 0.76 ^{cd}	0.45 $\pm$ 0.1 ^{cd}	140.42 ^{cd}	1.494 $\pm$ 0.19 ^{cd}	0.39 $\pm$ 0.176 ^{cd}	1.544 $\pm$ 0.058 ^{cd}	0.72 $\pm$ 0.02 ^{cd}	0.97 $\pm$ 0.6 ^{cd}	0.584 $\pm$ 0.29 ^{cd}	0.029	0.293
1-Hexanol	2.312 $\pm$ 0.33 ^{ab}	0.757 $\pm$ 0.084 ^d	2.211 $\pm$ 1.15 ^{cd}	0.771 $\pm$ 0.164 ^d	2.838 $\pm$ 0.57 ^a	1.671 $\pm$ 0.16 ^{cd}	1.131 $\pm$ 0.12 ^{cd}	0.552 $\pm$ 0.022 ^d	0.911 $\pm$ 0.45 ^{cd}	0.526 $\pm$ 0.13 ^d	0.861 $\pm$ 0.25 ^d	0.713 $\pm$ 0.14 ^d	0.021	0.003
2-Heptanoic	5.93 $\pm$ 0.06 ^d	6.547 $\pm$ 1.83 ^{ab}	5.66 $\pm$ 0.83 ^{bc}	4.634 $\pm$ 1.9 ^{bc}	8.11 $\pm$ 0.27 ^a	1.958 $\pm$ 0 ^d	1.476 $\pm$ 0.33 ^d	1.117 $\pm$ 0.047 ^d	1.243 $\pm$ 0.11 ^d	0.939 $\pm$ 0 ^d	1 $\pm$ 0.182 ^d	1.195 $\pm$ 0 ^d	0.000	0.035
Heptanol	0.922 $\pm$ 0.07 ^a	0.674 $\pm$ 0.02 ^d	0.988 $\pm$ 0.08 ^a	1.06340 $\pm$ 5 ^a	0.77940 $\pm$ 08 ^a	0.892 $\pm$ 0.31 ^a	0.889 $\pm$ 0.11 ^a	0.946 $\pm$ 0.06 ^a	0.939 $\pm$ 0.02 ^a	1.11 $\pm$ 0.06 ^a	0.923 $\pm$ 0.03 ^a	0.978 $\pm$ 0.09 ^a	0.802	0.761
1-hexanol, 2-butyny-	0.4184 $\pm$ 0.03 ^{ab}	0.3611 $\pm$ 0.06 ^{ab}	0.3511 $\pm$ 0.01 ^{ab}	0.4534 $\pm$ 0.06 ^{ab}	0.4734 $\pm$ 0.2 ^{ab}	0.4884 $\pm$ 0.08 ^a	0.3834 $\pm$ 0.07 ^{ab}	-	0.7484 $\pm$ 0.01 ^b	0.2474 $\pm$ 0.04 ^b	0.764 $\pm$ 0.08 ^{ab}	0.3854 $\pm$ 0 ^{ab}	0.033	0.447
Benzaldehyde	1.565 $\pm$ 0.79 ^a	1.4884 $\pm$ 0.35 ^{ab}	1.421 $\pm$ 0.08 ^d	2.5184 $\pm$ 0.51 ^a	1.4954 $\pm$ 0.49 ^a	2.793 $\pm$ 0.44 ^a	3.059 $\pm$ 0.26 ^a	2.2784 $\pm$ 0.05 ^b	3.6114 $\pm$ 1.16 ^a	1.8814 $\pm$ 0.33 ^b	3.44 $\pm$ 5.07 ^a	1.7574 $\pm$ 0.6 ^a	0.544	0.400
Hexanoic acid	1.891 $\pm$ 0.73 ^d	1.379 $\pm$ 0.16 ^d	0.09 $\pm$ 0.82 ^{cd}	3.1211 $\pm$ 0.96 ^{cd}	2.169 $\pm$ 0.239 ^{cd}	3.879 $\pm$ 1.74 ^{cd}	4.1510 $\pm$ 25 ^{cd}	5.761 $\pm$ 1.77 ^{cd}	6.935 $\pm$ 0.08 ^{ab}	6.0810 $\pm$ 0.043 ^{cd}	6.3110 $\pm$ 0.53 ^{cd}	5.616 $\pm$ 0.99 ^{cd}	0.002	0.970
1-Octen-3-ol	3.589 $\pm$ 0.45 ^{ab}	2.108 $\pm$ 0.52 ^{cd}	3.463 $\pm$ 0.09 ^{ab}	2.83 $\pm$ 1.13 ^{ab}	3.094 $\pm$ 0.15 ^{ab}	2.683 $\pm$ 0.26 ^{ab}	3.526 $\pm$ 0.32 ^{ab}	3.591 $\pm$ 0.24 ^{ab}	4.166 $\pm$ 0.31 ^a	3.652 $\pm$ 0.044 ^{ab}	2.74 $\pm$ 0.23 ^{ab}	3.641 $\pm$ 1.03 ^{ab}	0.460	0.294
Formic, 2-pentyl-	0.541 $\pm$ 0.033 ^a	0.513 $\pm$ 0.15 ^a	0.594 $\pm$ 0.08 ^{cd}	0.944 $\pm$ 0.06 ^{cd}	0.512 $\pm$ 0 ^d	1.286 $\pm$ 0.34 ^a	0.499 $\pm$ 0.05 ^d	0.712 $\pm$ 0.12 ^{cd}	0.87 $\pm$ 0.13 ^{cd}	0.7874 $\pm$ 0.01 ^{cd}	0.67 $\pm$ 0.05 ^{cd}	0.7354 $\pm$ 0.04 ^{cd}	0.102	0.013
Benzyl alcohol	1.0834 $\pm$ 0.67 ^a	4.0854 $\pm$ 0.719 ^a	0.7684 $\pm$ 0.41 ^{ab}	4.97411 $\pm$ 0.9 ^a	0.407 $\pm$ 0 ^b	6.175 $\pm$ 1.29 ^a	0.315 $\pm$ 0 ^b	1.1434 $\pm$ 0.1 ^d	0.7984 $\pm$ 0.07 ^{ab}	1.2974 $\pm$ 0.25 ^b	0.3640 $\pm$ 0.01 ^d	1.744 $\pm$ 0.84 ^d	0.003	0.000
3-Octen-2-one	0.709 $\pm$ 0.28 ^{ab}	0.524 $\pm$ 0.024 ^d	0.927 $\pm$ 0 ^{cd}	1.1 $\pm$ 0.49 ^{cd}	0.941 $\pm$ 0 ^{ab}	0.376 $\pm$ 0 ^d	1.879 $\pm$ 0 ^{cd}	2.13 $\pm$ 0.074 ^{cd}	2.478 $\pm$ 0.76 ^d	1.97 $\pm$ 0.35 ^{cd}	1.840 $\pm$ 0.75 ^{cd}	1.0140 $\pm$ 0.4 ^{cd}	0.004	0.214

Benzeneacetaldehyde	0.663±0.003 ^a	1.018±0.189 ^{bc}	1.241±0.09 ^{cd}	2.823±1.11 ^a	1.231±0.24 ^{cd}	2.197±0.16 ^{cd}	2.183±0.18 ^{cd}	1.77±0.07 ^{bc}	1.25±0.073 ^{cd}	1.998±0.29 ^{cd}	1.210±0.73 ^{cd}	2.14±0.098 ^{cd}	0.071	0.007
2-Octenal (E)-	0.79±0.1 ^{abc}	0.425±0.018 ^a	0.94±0.01 ^{abc}	0.66±0.32 ^{abc}	0.962±0 ^{cd}	0.46±0.050 ^{bc}	1.3±0.130 ^{cd}	1.25±0.048 ^{cd}	1.632±0.19 ^a	1.1±0.097 ^{bc}	1.26±0.2 ^a	0.94±0.253 ^{bc}	0.001	0.003
3,5-Octadien-2-one	2.734±1.37 ^{cd}	4.403±0.9 ^{cd}	5.133±0.97 ^{cd}	7.62±2.02 ^a	3.081±0 ^{cd}	0.701±0 ^a	4.078±1.47 ^{cd}	5.43±0.088 ^{cd}	5.156±1.34 ^{cd}	5.682±0.23 ^{cd}	3.8±0.96 ^{cd}	5.99±0 ^{cd}	0.010	0.125
Nonanal	3.169±0.018 ^a	1.811±0.448 ^a	3.077±0.043 ^a	2.525±0.77 ^a	3.068±0.011 ^a	2.75±0.73 ^a	2.90±0.73 ^a	2.644±0.206 ^a	2.952±0.71 ^a	2.952±0.06 ^a	2.63±0.533 ^a	3.06±0.24 ^a	0.957	0.270
Phenylethyl Alcohol	0.285±0.07 ^{bc}	0.459±0.15 ^{cd}	0.41±0.12 ^{cd}	0.625±0 ^{cd}	0.344±0.063 ^{cd}	0.964±0.63 ^{bc}	2.799±0.49 ^a	0.39±0.054 ^{cd}	1.87±0.23 ^a	-	1.09±0.4 ^{cd}	1.15±0 ^{cd}	0.007	0.005

1 Two-way ANOVA. ^{a, b, c, d} Values within a row with different superscripts differ significantly at $p \leq 0.05$ (LSD Test).

2 SEM: standard error of mean.

3

4

5 The results show that the volatile composition of the hams was influenced by both the nitrifying curing salt formulations and the maturation time.

6 In particular, overripening increased the diversity and concentration of volatiles in all treatments, consistent with a higher degree of oxidative and

7 enzymatic reactions throughout the curing time. This suggests that during the final stage of curing, lipid oxidative processes (which generate

8 volatile fatty acids) and possibly residual fermentative processes that produce acetic acid are intensified [52]. Regarding the effect of salt

9 treatments, an interesting pattern was observed: hams produced with reduced nitrite/nitrate concentrations showed a higher proportion of certain

10 volatiles derived from lipid oxidation, while those with higher nitrite doses had relatively lower levels of these compounds. This is consistent

11 with the known antioxidant role of nitrite in cured meats [41,53]. By reducing the amount of nitrite available, it is possible that fat oxidation

12 increased during maturation, raising the production of aldehydes and short-chain fatty acids responsible for rancid-sweet aromas. In this regard, it

13 has also been reported that high nitrate levels in other cured products, such as pastirma, reduce the formation of volatile compounds derived from

14 lipid oxidation without significantly altering color or residual nitrite [54], which is consistent with the findings of our study. It should be noted

that salinity can also modulate the formation of volatiles. High concentrations of NaCl can promote lipid peroxidation in the early stages of ripening due to saline oxidative stress, but at the same time nitrite can act as a radical scavenger and slow down the spread of oxidation [55].

3.4 Instrumental Color and Texture Profile

Table 6 shows the results obtained after measuring the instrumental color of the different ham samples analysed. The different salt treatments used significantly affected almost all of the color parameters analysed (L^* , a^* , b^* , h^*). The same occurred with overripening, which significantly affected almost all of the color values analysed (L^* , a^* , C^* , h^*).

Table 6. Effect of different salt treatments and the curing process instrumental color obtained from the samples of Spanish Dry-Cured Hams. Results are expressed as means values $\pm$ SEM*.

Salt Treatment	Processing Stage	L^*	a^*	b^*	C^*	h^*
I	4	49.64 $\pm$ 2.23 ^{bg}	24.31 $\pm$ 1.55 ^c	27.49 $\pm$ 1.03 ^c	36.84 $\pm$ 0.64 ^{abce}	48.45 $\pm$ 2.64 ^{abce}
I	5	38.11 $\pm$ 1.61 ^a	26.97 $\pm$ 0.46 ^{bc}	21.33 $\pm$ 1.28 ^a	34.44 $\pm$ 1.02 ^c	38.11 $\pm$ 1.54 ^e
III	4	59.21 $\pm$ 1.35 ^{de}	18.59 $\pm$ 1.38 ^a	28.88 $\pm$ 0.4 ^c	34.59 $\pm$ 0.68 ^c	57.45 $\pm$ 2.12 ^e
III	5	44.73 $\pm$ 0.56 ^c	29.07 $\pm$ 1.27 ^b	31.53 $\pm$ 2.52 ^{bc}	42.96 $\pm$ 2.68 ^d	46.99 $\pm$ 1.17 ^d
VI	4	53.27 $\pm$ 0.62 ^{fg}	24.71 $\pm$ 0.19 ^c	30.08 $\pm$ 0.84 ^{bc}	39 $\pm$ 0.79 ^{abcde}	50.56 $\pm$ 0.59 ^{abcde}
VI	5	45.35 $\pm$ 1.66 ^{bc}	26.91 $\pm$ 0.35 ^{bc}	30.18 $\pm$ 1.31 ^{bc}	40.56 $\pm$ 0.73 ^{abd}	48.1 $\pm$ 1.65 ^{abd}
VII	4	59.87 $\pm$ 1.46 ^{de}	19.37 $\pm$ 1.12 ^a	29.08 $\pm$ 1.32 ^{bc}	35.1 $\pm$ 1.06 ^{ce}	56.35 $\pm$ 2.11 ^{cc}
VII	5	52.77 $\pm$ 3.31 ^g	25.78 $\pm$ 2.3 ^{bc}	32.68 $\pm$ 1.47 ^{bc}	42.01 $\pm$ 0.67 ^{ad}	51.75 $\pm$ 3.53 ^{ad}
VIII	4	57.73 $\pm$ 1.13 ^{ef}	19.83 $\pm$ 0.88 ^a	29.44 $\pm$ 0.24 ^{bc}	35.58 $\pm$ 0.51 ^{bce}	56.18 $\pm$ 1.18 ^{bce}
VIII	5	52.91 $\pm$ 1.82 ^{fg}	25.48 $\pm$ 1.06 ^{bc}	30.67 $\pm$ 1.56 ^{bc}	40 $\pm$ 0.85 ^{abcd}	50.35 $\pm$ 2.42 ^{abcd}
IX	4	63.18 $\pm$ 2.25 ^d	16.69 $\pm$ 1.79 ^a	30.08 $\pm$ 1.27 ^{bc}	34.46 $\pm$ 1.82 ^e	61.12 $\pm$ 2.02 ^e
IX	5	52.44 $\pm$ 0.35 ^g	25.86 $\pm$ 0.42 ^{bc}	33.54 $\pm$ 2.18 ^b	42.46 $\pm$ 1.92 ^d	52.23 $\pm$ 1.48 ^d
<i>p-value</i>						
Salt Treatment		0.000	0.023	0.006	0.334	0.000
Processing Stage		0.000	0.000	0.429	0.000	0.000

Two-way ANOVA. ^{a, b, c, d} Values within a column with different superscripts differ significantly at $p \leq 0.05$ (Fisher LSD Test).

SEM: standard error of mean.

1 Results show significant differences in most of the parameters analysed, both be-cause
2 of using different nitrifying salt treatments and due to the maturation time. In general,
3 hams made with higher concentrations of nitrifying salts exhibited more in-tense red
4 colours (higher a^* values), while overripening tended to slightly darken the product
5 (reduction in L^*) while accentuating yellowish hues (b^* increased) and re-duc-ing
6 saturation in some cases.

7 L^* values (lightness) ranged from 38.11 (I, 5) to 63.18 (IX, 4). The ham with re-duced
8 nitrite and longer maturation was visibly darker than the ham with full salt/nitrite and
9 shorter curing time. This is consistent with the expectation that a lower proportion of
10 nitrite may result in less bright coloration, and that prolonged oxidation during
11 maturation may slightly brown the product [56,57]. A^* (redness) ranged from 16.69
12 (IX, 4) to 29.07 (III, 5). The treatments with the highest residual nitrate concen-tration
13 (I: 213-227 mg/kg; IX: 105-181 mg/kg) had the highest a^* values (red intensity, up to
14 29.07 in treatment III and 27-25.9 respectively in I/IX), reflecting greater for-mation of
15 stable nitrosomyoglobin. In contrast, treatments with lower nitrification inputs (VII and
16 VI, residual nitrate ~100 mg/kg or less) achieved reduced a^* values (19-24
17 respectively), showing less reddish and more muted tones. It should be noted that, in the
18 absence of nitrifying agents, the accumulation of zinc protoporphyrin IX (ZnPP) has
19 been described as responsible for the stable red color in cured hams [58], which could
20 explain the reddish hue observed even in treatments with reduced ni-trate/nitrite levels
21 in overripening hams. Component b^* ranged between 21.33 (I, 5) and 33.54 (IX, 5). In
22 phase 5, all treatments had higher b values than in phase 4, indicating a slight shift
23 towards warmer/yellowish tones with maturation (possibly due to lipid oxidation and
24 brown pigment formation). The chroma (C^*) values ranged from 34.44 (I, 5) to 42.96
25 (III, 5), suggesting this results those formulations with sufficient nitrite allow for a purer

1 and more intense color, while a lack of nitrite can result in a slightly less saturated
 2 color. Finally, the hue angle (h*) varied from 38.11 (I, 5) to 61.12 (IX, 4).
 3 Table 7 shows the results obtained after measuring the instrumental texture of the
 4 different ham samples analysed. The different nitrifying salt treatments used
 5 significantly affected the adhesiveness of the samples analysed. The different
 6 processing stages analysed significantly affected all the texture parameters analysed.

7 **Table 7.** Effect of different nitrifying salt treatments and the curing process on the instrumental texture
 8 obtained from the samples of Spanish Dry-Cured Hams. Results are expressed as means values $\pm$ SEM*.

Salt Treatment	Processing Stage	C1-H (N)	C1 H-D	C1-A (mJ)	C2-C	C2-RD (mm)	C2-S	C2-G (N)	C2-CH (mJ)	C2-H (N)
I	4	8.2 $\pm$ 1.05 ^{ac}	2.97 $\pm$ 0 ^{bcd}	0.1 $\pm$ 0.04 ^{de}	7.72 $\pm$ 1 ^{ac}	0.7 $\pm$ 0.1 ^{ab}	1.3 $\pm$ 0.14 ^{ab}	2.1 $\pm$ 0.1 ^{abc}	5.3 $\pm$ 0.9 ^{ac}	11 $\pm$ 2.08 ^{ac}
I	5	15.86 $\pm$ 3.3 ^b	2.98 $\pm$ 0 ^{ad}	0.1 $\pm$ 0.02 ^{de}	14.9 $\pm$ 3.1 ^b	0.68 $\pm$ 0 ^{ab}	1.6 $\pm$ 0.03 ^b	2.4 $\pm$ 0.04 ^{bc}	10.8 $\pm$ 2.1 ^b	25.8 $\pm$ 4.8 ^b
III	4	5.02 $\pm$ 0.74 ^c	2.96 $\pm$ 0 ^c	0.5 $\pm$ 0.2 ^a	4.6 $\pm$ 0.69 ^c	0.63 $\pm$ 0 ^{ab}	1.3 $\pm$ 0.04 ^{ab}	2.14 $\pm$ 0 ^{abc}	3.17 $\pm$ 0.4 ^c	6.83 $\pm$ 0.9 ^c
III	5	15.43 $\pm$ 0.7 ^b	2.98 $\pm$ 0 ^{ad}	0.18 $\pm$ 0.1 ^{de}	14.5 $\pm$ 0.6 ^b	0.7 $\pm$ 0.03 ^b	1.61 $\pm$ 0.1 ^b	2.4 $\pm$ 0.06 ^{bc}	11 $\pm$ 0.5 ^b	26.7 $\pm$ 1.8 ^b
VI	4	5.13 $\pm$ 0.3 ^c	2.97 $\pm$ 0 ^{abcd}	0.3 $\pm$ 0.1 ^{abcd}	4.8 $\pm$ 0.29 ^c	0.64 $\pm$ 0 ^{ab}	1.3 $\pm$ 0.06 ^{ab}	2.1 $\pm$ 0.03 ^{abc}	3.28 $\pm$ 0.2 ^c	6.94 $\pm$ 0.5 ^c
VI	5	15.3 $\pm$ 2.17 ^b	2.98 $\pm$ 0 ^{abd}	0.47 $\pm$ 0.2 ^{ab}	14.1 $\pm$ 1.9 ^b	0.6 $\pm$ 0.1 ^{ab}	1.3 $\pm$ 0.22 ^{ab}	2.1 $\pm$ 0.23 ^{abc}	9.3 $\pm$ 1.84 ^{ab}	20.3 $\pm$ 6 ^{ab}
VII	4	3.66 $\pm$ 0.22 ^c	2.97 $\pm$ 0 ^{bcd}	0.2 $\pm$ 0 ^{bcd}	3.43 $\pm$ 0.22 ^c	0.64 $\pm$ 0 ^{ab}	1.29 $\pm$ 0.1 ^{ab}	2 $\pm$ 0.1 ^{ac}	2.3 $\pm$ 0.09 ^c	4.6 $\pm$ 0.32 ^c
VII	5	16.65 $\pm$ 3.5 ^b	2.99 $\pm$ 0 ^a	0.18 $\pm$ 0.1 ^{de}	15.7 $\pm$ 3.2 ^b	0.71 $\pm$ 0 ^b	1.7 $\pm$ 0.06 ^b	2.5 $\pm$ 0.04 ^b	11.8 $\pm$ 2.3 ^b	29.6 $\pm$ 5.8 ^b
VIII	4	5.17 $\pm$ 1.16 ^c	3 $\pm$ 0.01 ^{bcd}	0.25 $\pm$ 0 ^{abde}	4.79 $\pm$ 1.06 ^c	0.6 $\pm$ 0 ^{ab}	1.3 $\pm$ 0.13 ^{ab}	2 $\pm$ 0.2 ^{ac}	3.3 $\pm$ 0.82 ^c	6.6 $\pm$ 2 ^c
VIII	5	13.2 $\pm$ 2.7 ^{ab}	2.97 $\pm$ 0 ^{bcd}	0.05 $\pm$ 0.01 ^e	12.4 $\pm$ 2.5 ^{ab}	0.69 $\pm$ 0 ^{ab}	1.6 $\pm$ 0.13 ^b	2.32 $\pm$ 0.1 ^{bc}	9 $\pm$ 1.58 ^{ab}	20.7 $\pm$ 3.5 ^{ab}
IX	4	4.59 $\pm$ 1.14 ^c	2.96 $\pm$ 0 ^{bc}	0.4 $\pm$ 0.1 ^{abc}	4.23 $\pm$ 1.06 ^c	0.6 $\pm$ 0.12 ^a	1 $\pm$ 0.34 ^a	1.7 $\pm$ 0.4 ^a	2.74 $\pm$ 1.0 ^c	5.65 $\pm$ 2.5 ^c
IX	5	12.6 $\pm$ 3.2 ^{ab}	2.98 $\pm$ 0 ^{ad}	0.1 $\pm$ 0.1 ^{de}	11.9 $\pm$ 3.1 ^{ab}	0.7 $\pm$ 0.1 ^b	1.7 $\pm$ 0.22 ^b	2.4 $\pm$ 0.2 ^{bc}	9.5 $\pm$ 2.8 ^{ab}	24.5 $\pm$ 8.3 ^{ab}
<i>p-value</i>										
Salt Treatment		0.000	0.637	0.047	0.645	0.802	0.840	0.731	0.756	0.030
Processing Stage		0.000	0.000	0.021	0.000	0.035	0.001	0.001	0.000	0.000

9 *Two-way ANOVA. a, c, d Values within a row with different superscripts differ significantly at $p \leq 0.05$ (Fisher LSD*
 10 *Test).*

11 *SEM: standard error of mean.*

12 **C1-H (N): C1 Hardness (N); C1-HD: C1 Hardness Deformation; C1-A (mJ): C1 Adhesiveness (mJ); C2-C: C2*
 13 *Cohesiveness; C2-RD (mm): C2 Recoverable deformation (mm); C2-S: C2 Springiness; C2-G (N): C2 Gumminess*
 14 *(N); C2-CH (mJ): C2 Chewiness (mJ); C2-H (N): C2 Hardness (N).*

16 Hardness (C1-H) ranged from 3.66 N (VII. IV) to 16.65 N (VII. V). Adhesiveness (C1-
 17 A) ranged from 0.05 mJ (VIII. V) to 0.5 mJ (III. IV). Cohesiveness (C2-C) ranged from
 18 3.43 (VII. IV) to 15.7 (VII. V). Gumminess (C2-G) ranged from 1.7 N (IX. IV) to 2.5 N
 19 (VII. V). Chewiness (C2-CH) ranged from 2.3 mJ (VII. IV) to 11.8 mJ (VII. V). There

1 was a marked increase in hardness and chewiness in overripening hams, reflecting
2 dehydration and compaction of the matrix. The differences between nitrifying salt
3 treatments were less clear, confirming that curing time has a greater impact on texture.
4 These findings regarding texture are consistent with studies such as that by Coll-Brasas
5 et al. [15] in Fenalår, who reported a decrease in hardness when reducing the amount of
6 salt added, but also warned that without nitrite, the matrix can be altered. In our case, all
7 hams were within a typical hardness range after complete maturation (10–17 N). The
8 differences between treatments, although statistically significant in adhesiveness, were
9 clear: no treatment produced a ham that was excessively soft or excessively elastic
10 compared to another, suggesting that the variation in nitrifying salts did not seriously
11 affect the texture. The influence of prolonged drying was noticed: all overripening hams
12 were harder and chewier, reflecting the typical greater “dryness” of a ham that has been
13 aged for longer. Technologically, an acceptable texture was obtained, although possibly
14 slightly less cohesive in the reduced formulations, which could even be preferable to
15 facilitate chewability. Despite this, parameters related to the acceptability and sensory
16 quality of the ham were not subsequently affected.

17

18 **3.5 Sensory analysis**

19 In order to evaluate how differences influence the sensory acceptance of white boneless
20 hams a consumer tasting was conducted focusing on the acceptability of various
21 attributes. The results obtained for the different samples analysed on the sensory
22 acceptability parameters by the consumer panel are presented in Figure 3. Due to the
23 similarity in appearance, color, and texture to normal ham, it was decided that the best
24 samples for the consumer panel would be those from the overripening phase (5).

1 Therefore, the panel was given samples from this phase with the different nitrifying salt
2 treatments used.

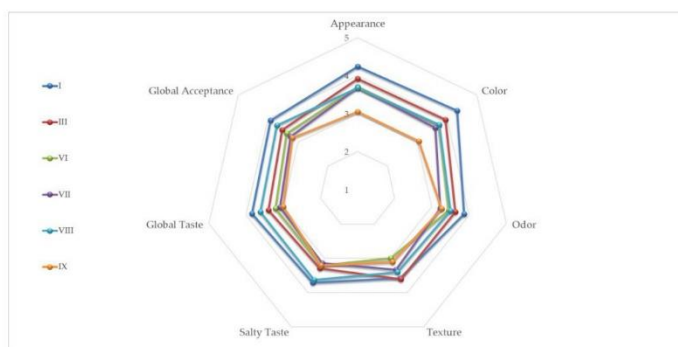


Figure 3. Sensory evaluation of overripening dry-cured hams according to nitrifying salt treatment.

6 The results indicate that all formulations obtained relatively similar acceptability
7 ratings, with no marked rejections. However, trends were observed in several attributes
8 (Sup. Table 2). Appearance was rated highest in treatment I (4.24) and lowest in IX
9 (3.05). Color reached 4.34 in I and dropped to 3.05 in IX. Smell ranged from 3.87 (I) to
10 3.22 (VII). Texture was 3.61 (III) compared to 3.0 (VI). Salty taste was perceived as
11 3.71 in I and 3.14 in VII. Overall taste reached 3.84 in I and 3.0 in IX. Overall
12 acceptance was highest in I (3.92) and lowest in IX (3.18). Treatment I, with a higher
13 residual nitrate concentration (213–227 mg/kg), had a firmer texture and a more intense
14 and stable color (a^* ~24–27), which coincided with the highest consumer acceptance
15 (overall acceptance 3.92). This correlation suggests that the combination of color
16 stability and compact texture was more appealing to the panel, compared to treatments
17 with higher proteolysis or salinity that did not achieve the same level of rating.
18 The panel perceived less salinity and color in the treatments with less nitrifying salts.
19 although overall acceptability remained close to the control [24]. This coincides with
20 Armenteros et al., 2012 [16] who demonstrated that partial substitutions of NaCl with

1 other chloride salts just affect the taste but were in general better valued. In any case, it
2 should be remembered that nitrifying agents perform multiple functions such as color,
3 flavor, microbiological safety, and oxidative stability, which are difficult to replace
4 completely with a single ingredient, meaning that any reduction strategy must balance
5 safety and sensory quality [59].

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CONCLUSIONS

The results obtained showed that variations in the concentration of nitrifying salts, together with the degree of maturation, had a decisive effect on the biochemistry and sensory properties of cured ham.

In terms of composition, overripening significantly reduced moisture and aw, while the formulations modulated pH and salt concentration. Treatment VIII stood out for its higher NaCl content, compared to VI and VII with lower values. Biochemically, over-maturation increased the proteolysis index and FAA release in all cases, especially in treatments with fewer nitrifying agents (VI and VII), which accumulated higher levels of sweet and bitter amino acids. In contrast, treatment I, with more residual nitrate, showed more moderate proteolysis.

During phase 5, oxidation processes intensified, with an increase in aldehydes and volatile acids. Treatments with less nitrite/nitrate generated a higher proportion of oxidation compounds, while those with higher concentrations kept them lower, confirming the antioxidant effect of nitrite. In terms of color, over-maturation slightly darkened the hams and accentuated the yellow tones, while treatments with more nitrate/nitrite had higher a^* (reddish) values, compared to VI and VII, which had more muted colors.

In terms of texture, overripening increased hardness and chewiness. Treatment I stood out for its firmness associated with greater dehydration, while VI and VII were softer and more cohesive. Sensory evaluation showed that, although overripening partially homogenized acceptance, treatment I was the highest rated in terms of appearance, color, and overall acceptance, while IX received the lowest scores, linked to its higher salinity and lower visual appeal.

1 In terms of safety, the partial reduction of nitrites and nitrates did not
2 compromise the stability of the product, as the aw values achieved would prevent the
3 development of pathogens such as *Clostridium botulinum*.

4 Overall, this study demonstrates that it is possible to partially reduce nitrifying
5 salts in Spanish cured ham while maintaining technological and sensory quality and
6 safety. All treatments were acceptable to the panel (>2.5 points), but treatment I
7 obtained the highest acceptance (3.92), while IX obtained the lowest (3.18). This
8 indicates that the reduction of nitrites/nitrates does not excessively decrease acceptance
9 as long as an adequate balance between composition and maturation is achieved.

10 These results support the viability of healthier and more sustainable curing
11 strategies and point to treatment I as the most recommended formulation, as it best
12 combines technological stability, safety, and consumer preference.

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4 *review and editing, visualization. G.M.V.: methodology, formal analysis, investigation. A.A.: supervision,*
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17

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26

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1 SUPPLEMENTARY MATERIAL

2 **Sup. Table 1.** Effect of different salt treatments and the curing process on the biochemical parameters obtained from
 3 the samples of Spanish Dry-Cured Hams. Results are expressed as means values $\pm$ SEM*.

Salt Treatment	Processing Stage	TN	NPN	Proteolysis Index
I	4	4.96 $\pm$ 0.02 ^{bcd}	0.64 $\pm$ 0.08 ^a	12.99 $\pm$ 1.58 ^a
I	5	5.66 $\pm$ 0.14 ^a	0.99 $\pm$ 0.05 ^{defg}	17.68 $\pm$ 1.16 ^{de}
III	4	4.57 $\pm$ 0.08 ^c	0.83 $\pm$ 0.05 ^{eg}	18.29 $\pm$ 1.15 ^{de}
III	5	4.98 $\pm$ 0.15 ^{bcd}	1.06 $\pm$ 0.06 ^{bde}	21.36 $\pm$ 1.19 ^{bc}
VI	4	5.25 $\pm$ 0.12 ^{abd}	0.99 $\pm$ 0.04 ^{defg}	18.9 $\pm$ 0.56 ^{cde}
VI	5	5.33 $\pm$ 0.42 ^{abd}	1.23 $\pm$ 0.06 ^b	23.25 $\pm$ 1.32 ^b
VII	4	4.49 $\pm$ 0.36 ^c	0.87 $\pm$ 0.06 ^{efg}	19.51 $\pm$ 0.89 ^{bcd}
VII	5	5.57 $\pm$ 0.16 ^{af}	1.22 $\pm$ 0.05 ^b	21.81 $\pm$ 0.44 ^{bcd}
VIII	4	4.44 $\pm$ 0.18 ^c	0.77 $\pm$ 0.04 ^{ac}	17.49 $\pm$ 1.54 ^e
VIII	5	5.23 $\pm$ 0.49 ^{abd}	1.01 $\pm$ 0.05 ^{def}	19.75 $\pm$ 2.03 ^{bcd}
IX	4	4.68 $\pm$ 0.22 ^{bc}	0.91 $\pm$ 0.06 ^{cefg}	19.48 $\pm$ 1.05 ^{bcd}
IX	5	5.41 $\pm$ 0.21 ^{ad}	1.11 $\pm$ 0.1 ^{bd}	20.51 $\pm$ 1.74 ^{bcd}
<i>p-value</i>				
Salt Treatment		0.084	0.002	0.004
Processing Stage		0.000	0.000	0.000

4 Two-way ANOVA. *a, b, c, d* Values within a column with different superscripts differ significantly at $p \leq 0.05$ (Fisher
 5 LSD Test).

6 SEM: standard error of mean.

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1 **Sup. Table 2.** Effect of different salt treatments during the curing process on the consumer acceptability by a
 2 consumer panel. Results are expressed as means values $\pm$ SEM*.

	Salt Treatment						p-value Salt Conditions
	I	III	VI	VII	VIII	IX	
Appearance	4.24 $\pm$ 0.88 ^b	3.92 $\pm$ 0.97 ^{bc}	3.71 $\pm$ 1.18 ^c	3.69 $\pm$ 1.04 ^c	3.68 $\pm$ 1.14 ^c	3.05 $\pm$ 1.09 ^a	0.000
Color	4.34 $\pm$ 0.75 ^b	3.95 $\pm$ 0.93 ^{ab}	3.73 $\pm$ 1.10 ^c	3.61 $\pm$ 1.10 ^c	3.74 $\pm$ 1.08 ^c	3.05 $\pm$ 1.14 ^c	0.000
Odor	3.87 $\pm$ 0.78 ^a	3.63 $\pm$ 0.82 ^{ab}	3.45 $\pm$ 1.01 ^b	3.22 $\pm$ 1.15 ^b	3.5 $\pm$ 0.89 ^{ab}	3.26 $\pm$ 0.89 ^b	0.031
Texture	3.58 $\pm$ 1.13 ^b	3.61 $\pm$ 1.10 ^b	3 $\pm$ 1.12 ^a	3.33 $\pm$ 0.96 ^{ab}	3.4 $\pm$ 1.26 ^{ab}	3.11 $\pm$ 1.3 ^{ab}	0.129
Salty Taste	3.71 $\pm$ 0.9 ^a	3.29 $\pm$ 0.96 ^{ab}	3.21 $\pm$ 1.12 ^b	3.14 $\pm$ 1.2 ^b	3.63 $\pm$ 1.17 ^{ab}	3.21 $\pm$ 1.23 ^b	0.109
Global Taste	3.84 $\pm$ 0.92 ^a	3.4 $\pm$ 0.79 ^{abc}	3.21 $\pm$ 0.99 ^{bc}	3.08 $\pm$ 1.08 ^c	3.61 $\pm$ 1.31 ^{ab}	3 $\pm$ 1.19 ^c	0.004
Global Acceptance	3.92 $\pm$ 0.75 ^a	3.53 $\pm$ 0.83 ^{abc}	3.37 $\pm$ 0.82 ^{bc}	3.25 $\pm$ 1.27 ^{bc}	3.71 $\pm$ 1.25 ^{ab}	3.184 $\pm$ 1.27 ^c	0.021

p-value Processing: One-way ANOVA between different processing conditions (*p*-value significant at $p \leq 0.05$).
 Attributes were scored assigning a numerical value through a verbal hedonic scale between 1 (I dislike very much)
 and 5 (I like very much).

*SEM: standard error of mean.

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ARTICLE 5:

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

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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 (Correa 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

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related to the different phases that take place during the curing process of the ham (Gallego et al., 2019; Heres, Gallego, et al., 2022; Hernández Correás 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; Hernández Correás 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 4000g 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:

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

*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

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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 inhibitory activity of 50 %, i.e. $IC_{50} = (50 - b)/a$, 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 μ L of ethanol, 500 μ L of sample, and 125 μ L of 0.02 % (w/v) DPPH solution were added. For the blank, 500 μ L of ethanol, 500 μ L of water, and 125 μ L of DPPH were used. Samples were incubated for 1 h in the dark at room temperature. The samples were then centrifuged at 10,000g for 2 min and, their absorbance was measured at 517 nm.

% DPPH Radical Scavenging Assay (RSA) = (Abs Blank - Abs Sample) / Abs Blank $\times$ 100.

To determine the IC_{50} — 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 μ m $\times$ 50 cm) Acclaim Pepmap column (Thermo Scientific). The previously obtained fraction was loaded onto a 300 μ m $\times$ 5 mm Acclaim Pepmap precolumn (Thermo Scientific) in a 2 % acetonitrile/0.05 % TFA mixture for 5 min at a flow rate of 5 μ L/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 UniProtKB 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 $\geq$ 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, 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/metascreeener>). 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 μ L of CaCl_2 (0.3 M) and 975 μ L of H₂O are added. The mixture is incubated at 37 $^\circ\text{C}$ 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 μ L of CaCl_2 (0.3 M) and 695 μ L of H₂O are added. The pH is adjusted to 3 by HCl. The mixture is incubated at 37 $^\circ\text{C}$ 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 μ L of CaCl_2 (0.3 M), 1.31 mL of H₂O and pH adjusted to 7 with NaOH are added. Incubation at 37 $^\circ\text{C}$ 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 μ L of 0.1 % (v/v) TFA. 10 μ L of each sample was cleaned and concentrated using standard bed format Zip-Tip C18 tip cartridges from Millipore Corporation. 4 μ L 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 μ L/min at 30 $^\circ\text{C}$.

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. S1 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.

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

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

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

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, & Toldrà, 2013; Sentandreu & Toldrà, 2001; Sentandreu & Toldrà, 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 Correia 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

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

DPPH (IC50)		
Salt Treatment	Processing Stage	
	4	5
I	1.079 $\pm$ 0.076 ^{cd}	0.927 $\pm$ 172.09 ^{defg}
III	0.906 $\pm$ 0.109 ^{defg}	0.879 $\pm$ 0.047 ^{bcde}
IV	0.682 $\pm$ 0.114 ^c	0.976 $\pm$ 0.036 ^{abdef}
VI	0.861 $\pm$ 0.046 ^{bedefg}	1.103 $\pm$ 0.226 ^g
VII	0.682 $\pm$ 0.1 ^{bcg}	0.795 $\pm$ 0.049 ^{bcde}
IX	1.05 $\pm$ 0.25 ^{ade}	0.692 $\pm$ 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 $p \leq 0.05$).

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 Correia 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.

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 Correia 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 (Hamedy 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	THI	TIV	TVI	TVII	TIIX
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. 1 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 biopeptides 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).

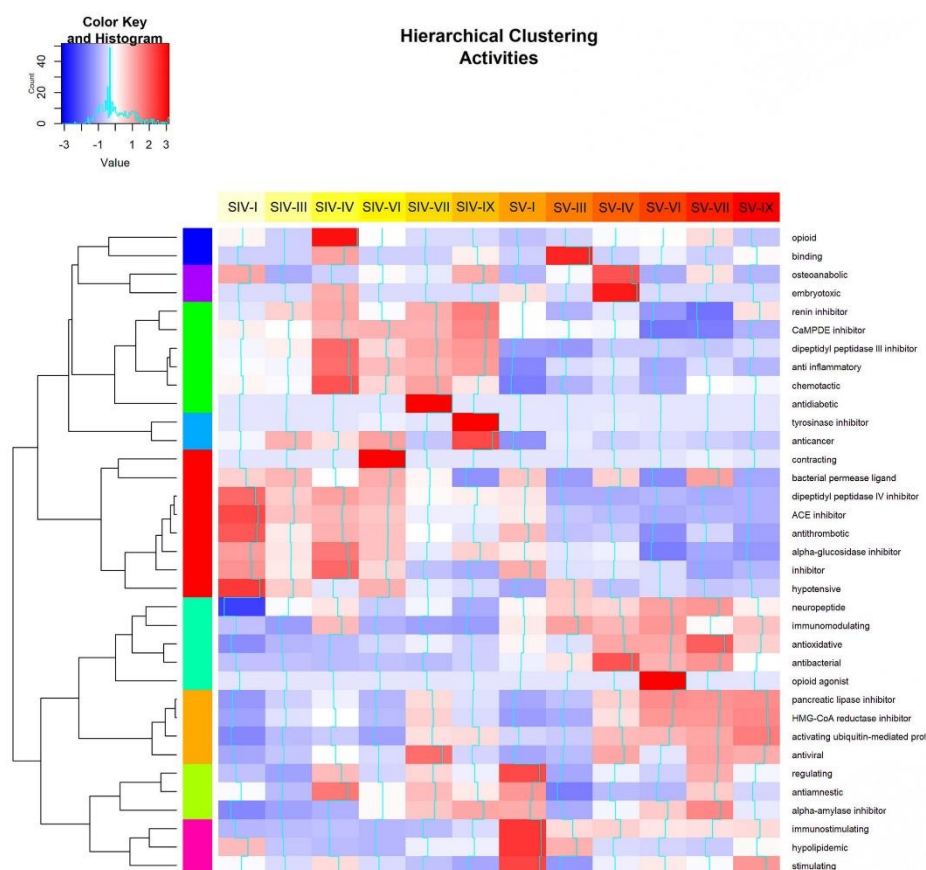


Fig. 1. Heatmap and dendrogram of bioactivities of the different ham samples studied. Quantification of bioactivity is regarding the mean. The grouping relationship between the groups of activities is defined.

Regarding the bioactivities evaluated *in vitro*, specifically the anti-hypertensive and antioxidant activities, it is highlighted that the anti-hypertensive 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 (Hamedy 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 FLGFPTTKTYFPHE, 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,

Table 4
Bioactivities predicted for complete peptides.

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

antioxidative activity was detected in the peptide LEQQVDDLEGSLEQEKK.

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

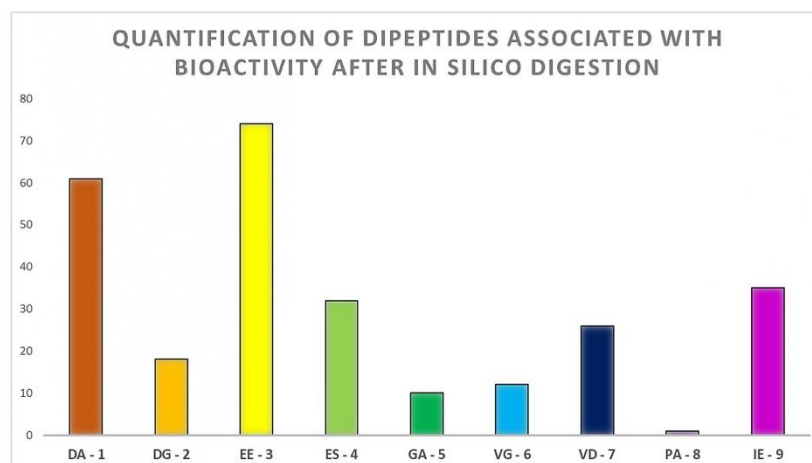


Fig. 2. Quantification of dipeptides associated with bioactivity after in silico digestion.

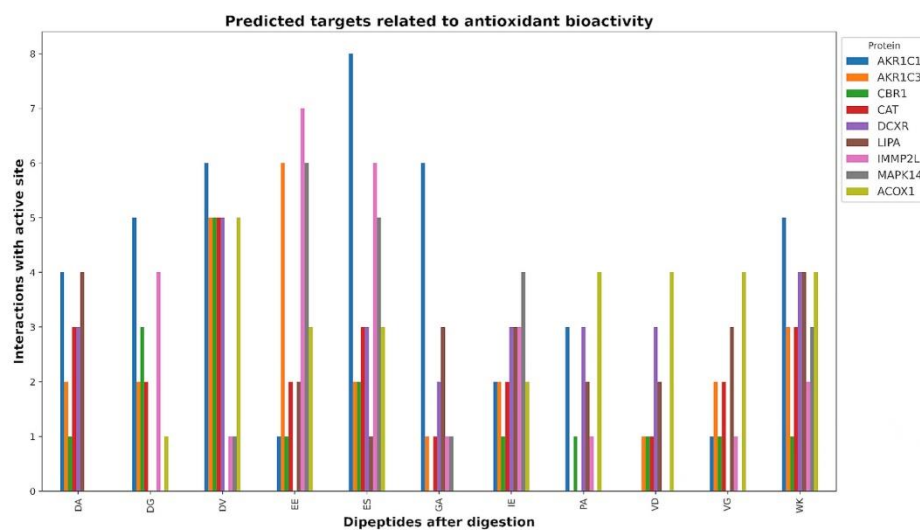


Fig. 3. Predicted targets related to antioxidant bioactivity.

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

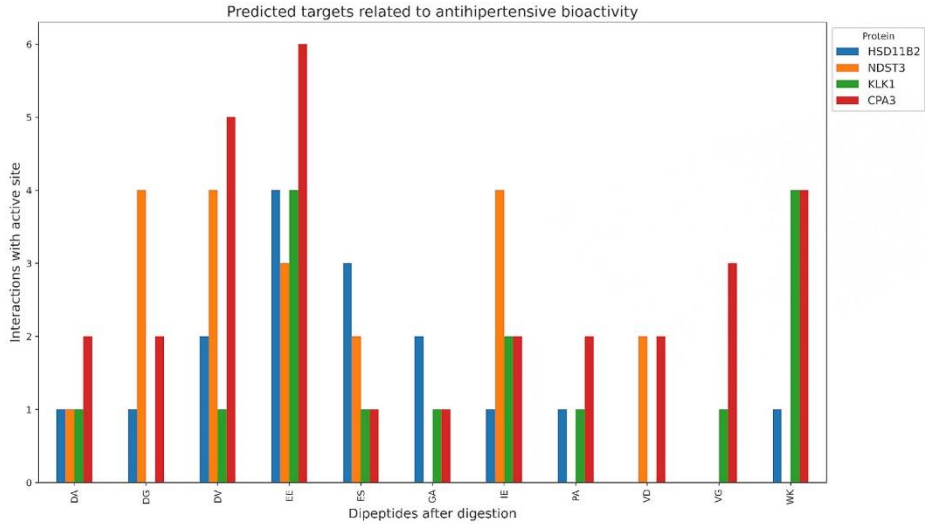


Fig. 4. Predicted targets related to antihypertensive bioactivity.

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
		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
		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
		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
		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
		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
		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
p-value	Salt Treatment	0.095	0.052	0.028	0.113	0.016	0.000	0.150	0.421	0.040	0.000
	Processing Stage	0.000	0.000	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.

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CRedit authorship contribution statement

Noelia Hernández Correás: 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.

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Declaration of competing interest

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

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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.

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ARTICLE 6:

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1 **Title:** Curing strategies for the production of a Spanish boneless dry-cured ham with up
2 to 50% less sodium: Effect on the physicochemical profile, mineral composition and on
3 sensory quality and acceptability.

4

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26 ABSTRACT

27 This study evaluated the feasibility of producing boneless dry-cured ham with reduced
28 sodium content by applying curing salts formulated with less sodium and using different
29 particle sizes. Three batches were produced: batch 1 (coarse salt, 50% sodium-reduced
30 formulation), batch 2 (medium-grain salt, 50% sodium-reduced formulation), and batch
31 3 (control, traditional curing treatment). The objective was to obtain a product with a 50%
32 reduction in sodium compared with conventional white dry-cured hams (2.1–2.35
33 g/100g). Batch 1 achieved a sodium content of 1.69 g/100 g ($\approx$ 40% reduction), whereas
34 batch 2 reached 1.19 g/100 g, corresponding to a reduction of nearly 50%. Reduced-
35 sodium hams maintained safe water activity (<0.91) and showed enhanced proteolysis
36 and release of free amino acids, particularly glutamate, leucine, glycine and proline,
37 compounds directly linked to umami and sweet taste. Textural changes were limited:
38 batch 1 showed lower cohesiveness, while batch 2 preserved a texture profile comparable
39 to the control. Sensory analysis revealed that sodium reduction did not compromise
40 consumer acceptance. Appearance and color were rated significantly higher in the
41 reduced-sodium batches, while no differences were found in odor, flavor, texture or
42 overall acceptability. These findings confirm that sodium reduction of up to 50% in
43 boneless dry-cured ham is technologically viable without the use of salt substitutes, while
44 maintaining product safety, stability and sensory quality.

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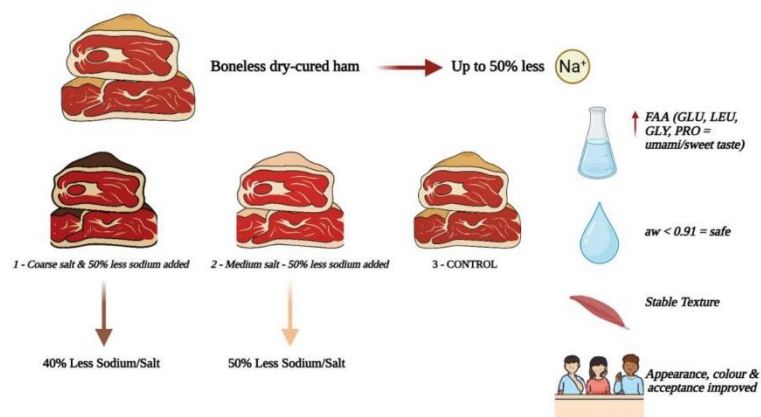
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51 GRAPHICAL ABSTRACT

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56 **Keywords:**

57 Boneless dry-cured ham; sodium reduction; salt particle size; physicochemical quality;

58 proteolysis; texture; sensory analysis.

59

60 INTRODUCTION

61 Traditional dry-cured ham is characterised by a high sodium content due to the intensive
62 use of common salt (NaCl) during its production. Salt performs multiple technological
63 functions: it acts as a preservative by reducing water activity (a_w) and inhibiting the
64 growth of microorganisms, contributes to the development of the characteristic salty
65 flavour, promotes the stability of the cured colour (through nitrite/nitrate nitrifying salts)
66 and affects texture by influencing the solubilisation and denaturation of myofibrillar
67 proteins (Bidlas & Lambert, 2008; Blesa et al., 2008). However, the high sodium content
68 in cured ham has been associated with negative health effects (hypertension and
69 cardiovascular disease), which has prompted efforts by the meat industry and the
70 scientific community to reduce the amount of salt added to these products (Desmond,
71 2006; Schivazappa & Virgili, 2020a). International health authorities recommend
72 reducing sodium intake in the population, and producing cured hams with less salt could
73 contribute to this goal without sacrificing a food of high gastronomic value.

74

75 Reducing salt in cured ham is a considerable challenge, as a decrease in NaCl can
76 compromise microbiological safety and alter quality properties such as flavour, aroma,
77 texture and colour (Andrés et al., 2004; Armenteros et al., 2012; Schivazappa & Virgili,
78 2020a). Despite this, various strategies have been explored in recent decades to achieve
79 moderate reductions in sodium in cured meat products. Studies on Iberian dry-cured ham
80 have shown that it is feasible to reduce the salt content by at least 25% by adjusting the
81 processing conditions without added nitrites, maintaining safety and obtaining a product
82 with a colour similar to the traditional one (Hernández Correás et al., 2024; Muñoz-
83 Rosique et al., 2022). Another common strategy has been the partial replacement of NaCl
84 with other salts less associated with health risks, mainly potassium chloride (KCl).

85 Controlled studies have reported that up to 50% of NaCl can be replaced by KCl in the
86 curing of hams, achieving safe and sensorially acceptable products, albeit with slight
87 bitter notes due to the potassium (Armenteros et al., 2012). Likewise, the addition of small
88 amounts of calcium and magnesium chlorides has been evaluated, although with adverse
89 effects on sensory properties when used in excess (Lorenzo et al., 2015). In general, these
90 previous studies with partial reductions (around 25%) indicate that it is possible to reduce
91 sodium in cured hams to a certain extent without seriously compromising quality,
92 especially if technological adjustments are made, such as prolonging the drying time to
93 compensate for the lower osmotic strength of the salt (Blesa et al., 2008; Pinna et al.,
94 2020). However, no scientific references have been found regarding reductions of around
95 50% in sodium in boneless cured hams, which highlights the innovation and relevance of
96 the present study.

97

98 In addition to the absolute salt content, another factor that has been little explored in the
99 literature is the grain size of the salt used during the curing process when burying the
100 hams and its possible influence on the process and the quality of the ham. In traditional
101 practice, coarse-grained salt is usually used for salting hams, as it is considered that fine
102 salt could penetrate too quickly, over-salting the surface and causing undesirable salt
103 gradients (Aheto et al., 2019). The particle size of salt affects its rate of dissolution and
104 diffusion in food. The speed at which salt penetrates ham meat depends largely on the
105 size of the salt crystals. Fine-grained salts, having a larger surface area, dissolve much
106 more quickly and penetrate the muscle faster than coarse-grained salts. This may indicate
107 that with fine salt it is possible to achieve the internal NaCl concentration necessary to
108 stabilize the ham more quickly, even allowing the use of a lower total amount of salt
109 without compromising preservation. On the other hand, coarse salt, which dissolves

110 slowly, usually requires higher doses or longer salting times to achieve the same final
111 salinity in the piece. In fact, studies on sodium reduction in meat products indicate that
112 “finer crystal salts can be used in lower concentrations” than conventional-sized salts.

113

114 The usual trend is to use coarse salt in traditional curing processes for more gradual and
115 controlled salting, although modern techniques using fine salt aim to accelerate diffusion
116 and reduce the final salt content in the product. With certain technologies, it is possible
117 to obtain very small crystals with a particular crystalline shape (cubic, dendritic, etc.) and
118 a large contact or exposed surface area (R.e, 1983, p. 2015). Aheto et al. (2019)
119 demonstrated using hyperspectral imaging techniques that fine salt crystals dissolve
120 quickly and achieve greater NaCl penetration in dry-cured pork than larger crystals. This
121 suggests that a fine-grained salt could accelerate the salting and dehydration process of
122 ham, while a coarse-grained salt would slow it down. The salting rate in turn influences
123 the kinetics of water loss and the activity of enzymes and microorganisms during curing.
124 For example, slower salting (coarse salt) means that the muscle remains at a higher water
125 activity for a longer initial period, which could allow for greater proteolysis and lipolysis
126 before the product dries completely; conversely, rapid salting (fine salt) can cause an
127 earlier drop in aw, slowing down enzymatic and microbial action sooner. Despite its
128 potential importance, there is little specific scientific literature on the effect of salt particle
129 size on cured meat products. Most studies on sodium reduction have focused on salt
130 substitutes or process adjustments rather than the physical form of salt. Therefore, this
131 work also seeks to provide novel data on how salt crystal size could modulate the ham
132 curing process.

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134 Therefore, the main objective of this study was to obtain a cured ham with a “reduced
135 sodium” nutritional profile (50% less Na). To this end, two technological strategies were
136 applied: firstly, reducing the sodium content in the formulation of the curing salt mixture
137 and, secondly, varying the grain size of the salt used during salting. These experimental
138 hams were compared with a reference product cured with a 25% reduction in sodium, a
139 formulation that is already considered feasible in the industry and has been described in
140 previous studies.

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159 MATERIAL AND METHODS

160 2.1 Sample preparation and collection

161 Dry-cured ham samples used in this study were provided by
162 AROMAIBERICASERRANA S.L., located in Balsapintada (Murcia, Spain). All hams
163 came from pigs with a genetic line of 50% White and 50% Duroc.

164 In order to study the effect of different levels of reduction in nitrite salt content and to
165 carry out a pre-industrial validation of the process, three experimental batches were
166 produced, each consisting of 450 hams (n = 150 per batch), with the following
167 characteristics:

168

169 **BATCH 1:** Nitrifying agents are used at a ratio of 0.7 g/kg (reduced to 50% compared to
170 standard processing), with the following composition: 64.3% sodium nitrite, 21.4%
171 sodium isoascorbate, 8.6% potassium nitrate, and 5.7% dextrose. The remaining 4.3 g/kg
172 of nitrifying salt to make up the 5 g/kg used is sodium-reduced NaCl. During the salting
173 process, the hams are buried in coarse-grained salt with 25% less sodium. The salting
174 process lasts for 0.8 days per kilogram of ham weight.

175

176 **BATCH 2:** Nitrifying agents are used at a ratio of 0.7 g/kg (reduced to 50% compared to
177 standard processing), with the following composition: 64.3% sodium nitrite, 21.4%
178 sodium isoascorbate, 8.6% potassium nitrate, and 5.7% dextrose. The remaining 4.3 g/kg
179 of nitrifying salt to make up the 5 g/kg used is sodium-reduced NaCl. During the salting
180 process, a salt with 25% less sodium and medium grain size is used to bury the hams. The
181 salting process lasts for 0.8 days per kilogram of ham weight.

182

BATCH 3 - CONTROL: Nitrifying agents are used at a ratio of 0.7 g/kg (reduced to 50% compared to standard processing), with the following composition: 64.3% sodium nitrite, 21.4% sodium isoascorbate, 8.6% potassium nitrate, and 5.7% dextrose. The remaining 4.3 g/kg of nitrifying salt to make up the 5 g/kg used is sodium-reduced NaCl. During the salting process, standard coarse-grained salt is used to bury the hams. The salting process takes place over 1 day per kilogram of ham weight. Batch 3 was used as a control, as it is the standard reference cured product.

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All hams were cured following the standard curing methodology used by the company, which has already been described in other studies (Hernández Correias et al., 2024; Muñoz-Rosique et al., 2022). Finally, 20 hams were selected from each batch (60 hams). Samples were taken at different stages, but the analyses presented correspond to samples taken from the final product, when the hams had lost between 38% and 40% of their initial fresh weight. Table 1 below summarises the curing conditions used.

Table 1. Summary of curing treatments used in the different batches.

	<i>BATCH 1</i>	<i>BATCH 2</i>	<i>BATCH 3</i>
NITRIFYING SALTS COMPOSITION	0.7g/Kg Nitrifying Agents 4.3g/Kg NaCl – 25% Sodium Reduced	0.7g/Kg Nitrifying Agents 4.3g/Kg NaCl – 25% Sodium Reduced	0.7g/Kg Nitrifying Agents 4.3g/Kg NaCl – 25% Sodium Reduced
BURYING SALT	Coarse-Grained 25% Less Sodium	Medium-Grained 25% Less Sodium	Coarse-Grained Standard Composition

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2.2 Physical-chemical analyses

The physicochemical parameters of the samples were analysed in triplicate for each of the selected samples. The moisture content was determined using the procedure described by Molina-Valero et al., 2024. The salt content was evaluated by applying the methodology used by Abellán et al., 2018. The Folch method was used to determine the

intramuscular fat of the Iberian hams Tejada et al., 2021. Water activity was measured by gravimetry following the procedure used by Muñoz-Rosique et al., 2022.

2.3 Proteolytic profile

The quantification of total nitrogen (TN) and non-protein nitrogen (NPN) was carried out using the Kjeldahl method described by Tejada et al., 2021. For the preparation of the NNP extract, we followed the methodology proposed by Correás et al., 2025. Crude protein value was calculated by multiplying the NT by the factor 6.25. The proteolysis index was obtained by expressing the ratio of non-protein nitrogen to total nitrogen as a percentage. The concentration of free amino acids (FAA) was determined according to the method described by Abellán et al., 2012. Nitrate and nitrite content was determined in SIPA by a validated method based on the Griess reaction, after reduction of nitrate to nitrite, according to ISO 14673-1/AOAC for meat products. The results were expressed in mg/kg sample.

2.4 Mineral composition

The mineral composition of the samples was analysed according to the methodology proposed by Tejada et al., 2021. For this purpose, 0.5 g of dry-cured ham (previously homogenised) was digested in a microwave system (Milestone 1200, FVK, Bergamo, Italy) with 3 mL of nitric acid (Romil Ltd., Cambridge, UK) and 0.5 mL of 30% Suprapur hydrogen peroxide (Merck, Germany). Afterwards, the digested material was brought to

a final volume of 50 mL with Milli-Q water and analysed at the corresponding wavelengths.

228

2.6 Instrumental Colour Determination and Texture Profile Analysis (TPA)

Instrumental colour determination was carried out by colorimetry using a colorimeter (HunterLab, Colorflex) under the CIELab system. The results were expressed in the coordinates L*, a* and b*, which correspond to the lightness, red-green scale and yellow-blue scale, respectively. Chroma (colour intensity) and hue angle (h*) values were also calculated. For each sample, the results were obtained as an average of three measurements. For instrumental texture analysis, a QTS-25 texturimeter (Brookfield CNS Farnell, Borehamwood, Hertfordshire, England), equipped with a 25 kg load cell and a 10 mm diameter probe, was used. Data interpretation was performed using Texture Pro v. 2.1. software. The biceps femoris muscle was cut into 10 x 10 x 10 mm blocks, taking three blocks per sample. The tests were carried out at a controlled room temperature of 20 °C. The procedure consisted of subjecting each block to two consecutive compression cycles, applied perpendicularly to the orientation of the muscle fibres, with a deformation of 50 % and at a constant speed of 30 mm/s. Finally, a texture profile analysis (TPA) was carried out to determine the parameters of hardness, deformation associated with hardness, adhesiveness, cohesiveness, elasticity (recoverable deformation), gumminess and chewiness.

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2.7 Sensory Analysis

For the sensory analysis of the samples, a consumer test was carried out with a panel of untrained tasters. The evaluation was carried out following the methodology proposed by (Salazar et al., 2014) with some adaptations. A total of 200 anonymous participants took

part on a voluntary basis, without having received any prior information or specific training. The study was carried out in a single session, in which the participants evaluated three different batches of ham, considering an age range between 18 and 55 years. The participants evaluated the samples using a questionnaire that included the hedonic evaluation of different attributes: appearance, colour, smell, texture, salty taste, overall taste and general acceptability. Each characteristic was scored on a verbal hedonic scale from 1 (I dislike very much) to 5 (I like very much). The samples consisted of whole slices of the cross-section of the piece, prepared according to the standards (UNE-ISO 6658:2019 *Análisis sensorial. Metodología. Guía general.*, 2023) and (UNE-ISO 4121:2006 *Análisis sensorial. Directrices para la util...*, 2023) and were identified with a three-digit random number. The results were obtained by calculating the average score given to each attribute by consumers. Likewise, to determine the preference between the three samples, the percentages of choice recorded by the consumer panel for each type of cured ham evaluated were calculated.

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2.8 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). The effect of salt reduction and particle size used during ham curing was obtained using a two-way ANOVA (*p-value interaction*). When the effect was significant ($p < 0.05$), the results were compared using Fisher's LSD test.

To evaluate how salt reduction affected the different analyses, a one-way ANOVA was performed between hams from batches 1 and 3 (*p-value salt reduction*). The effect of particle size was also evaluated using a one-way ANOVA between hams from batches 1 and 2 (*p-value salt size*).

276 RESULTS AND DISCUSSION

277 *3.1 Analysis of physicochemical and compositional parameters and proteolysis* 278 *evaluated in hams produced with different sodium concentrations and salt sizes.*

279 **Table 2** shows the results of the physicochemical, compositional and proteolysis
280 parameters of the hams produced with different salt formulations and particle sizes. The
281 parameters analysed of dry-cured ham showed notable changes associated with both
282 sodium reduction and salt particle size. Firstly, the interaction of both factors affected
283 water activity, sodium chloride percentage, non-protein nitrogen and proteolysis index.
284 Moisture content did not show significant differences ($p>0.05$) between treatments, with
285 values ranging from 43.7 to 46.4%. These results agree with previous reports on reduced-
286 salt hams, where changes in drying kinetics did not always result in marked differences
287 in final moisture (Muñoz-Rosique et al., 2022).

288 Respect to NaCl %, the batch with intermediate grain size salt and 50% sodium reduction
289 (Batch 2) had a final NaCl content of 2.17 g/100 g, compared to the usual range for white
290 cured hams produced using conventional methods (5.5–6 g/100 g, ANICE, 2017;
291 Schivazappa & Virgili, 2020). In batch 1 (coarse salt with 50% sodium reduction), the
292 final NaCl content was 3.16 g/100 g, representing a decrease of close to 40% compared
293 to conventional white cured hams. This decrease confirms that the strategy applied
294 (reformulation of the salting salt with a lower sodium concentration) allows the nutritional
295 claim of “reduced sodium content” ($\geq 25\%$ reduction) to be achieved, exceeding it by up
296 to 50% in batch 2 without the need for substitute salts. In contrast, in the control (batch
297 3), the reduction in salt cannot be considered significant because, although nitrifying
298 rubbing salts with 25% less sodium were used, the curing process was carried out
299 following the traditional methodology with standard coarse salt, resulting in final NaCl
300 values (4.56 g/100 g) characteristic of conventional ham.

Regarding water activity, this was predictable, since a lower incorporation of NaCl reduces the osmotic force that extracts water from muscle tissue. In fact, Blesa et al. (2008) observed that reduced hams required more time in post-salting to reach aw values like those of standard salted hams. In our study, at the end of maturation, the water activity (aw) of the hams with the most sodium reduction (batches 1 and 2) fell to safe levels (0.91 and 0.89 respectively), although it remained slightly higher than that of the control (0.87). This threshold is considered safe for inhibiting the growth of pathogens in dry-cured hams (Blesa et al., 2008; Schivazappa & Virgili, 2020). This slight variation in water activity between the control and the hams with lower sodium content (1 and 2) is consistent with the findings reported by Pinna et al. (2020), who, in order to achieve the same water activity as traditional ham and thus guarantee product safety, had to prolong the cold drying phase in hams cured with 25% less salt.

Table 2. Effect of different salt treatments on physicochemical parameters, compositionals and proteolysis of the ham during the final stage. Results are expressed in g/100 g of product (means values $\pm$ SEM).

	Salt Treatment			Salt Reduction	<i>p-value</i>	
	1	2	3		Salt Size	Interaction
Moisture	46.423 $\pm$ 3.6 ^a	44.543 $\pm$ 4.50 ^a	43.70 $\pm$ 2.56 ^a	0.257	0.179	0.229
NaCl	3.16 $\pm$ 0.316 ^b	2.788 $\pm$ 0.364 ^c	4.563 $\pm$ 0.35 ^a	0.000	0.045	0.000
Aw	0.915 $\pm$ 0.01 ^a	0.890 $\pm$ 0.010 ^b	0.87 $\pm$ 0.011 ^b	0.003	0.015	0.000
Ph	6.209 $\pm$ 0.18 ^a	6.038 $\pm$ 0.107 ^a	6.04 $\pm$ 0.085 ^a	0.083	0.075	0.123
Fat	14.94 $\pm$ 3.25 ^b	16.70 $\pm$ 3.446 ^a	17.64 $\pm$ 3.50 ^a	0.046	0.508	0.294
Protein	30.59 $\pm$ 1.98 ^a	30.81 $\pm$ 5.696 ^a	31.29 $\pm$ 3.14 ^a	0.362	0.920	0.935
Non-Protein Nitrogen	0.578 $\pm$ 0.09 ^b	0.683 $\pm$ 0.064 ^a	0.736 $\pm$ 0.07 ^a	0.010	0.041	0.009
Proteolysis Index (%)	11.81 $\pm$ 0.18 ^b	14.96 $\pm$ 0.24 ^a	14.84 $\pm$ 0.07 ^a	0.000	0.021	0.000

SEM*: standard error of mean.

p-value Salt Reduction: One-way ANOVA between 1 and 3; *p-value* Salt Size: One-way ANOVA between 1 and 2; *p-value* Interaction: two-way ANOVA between 1, 2 and 3.

313

314 In terms of pH, no significant differences ($p>0.05$) were observed between treatments.

315 The final pH of all hams was around 5.8–6.0, consistent with typical values for dry-cured

316 ham (Hernández Correás et al., 2024). The reduction in salt did not substantially alter the
317 muscle pH, which was to be expected given that the pH in ham depends more on initial
318 post-mortem acidity and minor fermentation processes than on salt concentration. A slight
319 decrease in pH was recorded between all batches, attributable to the production of acidic
320 compounds (e.g., conversion of nitrate to nitrite and formation of nitric acid, or release of
321 acidic amino acids during proteolysis). However, the reduction in sodium and particle
322 size did not significantly affect pH, suggesting that the biochemical processes that
323 modulate pH occurred similarly in both treatments. Fat content differed significantly
324 ($p<0.05$), with the control showing the highest intramuscular fat content (17.6%),
325 followed by batch 2 (16.7%) and batch 1 (14.9%). These differences may reflect raw
326 material variability rather than a direct effect of the salting formulation, as lipid content
327 is not strongly influenced by sodium concentration. Protein content did not differ
328 significantly between treatments ($p>0.05$), with average values between 30 and 31%, in
329 agreement with previous studies in which reduced salt did not alter the overall protein
330 composition of cured ham (Muñoz-Rosique et al., 2022).

331 Non-protein nitrogen (NPN) was significantly affected by sodium concentration
332 ($p<0.05$). Batch 1 showed the lowest value (0.578), whereas batch 2 (0.683) and the
333 control (0.736) presented higher levels. This reflects the influence of reduced sodium on
334 proteolytic enzyme activity, which is less inhibited under lower salinity conditions.

335 Finally, proteolysis index showed significant differences ($p<0.05$). Batch 1 (50% less
336 sodium with coarse salt) had a significantly lower value (11.8%), while both the control
337 (14.8%) and batch 2 (14.9%) showed higher levels. This indicates that the main effect
338 comes from the sodium concentration, since the control and the reduced batch with
339 medium-grain salt achieved practically the same degree of proteolysis. Therefore, our
340 results do not show a clear effect attributable to salt particle size on proteolysis, but rather

341 to the overall reduction in NaCl. These results are particularly relevant, since although
342 the proteolysis index was lower in batch 1, the hams from batch 2 exhibited proteolysis
343 values comparable to those of the control. This indicates that the reduction in sodium did
344 not promote excessive proteolysis and, consequently, is unlikely to generate undesirable
345 texture defects typically associated with extensive protein degradation in low-salt muscles
346 (Pinna et al., 2020).

347

348 *3.2 Analysis of mineral content in hams produced under different sodium*
349 *concentrations and salt sizes.*

350 The results for mineral content are shown in **Table 3**. The mineral profile of the hams
351 clearly reflected the modifications in the curing salts. Clear differences were observed
352 between treatments (Table 3) in the sodium (Na) content on the final product. In the
353 control (batch 3, traditional coarse salt), sodium concentration reached 2.17 g/100 g,
354 which is consistent with the values commonly reported for conventional white hams (2.1–
355 2.35 g/100 g; Armenteros et al., 2012). In batch 1 (coarse salt with 50% sodium
356 reduction), sodium levels decreased to 1.69 g/100 g, corresponding to an approximate
357 reduction of 25–30% compared with the conventional range. In batch 2 (medium-grain
358 salt with 50% sodium reduction), sodium concentration was reduced even further to 1.19
359 g/100 g, which represents a reduction close to 45–50%. These results confirm that the
360 nutritional claim of “reduced sodium content” ($\geq 25\%$ reduction compared with a
361 conventional product) was achieved in both experimental batches, with batch 2 reaching
362 the targeted 50% reduction. Previous studies had achieved smaller reductions: for
363 example, Muñoz-Rosique et al., 2022 achieved about 25% less sodium in boneless Iberian
364 hams through process adjustments, and Pinna et al. 2020 reported a 25% reduction in

Italian ham by adjusting cold stages, but there are no published data on reductions of the order of 50%.

Table 3. Effect of different salt treatments on minerals content during the final stage of the ham. Results are expressed as means values $\pm$ SEM*.

	Salt Treatment			<i>p-value</i>		
	1	2	3	Salt Reduction	Salt Size	Interaction
Na ¹	1.685 $\pm$ 0.46 ^{ab}	1.19 $\pm$ 0.372 ^b	2.17 $\pm$ 0.453 ^a	0.016	0.012	0.032
K ¹	0.618 $\pm$ 0.16 ^a	0.305 $\pm$ 0.075 ^b	0.52 $\pm$ 0.258 ^{ab}	0.015	0.147	0.092
Ca ¹	0.013 $\pm$ 0.005 ^a	0.01 $\pm$ 0 ^a	0.01 $\pm$ 0 ^a	0.160	0.012	0.405
Mg ¹	0.03 $\pm$ 0 ^a	0.03 $\pm$ 0 ^a	0.038 $\pm$ 0.01 ^a	-	0.356	0.141
P ¹	0.245 $\pm$ 0.006 ^a	0.203 $\pm$ 0.041 ^a	0.255 $\pm$ 0.044 ^a	0.168	-	0.131
Fe ²	16.23 $\pm$ 3.51 ^{ab}	13.98 $\pm$ 1.473 ^b	18.15 $\pm$ 2.037 ^a	0.130	0.086	0.113
Cu ²	1.953 $\pm$ 0.494 ^a	2.325 $\pm$ 1.022 ^a	2.503 $\pm$ 0.589 ^a	0.016	0.282	0.580
Mn ²	0.173 $\pm$ 0.058 ^a	0.173 $\pm$ 0.017 ^a	0.215 $\pm$ 0.019 ^a	0.774	0.536	0.225
Zn ²	21.525 $\pm$ 3.5 ^a	22.375 $\pm$ 4.277 ^a	27.075 $\pm$ 6.76 ^a	0.016	1	0.294
B ²	0.675 $\pm$ 0.275 ^a	0.785 $\pm$ 0.436 ^a	0.7 $\pm$ 0.182 ^a	0.284	0.768	0.877
Total Minerals	6.59 $\pm$ 0.594 ^a	5.33 $\pm$ 0.393 ^a	7.085 $\pm$ 0.983 ^a	0.731	0.684	0.017

SEM: standard error of mean.

¹ Results are expressed in g/100 g of product

² Results are expressed in mg/100 g of product

p-value Processing: One-way ANOVA between different salt treatments (p-value significant at $p \leq 0.05$).

Along with the decrease in sodium, the size of the salt significantly affected the concentration of potassium ions (K), which increased significantly in hams with intermediate grain size (Batch 2). Although KCl was not added as a substitute for NaCl, it is possible that the size of the salt increased the perfusion of other ions, and it is also possible that the presence of potassium nitrites raised K. This increase is technologically beneficial because potassium, like sodium, contributes to low water activity and microbiological stability (Bidlas & Lambert, 2008). From a nutritional point of view, a higher potassium content can be beneficial, as K counteracts some of the effects of sodium in the diet. No significant differences were found in other major minerals such as calcium (Ca) and magnesium (Mg) between the treatments. The Ca and Mg values in all hams remained low, with no changes attributable to the formulation or particle size of the salt. This was to be expected given that no calcium salts were added.

379 The particle size of the burial salt did not have a marked influence on the final mineral
380 composition of the hams. The decrease in sodium did affect copper (Cu) levels, which
381 increased (Batch 3), and zinc (Zn) levels, which also increased significantly.

382

383 *3.3 Analysis of free amino acids*

384 The results obtained in the free amino acid analysis are shown in **Table 4**. The content
385 and profile of free amino acids in dry-cured ham can be considered a direct indicator of
386 the extent of proteolysis during maturation, since endogenous proteases progressively
387 degrade muscle proteins into peptides and free amino acids. In our study, both reduced-
388 sodium batches (batches 1 and 2) showed higher total FAA concentrations compared with
389 the control, with batch 2 (50% sodium reduction with medium-grain salt) presenting the
390 highest levels. Interestingly, batch 1 (50% sodium reduction with coarse salt) displayed a
391 lower proteolysis index than the other treatments (Table 2) but still accumulated more
392 FAA than the control. This indicates that the release of amino acids does not depend
393 exclusively on the overall proteolysis index, but also on the specific enzymatic pathways
394 involved and the salting dynamics. It should also be considered that part of the FAA
395 accumulation may result from microbial activity, since bacterial peptidases are active
396 during ham maturation and can contribute to peptide hydrolysis (Heres et al., 2023;
397 Lorenzo et al., 2015; Toldrá et al., 2000). Thus, the combined action of endogenous
398 enzymes and the microbiota explains the variability observed among treatments.
399 Importantly, in none of the batches did proteolysis reach excessive levels that could
400 compromise texture or cause the accumulation of undesirable bitter amino acids.

401 The content and profile of free amino acids (FAA) in dry-cured ham are intimately
402 associated with the development of umami flavor and the characteristic matured aroma
403 of the final product. (You et al., 2024). Among them, glutamate is recognised as a key

umami contributor, while other amino acids such as lysine and leucine, together with small peptides, modulate taste intensity and overall flavour complexity (Mora & Toldrá, 2025). In our study, the higher FAA concentrations found in batches 1 and 2 are particularly relevant, since compounds such as glutamate, leucine and lysine are directly linked to umami perception and flavour intensity in cured meats (Ju et al., 2024; Mora & Toldrá, 2025). The accumulation of these compounds under reduced-salt conditions can therefore contribute to the overall acceptability of the product, compensating for the decrease in saltiness by enhancing other flavour dimensions.

Table 4. Effect of different salt treatments on free amino acids content during the final stage of the ham. Results are expressed in $\mu\text{mol/g}$ of dry matter as means values $\pm$ SEM*.

	Salt Treatment			Salt Reduction	<i>p-value</i>	
	1	2	3		Salt Size	Interaction
Ala	2 $\pm$ 0.318 ^a	1.953 $\pm$ 0.314 ^a	1.635 $\pm$ 0.298 ^a	0.145	0.838	0.247
Arg	0.613 $\pm$ 0.144 ^a	0.598 $\pm$ 0.113 ^a	0.6 $\pm$ 0.166 ^a	0.913	0.875	0.987
Asn	0.863 $\pm$ 0.079 ^a	0.835 $\pm$ 0.058 ^a	0.873 $\pm$ 0.069 ^a	0.855	0.595	0.739
Asp	0.503 $\pm$ 0.092 ^a	0.53 $\pm$ 0.08 ^a	0.54 $\pm$ 0.088 ^a	0.576	0.667	0.821
Cys	< 2.5	< 2.5	< 2.5	-	-	-
Glu	0.623 $\pm$ 0.086 ^a	0.59 $\pm$ 0.109 ^a	0.578 $\pm$ 0.09 ^a	0.497	0.656	0.794
Gln	0.738 $\pm$ 0.104 ^b	0.75 $\pm$ 0.032 ^b	0.875 $\pm$ 0.024 ^a	0.041	0.825	0.026
Gly	0.58 $\pm$ 0.047 ^a	0.628 $\pm$ 0.028 ^a	0.54 $\pm$ 0.091 ^a	0.465	0.131	0.186
His	0.5 $\pm$ 0.016 ^a	0.505 $\pm$ 0.026 ^a	0.453 $\pm$ 0.051 ^a	0.127	0.758	0.113
Ile	0.875 $\pm$ 0.033 ^b	0.903 $\pm$ 0.043 ^b	0.738 $\pm$ 0.101 ^a	0.041	0.348	0.014
Leu	1.21 $\pm$ 0.082 ^b	1.235 $\pm$ 0.058 ^b	1.045 $\pm$ 0.129 ^a	0.073	0.636	0.038
Lys	0.518 $\pm$ 0.056 ^a	0.495 $\pm$ 0.079 ^a	0.43 $\pm$ 0.074 ^a	0.109	0.658	0.243
Met	0.54 $\pm$ 0.045 ^{ab}	0.575 $\pm$ 0.045 ^a	0.465 $\pm$ 0.068 ^b	0.115	0.316	0.047
Phe	0.71 $\pm$ 0.079 ^{ab}	0.745 $\pm$ 0.07 ^a	0.61 $\pm$ 0.098 ^b	0.163	0.532	0.111
Pro	0.955 $\pm$ 0.044 ^a	0.898 $\pm$ 0.058 ^{ab}	0.83 $\pm$ 0.062 ^b	0.016	0.168	0.033
Ser	0.433 $\pm$ 0.048 ^a	0.468 $\pm$ 0.059 ^a	0.458 $\pm$ 0.059 ^a	0.532	0.392	0.667
Thr	0.428 $\pm$ 0.077 ^a	0.48 $\pm$ 0.073 ^a	0.368 $\pm$ 0.085 ^a	0.336	0.361	0.185
Trp	0.903 $\pm$ 0.064 ^{ab}	1.083 $\pm$ 0.091 ^a	0.863 $\pm$ 0.162 ^b	0.662	0.017	0.050
Tyr	0.518 $\pm$ 0.049 ^a	0.415 $\pm$ 0.113 ^a	0.403 $\pm$ 0.095 ^a	0.075	0.146	0.195
Val	1.135 $\pm$ 0.024 ^a	1 $\pm$ 0.142 ^{ab}	0.908 $\pm$ 0.128 ^b	0.013	0.109	0.051
Total FAA	14.65	14.67	13.21	0.012	0.593	0.261

SEM*: standard error of mean.

p-value Salt Reduction: One-way ANOVA between 1 and 3; *p-value* Salt Size: One-way ANOVA between 1 and 2; *p value* Interaction: two-way ANOVA between 1, 2 and 3.

413 When analysing individual FAA, several compounds with a well-established role in taste
414 perception showed significant differences between treatments. Salt reduction had a
415 significant effect ($p < 0.05$) on glycine, isoleucine, proline and valine, all of which are
416 associated with sweet or umami-related notes that contribute to flavour balance in dry-
417 cured hams. Glycine, for example, has been reported to provide mild sweetness and
418 participate in meaty flavour development through Maillard reactions (Ma et al., 2020),
419 while isoleucine and valine, two branched-chain amino acids, are recognised contributors
420 to flavour intensity and complexity (You et al., 2024). Proline, beyond its structural role,
421 also contributes to a mild sweet taste in cured meats (López-Pedrouso et al., 2019). In
422 addition to the umami-related amino acids, the reduced-sodium hams also showed a
423 higher proportion of neutral and sweet-tasting amino acids (Batch 1 and 2), including
424 glycine (aforementioned), alanine, serine and threonine. These compounds are known to
425 contribute to a sweeter or milder flavour perception, which may partly compensate for
426 the reduction in saltiness. This trend has been reported previously in dry-cured hams with
427 reduced salt content, where proteolytic intensification increased the concentration of
428 sweet amino acids and slightly attenuated the overall perception of reduced saltiness
429 (Fang & Zhu, 2025; Toldrá et al., 2000). Moreover, the interaction between sodium
430 reduction and salt particle size was significant ($p < 0.05$) for isoleucine, leucine,
431 methionine and proline, indicating that in these cases both sodium level and diffusion
432 dynamics influenced amino acid release. Notably, leucine and isoleucine were
433 consistently higher in the reduced-salt treatments, reinforcing their contribution to umami
434 and overall flavour intensity, as has also been described in recent studies on dry-cured
435 hams (Furuhashi & Takeuchi, 2025; Tian et al., 2022).

436 The effect of salt particle size alone was limited, with significant differences ($p < 0.05$)
437 detected only for tryptophan, although its concentration remained within the normal range

438 for dry-cured hams. In contrast, amino acids typically associated with bitter notes, such
439 as histidine and arginine, did not show significant changes between treatments and did
440 not accumulate to levels that could negatively affect flavour. This confirms that the
441 increase in FAA observed in the reduced-sodium batches corresponded to a favourable
442 amino acid profile, enhancing umami, sweetness and flavour complexity, without leading
443 to undesirable sensory attributes (Mora & Toldrá, 2025).

444

445 Results for nitrate and nitrite content are presented in **Table 5**. Nitrite salts are essential
446 additives in ham curing, responsible for the formation of a stable pink colour and the
447 inhibition of pathogens such as *Clostridium botulinum*. In this study, the reformulation
448 included a reduction in sodium in the dose of nitrifying salts added at the beginning of
449 the process, as well as a reduction in sodium in the coating salt and a change in particle
450 size. Analyses of residual nitrite (NaNO_2) and nitrate (NaNO_3) at the end of the process
451 showed significantly lower levels in hams with lower sodium content (Batch 1 and 2).
452 These final concentrations comply with the food regulation to be implemented from
453 October 2025, which will limit residual nitrite (NaNO_2) to <65 mg/kg and 150 mg/kg
454 for nitrate (NaNO_3) in the final product (*Commission Decision (EU) 2024/1225 of*
455 *30 April 2024 Concerning National Provisions Notified by Denmark on the Addition of*
456 *Nitrite to Certain Meat Products (Notified under Document C(2024) 2669), s. f.*).

457 The marked reduction in residual nitrate can be attributed to greater microbial conversion
458 of nitrate to nitrite during curing in a low-salt environment. In environments with lower
459 salinity, nitrate-reducing bacteria (genera *Micrococcus*, coagulase-negative
460 staphylococci, etc.) may be more active, consuming nitrate more rapidly (Desmond,
461 2006). In addition, a slightly higher a_w in hams with lower sodium content at the outset
462 may have favored this early biological reduction in nitrate.

Table 5. Effect of different salt treatments on NaNO₂ during the final stage of the ham. Results are expressed in mg/Kg of dry matter as means values $\pm$ SEM*.

	Salt Treatment			<i>p-value</i>		
	1	2	3	Salt Reduction	Salt Size	Interaction
NaNO ₂	< 2.5	< 2.5	< 2.5	-	-	-
NaNO ₃	49.75 $\pm$ 0.975a	42.25 $\pm$ 0.47b	54.25 $\pm$ 0.92a	0.010	0.000	0.000

SEM: standard error of mean.

p-value Salt Reduction: One-way ANOVA between 1 and 3; *p-value* Salt Size: One-way ANOVA between 1 and 2; *p value* Interaction: two-way ANOVA between 1, 2 and 3.

463

464 As for residual nitrite, all hams showed very low values after long maturation (several
 465 months), as the added or generated nitrite is consumed in colour-fixing reactions and
 466 natural decay (Toldrá et al., 2009). This suggests that the nitrite dynamics reached their
 467 full course in all cases. Pinna et al. 2020 reported a similar finding: they achieved hams
 468 with normal colour without adding nitrite (using only nitrate and prolonging drying)
 469 despite having reduced salt. This supports the idea that, with optimised processes, it is
 470 possible to reduce the amount of nitrifying salts without sacrificing colour.

471

472 3.2 Instrumental Color and Texture Profile

473 The instrumental colour results are presented in Table 6. Colour is a critical attribute of
 474 cured ham, both for its visual appeal (that characteristic red-pink hue) and for its
 475 association with proper curing and product safety. The results showed that sodium
 476 reduction only significantly affected one of the defined parameters. Specifically, no
 477 significant differences were observed in the a* (red) value, with similar results obtained
 478 in all three batches. This indicates that the development of the pink nitrous pigment
 479 (nitrosylhaemoglobin) occurred adequately even with a lower sodium content and the
 480 particle salt size.

481

Table 6. Effect of different salt treatments on instrumental color during the final stage of the ham. Results are expressed in $\mu\text{mol/g}$ of dry matter as means values $\pm$ SEM*.

	Salt Treatment			<i>p-value</i>		
	1	2	3	Salt Reduction	Salt Size	Interaction
Lightness (L*)	51.33 $\pm$ 3.75 ^a	50.35 $\pm$ 2.96 ^a	48.61 $\pm$ 5.77 ^a	0.283	0.572	0.459
Redness (a*)	22.65 $\pm$ 2.732 ^a	23.91 $\pm$ 2.17 ^a	24.89 $\pm$ 1.39 ^a	0.057	0.324	0.142
Yellowness (b*)	24.33 $\pm$ 3.468 ^a	24.85 $\pm$ 2.32 ^a	23.534 $\pm$ 3.34 ^a	0.648	0.731	0.697
Chroma (C*)	33.62 $\pm$ 2.89 ^a	34.75 $\pm$ 2.53 ^a	34.64 $\pm$ 1.747 ^a	0.407	0.419	0.599
Hue angle (h°)	46.31 $\pm$ 5.5 ^a	45.79 $\pm$ 3.51 ^a	42.898 $\pm$ 5.23 ^a	0.224	0.827	0.332

SEM*: standard error of mean.

p-value Salt Reduction: One-way ANOVA between 1 and 3; *p-value* Salt Size: One-way ANOVA between 1 and 2; *p-value* Interaction: two-way ANOVA between 1, 2 and 3.

482

483 Pinna et al. (2020) had already observed that hams with 25% less salt and cured without
 484 nitrite had a colour equivalent to that of conventional hams, attributing the success to an
 485 adjustment in the process (drying extension) that allowed the colour curing reactions to
 486 be completed (Costa-Corredor et al., 2009). The same occurred in other studies where,
 487 despite the reduction in salt, the values were not significantly altered (Hernández Correas
 488 et al., 2024; Muñoz-Rosique et al., 2022). Certain studies have reported slight changes in
 489 hue with salt reduction: for example, Schivazappa and Virgili., 2020 found that salt
 490 reduction tended to increase lightness (L) and slightly decrease redness (a), possibly due
 491 to a higher water content in the reduced-salt product. In our study, this trend was not
 492 observed; rather, the variations were the opposite. Aliño et al., 2010 mentioned that the
 493 color differences recorded when modifying the curing salt were mainly due to differences
 494 in the water content of the hams. Our findings agree with this explanation: the subtle color
 495 variations between treatments seem to be related to different water contents (and,
 496 therefore, pigment concentration) rather than to the presence or absence of salt.
 497 The grain size of the salt had no significant effect on the final instrumental colour of the
 498 ham. Both hams cured with fine salt and those cured with coarse salt achieved a uniform

internal colour. These results are important because they demonstrate that it is possible to maintain the colour quality of cured ham even with a drastic reduction in salt. Results obtained from the instrumental texture measurement are presented in **Table 7**. The texture measurements revealed clear differences between hams with different salt treatments and particle sizes.

Table 7. Effect of different salt treatments on instrumental texture of the ham during the final stage. Results are expressed as means values $\pm$ SEM*

	Salt Treatment			Salt Reduction	<i>p-value</i>	
	1	2	3		Salt Size	Interaction
C1 Hardness (N)	7.207 $\pm$ 1.86 ^b	6.15 $\pm$ 2.2 ^a	6.81 $\pm$ 2.02 ^{ab}	0.059	0.048	0.111
C1 Hardness Deformation	3.368 $\pm$ 0.12 ^a	2.98 $\pm$ 0.006 ^a	2.97 $\pm$ 0.007 ^a	0.336	0.419	0.415
C1 Adhesiveness (mJ)	0.56 $\pm$ 0.994 ^a	0.48 $\pm$ 0.447 ^a	0.509 $\pm$ 0.14 ^a	0.339	0.650	0.535
C2 Cohesiveness	6.07 $\pm$ 3.55 ^b	7.83 $\pm$ 2.03 ^{ab}	7.1 $\pm$ 3.79 ^a	0.046	0.147	0.091
C2 Recoverable deformation (mm)	0.73 $\pm$ 1.793 ^a	0.61 $\pm$ 0.048 ^a	0.638 $\pm$ 0.04 ^a	0.314	0.488	0.364
C2 Springiness	1.359 $\pm$ 0.32 ^a	1.279 $\pm$ 0.18 ^a	1.33 $\pm$ 0.12 ^a	0.817	0.301	0.790
C2 Gumminess (N)	2.1 $\pm$ 0.318 ^a	2.1 $\pm$ 0.145 ^a	2.11 $\pm$ 0.146 ^a	0.874	0.525	0.978
C2 Chewiness (mJ)	7.56 $\pm$ 2.116 ^a	8.88 $\pm$ 2.297 ^a	7.02 $\pm$ 2.747 ^a	0.064	0.161	0.137
C2 Hardness (N)	10.11 $\pm$ 0.13 ^a	11.17 $\pm$ 0.2 ^a	9.49 $\pm$ 0.69 ^a	0.094	0.111	0.180

SEM*: standard error of mean.

p-value Salt Reduction: One-way ANOVA between 1 and 3; *p-value* Salt Size: One-way ANOVA between 1 and 2; *p value* Interaction: two-way ANOVA between 1, 2 and 3.

Hardness (cycle 1) showed a slight trend towards lower values in the reduced-sodium batches, particularly batch 2 (6.15 N), compared with batch 1 (7.21 N) and the control (6.81 N), although the difference was only marginally significant ($p = 0.059$). This suggests that the reduction in salt may have led to a softer texture, in line with the higher moisture contents observed in the reduced-sodium hams. Studies in boneless, salt-reduced hams report that salt reduction and deboning can affect hardness/elasticity, yet without compromising consumer acceptance, aligning with our observation (Muñoz-Rosique et al., 2022). Cohesiveness was significantly affected ($p < 0.05$) by the salt reduction, with

batch 1 (6.07) presenting lower values than both batch 2 (7.83) and the control (7.10). This indicates that the 50% sodium reduction with coarse salt reduced the structural integrity of the product to a greater extent than the other treatments. Classic studies linking NaCl, water content, and proteolysis to texture have shown that muscles with lower salt content tend to have lower hardness, cohesion, and elasticity, especially at higher pH levels, and that greater proteolysis is associated with softer and more cohesive textures, which fits our trend (Ruiz-Ramírez et al., 2006; Serra et al., 2005). By contrast, parameters such as gumminess, chewiness, springiness and adhesiveness did not show significant differences ($p > 0.05$) between batches, suggesting that the global texture profile was preserved despite the reduction in sodium. Most recent studies also indicate that a moderate reduction in NaCl may even improve consumer acceptance, while only subtly modifying the instrumental texture, consistent with our finding that the overall texture profile was largely preserved despite a 50% reduction in sodium in the experimental batches (Schivazappa & Virgili, 2020b).

The effect of salt particle size alone was minor. The only significant difference ($p < 0.05$) attributable to granulometry was observed in hardness during the first compression cycle, where hams from batch 2 (medium grain salt) were softer than those from batch 1 (coarse salt). This could be related to slightly faster NaCl diffusion with medium-grain salt, leading to more homogeneous dehydration and a less firm structure. However, other parameters remained unaffected, confirming that salt particle size did not substantially alter the texture once the curing time was completed.

Finally, comparative studies on low-salt cured sausages highlight the fundamental role played by water activity, moisture, and proteolysis in changes in TPA. Under controlled processing conditions, a significant reduction in sodium does not necessarily result in an undesirable texture, supporting our observation that the softening effect

(hardness/cohesion) is moderate and manageable within standard curing times (Martuscelli et al., 2015)

3.3 Sensory analysis

The results of the sensory analysis are presented in Figure 2. Overall consumer acceptance was not negatively affected by the curing conditions used, which is a very relevant finding of this study. Results showed that consumer perception of appearance and color was significantly affected by salt reduction ($p < 0.05$). Both reduced-sodium batches (1 and 2) received higher scores for appearance and color compared with the control. This suggests that reducing sodium improved the visual attributes of the product, possibly due to differences in water retention and surface colour development during curing, as previously reported in salt-reduced dry-cured hams (Lorenzo et al., 2015). In contrast, no significant differences ($p > 0.05$) were observed among treatments for odor, texture, salty taste, global taste or overall acceptability. Nevertheless, the reduced-sodium batches consistently obtained equal or slightly higher mean scores than the control in most of these attributes, indicating that a 50% sodium reduction did not compromise, and may even have improved, certain sensory perceptions. This is consistent with recent trends: contemporary consumers are increasingly receptive to less salty meat products, valuing balanced palatability over the intense saltiness of older formulations (Huang et al., s. f.; Taormina, 2010).

Importantly, global taste and overall acceptance remained stable across treatments (3.8–3.9), confirming that the reduction strategy was compatible with consumer liking. Similar observations have been reported in dry-cured ham and other cured meat products, where moderate sodium reduction was not perceived negatively by consumers, particularly when compensatory flavour compounds such as umami- and sweet-tasting amino acids

were increased (Ju et al., 2024; Mora & Toldrá, 2025; Toldrá et al., 2000). It is possible too that the slight decrease in salinity was compensated for by the increase in flavour compounds derived from greater proteolysis, which contributed to the overall flavour (Fang & Zhu, 2025).

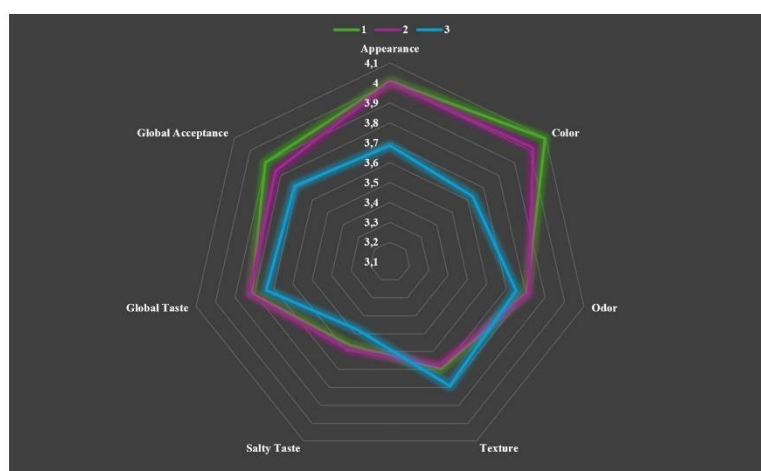


Figure 1. Sensory profile of cured hams according to consumer assessment ($n = 200$).

Hernández Correas et al., 2024 & Muñoz-Rosique et al., 2022 had already observed in a tasting panel that a moderate level of NaCl in dry-cured ham obtained better flavour and texture scores than traditional higher levels, enhancing juiciness and natural flavour qualities. In line with this, other study on cured meat products has shown that gradual reductions in sodium and nitrite can be achieved while maintaining acceptability, especially if other aspects of the process are optimised, such as maturation time or the use of natural flavour enhancers (Lorenzo et al., 2015).

3.4 Multivariate analysis of cured ham quality parameters. Integration of physicochemical, biochemical and sensory parameters using PCA.

Figure 2 shows a principal component analysis that includes some of the most notable parameters of hams produced with different sodium reduction treatments and salt particle sizes. Specifically, moisture, aw, proteolysis index, sodium, total FAA, key individual amino acids (glutamate, valine), nitrite, color parameters (*a, L*) and the main sensory attributes (global acceptance and salty taste) were used. These parameters were selected because they capture the main quality dimensions of dry-cured ham and, importantly, allow the relationships between biochemical changes and sensory perception to be explored.

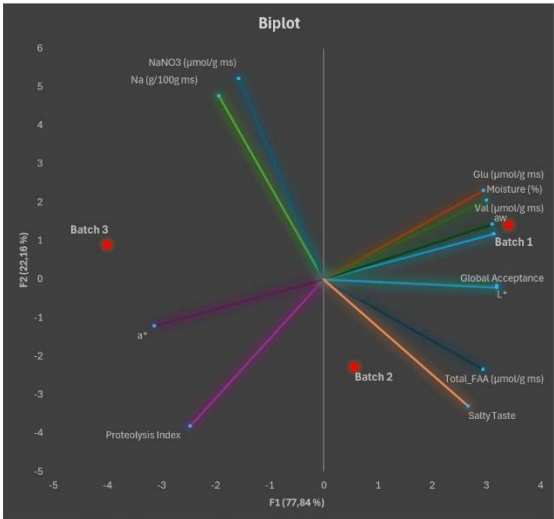


Figure 2. Principal component analysis among the most notable parameters evaluated in the three batches

592 The first two components together explained more than 100% of the variability (PC1:
593 77.8%; PC2: 22.2%), confirming that the model adequately summarises the effects of
594 the treatments.

595 The PC1 axis mainly represented the salinity gradient. On this axis, batch 3 (control)
596 was located at the positive end, associated with higher values of Na and residual
597 nitrates, while Batches 1 and 2 (50% less sodium) were located on the opposite side,
598 correlated with higher moisture content, lower water activity content, higher
599 concentration of free amino acids, and higher overall acceptance. This pattern confirms
600 that sodium reduction was the main factor structuring the differences between
601 treatments.

602 The PC2 axis was associated with minor variations related to colour and specific
603 sensory attributes. In this regard, Batch 1 and 2, both with 50% less sodium, differed
604 slightly from each other: Batch 1, with reduced coarse salt, was associated with higher
605 moisture and milder flavour notes; while Batch 2, with intermediate grain size salt, was
606 more closely linked to sensory flavour attributes. However, these differences were small
607 compared to the clear separation marked by PC1.

608 Overall, the PCA confirms the individual findings in the tables: the 50% reduction in
609 sodium does not compromise the safety or sensory quality of the product but rather
610 enhances the release of flavour compounds derived from proteolysis. The salt particle
611 size had only a minor influence, introducing slight variations in texture and sensory
612 perception, but without altering overall acceptance. The batches with lower sodium
613 content are grouped into a distinct and consistent profile, reinforcing the idea that it is
614 feasible to produce cured hams with half the sodium while maintaining their
615 technological and organoleptic properties.

616

617 CONCLUSIONS

618 The results of this study confirm that it is technologically feasible to reduce the sodium
619 content of boneless dry-cured ham by up to 50% without compromising product safety,
620 stability or sensory quality. The reduction strategies applied in the curing process led to
621 different outcomes depending on the salt particle size used.

622

623 Batch 1 (coarse salt with 25% sodium-reduced formulation) achieved a sodium content
624 of 1.69 g/100 g, which represents a reduction of approximately 28% compared with
625 conventional Respect to %NaCl, the final content was 3.16 g/100 g, representing a
626 decrease close to 43-53% compared to conventional white cured hams.

627 Batch 2 (medium-grain salt with 25% sodium-reduced formulation) reached 1.19 g/100
628 g, corresponding to a reduction close to 50%, thus fulfilling the main objective of this
629 work. Respect to NaCl %, the final content was 2.17 g/100 g, representing a decrease
630 close to 60-64% compared to the usual range for white cured hams (5.5–6 g/100 g)

631

632 From a biochemical perspective, the 50% sodium-reduced hams maintained safe water
633 activity values and showed increased release of free amino acids, particularly those
634 related to umami and sweet taste (glutamate, leucine, glycine, proline), which likely
635 contributed to preserving flavour intensity despite the lower saltiness. The proteolysis
636 index was lower in batch 1, while batch 2 showed values comparable to the control,
637 indicating that the medium salt grain compensated for the reduction by promoting
638 proteolytic activity. Instrumental texture was only slightly affected: batch 1 displayed
639 lower cohesiveness, whereas batch 2 maintained a texture profile very similar to that of
640 the control.

641

642 From a sensory standpoint, reducing sodium by up to 50% did not compromise consumer
643 acceptance. Appearance and colour were even rated significantly higher in the reduced-
644 sodium batches, while no significant differences were found in flavour, aroma, texture or
645 overall acceptance. These findings demonstrate that it is possible to produce boneless dry-
646 cured hams with half the sodium of conventional products, without using salt substitutes
647 or altering the dose of curing agents, and while maintaining the technological and
648 organoleptic qualities that characterise this traditional product.

649

650 *According to the EU Regulation 1924/2006 on nutrition claims, both reduced-sodium*
651 *batches (40–50% less sodium) qualify for the claim “reduced sodium content”, since they*
652 *exceed the minimum 25% reduction required compared with conventional dry-cured*
653 *hams. However, the stricter claims “low sodium” or “sodium-free” are not applicable to*
654 *this type of product due to its intrinsic composition.*

655

656 This results in a product that is healthier from a nutritional point of view while
657 maintaining food safety, colour and characteristic organoleptic quality. This finding is
658 highly innovative, as it demonstrates for the first time the technological feasibility of
659 drastically reducing salt in cured hams without compromising their quality, paving the
660 way for the development of cured meat products with a better nutritional profile in line
661 with current recommendations for reducing sodium in the diet.

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680

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682 *in the study.*

683

684 *Data Availability Statement: The original contributions presented in the study are*
685 *included in the article, further inquiries can be directed to the corresponding author.*

686

687 *Conflicts of Interest: The authors declare that they have no known competing financial*
688 *interests or personal relationships that could have appeared to influence the work*
689 *reported in this paper.*

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835 **FIGURE LEGENDS.**836 **Figure 1.** Sensory profile of cured hams according to consumer assessment (n = 200).837 **Figure 2.** Principal component analysis among the most notable parameters evaluated in
838 the three batches

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860 SUPPLEMENTARY TABLE

Table Sup 1. Effect of different salt treatments during final stage of ham on consumer acceptability by a consumer panel. Results are expressed as means values $\pm$ SEM*.

	Salt Treatment			<i>p-value</i>		Interaction
	1	2	3	Salt Reduction	Salt Size	
Appearance	4.01 $\pm$ 0.883 ^b	4 $\pm$ 0.839 ^b	3.685 $\pm$ 1.02 ^a	0.014	0.128	0.024
Color	4.098 $\pm$ 0.85 ^b	4.022 $\pm$ 0.78 ^b	3.63 $\pm$ 1.002 ^a	0.024	0.221	0.001
Odor	3.804 $\pm$ 1.03 ^a	3.815 $\pm$ 0.89 ^a	3.75 $\pm$ 0.86 ^a	0.431	0.762	0.878
Texture	3.696 $\pm$ 0.87 ^a	3.674 $\pm$ 0.95 ^a	3.791 $\pm$ 0.84 ^a	0.492	0.323	0.638
Salty Taste	3.565 $\pm$ 0.93 ^a	3.587 $\pm$ 0.88 ^a	3.478 $\pm$ 1.06 ^a	0.714	0.533	0.719
Global Taste	3.815 $\pm$ 0.91 ^a	3.826 $\pm$ 0.81 ^a	3.74 $\pm$ 0.936 ^a	0.198	0.638	0.769
Global Acceptance	3.901 $\pm$ 0.78 ^a	3.835 $\pm$ 0.76 ^a	3.714 $\pm$ 0.87 ^a	0.266	0.317	0.286

SEM*: standard error of mean.

p-value Salt Reduction: One-way ANOVA between 1 and 3; *p-value* Salt Size: One-way ANOVA between 1 and 2; *p value* Interaction: two-way ANOVA between 1, 2 and 3.

Attributes were scored assigning a numerical value through a verbal hedonic scale between 1 (I dislike very much) and 5 (I like very much); SEM: standard error of mean.

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ARTICLE 7:

Hernández-Correas, N., Rodríguez-Martínez, A., Tejada, L., Pérez-Sánchez, H., & Abellán, A. (2025). Curing strategies for obtaining ham with up to 50% less sodium. Effect on the generation of bioactive peptides. *In vitro* evaluation. (Under Development)

Title: Curing strategies for obtaining ham with up to 50% less sodium. Effect on the generation of bioactive peptides. In vitro evaluation.

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27 **ABSTRACT**

28 This study evaluated how sodium reduction and salt particle size during curing
29 influence peptide formation and bioactivity in dry-cured ham. Three batches were
30 produced: two experimental hams cured with sodium-reduced salts (40% and 50% final
31 sodium reduction) using coarse or intermediate grain size, and a standard control.
32 Peptide fractions were analyzed to determine angiotensin-converting enzyme (ACE-I)
33 inhibitory activity and antioxidant activity, peptide sequences were identified by LC-
34 MS/MS, and the stability of bioactive peptides was evaluated by bioinformatic analysis
35 and simulated gastrointestinal digestion. All hams showed strong ACE-I activity, while
36 antioxidant capacity was significantly higher in hams with 50% sodium reduction. Mass
37 spectrometry revealed a higher number of low molecular weight peptides (< 1.5 kDa) in
38 the sodium-reduced batches, enriched in hydrophobic and aromatic residues (Tyr, Trp)
39 associated with antioxidant and antihypertensive potential. Key bioactive motifs
40 (KPVAAP, KAAAAP, Ala-Ala, Trp-Tyr) were identified, and dipeptide quantification
41 confirmed that DG (Asp-Gly) and ES (Glu-Ser) increased with sodium-reduced curing,
42 while EE (Glu-Glu), IE (Ile-Glu), and VD (Val-Asp) predominated in the control.

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44 **Keywords:** bioactive peptides; sodium reduction; salt particle size; antioxidant;
45 angiotensin-I converting enzyme (ACE); in vitro digestion.

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47

1.-Introduction

Cured ham is a traditional product of high gastronomic value, whose production involves a lengthy process of salting and maturing under controlled conditions. Salt plays a fundamental role in the preservation and development of the characteristics of ham: it influences water activity, texture, flavour, pH and, especially, the dynamics of proteolysis during curing (Martín et al., 1998; Wang et al., 2011). However, the high sodium content of cured hams has raised health concerns, as excessive salt consumption is directly associated with high blood pressure and increased cardiovascular risk (Strazzullo et al., 2009; Uetake et al., 2015). For this reason, health authorities and the meat industry have emphasised the need to reduce the salt content of cured products to improve their nutritional profile while maintaining safety and quality (Nutrition, 2022). Reducing salt in cured ham is a technological challenge given the multifaceted role of NaCl in the process. A decrease in salt can alter dehydration and promote the activity of endogenous proteases, increasing proteolysis (Costa-Corredor et al., 2009; Muñoz-Rosique et al., 2022). This can be beneficial to a certain extent, as it enhances the release of flavour compounds and bioactive peptides, but excessive proteolysis could negatively affect the texture (softening) and aroma of the product (Pérez-Santaescolástica et al., 2018; Ruiz et al., 2006). Strategies such as modifying the salt particle size (using finer or coarser crystals) and adjusting salting times have been proposed to optimise salt penetration and ensure uniform distribution, thereby avoiding curing defects. The use of coarse-grained salt, for example, traditionally aims for slow and homogeneous diffusion, while fine-grained salts could accelerate salt penetration when the total amount of salt is reduced, partially compensating for the lower dose of NaCl. These modifications to the salting process could allow for substantial reductions

72 in salt content without compromising microbiological stability or sensory properties,
73 although their effects must be carefully evaluated in each case.

74 At the same time, over the last two decades there has been growing scientific interest in
75 the bioactive peptides present in fermented and cured meat products. In dry-cured ham,
76 muscle proteins (myosin, actin, troponins, titin, among others) and sarcoplasmic
77 proteins are progressively fragmented under the action of endogenous proteases
78 (cathepsins, calpains) and microbial proteases, releasing a wide variety of low
79 molecular weight peptides throughout the maturation process (Heres et al., 2023; Toldrá
80 et al., 2020). Several studies have demonstrated multiple bioactive activities of these
81 ham-derived peptides in vitro: notably, antihypertensive activities through inhibition of
82 angiotensin-converting enzyme I (ACE) (Mora et al., 2015), antioxidant activities with
83 free radical scavenging capacity (Escudero et al., 2013), as well as antimicrobial
84 (Corrêa et al., 2023), antidiabetic (dipeptidyl peptidase IV inhibitors) (Xu et al., 2025)
85 and even anti-inflammatory effects (L. Fu et al., 2021). For example, specific sequences
86 with potent ACE-inhibitory activity have been identified, such as AAPLAP, KAAAAP
87 or KPVAAP, derived from the degradation of myofibrillar proteins and collagen,
88 capable of blocking ACE in a manner comparable to bioactive peptides from other
89 sources (Hernández Correás et al., 2025). Similarly, short peptides such as SNAAC or
90 GKFNV show high antioxidant capacity by donating hydrogen or chelating pro-oxidant
91 metals (Gallego, Mora, Reig, et al., 2018). Other peptides identified in Spanish hams
92 include RHGYM, which has an antimicrobial effect, and fragments such as
93 AEEEYPDL or LGVGG, which inhibit enzymes related to carbohydrate metabolism
94 (DPP-IV, α -glucosidase) (Hernández Correás et al., 2025). Dry-cured ham has been
95 identified as a notable source of this type of peptide, to the extent that preliminary
96 studies in humans suggest beneficial effects: regular consumption of ham enriched with

97 bioactive peptides was shown to improve blood glucose and lipid profiles in healthy
98 adults, indicating a positive influence on cardiovascular risk factors (Montoro-García et
99 al., 2017, 2022).

100 Despite advances in the characterisation of bioactive peptides in cured ham, there has
101 been no in-depth research into how modifications to the curing process, particularly the
102 reduction in salt content and the particle size used, affect the generation of these
103 peptides.

104 Salt could influence not only the amount of peptides released (by modulating enzyme
105 activity) but also the type of fragments produced, given its impact on the rate of
106 proteolysis and the product's microbiota. It is also unclear whether a drastic reduction in
107 salt could compromise or improve the final bioactive activities of ham, balancing the
108 traditional negative effect of sodium on blood pressure with the presence of endogenous
109 antihypertensive peptides (ref). In this context, the present study aims to evaluate the
110 impact of a 50% reduction in the final sodium concentration in ham, together with an
111 adjustment in salt particle size, on the generation of bioactive peptides with antioxidant
112 and antihypertensive activity. It is proposed that the controlled reduction in salt will
113 enhance proteolysis and the release of small peptides, increasing the ACE-inhibitory
114 and antioxidant activities of the peptide extracts from the ham, without detriment to the
115 overall quality. This study provides novel insights into the relationship between the
116 salting conditions of ham and its functional properties, with a view to developing
117 healthier and more functional cured meat products.

118

119

120

121

122 2.-Materials and Methods

123 Dry-cured ham samples used in this study were provided by
124 AROMAIBERICASERRANA S.L., located in Balsapintada (Murcia, Spain). All hams
125 came from pigs with a genetic line of 50% White and 50% Duroc.

126 In order to study the effect of different levels of reduction in nitrite salt content and to
127 carry out a pre-industrial validation of the process, three experimental batches were
128 produced, each consisting of 450 hams (n = 150 per batch), with the following
129 characteristics:

130 **BATCH 1:** Nitrifying agents are used at a ratio of 0.7 g/kg (reduced to 50% compared
131 to standard processing), with the following composition: 64.3% sodium nitrite, 21.4%
132 sodium isoascorbate, 8.6% potassium nitrate, and 5.7% dextrose. The remaining 4.3
133 g/kg of nitrifying salt to make up the 5 g/kg used is sodium-reduced NaCl. During the
134 salting process, the hams are buried in coarse-grained salt with 25% less sodium. The
135 salting process lasts for 0.8 days per kilogram of ham weight.

136 **BATCH 2:** Nitrifying agents are used at a ratio of 0.7 g/kg (reduced to 50% compared
137 to standard processing), with the following composition: 64.3% sodium nitrite, 21.4%
138 sodium isoascorbate, 8.6% potassium nitrate, and 5.7% dextrose. The remaining 4.3
139 g/kg of nitrifying salt to make up the 5 g/kg used is sodium-reduced NaCl. During the
140 salting process, a salt with 25% less sodium and medium grain size is used to bury the
141 hams. The salting process lasts for 0.8 days per kilogram of ham weight.

142 **BATCH 3 - CONTROL:** Nitrifying agents are used at a ratio of 0.7 g/kg (reduced to
143 50% compared to standard processing), with the following composition: 64.3% sodium
144 nitrite, 21.4% sodium isoascorbate, 8.6% potassium nitrate, and 5.7% dextrose. The
145 remaining 4.3 g/kg of nitrifying salt to make up the 5 g/kg used is sodium-reduced
146 NaCl. During the salting process, standard coarse-grained salt is used to bury the hams.

The salting process takes place over 1 day per kilogram of ham weight. Batch 3 was used as a control, as it is the standard reference cured product. All hams were cured following the standard curing methodology used by the company, which has already been described in other studies (Hernández Correas et al., 2024; Muñoz-Rosique et al., 2022). Finally, 20 hams were selected from each batch (60 hams). Samples were taken at different stages, but the analyses presented correspond to samples taken from the final product, when the hams had lost between 38% and 40% of their initial fresh weight.

Table 1. Summary of curing treatments used in the different batches.

	BATCH 1	BATCH 2	BATCH 3
NITRIFYING SALTS COMPOSITION	0.7g/Kg Nitriding Agents 4.3g/Kg NaCl – 25% Sodium Reduced	0.7g/Kg Nitriding Agents 4.3g/Kg NaCl – 25% Sodium Reduced	0.7g/Kg Nitriding Agents 4.3g/Kg NaCl – 25% Sodium Reduced
BURYING SALT	Coarse-Grained 25% Less Sodium	Medium-Grained 25% Less Sodium	Coarse-Grained Standard Composition

The aim of the curing strategy was to obtain hams with a significant reduction in sodium content while maintaining product quality and safety. This objective was achieved by combining the use of sodium-reduced curing salts and different salt particle sizes during the salting stage. As a result, the experimental hams reached a final sodium reduction of approximately 40% in Batch 1 and around 50% in Batch 2 compared with the standard control ham (Batch 3). The sodium content of the final products was confirmed in previous analytical determinations (Chapter 6 – Thesis).

2.1. Extraction of peptides – obtaining the fraction

Peptide extraction was performed following the methodology used by (Correas et al., 2025) with slight modifications. Eight grams of sample were homogenised in 30 mL of distilled water, stirring for 15–20 min. Subsequently, 15 mL of 15% trichloroacetic acid was added, stirred for 10 min and filtered into a 50 mL volumetric flask. Finally, it was

171 topped up with distilled water. The quantification of non-protein nitrogen (NPN) was
172 carried out using the Kjeldahl method described by Tejada et al., 2021.

173

174 **2.2. *In vitro* assays**

175 **2.2.1. *Angiotensin -I- converting enzyme inhibitory activity***

176 The spectrophotometric method described (Muñoz-Rosique et al., 2023) was applied.
177 Forty μL of extract was incubated with 100 μL of HHL (5 mM in 0.1 M borate buffer
178 with 0.3 M NaCl, pH 8.3) at 37 °C. Then, 2 mU of ACE was added and, after 30 min,
179 150 μL of 1 M HCl. The hippuric acid generated was extracted with ethyl acetate,
180 evaporated at 95 °C, resuspended in water and measured at 228 nm. Activity was
181 expressed as a percentage of ACE inhibition relative to controls, and the IC₅₀ was
182 calculated by linear regression. Captopril (1 mM) was used as a reference.

183

184 **2.2.2. *Antioxidant activity***

185 Antioxidant capacity was evaluated using the DPPH assay (Hernández-Correas et al.,
186 2024). 500 μL of ethanol, 500 μL of sample and 125 μL of DPPH (0.02%) were mixed.
187 After 1 h in darkness, the mixture was centrifuged (10,000 g, 2 min) and the absorbance
188 was measured at 517 nm. Inhibition was calculated as %RSA and the IC₅₀ was
189 determined against the Trolox standard.

190

191 **2.3 *Peptide identification and bioactivity analysis***

192 The methodology used followed the protocol described by (Bueno-Gavilá et al., 2021)
193 with slight modifications. Peptide identification was performed using tandem mass
194 spectrometry (MS/MS) coupled with nano-liquid chromatography (nLC), based on the
195 peptide fraction obtained in Section 2.1. Chromatographic separation was performed on

196 a Dionex Ultimate 3000 nano UPLC system (Thermo Scientific), using a C18 Acclaim
197 Pepmap column (75 μm $\times$ 50 cm, Thermo Scientific). The samples were preloaded onto
198 an Acclaim Pepmap precolumn (300 μm $\times$ 5 mm, Thermo Scientific) using a 2%
199 acetonitrile and 0.05% TFA solution, with a flow rate of 5 $\mu\text{L}/\text{min}$ for 5 minutes.
200 Peptide separation was performed at 40 $^{\circ}\text{C}$ for all samples.
201 Mobile phase A consisted of water with 0.1% formic acid, while mobile phase B
202 contained 20% acetonitrile and 0.1% formic acid. The elution gradient was developed at
203 a flow rate of 300 nL/min, increasing phase B from 4% to 45% in 60 min, from 45% to
204 90% in 1 min, followed by a 5-min wash at 90% and a final 15-min re-equilibration at
205 4%. The total chromatographic analysis time was 85 min.
206 The identification of the peptide sequences corresponding to the different fractions was
207 carried out at the Proteomics and Bioinformatics Unit of the University of Córdoba
208 (Spain). The MS/MS spectra were processed using the SEQUEST HT algorithm, using
209 the UniProtKB database as a reference.
210 The peptides were identified and quantified based on peptide spectral matches (PSM),
211 normalizing the quantification values according to the total number of PSM in each
212 sample, which allowed the relative abundance of the same peptide to be compared
213 between different samples. Subsequently, a search was performed for the identified
214 sequences in the BIOPEP-UWM database (Minkiewicz et al., 2019), with the aim of
215 detecting both previously described bioactive peptides and potential peptides containing
216 fragments with recognized biological activity. In addition, the PeptideRanker web tool
217 (Mooney et al., 2012) was employed to estimate the probability of bioactivity of each
218 identified sequence, providing a complementary *in silico* prediction to the database
219 search.”

220 2.4. Simulated gastrointestinal digestion of ham peptides

221 To reproduce the human digestive process, the standardised protocol of Minekus et al.
222 (2014) modified by (Correas et al., 2025) was followed. Five grams of biceps femoris
223 muscle sample, previously defatted and homogenised, was used.

224 Oral phase: the sample was mixed with 3.5 mL of simulated salivary fluid (SSF), 0.5
225 mL of α -amylase (1500 U/mL), 25 μ L of 0.3 M CaCl_2 and 975 μ L of water. The
226 mixture was incubated for 2 minutes at 37 °C under agitation, reproducing chewing and
227 the initial action of the enzyme.

228 Gastric phase: simulated gastric fluid (SGF) was added until a 1:1 ratio with the sample
229 was obtained. The mixture contained 7.5 mL of SGF, 1.6 mL of porcine pepsin (25,000
230 U/mL), 5 μ L of 0.3 M CaCl_2 and 695 μ L of water. The pH was adjusted to 3 with HCl,
231 and incubation was maintained for 2 h at 37 °C with continuous stirring.

232 Intestinal phase: the resulting chyme was combined with an equal volume of simulated
233 intestinal fluid (SIF). Eleven mL of SIF, 5 mL of porcine pancreatin (trypsin activity
234 800 U/mL), 2.5 mL of bile salts (160 mM), 40 μ L of 0.3 M CaCl_2 , and 1.31 mL of
235 water were added. The pH was adjusted to 7 with NaOH and the mixture was incubated
236 for 2 h at 37 °C.

237 Each stage was carefully controlled in terms of pH and incubation conditions in order to
238 obtain a reliable and reproducible model of the digestibility and bioaccessibility of the
239 compounds present in ham.

240

241 **2.5. Quantification of dipeptides**

242 The peptide fractions (2.1) and the digested fractions (2.4) were analysed using a nano-
243 LC system (Eksigent Nano-LC Ultra 1D Plus, AB Sciex) coupled to a TripleTOF 56001
244 Q/TOF mass spectrometer with a nanoelectrospray source. The quantification method
245 used followed the procedure previously validated by (Correas et al., 2025), applying

246 minor modifications to adapt it to the current experimental design. Quantification was
247 performed using synthetic peptide standards previously synthesised and characterised
248 (DA, DG, DV, GA, PA, VG, WK, EE, ES, IE, and VD), which were used as reference
249 compounds for calibration and method validation. These peptides were selected because
250 they had been previously identified in cured ham digests and associated with bioactive
251 potential in vitro. In this stage, only these target dipeptides were quantified, rather than
252 performing a complete sequencing of all digestion products. The samples, previously
253 lyophilised, were resuspended in 100 µL of 0.1% TFA and cleaned with Zip-Tip C18
254 cartridges. They were then loaded onto a C18 trap column and separated on a capillary
255 nano-HPLC column, applying a mobile phase gradient (water/formic acid vs.
256 acetonitrile/formic acid). The effluent was analysed in positive mode with information-
257 dependent acquisition: an initial scan (TOF MS, 250 ms, range 300–1250 m/z) followed
258 by product ion analysis (50 ms, 100–1500 m/z) of the 50 most intense ions.
259 The quantification of the peptide sequences was carried out at the SCAI Metabolomics
260 facilities (University of Córdoba, Spain), which allowed the concentration of dipeptides
261 released after simulated digestion to be determined accurately.

262

263 **2.6. Statistical analysis**

264 All experimental tests were performed in triplicate. The results were analysed using
265 SPSS v21.0 software (IBM, Armonk, NY, USA). A one-way analysis of variance
266 (ANOVA) was used to study the influence of the different curing treatments. When
267 significant differences were observed ($p < 0.05$), a comparison of means was performed
268 using Fisher's LSD test. This statistical approach allowed the effects attributable to the
269 curing salt treatment and the salt particle size used to be reliably identified.

270

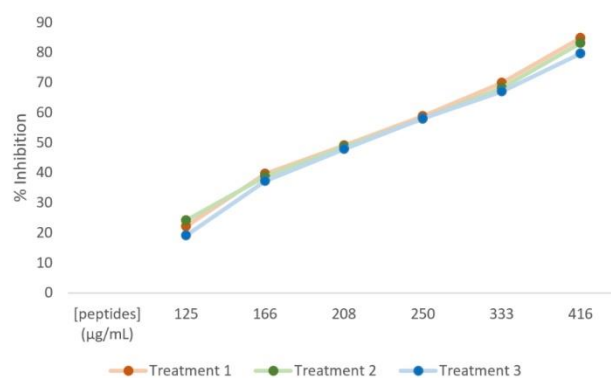
271 3.- Results and discussion

272 3.1. *In vitro* assays

273 3.1.1. *Evaluation of antihypertensive activity (ACE-I) in hams with different curing treatments.*

275 Peptide concentration used for ACE-I assays was standardised based on the non-protein
 276 nitrogen (NPN) content of each extract, determined by the Kjeldahl method, ensuring
 277 comparable peptide levels among treatments. Peptide extracts from dry-cured ham from
 278 the three batches showed concentration-dependent ACE inhibitory activity in vitro. As
 279 the peptide concentration increased, ACE inhibition increased progressively, reaching
 280 values close to 90% at the highest concentrations analysed.

281

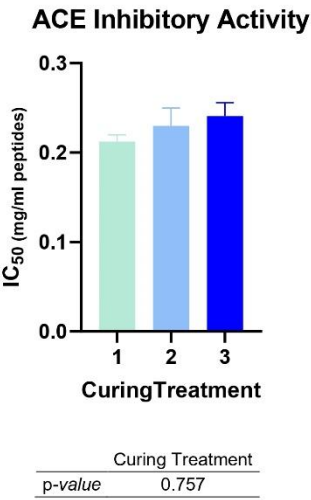


282

283 **Figure 1.** *Evolution of ACE-I activity as a function of peptide concentration in hams cured with different salt*
 284 *treatments.*

285 In terms of IC₅₀ (peptide concentration required to inhibit 50% of ACE activity), the
 286 results obtained are presented in Figure 1. The IC₅₀ values obtained indicated a tendency
 287 towards greater inhibitory potency in sodium-reduced hams (Batch 1 and 2) compared
 288 to the control, although this difference was not statistically significant ($p > 0.05$). Both
 289 experimental batches were produced using less sodium, resulting in an overall reduction

290 of approximately 40% (batch 1) and 50% (batch 2) of sodium in the final product
291 compared to the control (Chapter 6 - Thesis). These findings are consistent with
292 previous studies that highlight the presence of low molecular weight peptides (<3 kDa)
293 responsible for ACE activity in cured hams (Heres et al., 2021). In fact, many of these
294 small peptides are known to be generated by the intense proteolysis of the curing
295 process and are capable of inhibiting ACE (Mora et al., 2015). In this sense, the
296 decrease in salt content favours this proteolysis: a recent study reported that salt
297 reduction enhances enzyme activity, increasing the generation of bioactive peptides in
298 cured ham (Muñoz-Rosique et al., 2023). Our results suggest that hams with lower
299 sodium tended to contain more of these antihypertensive peptides, which is consistent
300 with the literature on blood pressure, where the consumption of ham peptides is
301 associated with blood pressure-regulating effects (Abachi et al., 2019; Montoro-García
302 et al., 2017)

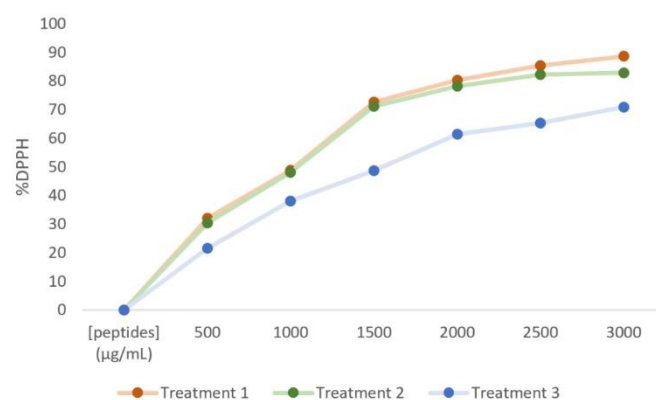


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304
305

Figure 2. Effect of sodium concentration and particle size on ACE inhibitory activity in boneless cured hams.

3.1.2. Antioxidant activity

The antioxidant capacity of the peptide fraction was evaluated using the DPPH assay. All peptide extracts showed radical scavenging activity, which increased as the peptide concentration increased, reaching inhibitions of close to 80–90% at the highest concentrations analysed (**Figure 3**). The antioxidant capacity of each extract was expressed as IC₅₀ (mg of peptides/mL) and as Trolox equivalent antioxidant capacity (TEAC), calculated from the calibration curve of Trolox used as a reference standard.



313

Figure 3. Evolution of DPPH activity as a function of peptide concentration in hams cured with different salt treatments.

This confirms that cured ham provides peptides with appreciable antioxidant activity in sufficient quantities even under conditions of lower salinity. Previous studies have identified cured ham as a natural source of antioxidant peptides, so our results are consistent with those reports. In our work, the batches with reduced sodium tended to show a slight improvement in antioxidant activity compared to the control. In fact, batch 2 had the highest average antioxidant capacity, with a significant difference between treatments ($p < 0.05$) in favour of hams with 50% less sodium (**Fig.1**). This can be attributed to a greater release of peptides rich in aromatic amino acids during longer

curing and with less sodium(Correas et al., 2025; Hernández Correas et al., 2025). In line with this, Hernández-Correas et al. (2025) also found that more mature hams (greater mass loss) showed an abundance of peptides <1.5 kDa with notable antioxidant effects. Taken together, these results indicate that the sodium reduction strategy may contribute to increased accumulation of antioxidant peptides during ham maturation.

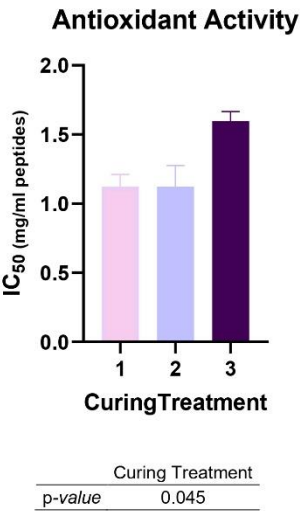


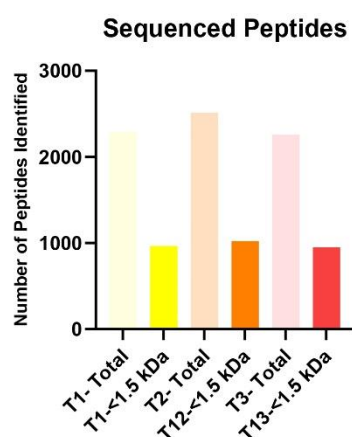
Figure 4. Effect of sodium concentration and particle size on the antioxidant inhibitory activity of boneless cured hams.

3.2. Bioactive peptide identification

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

Figure 5 shows the total number of peptides identified in the different cured ham samples processed with the three different treatments. In addition, the number of peptides identified with a mass below 1.5 kDa is specified. Peptides with bioactive

potential were identified in all samples, with a higher proportion of peptides <1.5 kDa in treatment 2 (1023) and 1 (967), representing a slight increase compared to treatment 1 (951). These results verify the activity of proteolytic enzymes with decreasing salt content. Peptides <1.5 kDa have greater bioavailability and functional activity, highlighting the importance of treatments that promote their generation. The results obtained by (Li et al., 2022), showed that the oxidation of peptides could be related to their molecular weight, such that a greater number of fractions with a small molecular weight showed greater antioxidant activity. Other studies reported that molecular size and amino acid composition play an important role in the antioxidant activities of protein hydrolysates (Harnedy et al., 2017).



350

351 *Figure 5. Number of peptides identified in the different dry-cured ham peptide fractions.*

352

353 3.2.2. Comparative study of peptides identified in each peptide fraction from hams 354 produced with different salt treatments with putative activity

355 To compare the overall bioactivity profile between treatments, the results were
356 normalised using Z statistics and represented in a hierarchical heat map (Fig. 1). In this

map, each row corresponds to a type of activity (antihypertensive, antioxidant, etc.) and each column to one of the batches; values in red indicate a higher relative expression of that activity in the corresponding sample compared to the average. This analysis revealed slight differences in the distribution of potential bioactive activities: for example, antihypertensive and antioxidant activities showed higher relative intensities in hams with 50% sodium reduction (Batches 1 and 2). At the same time, an exhaustive review of databases (BIOPEP, PeptideRanker) was carried out to identify regions in the precursor protein sequences that are likely to release active peptides after digestion.

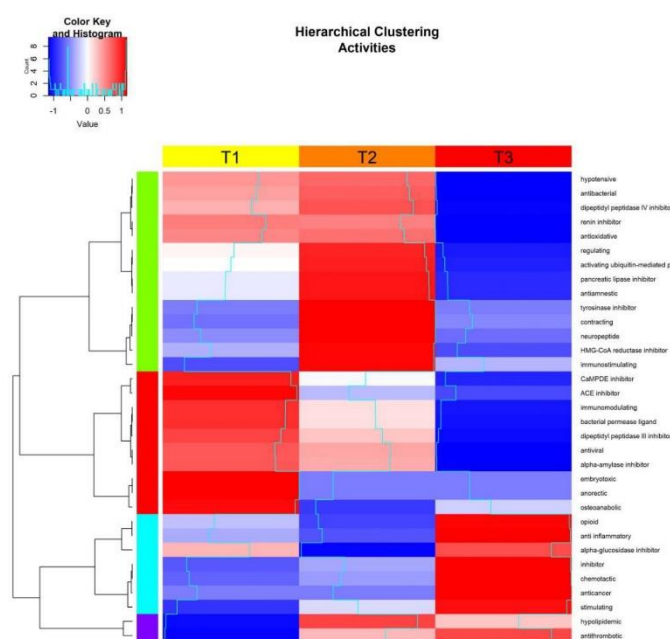


Figure 6. Heatmap and dendrogram of the bioactivities of the different ham samples studied. The quantification of bioactivity refers to the mean. The grouping relationship between the activity groups is defined.

This in silico approach complements traditional proteomic detection, allowing the prediction of very short fragments (≤ 6 aa) that could escape experimental identification.

Overall, the comparative analysis indicates that sodium reduction subtly alters the profile of potential bioactive peptides, aligning with the changes observed in overall peptide generation.

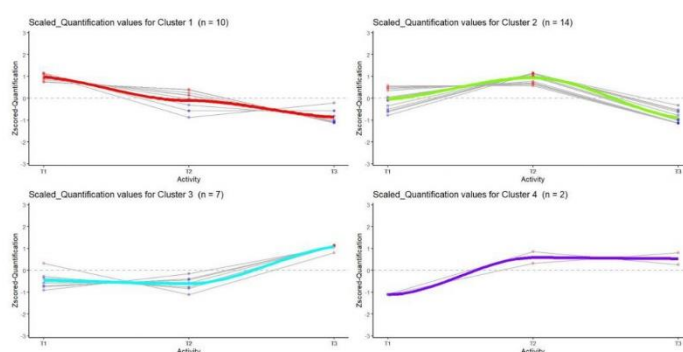


Figure 7. Representation of the bioactivity in each sample for each cluster of activities.

3.3. Bioactivity analysis based on amino acid composition

The analysis of the amino acid composition of the peptides identified in the different treatments revealed a predominance of hydrophobic and aromatic residues, mainly Val, Ala, Leu, Tyr, and Trp. These amino acids are frequently associated with enhanced biological activity due to their ability to interact with hydrophobic pockets of enzymes such as ACE or to donate electrons and scavenge reactive oxygen species (Escudero et al., 2013; Hamedy et al., 2017; Heres et al., 2023). In particular, the abundance of terminal aromatic residues (Tyr, Trp, Phe) is consistent with the antioxidant activity observed in vitro, as these residues play a key role in hydrogen donation and radical neutralization (Gallego, Mora, Reig, et al., 2018; Gallego, Mora, & Toldrá, 2018; Hernández Correás et al., 2025). The selection of the 20 most relevant peptides (mass < 1.5 kDa) from each treatment was based on three complementary criteria:

- A high *PeptideRanker* score (>0.8).

389 - *The presence of amino acid motifs previously described as bioactive —such as*
390 *KPVAAP, KAAAAAP, Ala-Ala, Trp-Tyr, or Ala-Trp (Mora et al., 2015; Toldrá et al.,*
391 *2023).*

392 - *Their relative abundance in LC–MS/MS analyses. These peptides therefore represent*
393 *the sequences with the greatest theoretical potential to contribute to the biological*
394 *effects measured in vitro.*

395 The comparative amino acid profile showed that hams produced with lower sodium
396 content (Batches 1 and 2) contained a higher proportion of peptides rich in hydrophobic
397 and aromatic residues. This pattern aligns with previous studies reporting that low-
398 sodium curing promotes the release of short peptides (<1.5 kDa) enriched in these
399 residues, which exhibit strong antioxidant and antihypertensive properties (Li et al.,
400 2022; Muñoz-Rosique et al., 2023). Furthermore, the prevalence of Ala–Ala and Pro–
401 Lys–Pro motifs supports the enhanced ACE-inhibitory potential observed in these
402 samples, as such dipeptides have been associated with reductions in systolic blood
403 pressure in both in vitro and in vivo models (Abachi et al., 2019; Heres et al., 2021).

404 For all these, the 20 most relevant peptides (mass < 1500 Da) from each treatment are
405 summarised below, with their sequence, mass, potential activity (potential or
406 demonstrated), tool/database consulted, and a brief comment on the mechanism by
407 which they may exert their bioactivity. These results corroborate the presence of
408 peptides that, after digestion, can give rise to smaller peptides (dipeptides) that can exert
409 bioactivity.

410 **Table 1.** Bioactivity derived from peptides identified in hams from batch 1.

Sequence	Mass (<1.5 kDa)	Potential Bioactivity	Tool / Source	Comment
MTATKAAAPK	1115.56	Antihypertensive (ACE inhibitor)	BIOPEP-UWM, Bibliography	Contains the KAAAP motif (myosin light chain 3) identified as an ACE inhibitor.
NKPVAAPPQLSK	1361.80	Antihypertensive (ACE inhibitor)	BIOPEP-UWM, Bibliography	Contains KPVAAP (myosin XV) associated with potent ACE inhibition.
NKPVAAPPQLK	1274.77	Antihypertensive (ACE inhibitor)	BIOPEP-UWM, Bibliography	Similar to the previous one, it contains KPVAAP, a known antihypertensive agent.
NKPVAAPPKTGIIVR	1474.86	Antihypertensive (ACE inhibitor)	BIOPEP-UWM, Bibliography	Contains KPVAAP (same motif) in its chain, implying anti-FCA activity.
RITSLPVKPVAAAPK	1499.89	Antihypertensive (ACE inhibitor)	BIOPEP-UWM, Bibliography	Includes KPVAAP; peptide with penultimate proline and terminal lysine characteristic of ACE inhibitors.
AWAPPPDGK	937.47	Antioxidant (Potential)	PeptideRanker, BIOPEP-UWM	Contains AW (Ala-Tip), a cardioprotective dipeptide found in ham; its Tip promotes antioxidant activity.
FPGWMLYK	1040.52	Antioxidant (Potential)	PeptideRanker	Contains aromatic Trp-Tyr; peptides with these residues in ham (e.g. SNAAC) showed high antioxidant activity.
ITLPGGINWK	1044.53	Antioxidant (Potential)	PeptideRanker	It possesses Trp and a hydrophobic sequence; aromatic residues at the C-terminal end are associated with potent antioxidant activity.
KPIFIWLK	1019.58	Antioxidant (Potential)	PeptideRanker	Contains Trp (W) at the end; final aromatic residues promote antioxidant capacity.
QNGVAAKPAK	982.56	Antihypertensive (ACE inhibitor)	BIOPEP-UWM	Includes di-Ala (AA) in the internal position; Ala-Ala is a common dipeptide in antihypertensive peptides from ham.
AAFKTFKK	967.55	Antihypertensive (ACE inhibitor)	BIOPEP-UWM	Contains AA (n-terminal Ala-Ala); studies indicate that peptides rich in AA exhibit a blood pressure-lowering effect.
AWLGADLEK	1001.52	Antioxidant (Potential)	PeptideRanker	Includes di-Ala-Tip (AW); peptides with AW have demonstrated cardioprotective and antioxidant activity.
GPAAHVVVVR	1003.59	Antihypertensive (Potential)	PeptideRanker, BIOPEP-UWM	It contains an AA motif and ends in a hydrophobic Val; peptides with this composition (hydrophobic C-terminal) tend to inhibit ACE.
VVGAWCYK	924.45	Antioxidant (Potential)	PeptideRanker	It possesses aromatic Tyr-Trp and nonpolar chains; similar to antioxidant peptides reported in ham.
SSTLVQIHWK	1199.59	Antioxidant (Potential)	PeptideRanker	Trp in an almost terminal position; the presence of Trp usually confers high antioxidant capacity.
LTAATVGLR	997.59	Antihypertensive (ACE inhibitor)	PeptideRanker, BIOPEP-UWM	Contains AA (in the middle of the sequence); the Ala-Ala motif has been quantified with a notable pressure-reducing effect in ham.
TLVKRYK	906.57	Antioxidant (Potential)	PeptideRanker	Ends in Tyr; terminal aromatic residues (e.g. Tyr) frequently identified in antioxidant peptides in ham.
DIKUTTYNK	1094.60	Antioxidant (Potential)	PeptideRanker	Contains Tyr in the terminal position; peptides with terminal Tyr tend to have significant antioxidant activity.
GMEVPPYK	919.45	Antioxidant (Potential)	PeptideRanker	It has a Tyr terminal; similar to other antioxidant peptides in ham that have already been identified.
LYPKIOSYK	987.58	Antioxidant (Potential)	PeptideRanker	It has a Tyr terminal; similar to other antioxidant peptides in ham that have already been identified.

411 **Table 2.** Bioactivity derived from peptides identified in hams from batch 2.

Sequence	Mass (<1.5 kDa)	Potential Bioactivity	Test / Source	Comment
NKPVAAPPALGLK	1274.77	Antihypertensive (ACE inhibitor)	BIOPEP-UWM, Isocrania	Contains KPVAAAP (myosin XV), ACE motif.
SGPFAPVTTAWR	1288.66	Antioxidant (Potential)	PeptideRanker	Ends in Trp (W); ham peptides with terminal W show high antioxidant capacity.
FTWNTAWVDQR	1422.67	Antioxidant (Potential)	PeptideRanker	It contains the AW dimer; Ala-Trp is a cardioprotective dipeptide found in ham.
DPTAWAWKVLTR	1341.72	Antioxidant (Potential)	PeptideRanker	It contains twice the amount of AW; its high Trp content makes it a potent antioxidant candidate.
AWEDDWDEPPR	1428.59	Antioxidant (Potential)	PeptideRanker	It has an AW sequence and several acidic residues; peptides with AW are recognised antioxidants.
TYPLSRMAWR	1392.70	Antioxidant (Potential)	PeptideRanker	Ends in AW; Ala-Trp and Trp are typical terminals in antioxidant peptides in ham.
WDMASACAWRPK	1420.64	Antioxidant (Potential)	PeptideRanker	Contains AW and Trp; similar to other peptides with terminal Trp detected with antioxidant activity.
DLAGGVFAWLIR	1463.69	Antioxidant (Potential)	PeptideRanker	Contains W in its chain; antioxidant activity is expected due to the aromatic residue.
WVAAVAVALNPEK	1367.74	Antioxidant (Potential)	PeptideRanker	It has W in the internal position; peptides with internal Trp can also act as antioxidants.
LKEWGYCCCK	1231.52	Antioxidant (Potential)	PeptideRanker	Includes WGY motif with Trp and Tyr; analogous aromatic sequences are antioxidants in ham.
NGPSNTEFWK	1191.49	Antioxidant (Potential)	PeptideRanker	Ends in Trp; peptides with W at the end show strong antioxidant activity.
VEQSQLPWQR	1270.63	Antioxidant (Potential)	PeptideRanker	Contains internal W; the presence of Trp suggests antioxidant functionality.
TEATAAALRK	1177.61	Antihypertensive (ACE inhibitor)	BIOPEP-UWM, PeptideRanker	It contains repeated AA (Ala-Ala), known for its antihypertensive effect in ham peptides.
TMEATAAALR	1049.52	Antihypertensive (ACE inhibitor)	BIOPEP-UWM	It has internal AA; similar to the previous one, it suggests ACE inhibitory potential (relevant Ala-Ala).
APPKRPVPEFK	1218.70	Antihypertensive (Potential)	PeptideRanker	Termination at Lys with PPKK sequence; peptides with Lys-Pro as the penultimate amino acid tend to inhibit ACE.
KPVVEKLEK	1068.65	Antihypertensive (Potential)	PeptideRanker	It possesses K-P-V at the beginning; the presence of Pro and basic residues at the C-terminus facilitates binding to ACE.
YMALQDACNK	1155.51	Antioxidant (Potential)	PeptideRanker	Ends in Tyr (Y); terminal aromatic residues are characteristic of antioxidant peptides.
FDGTRTGTGYR	1269.61	Antioxidant (Potential)	PeptideRanker	Contains Tyr terminal; similar to other antioxidant peptides (e.g. SNAAC) in ham.
SPSTDVAGLEYCPR	1493.68	Antioxidant (Potential)	PeptideRanker	It possesses Tyr at the end and hydrophobic chains; antioxidant activity is predicted due to its aromatic composition.
VWPFVPLAGYKK	1432.85	Antioxidant (Potential)	PeptideRanker	Contains Tyr (Y) and several Val; peptides with similar Tyr and aliphatic structures have shown antioxidant properties.

412 **Table 3.** Bioactivity derived from peptides identified in hams from batch 3.

Sequence	Mass (<1.5 kDa)	Potential Bioactivity	Tool / Source	Comment
FPAGSWKGLIR	1254.66	Antioxidant (Potential)	PeptideRanker	It has Trp (W) and a mixed structure; peptides with terminal W are antioxidants in ham.
TEGTLPWRTIPR	1312.69	Antioxidant (Potential)	PeptideRanker	Includes WR (Trp in antepenultimate position); similar compounds containing W were identified as antioxidants.
WVPLNKDYK	1161.62	Antioxidant (Potential)	PeptideRanker	Ends in Tyr (Y) with internal W; typical aromatic combination of antioxidant peptides.
TAPGALWTSHPK	1264.66	Antioxidant (Potential)	PeptideRanker	Contains LWTSII (Trp-Tyr); multiple aromatic residues promote antioxidant activity.
LALTQGGGTWR	1158.61	Antioxidant (Potential)	PeptideRanker	Ends in Trp (W); similar peptides (e.g. TGGTW) behave as antioxidants in ham.
MIIEEFVVEFK	1398.64	Antidiabetic (potential)	PeptideRanker	Very rich in Glu (E); sequences with multiple E (AETTY...) are inhibitors of DPP-IV/ α -glucosidase.
MTEPEHVENSK	1214.52	Antidiabetic (potential)	PeptideRanker	Contains EEIVEN sequences; repeated Glu motifs suggest DPP-IV inhibition.
EFVTEEFVEFK	1430.66	Antidiabetic (potential)	PeptideRanker	Large accumulation of Glu (E) and Phe; similar to AEEEEYPDL (diabetes) described in ham.
MPVVENFK	1106.58	Antidiabetic (potential)	PeptideRanker	Reason VVEFF; repeated Val-Glu sequences are associated with DPP-IV/ α -glucosidase inhibition.
KVPELPKKPEEK	1420.83	Multifunctional (potential)	PeptideRanker	K(PK) KK sequence at the end; presence of proline and lysine suggests possible antihypertensive and anti-DPP-IV activity.
KPKPVPEK	1050.61	Multifunctional (potential)	PeptideRanker	Includes KPKPVP with various Lys; peptides rich in Lys and Pro have been linked to ACE inhibition.
EEPKPVPEK	1179.65	Multifunctional (potential)	PeptideRanker	Termination in Lys; the KPKVP fragment is analogous to other antihypertensive peptides (terminating in Pro/Lys).
VPPKPVLVQVAHK	1439.90	Multifunctional (potential)	PeptideRanker	VPPKKP repeat chain; Lys-Pro motifs in the centre may modulate blood pressure.
VPPKPVVDKAHK	1439.86	Multifunctional (potential)	PeptideRanker	Similar to the previous one with 'KPKPVP'; high Lys-Pro content indicates antihypertensive potential.
MTTATAAALRKK	1305.71	Antihypertensive (ACE inhibitor)	BIOPFP-IPWM	Contains repeated AA; peptides with Ala-Ala in sequence (e.g. AAAIP) have been shown to reduce systolic blood pressure (SIIP).
KVKEIPGNAAR	1165.66	Antihypertensive (Potential)	PeptideRanker	Secuencia rica en Lisinas; péptidos con C-ter Lys y Pro internos suelen inhibir la ECA (motivo conocido en jamón).
NCAALKKAGMCR	1264.62	Antihypertensive (Potential)	PeptideRanker	Sequence rich in lysines; peptides with internal C-terminal Lys and Pro often inhibit ACE (a known motif in ham).
YPAKGYSGISK	1169.61	Antioxidant (Potential)	PeptideRanker	Ends in Tyr; peptides with Tyr in the terminal position are typically antioxidants.
LTLQNLQGGFYK	1380.74	Antioxidant (Potential)	PeptideRanker	It contains Tyr near the end; similar to other antioxidant peptides with Tyr observed in ham.
FIFPYVCFVNR	1391.67	Antioxidant (Potential)	PeptideRanker	Contains internal Tyr (Y); aromatic residues promote free radical scavenging.

Bioactive activities were obtained through in silico prediction (PeptideRanker) and consultation of specialised databases (BIOPEP-UWM) and recent literature (Escudero et al., 2014; Heres et al., 2021, 2023) on ham peptides. The comments highlight reasons for activity (e.g., aromatic residues or known dipeptides) and previous studies on ham that support the selection of each peptide. Reviews and studies on cured ham peptides summarising known bioactive sequences (KPVAAP, KAAAAP, SNAAC, AEEEEYPDL, etc.) are cited, as well as reactivity analyses (BIOPEP, PeptideRanker) to support the activities reported in the tables (Hernández Correás et al., 2025; Hu et al., 2024; Toldrá et al., 2023). Each entry indicates the source of the relevant prediction or experimental finding.

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3.4. Quantification of dipeptides identified before and after in vitro digestion

The quantitative analysis of dipeptides identified in the peptide fractions (**Table 4**) and after simulated gastrointestinal digestion (**Table 5**) revealed clear differences associated with the curing treatment and the digestion process.

Before digestion, no significant differences ($p > 0.05$) were observed for DA, DV, GA, PA, WK, EE, IE and VD, indicating that their concentrations remained stable across different treatments applied. These peptides appear to be released in comparable amounts during curing, suggesting that their generation is not highly sensitive to variations in sodium content.

In contrast, significant differences were detected for DG, VG and ES ($p < 0.05$). The DG dipeptide (Asp–Gly) showed the highest concentration in Batch 1, followed by Batch 3 and Batch 2, suggesting that moderate sodium reduction (40%) favoured its accumulation. The VG dipeptide (Val–Gly), on the other hand, was markedly higher in Batch 3 (control), reaching a mean value of 7.746 mg/mL compared with 0.46 mg/mL

in Batch 2 and 0.075 mg/mL in Batch 1, indicating that high sodium content promotes the retention or stability of this peptide. Finally, ES (Glu-Ser) was significantly higher in Batch 2 than in Batches 1 and 3, demonstrating that the 50% sodium reduction enhanced the release of this dipeptide.

Table 4. Quantification of dipeptides identified in the peptide fraction. Results are expressed in mg/ml of sample (means values $\pm$ SEM*).

TT	[DA]	[DG]	[DV]	[GA]	[PA]	[VG]	[WK]	[EE]	[ES]	[IE]	[VD]
O	(mg/ml)	(mg/ml)	(mg/ml)	(mg/ml)	(mg/ml)	(mg/ml)	(mg/ml)	(mg/ml)	(mg/ml)	(mg/ml)	(mg/ml)
1	0.177 $\pm$ 0.062	0.339 $\pm$ 0.256	0.266 $\pm$ 0.045	0.585 $\pm$ 0.274	0.094 $\pm$ 0.011	0.075 $\pm$ 0.002	0.088 $\pm$ 0.002	0.406 $\pm$ 0.054	1.135 $\pm$ 0.029	0.201 $\pm$ 0.033	0.075 $\pm$ 0.003
2	0.143 $\pm$ 0.031	0.075 $\pm$ 0.039	0.247 $\pm$ 0.039	0.758 $\pm$ 0.522	0.103 $\pm$ 0.024	0.461 $\pm$ 0.09	0.121 $\pm$ 0.021	0.35 $\pm$ 0.065	1.717 $\pm$ 0.027	0.219 $\pm$ 0.012	0.078 $\pm$ 0.003
3	0.236 $\pm$ 0.03	0.075 $\pm$ 0.017	0.277 $\pm$ 0.017	0.833 $\pm$ 0.413	0.096 $\pm$ 0.021	7.746 $\pm$ 4.429	0.1 $\pm$ 0.015	0.381 $\pm$ 0.075	0.953 $\pm$ 0.035	0.182 $\pm$ 0.021	0.075 $\pm$ 0.003
P	0.357	0.000	0.843	0.210	0.939	0.000	0.121	0.066	0.000	0.487	0.405

SEM*: standard error of mean.

After digestion, several dipeptides exhibited notable changes. No significant differences were observed for DA, DV, GA, PA, and VG ($p > 0.05$), confirming that these fragments were stable during gastrointestinal hydrolysis across all treatments. However, significant effects of treatment were found for EE, ES, IE, and VD ($p < 0.05$). The EE dipeptide (Glu-Glu) showed a tendency to increase post-digestion, particularly in Batch 3, which reached the highest value. The ES dipeptide also displayed significantly higher concentrations in Batch than in the reduced-sodium batches, indicating that despite the lower sodium content favouring proteolysis during curing, traditional salting conditions may promote the persistence of certain acidic dipeptides after digestion. Likewise, IE (Ile-Glu) and VD (Val-Asp) were both significantly higher in the control treatment, suggesting that these fragments are more abundant in hams cured with standard salt composition. In the work of Paoletta et al. (2015) with Parma ham, after simulated gastrointestinal digestion, 21 dipeptides and 12 tripeptides were released within a set of 81 total peptides, confirming that digestion can generate additional short fragments from larger precursors. Studies on other protein sources indicate that, in the case of

dairy products, the antihypertensive peptides VPP and IPP, derived from casein, remain partially intact after in vitro digestion in some mature cheeses (Y. Fu et al., 2017). Dipeptides such as PK, EK, VE, and DE have also been identified in dairy products, contributing to flavor and, potentially, bioactivity (Keska & Stadnik, 2017). In soybean vegetable protein hydrolysates, increases in the proportion of dipeptides have been observed after fermentation, and many of these released dipeptides eventually act as ACE inhibitors (Zhang et al., 2022).

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Table 5. Quantification of dipeptides identified in the samples after in vitro digestion. Results are expressed in mg/ml of sample (means values $\pm$ SEM*).

TT	[DA]	[DG]	[DV]	[GA]	[PA]	[VG]	[WK]	[EE]	[ES]	[IE]	[VD]
O	(mg/ml)	(mg/ml)	(mg/ml)	(mg/ml)	((mg/ml)	(mg/ml)	(mg/ml)	(mg/ml)	(mg/ml)	(mg/ml)	(mg/ml)
1	0.214 $\pm$ 0.017	0.207 $\pm$ 0.013	0.446 $\pm$ 0.029	1.031 $\pm$ 0.039	0.121 $\pm$ 0.014	0.348 $\pm$ 0.044	ND	0.709 $\pm$ 0.041	1.001 $\pm$ 0.036	0.361 $\pm$ 0.037	0.307 $\pm$ 0.035
2	0.214 $\pm$ 0.015	0.208 $\pm$ 0.008	0.395 $\pm$ 0.017	0.973 $\pm$ 0.024	0.136 $\pm$ 0.009	0.333 $\pm$ 0.060	ND	0.684 $\pm$ 0.061	1.024 $\pm$ 0.042	0.441 $\pm$ 0.045	0.35 $\pm$ 0.030
3	0.199 $\pm$ 0.019	0.216 $\pm$ 0.017	0.459 $\pm$ 0.048	0.996 $\pm$ 0.043	0.119 $\pm$ 0.009	0.407 $\pm$ 0.060	ND	0.772 $\pm$ 0.065	1.217 $\pm$ 0.053	0.436 $\pm$ 0.049	0.408 $\pm$ 0.041
P	0.795	0.222	0.384	0.146	0.536	0.611	-	0.052	0.047	0.036	0.041

SEM*: standard error of mean.

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484 4.-Conclusions

485 The curing strategy based on the use of sodium-reduced salts and different salt particle
486 sizes proved to be an effective approach to obtain dry-cured hams with a 40–50%
487 reduction in final sodium content, without compromising the release or bioactivity of
488 peptides. The reduction of NaCl modified proteolytic dynamics, favouring the
489 generation of a higher number of low-molecular-weight peptides (<1.5 kDa),
490 particularly in the batch cured with intermediate-grained salt.

491

492 In vitro assays confirmed that all peptide extracts exhibited high angiotensin-converting
493 enzyme inhibitory (ACE-I) and antioxidant capacities. While ACE inhibition did not
494 differ significantly between treatments, the antioxidant activity increased notably in
495 hams with 50% sodium reduction, indicating that moderate salt reduction enhances the
496 formation of antioxidant peptides, likely enriched in aromatic residues such as Tyr and
497 Trp.

498

499 LC–MS/MS and bioinformatic analyses revealed the presence of characteristic bioactive
500 motifs (KPVAAP, KAAAAP, Ala–Ala, Trp–Tyr), consistent with sequences previously
501 reported as antihypertensive and antioxidant in cured meats. The comparative amino
502 acid profile supported these findings, showing a predominance of hydrophobic and
503 aromatic residues in sodium-reduced samples, which are known determinants of
504 biological activity.

505

506 Quantification of dipeptides before and after simulated gastrointestinal digestion
507 demonstrated that DG (Asp–Gly) and ES (Glu–Ser) were particularly influenced by
508 sodium reduction, whereas EE (Glu–Glu), IE (Ile–Glu) and VD (Val–Asp)

509 predominated in the control. These patterns indicate that salt concentration affects both
510 peptide release during curing and their stability during digestion. Acidic dipeptides,
511 especially DG and ES, increased after digestion and have been linked to antioxidant and
512 antihypertensive properties in ham and other protein matrices.

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548 *review & editing, Visualization, Validation, Methodology, Investigation, Formal*
549 *analysis. Alejandro Rodríguez Martínez: Writing – review & editing, Methodology,*
550 *Investigation, Formal analysis. Luis Tejada: Writing – original draft, Resources,*
551 *Investigation, Supervision, Project administration, Funding acquisition. Horacio Perez*
552 *Sanchez: Visualization, Software, Investigation, Data curation. Adela Abellán:*
553 *Supervision, Methodology, Project administration, Investigation, Conceptualization.*

554

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ARTICLE 8:

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24 **ABSTRACT**

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

42

43 **Keywords:** Dry-cured ham; Bioactive peptides; Antioxidants; *Caenorhabditis*
44 *elegans* model

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48

49 1. INTRODUCTION

50 Public awareness of the health risks associated with a diet consisting of highly processed
51 products rich in saturated fats has led to the beginning of a change in consumption habits,
52 increasingly demanding foods that, in addition to nourishing us, provide us with
53 additional benefits that can help improve or protect our health (Sharma et al., 2024).
54 Therefore, in recent years, there has been growing interest in functional compounds,
55 including bioactive peptides, which are small amino acids sequences derived from food
56 proteins with potential health benefits (Purohit et al., 2024). Bioactive peptides derived
57 from food, such as dry-cured ham, which is highly consumed by the population, have
58 attracted attention because of their unique composition and their numerous biological
59 activities (Toldrá et al., 2020). Spanish dry-cured ham undergoes a long maturation
60 process that improves its sensory properties and releases bioactive compounds such as
61 peptides (Benedini et al., 2012; Hernandez Correas et al., 2024; Hernández Correas et al.,
62 2024). These peptides are generated by the action of proteolytic phenomena that occur
63 during the ham curing process and are capable of degrading both the protein produced
64 and those already present in the muscle tissue (Rodríguez-Nuñez et al., 1995; Toldrá et
65 al., 2020).

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

2019), and immunomodulatory (Pavlicevic et al., 2022) properties. In 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 (H. 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

99 makes it easier to study genes related to antioxidant responses, allowing them to retain
100 may biological pathways with mammals that then facilitates the extrapolation of results
101 to other organisms, including humans (Miranda-Carrazco et al., 2025). Previous studies
102 have shown that dietary peptides can increase stress resistance and activate key regulatory
103 pathways associated with antioxidant defense mechanisms in *C. elegans* (Mudd et al.,
104 2022a; Wu et al., 2022; Yu et al., 2020). These activities have made bioactive peptides an
105 object of interest in the research and development of functional foods and new therapeutic
106 targets. Therefore, the main objective of this study was to evaluate the *in vivo* antioxidant
107 activity of peptides derived from Spanish dry-cured ham, using *C. elegans* as an *in vivo*
108 model. To the best of our knowledge, this is the first study to evaluate the potential of
109 dry-cured ham peptides in this model, providing information on their role in the
110 modulation of oxidative stress and possible health benefits.

111

112

113 2. MATERIAL AND METHODS

114 2.1. *Materials and reagents*

115 The cured ham samples used in this study were provided by
116 AROMAIBERICASERRANA S.L. (Balsapintada, Murcia, Spain). The hams came from
117 pigs with a genetic line of 50% Duroc, and 50% Iberian, and were subjected to the
118 traditional curing process developed by the company, with a reduction in salting time as
119 part of a strategy to reduce the total salt content by 25%. A total of 16 samples from 16
120 hams were used.

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

128

129 2.2. *Preparation of dry-cured ham-derived bioactive peptides*

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

137 mL volumetric flask. After filtration, the flask volume was brought to 50 mL using
138 distilled water.

139

140 **2.3. Simulated gastrointestinal digestion of ham peptides**

141 Ham samples were obtained from four pieces of dry-cured ham, produced under the same
142 conditions and from the same source. The analysis was performed in triplicate for each
143 sample. The Biceps femoris muscle, previously defatted to eliminate extramuscular fat,
144 was processed by grinding and subsequent homogeneous mixing. The *in vitro* digestion
145 was carried out following the protocol used in Hernández-Correas et al (2025) and
146 Minekus et al (2014), with some modifications, adapted to this study. To simulate
147 digestion, 5 g of the sample was subjected to physiological conditions that reproduced the
148 oral, gastric, and intestinal phases of the human digestive process. In the oral phase, the
149 sample was mixed with 3.5 mL of simulated salivary fluid (SSF), to which 0.5 mL of
150 amylase (1500 U/mL, dissolved in SSF), 25 μ L of CaCl₂ (0.3 mol/L) and 975 μ L of
151 distilled water were added. The mixture was incubated for 2.5 min at 37 °C under
152 agitation to simulate the mechanical effect of chewing and the initial enzymatic action.
153 During the gastric phase, simulated gastric fluid (SGF) was incorporated until a final ratio
154 of 1:1 (v/v) was added to the mixture. 7.5 mL of SGF, 1.6 mL of porcine pepsin (25,000
155 U/mL, dissolved in SGF), 5 μ L of CaCl₂ (0.3 mol/L), and 695 μ L of water. The pH of the
156 mixture was adjusted to 3 with HCl, and the mixture was incubated at 37 °C for 2 h with
157 continuous agitation, reproducing the acidic conditions of the stomach and the proteolytic
158 action of pepsin. Finally, in the intestinal phase, gastric contents (chyme) were combined
159 with simulated intestinal fluid (SIF) in a 1:1 ratio (v/v). 11 mL of SIF, 5 mL of porcine
160 pancreatin (800 U/mL trypsin activity), 2.5 mL of bile (160 mmol/L), 40 μ L of CaCl₂ (0.3
161 mol/L), 1.31 mL of water, and the pH was adjusted to 7.0 with NaOH. The mixture was

162 incubated for 2 h at 37 °C to allow for the joint action of pancreatic enzymes and bile
163 salts on proteins, lipids, and carbohydrates.

164

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

170

171 *2.4. Antioxidant capacity*

172 *2.4.1. 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS)*

173 *radical scavenging activity*

174 The ABTS assay was performed as described by Mudd et al. (2022 with slight
175 modifications. ABTS (7 mmol/L) was prepared using 2.45 mmol/L potassium persulfate
176 and was incubated at 25 °C for 16 h. ABTS working solution was prepared fresh each
177 day by diluting the stock solution to an absorbance of 0.700 ± 0.02 at 734 nm using
178 distilled water. The analyses were performed based on the unified fractions obtained in
179 sections 2.2 (HPF) and 2.3 (HDP). The reaction was carried out in a 96-well plate with
180 10 µL of HDP (1 mg/mL), 10 µL of HPF (1 mg/mL) or 10 µL of distilled water (control),
181 and 290 µL of ABTS working solution. This mixture was incubated for 10 min at 30 °C
182 and the absorbance was read on a Multiskan TM FC Microplate Photometer (Thermo
183 Fisher Scientific, Massachusetts, United States) at 734 nm. ABTS scavenging activity
184 was reported as the Trolox Equivalent (mmol/L TE/mg sample). In addition to calculating
185 the Trolox equivalent, the peptide concentration required for 50% inhibition (IC₅₀) was
186 calculated using four different concentrations (0.1, 0.25, 0.5, 1.0 mg/mL).

187 **2.4.2. 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity**

188 The DPPH free radical scavenging activity was determined following the method
189 described by Hernández-Correas et al. (2024). The analyses were performed based on the
190 unified fractions obtained in sections 2.2 (HPF) and 2.3 (HDP). The antioxidant activity
191 of the samples was determined using a 0.02 % (w/v) solution of the DPPH radical in
192 ethanol. In Eppendorf tubes, 500 µL of ethanol, 500 µL of the sample, and 125 µL of 0.02
193 % (w/v) DPPH solution were added. Ethanol (500 µL), water (500 µL) , and 125 µL
194 DPPH were used for the blank. The samples were incubated for 1 h in the dark at room
195 temperature. The samples were then centrifuged at 10,000 g for 2 min and their
196 absorbance was measured at 517 nm.

197 $\% \text{ DPPH Radical Scavenging Assay (RSA)} = (\text{Abs Blank} - \text{Abs Sample}) / \text{Abs Blank} \times$
198 100.

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

201

202 **2.4.3. Cell Viability Assay (MTT)**

203 To evaluate HDP cytotoxicity, a colorimetric MTT assay, based on the ability of active
204 mitochondria from viable cells to reduce MTT tetrazolium (3-(4,5-dimethylthiazol-2-yl)-
205 2,5-diphenyltetrazolium bromide) to purple formazan crystals, was performed as
206 described previously by Martínez-Sánchez et al., (2020) with slight modifications. Cells
207 were seeded in 96-well plates at a density of 15000 cells per well in DMEM without
208 antibiotics and supplemented with 10% fetal bovine serum (FBS). Seeding was performed
209 at a volume of 100 µL per well and incubated for 24 h at 37 °C in a 5% CO₂ atmosphere
210 to allow adhesion. After this time, the corresponding treatments, from the unified
211 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 μ L, thus doubling the total volume and ensuring adequate dilution of the compounds. The final concentrations were adjusted using the formula $C_i \cdot V_i = C_f \cdot V_f$, 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 μ L 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 μ L 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.

230

231 **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 24h, 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 μ M along with 10 μ L 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 μ L 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.

251

252 **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 24h, 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.

272

273 **2.5. *In vivo* study using *Caenorhabditis elegans***

274 **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.

287 **2.5.2. Survival analysis under acute oxidative stress**

288 The acute antioxidant activity assay was performed following the methodology of Mudd
289 and Liceaga (2022), for which previously synchronized worms in larval stage L4 were
290 washed three times with M9 buffer solution and subsequently incubated in a solution
291 containing HDP (from the unified fraction obtained in section 2.3) at concentrations of
292 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
293 were washed with M9 buffer three times to remove any remaining peptides. Twenty to
294 25 nematodes per well (ca. 100-125/treatment) were placed in a 96-well plate by adding
295 the same volume of a solution containing tert-butyl hydroperoxide (tBOOH) at a
296 concentration of 5 mmol/L. Live nematodes were counted every 30 min until all worms
297 died. Nematodes were observed under a compound microscope (Reichert- Jung Series
298 150 Microscope, Cambridge Instruments Inc, Buffalo, NY, USA). Worms were
299 considered dead if they were completely straight and no longer moved when probed with
300 a wire stick (Harrington and Harley, 1988).

301

302 **2.5.3. Survival analysis under chronic oxidative stress**

303 A chronic oxidative stress assay was performed according to the methodology described
304 by Mudd et al. (2022). For this assay the nematodes were previously synchronized and
305 grown until reaching they reach the L4 stage, after which they were washed with M9
306 buffer three times and then incubated for 24 or 48 h in the HDP solution (from the unified
307 fraction obtained in section 2.3) at concentrations of 0.25, 0.5, 1.0, 1.5 and 2.0 mg/mL.
308 After the incubation time had elapsed, the nematodes were washed three times with M9
309 buffer to remove all traces of the applied treatment. Nematodes were placed on top of a
310 24-well transwell plate, with approximately 25-30 nematodes per well (ca. 100-120
311 nematodes per treatment) along with 200 µL of paraquat solution (25 mmol/L) and 400

312 μL at the bottom. The number of live and dead nematodes was counted and recorded
313 every day (every 24 h) at the same time from the beginning until all were dead. Nematodes
314 were observed through a compound microscope (Reichert- Jung Series 150 Microscope,
315 Cambridge Instruments Inc, Buffalo, NY, USA).

316

317 **2.5.4. Reactive oxygen species measurement**

318 To quantify the generation of ROS, the methodology used by Zhao et al. (2018), modified
319 by Mudd et al. (2022), was followed. For this assay, the nematodes were previously
320 synchronized and reached the L4 stage before starting the assay. Once this stage was
321 reached the nematodes were washed 3 times with M9 buffer and incubated in HPF (the
322 unified fraction obtained in section 2.3) at 0.25; 0.5; 1; 1.5 or 2 mg/mL for 24 or 48 h.
323 After incubation, the worms were washed three times with M9 buffer and pre-incubated
324 with 15 mmol/L paraquat solution for 48 h to generate ROS. After this period, worms
325 were washed three times with M9 buffer and once more with 0.1% tween-20 PBS
326 solution. The solution containing the nematodes was frozen at $-80\text{ }^{\circ}\text{C}$ and sonicated for
327 30 min at 60% power on ice. The nematode lysate was centrifuged for 30 min, the
328 supernatant was collected, and the protein content of the supernatant was determined
329 using a Pierce TM bicinchoninic acid (BCA) protein determination kit (Thermo Fisher,
330 Waltham, MA). 50 μL of each sample lysate (previously adjusted to the lowest
331 concentration) were placed in a black-walled 96-well plate along with 50 μL of 2,7-
332 dichlorofluorescein (DCFH-DA) to a final concentration of 50 μM . The samples were
333 incubated for 90 min, at $37\text{ }^{\circ}\text{C}$ and the fluorescence level was measured with a
334 fluorometer (Fluoroskan Ascent FL Microplate Fluorometer and Luminometer, Thermo
335 Fisher Scientific, Waltham, MA, USA) at 485 nm excitation and 535 nm emission.

336

337 **2.5.5. *C. elegans* gene expression using real time qPCR**

338 L4 nematodes, that were previously synchronized, were exposed to the HDP fraction for
 339 24 h. The nematodes were then washed with M9 buffer and lysed using TRIzol reagent.
 340 RNA extraction was carried out following the protocols described by Mudd et al. (2022a).
 341 The extracted RNA was reverse-transcribed into cDNA using the Superscript™ Reverse
 342 Transcriptase kit, following the manufacturer's guidelines (ThermoFisher Scientific,
 343 Waltham, MA, USA). Quantitative Real-Time PCR was performed using a
 344 StepOnePlus™ Real-Time PCR system (Applied Biosystems Inc., Waltham, MA, USA)
 345 and SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA)
 346 as the detection method. The primer sequences used for real-time PCR were as follows:

<i>ACT-1</i>	F: 5' - TCCAAGAGAGGTATCCTTAC - 3' , R: 5' - CGGT-TAGCCTTTGGATTGAG - 3'
<i>DAF-16</i>	F: 5' - CTCAAGCCAATGC- CACTACC - 3' , R: 5' - GGAGATGAGTTGGATGTTGATAGC - 3'
<i>SOD-3</i>	F: 5' - CCAACCAGCGCTGAAATTCAATGG - 3' ; R: 5' - GGAACC-GAAGTCGCGCTTAATAGT - 3'
<i>SKN-1</i>	F: 5' - GACGTCAATTTATG- GAGTGTCG - 3' , R: 5' - GAAGATGTTTTGTCGTGATCCG - 3;
<i>GST-4</i>	F: 5' - TGGAGACTCATTGACTTGGG - 3' , R: 5' - TCCTTTCTTGTTGCCACG - 3'
<i>GST-10</i>	F: 5' - CGTGCCACAACCTTTACTACTTC - 3' , R: 5' - CAACT-GACCAAGGAGCATTC - 3'

347

348

349

350 **2.6. Statistical analysis**

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

358

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 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, Q. 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).

385 *Table 1. Results of the in vitro antioxidant activity of dry-cured ham peptides determined by ABTS,*
386 *measured as IC₅₀ (mg/mL) values ± SEM*.*

387

388 Similar results were obtained in the DPPH assay, where the IC₅₀ value of HDP (0.431 ±
389 0.092 mg/mL) was significantly lower than that of PF (0.873 ± 0.027 mg/mL) ($P < 0.05$).
390 This confirms that the peptides generated after simulated digestion have a greater capacity
391 to eliminate DPPH-type free radicals, reaffirming their superior antioxidant potential,
392 most likely owing to the presence of hydrophobic peptides (Liao et al., 2016). In other
393 studies conducted with corn protein hydrolysates, the DPPH radical scavenging activity
394 of digested peptide hydrolysates significantly increased their reducing power by up to
395 50% (Ren et al., 2018).

396

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

399

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

405

406 **3.1.3. Caco-2 cytotoxicity (MTT)**

407 The Caco-2 monolayer cell system is widely used as a model to study the cytotoxicity of
408 bioactive peptides (Hu et al., 2024). The results of the MTT assay in Caco-2 cells shown
409 in Fig. 1 indicate that HDP peptides do not exhibit significant cytotoxicity up to a
410 concentration of 2.5 mg/mL HDP, where there was an increase in absorbance due to the
411 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., 2022; Urbizo-Reyes et al., 2022; Q. Zhang et al., 2019). Recently other authors observed a decrease in cell viability when the concentration of peptides from Xuanwei ham 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.

420

Figure 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 ($P < 0.05$).

425

3.1.4. 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.

440

441 *Figure 2. Cellular antioxidant activity in Caco-2 cells, reported as ROS generation in DHE assay, for*
 442 *dry-cured ham peptides after simulated gastrointestinal digestion (HDP) at different concentrations*
 443 *(mg/mL). Antimycin (ANTI) was used as a positive control in ROS generation and N-acetylcysteine (NA)*
 444 *as a negative control. Values represent mean observations of three replicates $\pm$ SEM. Trials that do not*
 445 *share the same letter (a, b) are significantly different ($P < 0.05$).*
 446

447 This data aligns with other that have found similar results when examining peptides from
 448 other peptide matrices. The finding suggest that a fraction of peptides from canary seeds
 449 provided homogeneous and gradually increased cellular antioxidant protection, offering
 450 cellular antioxidant activity (CAA) of up to 80% (Urbizo-Reyes et al., 2022).

451

452 *Figure 3. Cellular antioxidant activity in Caco-2 cells, reported as ROS generation in DCFDA assay, for*
 453 *dry-cured ham peptides at different concentrations (mg/mL) after simulated gastrointestinal digestion*
 454 *(HDP). Pyocyanin (PYO) was used as a positive control in ROS generation and N-acetylcysteine (NA)*
 455 *as a negative control. Values represent mean observations of three replicates $\pm$ SEM. Trials that do not*
 456 *share the same letter (a, b) are significantly different ($P < 0.05$).*
 457

458 Xing et al. (2018) reported the ability of DLEE (a peptide isolated from ham) to prevent
 459 H₂O₂-induced ROS generation in Caco-2 cells. The relative antioxidant capacity of DLEE
 460 during the cell culture test was similar to that of glutathione, a natural cellular antioxidant.
 461 Therefore, DLEE could effectively function as an exogenous antioxidant to prevent the
 462 excessive intracellular accumulation of ROS. Other studies conducted on HhepG2 cells
 463 damaged by H₂O₂ showed that antioxidant peptides derived from Huanwei ham after
 464 simulated gastrointestinal digestion upregulated the activity of the antioxidant enzymes
 465 catalase (CAT), superoxide dismutase (SOD), and glutathione reductase (GR)(Hu et al.,
 466 2023). This upregulation of antioxidant enzymes suggests that these peptides may play a
 467 crucial role in mitigating oxidative stress and protecting cells from damage caused by
 468 reactive oxygen species.

469

470 **3.2. *In vivo* antioxidant capacity**

471 **3.2.1. Lifespan analysis of *C. elegans* under acute oxidative stress**

472 Tetrazolium hydroperoxide (t-BOOH) is an organic hydroperoxide commonly used to
473 induce oxidative stress in various cell types (Sestili et al., 1998). Its toxicity is due to the
474 generation of free radicals such as butoxyl radicals through the Fenton reaction, which
475 depletes intracellular thiols and glutathione reserves, thereby weakening antioxidant
476 systems and causing cell death (Baker & He, 1991). In this experiment, tBOOH was used
477 to generate acute oxidative stress in L4-stage *C. elegans* nematodes, that had been
478 previously treated with dry-cured ham digestion peptides (HDP) for 24 or 48 h.
479 Exposure of *C. elegans* to acute stress by t-BOOH demonstrated that pre-incubation with
480 HDP significantly increased ($P<0.05$) the survival rate as a function of time and dose
481 (Fig. 4A and 4B) than in those not exposed to HDP (control). Concentrations of 1.0 and
482 1.5 mg/mL were the most effective after 24 and 48 h of pretreatment. The mean survival
483 rate of nematodes exposed to a concentration of 1.0 mg/mL was approximately 630 min,
484 while in the control group it was 390 min, which the survival rate was less than 50%.
485 Similarly, nematodes treated with a concentration of 1.0 mg/mL HDP for 48 h showed a
486 mean survival of 600 min, compared to 390 min in the control treatment. This antioxidant
487 effect was enhanced by increasing the HDP concentration to 1.5 mg/mL. These findings
488 demonstrate that *C. elegans* is a useful model for demonstrating that peptides derived
489 from dry-cured ham can offer protection against acute oxidative stress in a living system.
490 Similar results were obtained for açai (*Euterpe oleracea* Mart.) extracts, and nematodes
491 treated with the extract survived significantly longer under tBOOH-induced oxidative
492 stress (Bonomo et al., 2014).
493 This suggests that HDP induces a state of greater resistance to oxidative stress, probably
494 by activating the endogenous antioxidant pathways. Several studies using digested
495 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 *Limnospira 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% (C. 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).

509

Figure 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.

513

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 5B). 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.

523

524 *Figure 5. Survival rate (in days) of C. elegans exposed to chronic oxidative stress, paraquat. Survival*
525 *rate after being exposed to different concentrations (ranging from 0.25 to 2.0 mg/mL) of ham digested*
526 *peptides for (A) 24 h and (B) 48 hours.*
527

528 The average survival rate of nematodes exposed to a concentration of 1.0 mg/mL was
529 approximately 4.5 days, while in the control group, it was 3 days, after which the survival
530 rate was less than 50%. Similarly, nematodes treated with a concentration of 1.0 mg/mL
531 HDP for 48 h showed an average survival of 5 days, compared to 3 days for the control.
532 This antioxidant effect was enhanced by increasing HDP concentration to 1.5 mg/mL. No
533 significant increase ($P>0.05$) in half-life was observed at higher HDP concentrations in
534 the 24- and 48-h assays. Several studies have shown that certain peptides can prolong the
535 lifespan of *C. elegans* under paraquat-induced oxidative stress. For example, peptides
536 from sea cucumbers (Wu et al., 2022), ginseng (Q. Wang et al., 2016), edible crickets
537 (Mudd et al., 2022b), canary seeds (Urbizo-Reyes et al., 2022), and purple sea urchin (S.
538 Zhao et al., 2018) and Asian hard clams (Jia et al., 2018) showed positive and protective
539 effects towards nematode survival rates. In another study using antioxidant peptides from
540 a hydrolysate of *Sepia esculenta*, superoxide dismutase (SOD) activity increased
541 significantly when worms were co-treated with paraquat and peptides, suggesting that
542 antioxidant peptides modulate intracellular antioxidant defence (Yu et al., 2020). Specific
543 amino acid residues of peptides, such as hydrophobic residues, are considered to play a
544 key role in enhancing the activity of antioxidant enzymes. In the future, the effect of these
545 amino acid residues could be confirmed by substitution to better understand their role in
546 antioxidant activity.
547 As paraquat causes oxidative stress through increased ROS generation (Blanco-Ayala et
548 al., 2014), ROS levels were measured to corroborate these results.
549

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- κ B, 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.

568

Figure 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 (dichlorofluorescein) 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;

579 Yu et al., 2020). A reduction in ROS levels was also observed in nematodes when pre-
580 incubated with 2.0 and 4.0 mg/mL peptides from *Angelica sinensis* protein hydrolysate
581 and exposed to paraquat (2 mmol/L) (Q. Wang et al., 2016).

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

595

596 3.2.4. Gene expression levels in *C. elegans*

597 To explore the molecular pathways associated with the antioxidant properties of these
598 peptides, the expression levels of various antioxidant-related genes (*DAF-16*, *SOD-3*,
599 *SKN-1*, *GST-4* and *GST-10*) were analyzed using real-time quantitative PCR (RT-qPCR)
600 after a 24 h exposure to different concentrations of HDP. Gene expression analysis (Fig.
601 7) revealed a significant increase ($P<0.05$) in the upregulation of genes (*GST-4*, *GST-10*,
602 *SKN-1*, and *SOD-3*) associated with the oxidative stress response after treatment with
603 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* (Z. Wang 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 defence 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 and 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-1* 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 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* (W. 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.

647

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

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

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

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

678 Although *C. elegans* provides a robust in vivo model sharing conserved antioxidant and
679 longevity pathways (IIS, MAPK, SKN-1/NRF2) with mammals, they are not identical,
680 with some differences existing when compared to the latter. Therefore, caution should be

681 exercised when extrapolating these findings, and future studies should validate the
682 antioxidant and health-promoting effects of ham-derived peptides in mammalian models,
683 as well as validating their efficacy in human clinical trials. Nevertheless, the presence of
684 bioactive peptides in functional food matrices such as from Spanish dry-cured ham
685 represents a promising avenue for preventive nutritional strategies based on scientific
686 evidence.

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723

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725 The authors declare that they have no known competing financial interests or personal
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727

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1 **TABLE 1**
2 **Table 1. Results of the *in vitro* antioxidant activity of dry-cured ham peptides**
3 **determined by ABTS, measured as IC₅₀ (mg/mL) values ± SEM*.**

ABTS	
Sample code ¹	IC ₅₀ (mg/mL)
PF	0.362 ^a ± 0.088
HDP	0.279 ^b ± 0.019
Control	-

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

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TABLE 2

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

DPPH	
Sample code ¹	IC ₅₀ (mg/mL)
PF	0.873 ^a $\pm$ 0.027
HDP	0.431 ^b $\pm$ 0.092
Control	-

¹ PF: Peptide Fraction

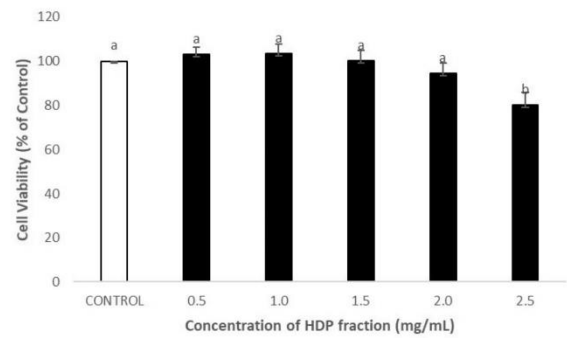
HDP: Ham Digested Peptides

Control: Distilled water. «-»: not detected / not applicable.

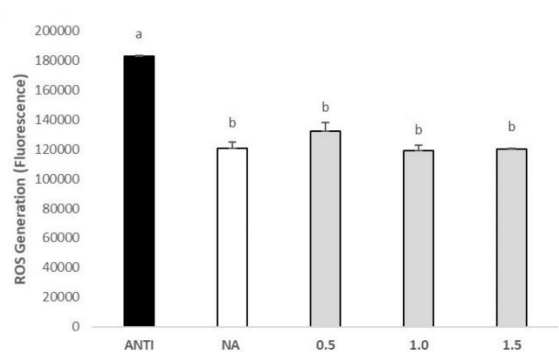
*SEM: standard error of mean.

Samples that do not share the same letter (a, b) are significantly different ($P < 0.05$).

1 **FIGURE 1**



2
3 **Figure 1.** Effect of simulated gastrointestinal ham digested peptides (HDP) on the viability
4 of Caco-2 cells, reported as Cell Viability % using the MTT assay. Control contained a
5 simulated digestion with water instead of ham peptides. Values represent mean observations
6 of three replicates $\pm$ SEM. Trials that do not share the same letter (a, b) are significantly
7 different ($P<0.05$).
8

1 **FIGURE 2**

2

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

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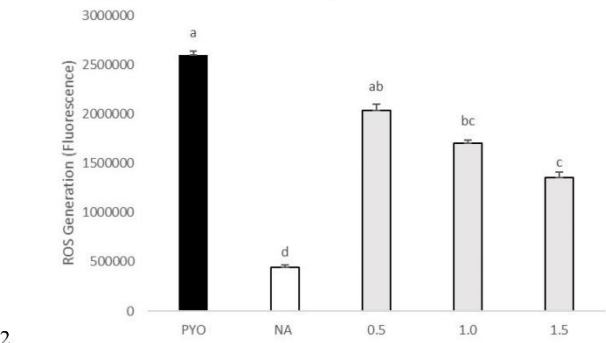
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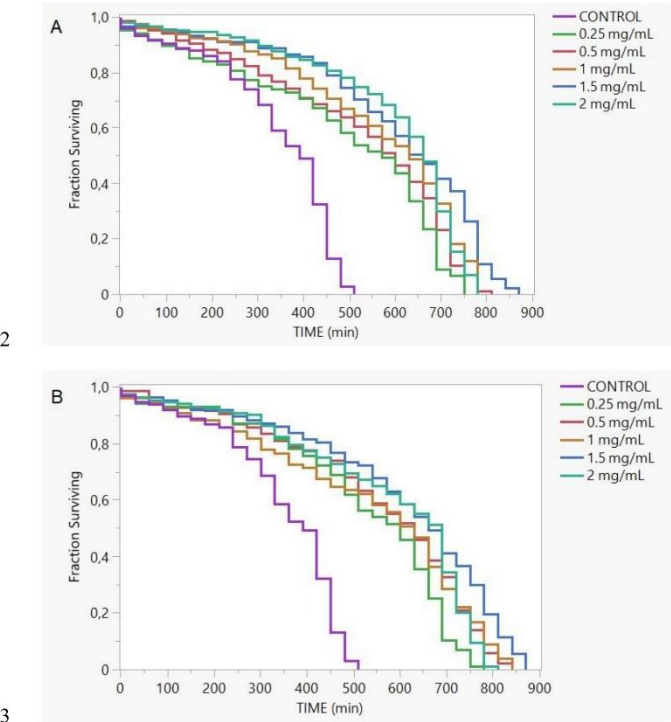
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1 **FIGURE 3**



2
3 **Figure 3. Cellular antioxidant activity in Caco-2 cells, reported as ROS generation in**
4 **DCFDA assay, for dry-cured ham peptides at different concentrations (mg/mL) after**
5 **simulated gastrointestinal digestion (HDP). Pyocyanin (PYO) was used as a positive control**
6 **in ROS generation and N-acetylcysteine (NA) as a negative control. Values represent mean**
7 **observations of three replicates $\pm$ SEM. Trials that do not share the same letter (a, b) are**
8 **significantly different ($P<0.05$).**
9

1 **FIGURE 4**



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

1 **FIGURE 5**

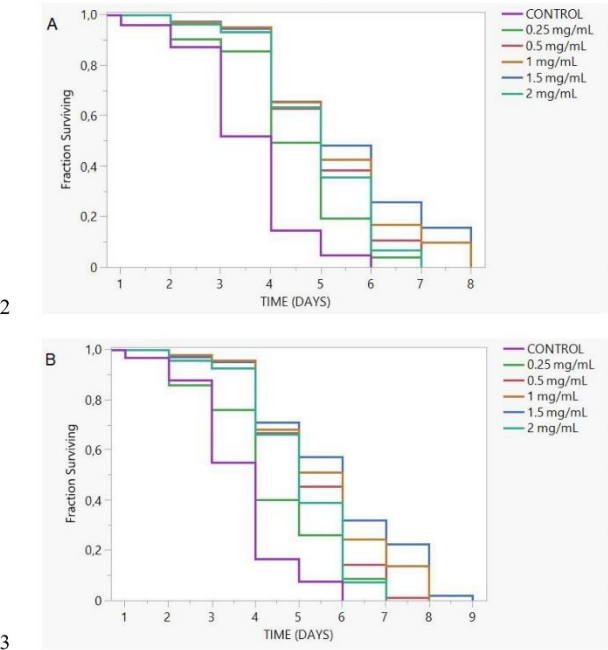
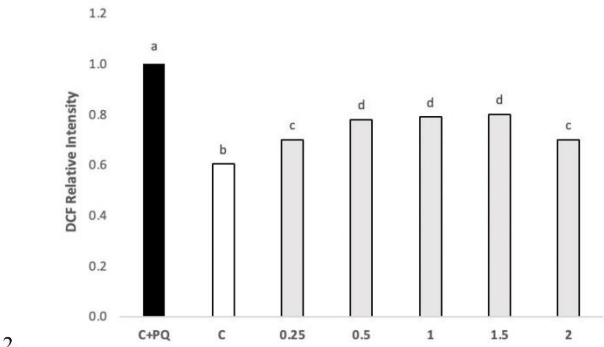


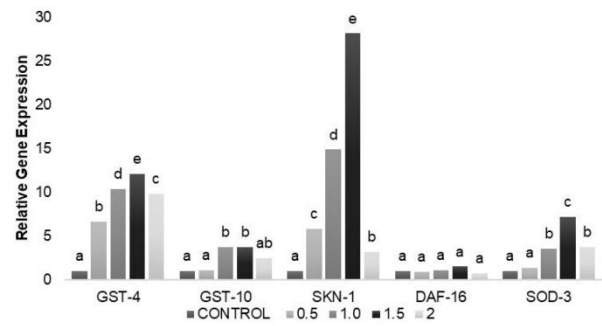
Figure 5. Survival rate (in days) of *C. elegans* exposed to chronic oxidative stress, paraquat. Survival rate after being exposed to different concentrations (ranging from 0.25 to 2.0 mg/mL) of ham digested peptides for (A) 24 h and (B) 48 hours.

1 **FIGURE 6**



2
3 **Figure 6. Reactive oxygen species (ROS) levels in *C. elegans* pretreated with different**
4 **concentrations of dry-cured ham digested peptides (at different concentrations in mg/mL)**
5 **and then exposed to paraquat (PQ). The DCF (dichlorofluorescein) intensity was determined**
6 **using a fluorometer at excitation 485 nm and emission 535 nm. C + PQ= control (with**
7 **paraquat, no peptides) , C= control (without paraquat and without peptides). The results**
8 **represent the adjusted fluorescence values from three independent assays repeated thrice,**
9 **those that do not share the same letter (a, b, c, d) are significantly different ($P<0.05$).**

1 **FIGURE 7**



2
3 **Figure 7. Oxidative stress-related genes analysis. Effect of simulated gastrointestinal dry-**
4 **cured ham digested peptides (HDP) on genes related to *C. elegans* oxidative stress response.**
5 **Trials for each gene that do not share the same letter (a, b, c, d, e) are significantly different**
6 **($P<0.05$).**
7

V – DISCUSSION

V - DISCUSSION

This doctoral thesis has evaluated the potential of boneless dry-cured ham as a functional food, focusing on the influence of different processing strategies; over-maturation and temperature control, modulation of nitrifying salts, and reduction of sodium content; on the generation of bioactive peptides. This approach has made it possible to evaluate dry-cured ham not only from its traditional and gastronomic perspective, but also as a matrix capable of providing benefits in the context of chronic diseases such as hypertension, oxidative stress, and metabolic syndrome. Although the endogenous proteolysis of ham releases a wide variety of peptides, clinical evidence in humans remains scarce, making it necessary to advance a multidisciplinary approach. Thus, this thesis integrates *in vitro* and *in silico* studies that have made it possible to characterize sequences with antioxidant and antihypertensive activity, as well as *in vivo* validations in simple models such as *Caenorhabditis elegans*, and sensory and technological evaluations aimed at consumer acceptability despite the different processing technologies used. All this forms an approach that combines peptidomic techniques, bioinformatic assays, cellular models, living organisms, and quality tests, with the aim of positioning reduced sodium boneless dry-cured ham as a healthier product with functional potential in the prevention of chronic diseases.

2.1. BEYOND ITS GASTRONOMIC VALUE: CURED HAM IS A FOOD OF INTEREST DUE TO ITS FUNCTIONAL PROPERTIES.

Dry-cured ham is an emblematic product of the Mediterranean diet, whose appeal has transcended purely gastronomic interests to enter the field of nutritional and functional research. The study of the peptides generated during curing has revealed bioactive potential with applications in the prevention of chronic diseases, especially those linked to metabolic syndrome. However, it is necessary to critically analyze these findings, considering both methodological limitations and comparison with other protein matrices of animal and plant origin.

Endogenous proteolysis processes, mediated by calpains, cathepsins, and exopeptidases, release a wide range of low molecular weight peptides, many of which have antihypertensive, antioxidant, antidiabetic, and antimicrobial properties (Hernández Correás et al., 2025; Toldrá et al., 2020). Among the most notable, angiotensin-converting enzyme (ACE) inhibitor peptides such as AAPLAP, KPVAAP, and KAAAAP have shown potencies comparable to those of peptides derived from milk caseins (such as VPP and IPP) widely used in functional foods (Dellafiora et al., 2015; van Platerink et al., 2008). However, unlike fermented dairy products, where peptides have been validated in extensive clinical studies and have achieved some commercial application, in the case of cured ham, evidence in humans remains incipient and limited to trials with small samples (Montoro-García et al., 2017, 2022).

Another relevant aspect is the gastrointestinal stability of peptides. Although significant activity has been observed *in vitro*, simulated digestion studies indicate that many long fragments degrade rapidly, releasing smaller di- and tripeptides that could be the real culprits behind the physiological effects (Paoletta et al., 2015). This coincides with observations in milk proteins and fish proteins, where antihypertensive and antioxidant activity is concentrated in fragments of 2–3 amino acids (Abachi et al., 2019; Liu et al., 2024). Therefore, beyond peptidomic identification, it is essential to evaluate actual bioavailability and metabolic profile under physiological conditions.

The multifunctionality of cured ham peptides, which are capable of acting simultaneously as ACE inhibitors, DPP-IV modulators, and antioxidants, is a particularly interesting characteristic (Gallego, Mora, Hayes, et al., 2019). However, this phenomenon is not exclusive to cured meats. In the case of milk proteins, peptides derived from β -lactoglobulin and α -casein also exhibit multifunctional effects, having even achieved nutraceutical developments approved in Japan and Europe (Udenigwe & Aluko, 2012). Similarly, fish hydrolysates represent another recognized source of bioactive peptides, with antihypertensive, antioxidant, and anti-inflammatory activities documented in both *in vitro* trials and animal models (Abachi et al., 2019; Ashraf et al., 2020).

Comparatively, cured ham presents both advantages and challenges. On the one hand, it is a traditional food with widespread cultural acceptance and a production process that favors prolonged and complex proteolysis, which is

difficult to replicate in other systems (Petrova et al., 2015). On the other hand, the high variability derived from curing conditions (time, salinity, humidity, pig genetics) introduces a degree of heterogeneity that can hinder the standardization of bioactivity, something less pronounced in dairy or marine products, where technological processes are more controlled.

The antioxidant peptides identified in cured ham, such as SNAAC or AEEEEYPDL, show remarkable potency, even superior to reduced glutathione in some trials (Gallego, Mora, Reig, et al., 2018; Gallego, Mora, & Toldrá, 2018a). However, a parallel can be observed with plant peptides, especially those derived from soybeans and legumes, where short sequences rich in proline and glycine have demonstrated similar antioxidant efficacy and metal chelation capacity (T.-T. Zhang et al., 2024). The fundamental difference lies in the fact that, while plant peptides are usually studied in controlled fermentation or enzymatic hydrolysis contexts, in cured ham the origin is a spontaneous and prolonged maturation process, which adds complexity but also uniqueness to the bioactive profile.

As for anti-inflammatory activity, studies on cured ham are still preliminary and mostly based on peptide mixtures (Xing et al., 2023). In contrast, in other animal proteins such as deer (velvet antler), specific peptides with clear and reproducible anti-inflammatory activity have already been isolated and identified (L. Zhao et al., 2016). This indicates that dry-cured ham indeed holds potential, but further work is needed regarding its isolation, characterization, and validation.

2.1.1. Nutraceutical prospects and technological challenges

The application of cured ham peptides in nutraceuticals or functional foods is promising but faces several challenges. Microencapsulation and nanoencapsulation have proven to be effective tools for improving the stability and bioavailability of peptides in dairy and marine matrices (Jiang et al., 2024; Sun et al., 2021). Their adaptation to cured ham seems feasible, although it will require adjusting techniques to protect peptides that are sensitive to pH or temperature variations.

One point of difference from other matrices is the possibility of valorizing by-products. Ham bones have been shown to be a source of hydrolyzed collagen with

anti-inflammatory peptides such as GPTGF (Hao et al., 2023), which opens the door to comprehensive use of the production chain, in line with sustainability. In comparison, this type of by-product utilization is already well established in the fishing industry (Liu et al., 2024), which could serve as a model for the meat sector.

2.2. PROCESSING TECHNOLOGIES – INFLUENCE ON THE QUALITY AND BIOACTIVE PEPTIDES GENERATION OF BONELESS CURED IBERIAN HAM

After reviewing the literature on bioactive peptides present in cured ham (Hernández-Correas et al., 2025), it became clear that further research was needed into the technological factors that modulate their generation. Although peptidomics has made it possible to identify multiple sequences with bioactive potential, their concentration and profile depend largely on processing conditions. In this context, the next logical step was to evaluate how controlled modifications in processing technology—in particular, sodium reduction and prolonged maturation (overripening) in this case in boneless Iberian dry cured hams—could influence not only proteolysis and the release of amino acids and bioactive peptides, but also the sensory quality and acceptability of the product (Hernández Correas et al., 2024)

The first trials were carried out on boneless Iberian hams. However, because the regulations governing Protected Designations of Origin (PDO) do not allow Iberian ham to be marketed in boneless form under that designation and considering the need to ensure the industrial viability of the proposal, it was necessary to change the raw material. Therefore, subsequent studies worked with white hams from animals with Duroc genetics (50% Duroc × 50% Landrace × Large White), a line recognized for providing characteristics similar to those of Iberian ham in terms of fat infiltration and technological quality.

This change is important because the animal's genetics, muscle microstructure, and intramuscular fat content directly influence the intensity of proteolysis, the composition and diversity of the peptide profile generated during maturation, and the thermal sensitivity of endogenous enzymes.

One of the most relevant findings was the increase in the proteolysis index (PI) and non-protein nitrogen (NPN) content in the batches subjected to longer

maturation times and, especially, to high temperatures (36 °C in the final phase). These results confirm that prolonging the processing favors protein degradation and the release of soluble nitrogen compounds, thereby generating bioactive peptides, coinciding with what has been previously described in Jinhua and Parma hams (Soresi Bordini, 2004; G. H. Zhou & Zhao, 2007). The intensification of proteolysis under these conditions has been attributed to increased activity of cathepsins B and L, enzymes whose function is modulated by pH, salt, and temperature (Toldrá, 2006; G. M. Zhao et al., 2005).

Sodium reduction has been shown to enhance proteolysis, in agreement with Armenteros et al., 2012, who demonstrated that partial replacement of NaCl with other chloride salts does not inhibit muscle peptidase activity. In fact, a lower sodium content may stimulate the activity of certain cathepsins (Chen et al., 2025; Harkouss et al., 2014; Yalınkılıç et al., 2025), which would explain the elevated NPN values observed in the reduced-salt ham batches.

The increase in maturation time and the application of higher temperatures promoted a significant accumulation of free amino acids (FAA)—notably glutamate, alanine, leucine, and proline (Hernández Correas et al., 2024). This trend is supported by investigations in Chato Murciano dry-cured hams showing FAA rising progressively during curing until advanced stages where their concentration stabilizes and even decreases (Salazar et al., 2020). Furthermore, in meat systems with modified salt conditions, low-sodium salt substitution was shown to enhance the protein hydrolysis index and to upregulate cathepsin B and L activities (J. Li et al., 2024). Additional evidence from activation strategies (e.g. electrical stimulation) confirms that cathepsin B and L activities may increase dramatically, accelerating proteolysis (X. Zhang et al., 2024). These free amino acids not only enrich the flavor profile (umami from glutamate, sweetness from alanine, slight bitterness from leucine) but also serve as essential precursors for the generation of bioactive peptides. Thus, elevated FAA levels in overripened hams under thermal intensification may be viewed as an indirect marker of enhanced functional potential.

The relationship between FAA and bioactivity has also been observed in other meat products. Guo et al., 2021 reported that increased FAA in cured lamb ham was associated with a higher content of peptides with antioxidant and antihypertensive properties. Similarly, Muñoz-Rosique et al., 2023 demonstrated

that sodium reduction in boneless Iberian dry-cured hams increased the bioactivity of peptide extracts, confirming that FAA can be considered markers of functionality.

Despite the increase in proteolysis, no significant changes were observed in the instrumental hardness, elasticity, or chewiness of the overripened hams. The only parameter that differed was adhesiveness, which was slightly higher in the batches subjected to longer maturation and higher temperatures. This result contrasts with that described in Italian studies, where excessive maturation periods led to textural defects (soft paste, stickiness) and a reduction in sensory acceptability (Benedini et al., 2012). The fact that no such defects were detected in this case may be linked to the boneless format and the controlled management of humidity and temperature, which prevent destabilizing proteolysis.

In terms of color, overripening caused a slight decrease in brightness (L^*), attributed to the concentration of pigments such as nitrosyl-myoglobin due to dehydration (Sanabria et al., 2004). This change did not significantly affect the a^* and b^* parameters, coinciding with what has been reported in previous studies on cured hams with different maturation times (Costa-Corredor et al., 2009; Pérez-Santaescolástica et al., 2018).

Consumer analysis showed that changes in processing methodology did not negatively affect overall acceptability. Only the color showed slight differences, while the flavor, smell, and texture were rated similarly between batches. These results coincide with those obtained by Cilla et al., 2005 in Serrano hams, where the sensory attributes remained stable during prolonged maturation as long as no textural defects appeared. Similarly, in Celta-type hams, Bermúdez et al., 2014 found that extending the curing time increased aromatic complexity without decreasing consumer acceptance.

This finding is particularly important in the current context of sodium reduction in meat products, where one of the main concerns is the loss of flavor and consumer acceptance (Hunter et al., 2022; Taormina, 2010). The results suggest that technological strategies such as overripening can compensate for these shortcomings by intensifying proteolysis and increasing FAA, while maintaining a positive sensory profile.

Although this study did not directly identify the peptides generated under the conditions applied, the increase in PI and FAA is a clear indication that overripening may favor the release of bioactive peptides, as has been documented in other studies (Escudero et al., 2013a; Gallego, Mora, & Toldrá, 2019). Furthermore, the use of controlled maturation conditions in reduced-sodium hams is in line with the need to produce healthier foods without sacrificing sensory quality, in accordance with innovation trends in the European meat industry (Petrova et al., 2015; Ventanas Barroso, 2012).

However, complementary peptidomic and *in vitro* studies are required to confirm that the peptides released under these conditions maintain bioactivity (ACE inhibition, antioxidant activity, DPP-IV modulation, etc.). It is also essential to move towards clinical trials to verify the efficacy of consuming reduced-sodium, overripening Iberian ham in modulating cardiovascular and metabolic parameters, as has already been suggested in preliminary trials with traditional cured ham (Montoro-García et al., 2017; 2022).

2.2.1. Bioactive Peptide Generation

After verifying that overripening and temperature control did not compromise the sensory quality or acceptability of boneless Iberian hams with reduced sodium content, and in line with the necessary complementary studies, it was decided to determine whether these same technological strategies could significantly enhance the release of bioactive peptides and improve their physiological functions. Thus, this study delved into the characterization and evaluation of specific biological activities (antioxidant and angiotensin-converting enzyme (ACE) inhibitor), in order to establish a direct link between processing conditions and the biofunctional profile of boneless reduced-sodium Iberian dry-cured ham (Hernández-Correas et al., 2024).

This study provided robust evidence on how overripening and moderate temperature increases modulate the generation of bioactive peptides in boneless reduced-sodium Iberian dry-cured hams, confirming the hypothesis that prolonged curing time favors proteolysis and the release of bioactive sequences (Mora, Gallego, et al., 2015). The results showed that both overripening at 30 °C

and at 36 °C significantly increased antioxidant capacity compared to the traditional control. Overripening boneless Iberian dry-cured ham at 30 °C stood out, with an IC₅₀ of 1,144 mg/mL, lower than that of traditional batches and values reported in other cured ham studies (Hu et al., 2023; P. Li et al., 2022). This high capacity is attributed to the abundance of hydrophobic amino acids (leucine, isoleucine, valine, alanine) in key positions, known to enhance antioxidant activity (Saiga et al., 2003; J. Wang et al., 2021).

The observation that excess temperature (36 °C) did not improve bioactivity is consistent with findings in other meat products, where overheating promotes more aggressive proteolysis, with degradation of functional peptides (Y. Fu et al., 2017; C.-Z. Zhu et al., 2013). In fact, studies on Jinhua ham have shown that high temperatures can accelerate proteolysis but do not always guarantee the stability of bioactive sequences (Luo et al., 2021). Thus, the data reinforces the idea that 30°C represents an optimal thermal window for preserving antioxidant functionality.

In addition, extracts from overripened hams showed a remarkable ability to inhibit ACE, with significantly lower IC₅₀ values in overripening boneless Iberian dry-cured ham at 30 °C (0.131 mg/mL) compared to the control (0.172 mg/mL). This indicates greater hypotensive potential, in line with previous studies that linked prolonged curing times to an increase in the concentration of dipeptides such as Ala-Ala (AA) and Val-Pro (VP), with ACE-inhibitory activity (Escudero et al., 2012, p. 2; Heres et al., 2021).

However, bioactivity was lower in overripening boneless Iberian dry-cured ham at 36 °C, suggesting that excess temperature may inactivate critical proteolytic enzymes or degrade the released peptides. The divergence may stem from inherent genetic and compositional differences: Iberian pork is characterized by substantially higher intramuscular fat, distinct muscle microstructure and associated microbiota, conditions that may render endogenous enzymes more heat-sensitive in this matrix (Abril et al., 2023; Kaur et al., 2021).

2.2.1.1. *Identification and biostatistical analysis*

nLC-MS/MS analysis revealed substantial differences in the number and diversity of peptides: 411 sequences in the control batch, 954 in overripening boneless Iberian dry-cured ham at 30 °C and 1,313 in overripening boneless Iberian dry-cured ham at 36 °C. The high diversity in overripening boneless Iberian dry-

cured ham at 36 °C confirms that high temperatures intensify proteolysis, although not necessarily to the benefit of bioactivity. Many of these fragments contained bioactive motifs described in the BIOPEP database, associated with antioxidant, anti-inflammatory, hypolipidemic, or enzyme-inhibiting activities such as DPP-IV and HMG-CoA reductase (Heres et al., 2021; Minkiewicz et al., 2022).

Among the relevant sequences, peptides derived from actin and myosin with ACE inhibitory (APAPAPPKE, AGFAGDDAPR) and antioxidant (AKEWGYAD, YETDAIQR) potential were identified, coinciding with what has been previously described in Serrano and Xuanwei hams (Escudero et al., 2013a; Nie et al., 2020). Fragments with possible anti-inflammatory properties (PA, DA, DG) were also detected, in line with recent studies in cell models (Xing et al., 2023).

Biostatistical analyses showed that overripening conditions favor not only classic bioactivities (antioxidant, anti-ACE), but also other less explored ones, such as immunomodulatory, hypolipidemic, anti-amnesic, or osteoanabolic activities. Although these associations are derived from bioinformatic predictions rather than direct testing, they suggest a much broader potential for Iberian ham as a functional food. This approach is in line with the current trend in food peptide research, where multifunctionality is considered key to nutraceutical applications (Peighambardoust et al., 2021; Zaky et al., 2022).

The results highlight the importance of fine temperature control: while 30°C favors maximum bioactivity, 36°C increases peptide diversity but with lower functionality. This observation raises a technological dilemma between peptide quantity and quality, making it necessary to prioritize strategies that optimize bioactivity rather than mere diversity.

This study confirms that overripening and thermal control are key tools for modulating the peptide bioactivity of boneless reduced-sodium Iberian dry-cured ham. Unlike previous work, which focused on physicochemical and sensory aspects, this study directly demonstrates that these conditions enhance antioxidant and antihypertensive activities, as well as expanding the diversity of potential bioactivities.

2.3. NITRIFYING SALTS – INFLUENCE ON THE QUALITY OF BONELESS CURED HAM AND BIOACTIVE PEPTIDES GENERATION ON BONELESS DRY-CURED HAMS

After confirming that overripening and increased temperature could enhance proteolysis and the release of bioactive peptides in boneless reduced-sodium Iberian dry-cured hams (Hernandez Correas et al., 2024) and, due to the turn of events that resulted from the change in the type of raw material used, another critical component in curing became apparent: nitrifying salts. Their role is essential in inhibiting microorganisms such as *Clostridium botulinum* (Torres-Baix et al., 2023), in stabilizing the typical pink color through the formation of nitrosylmyoglobin (Honikel, 2008; Toldrá et al., 2009) and in the development of aroma. However, their use also carries risks: nitrites are highly reactive, oxidize easily or convert to nitrates, and can lead to the formation of nitrosamines, compounds with recognized carcinogenic potential (Bedale et al., 2016; Demeyer et al., 2008; Herrmann et al., 2015; Sebranek & Bacus, 2007).

Although alternatives have been proposed in recent decades, such as partially replacing NaCl with KCl, MgCl₂, or CaCl₂ salts (Armenteros et al., 2012; Barat et al., 2013; Blesa et al., 2008), or applying emerging technologies (high pressure, ultrasound) to ensure safety in low-salt products (Coll-Brasas et al., 2019; Duranton et al., 2012; Gao et al., 2014), knowledge of how variations in nitrate and nitrite content specifically affect the physicochemical, biochemical, and sensory profile of cured ham remained limited. Furthermore, studies on the dynamics of nitrates (a more stable reservoir) and their conversion to nitrites by reducing bacteria (mainly staphylococci) have shown that this balance is decisive for both the safety and final quality of the product (Armenteros et al., 2012; Wakamatsu, 2022).

Therefore, an experimental study was designed on boneless white hams (50 % *Duroc* and 50 % *Landrace x Large White*), applying different formulations of nitrifying salts (with variations in nitrite, nitrate, and their combinations) and two maturation times (standard and overripening), with the aim of evaluating their impact on composition, proteolysis, free amino acid profile, the generation of volatile compounds, color, texture, and sensory acceptability.

The results obtained show that both the composition of the nitrifying salts and the degree of maturation significantly influence moisture, water activity (aw),

non-protein nitrogen (NPN), and the proteolysis index (PI). Overripening markedly reduced moisture and aw (<0.89 in all cases), values that are below the growth threshold of *Clostridium botulinum* and most pathogenic bacteria (Affairs, 2018; Torres-Baix et al., 2023), thus ensuring product safety even when the nitrite/nitrate concentration was reduced.

The increase in PI and NPN in hams with lower amounts of nitrifying salts, treatments VI (Standard nitrifying salt concentration without nitrates) and VII (Standard nitrifying salt concentration with higher nitrite proportion), confirms that these nitrifying salts have an inhibitory effect on muscle proteolytic enzymes (Toldrá, 2006; C.-Y. Zhou et al., 2019). A lower concentration of nitrifying salts favors the activity of cathepsins and aminopeptidases, increasing the release of peptides and free amino acids (Gratacós-Cubarsí et al., 2013; Muñoz-Rosique et al., 2022). This effect is comparable to that observed in other low-salt cured products, such as “cecina de potro” (Cittadini et al., 2020) or Jinhua-type hams (J. Zhang et al., 2011), where the reduction of protein metabolism inhibitors intensified proteolysis.

However, it is important to note that excessive proteolysis can lead to undesirable textural defects and bitter flavors, as reported in Italian hams that have been overripened (Benedini et al., 2012; Cilla et al., 2005). In this regard, treatment I (Standard concentration + 60%), with higher nitrate residue (213–227 mg/kg), presented a more balanced profile: lower FAA accumulation and a more moderate PI, but with the best overall sensory score, suggesting that some limitation of proteolysis may be positive from an organoleptic point of view.

The total free amino acid (FAA) content increased significantly with overripening, especially in treatments with a lower proportion of nitrifying bacteria. Among the most representative amino acids were alanine, proline, and serine (sweet flavors), as well as leucine, valine, and isoleucine (bitter flavors) (Toldrá et al., 2000; Virgili & Schivazappa, 2002). These FAAs, in addition to directly influencing flavor, are key precursors in the formation of volatile compounds derived from Maillard and Strecker reactions, which are responsible for the characteristic aroma of cured hams (C. Li et al., 2021). The higher concentration of FAAs in hams with reduced nitrates/nitrites indicates a potential increase in the formation of these compounds.

However, excess bitter-tasting FAA could compromise acceptance, as observed in some treatments VII and IX (Standard nitrifying salt concentration

with higher nitrite proportion), where the accumulation of valine and isoleucine was associated with lower flavor scores. This finding supports the hypothesis that the balance between sweet and bitter flavors is essential for sensory acceptability (Virgili & Schivazappa, 2002).

The analysis of volatiles showed that overripening increased the presence of aldehydes (hexanal, pentanal, heptanal), alcohols, and short-chain fatty acids, indicators of greater lipid oxidation. Hams with lower concentrations of nitrifying agents had higher levels of aldehydes, confirming the antioxidant role of nitrite, which is capable of slowing down the oxidation of lipids and myoglobin (Akköse et al., 2016; Sebranek & Bacus, 2007).

This protective effect had already been documented in products such as *pastirma*, where adequate levels of nitrate significantly reduced the accumulation of hexanal and rancid compounds (Akköse et al., 2016). In the present study, the treatments with higher nitrate residues, I (Standard concentration + 60%) and VIII (Double concentration) showed more balanced volatile profiles, with a predominance of notes associated with curing, while the reduced formulations tended to generate more oxidized profiles.

Color is one of the attributes most sensitive to the modification of nitrifying salts. Treatments with higher nitrate/nitrite levels had higher a^* values (redness intensity), attributable to greater formation of nitrosylmyoglobin (Honikel, 2008). In contrast, the batches with marked reduction showed less intense tones, which coincides with what has been described in Iberian hams with partial replacement of NaCl and nitrites by other salts (Armenteros et al., 2012; Barat et al., 2013). Overripening slightly reduced brightness (L^*), a result consistent with the progressive darkening reported in Parma and Celta hams during prolonged curing (Bermúdez et al., 2014).

In terms of texture, prolonged drying increased hardness and chewiness, associated with water loss and protein compaction (Ruiz et al., 2006). However, no significant differences were detected between nitrite salt treatments, suggesting that nitrite/nitrate modulation affects biochemistry and color more than mechanical texture.

Finally, sensory analysis revealed that all hams were acceptable, with average scores above 2.5 on a hedonic scale of 1 to 5. However, there were differences:

treatment I (Standard concentration + 60%) was the highest rated in overall acceptance (3.92), associated with a more attractive color and firm texture, while treatment IX (Standard nitrifying salt concentration with higher nitrite proportion) obtained the lowest ratings (3.18), due to lower color intensity and perceived excess salinity.

The reduction in nitrates/nitrites tended to decrease the rating of color and cured flavor, although it did not cause widespread rejection, which is consistent with the findings of Cilla et al. 2005 and Armenteros et al. 2012, who demonstrated that consumers tolerate formulations with fewer additives as long as overall quality is maintained.

2.3.1. Bioactive Peptide Generation

After analyzing the effect of reducing nitrifying salts and overripening on composition and sensory analysis, it was necessary to evaluate how these conditions directly influenced the generation of bioactive peptides and their potential physiological effects. Previous studies had shown that prolonged curing increases proteolysis and the release of free amino acids, suggesting an increase in bioactive potential (Gallego et al., 2015; Muñoz-Rosique et al., 2023; Virgili et al., 2007). Likewise, the partial replacement or reduction of nitrifying salts, although useful from a public health perspective, could modify the enzymatic profile and stability of the peptides generated (Armenteros et al., 2012; Barat et al., 2013).

However, to date, most studies have focused on characterizing sensory or compositional parameters, with little information on how these technological modifications affect biological functionality. Research on peptide bioactivity in cured ham has grown over the last decade (Escudero et al., 2012, 2013b; Gallego, Mora, Hayes, et al., 2019; Heres et al., 2021, 2022), but it has mostly been approached from a peptidomic or *in vitro* perspective, without integrating simulated digestion analysis or advanced molecular predictions. In this context, this new study represented an integrative effort by combining *in vitro* analysis (antihypertensive and antioxidant activity) with *in silico* approaches to digestion, docking, and pharmacophore similarity, as well as the quantification of dipeptides after simulated gastrointestinal digestion. This multidimensional approach allows

us to discuss not only which peptides are generated under different technological conditions, but also their actual viability as functional compounds in the body, a key aspect at the frontier between food science and clinical nutrition.

2.3.1.1. *In vitro analysis*

The inhibitory capacity of angiotensin-converting enzyme (ACE) in cured ham extracts depended mainly on the maturation time, rather than on the composition of the nitrifying salts used (nitrites and nitrates proportion). This observation is consistent with what was previously described by Escudero et al. 2012, 2013, who demonstrated that prolonged curing periods favor the action of muscle peptidases that release low molecular weight fragments with potent ACE-inhibitory activity. (M. A. Sentandreu & Toldrá, 2007; M. Sentandreu & Toldrá, 2001) had already pointed out the importance of endogenous dipeptidases in the generation of short peptides such as Ala-Ala or Val-Pro, which are responsible for much of this bioactivity.

Similarly, Heres et al., 2021 and Hernández-Ledesma et al., 2011 confirmed that prolonged maturation in meat products intensifies the accumulation of ACE inhibitors, comparable to bioactive peptides derived from fermented dairy products. Consequently, the formulation of nitrifying curing salts appears to play a secondary role in this regard, as antihypertensive activity is more closely linked to the advancement of natural proteolysis during curing.

In the case of antioxidant activity, the results showed more marked differences between nitrifying salt treatments and ripening stages. The variation in bioactivity according to nitrifying salt type coincides with the findings of Nan et al. 2024, who observed greater generation of peptides <3 kDa with antioxidant capacity in low-salt hams. The relevance of hydrophobic residues in the neutralization of free radicals (Tyr, Trp, Met, Cys) has been widely documented (J. Wang et al., 2021; Zheng et al., 2016), explaining why treatments and curing times that favor their release lead to greater antioxidant capacity.

The identification analysis revealed a significant increase in the amount of low molecular weight peptides (<1.5 kDa) in the prolonged maturation phases, which is consistent with the results of Heres et al., 2023 and P. Li et al., 2022, who

associated these fragments with greater bioavailability and functional activity. (Harnedy et al., 2017) also pointed out that both size and amino acid composition are determinants of the antioxidant and antihypertensive potential of protein hydrolysates, reinforcing the importance of this finding.

The abundance of dipeptides such as EE, VG, VD, DA, and DG, already described in other studies by Gallego et al. 2019, 2022 and Heres et al. 2021, 2022, suggests multifunctionality that may range from ACE inhibition to the modulation of metabolic enzymes such as HMG-CoA reductase or DPP-IV, with direct implications for the prevention of cardiovascular and metabolic diseases (Bailey et al., 2023; Nie et al., 2020).

2.3.1.2. *In silico analysis*

Simulation of gastrointestinal digestion and *in silico* analysis provided additional information on the potential of the peptides generated. Dipeptides such as DG and DA were identified as potential ACE inhibitors and antioxidants, while VD and PA were associated with DPP-IV inhibition, suggesting a relevant role in glycemic regulation (Makrilakis, 2019; T. Zhang et al., 2021).

Predicted molecular interactions with proteins such as Hsd11b2, KLK1, or CPA3 are consistent with physiological mechanisms involved in blood pressure regulation (Hunter et al., 2022; Sotiropoulou et al., 2009). In the antioxidant field, interactions with enzymes that generate reactive oxygen species (AKR1C1, ACOX1) have been described, whose inhibition could have protective effects against oxidative stress (Matsunaga et al., 2013; Zeng et al., 2017).

Although these approaches are valuable, it is important to highlight their limitations: *in silico* predictions require experimental validation in animal or human models. Previous studies on ham (Paolella et al., 2015) showed that many long peptides are degraded during digestion, with di- and tripeptides actually reaching the systemic circulation. Simulated gastrointestinal digestion corroborated the stability of key peptides such as EE, VG, and VD after passing through gastrointestinal conditions, with relevant concentrations exceeding 10 mg/mL in prolonged stages. These data coincide with the observations of Paolella et al. (2015), who showed that longer curing times increase the release and bioavailability of

dipeptides in digestion simulations. Likewise, the stability of these fragments during digestion is consistent with that described by Escudero et al. (2014), who demonstrated that some ACE inhibitors derived from ham are resistant to gastric and pancreatic hydrolysis, retaining their bioactivity.

This study represents a breakthrough by integrating peptidomics, *in vitro* and *in silico* assays to link different formulations of nitrifying salts (with variations in nitrite, nitrate, and their combinations) and two maturation times (standard and overripening) with functional bioactivity. However, it should be noted that most of the evidence comes from prediction models and simulated digestion. To confirm the clinical relevance of these results, clinical trials would be necessary.

Compared to other protein sources, such as fermented dairy products (Udenigwe & Aluko, 2012) or fish hydrolysates (Abachi et al., 2019; Ashraf et al., 2020), cured ham offers the advantage of being a traditional food where bioactivity arises from the maturation process itself, without the need for external enzymatic hydrolysis. However, the variability introduced by genetic and technological factors (del Olmo et al., 2015, p. 20; Mora et al., 2016) requires standardization strategies if nutraceutical applications are to be developed.

2.4. UP TO 50% REDUCTION IN SODIUM CONTENT IN BONELESS CURED HAM. EFFECT ON QUALITY, SENSORY ACCEPTANCE AND BIOACTIVE PEPTIDES GENERATION.

Following previous studies that confirmed that technological modifications such as overripening, controlled temperature increase, and reduction of nitrifying salts could modulate proteolysis and enhance the release of bioactive peptides, the next challenge was to directly address the main nutritional risk factor of cured ham: its high sodium content. Excessive NaCl intake is strongly associated with hypertension and cardiovascular disease (He & MacGregor, 2009; Desmond, 2006), which has prompted the meat industry to explore progressive salt reductions. Until now, most studies on cured ham had demonstrated reductions of around 25% without compromising safety or quality (Muñoz-Rosique et al., 2022; Pinna et al., 2020), but there was no clear evidence on the feasibility of reductions close to 50%. In addition, the impact of salt particle size had hardly been studied, despite the fact that this factor influences the rate of dissolution, diffusion, and, therefore, the

dehydration and enzymatic kinetics of the process (Aheto et al., 2019). The study presented here evaluated these two strategies in boneless cured hams, analyzing their effect on physicochemical parameters, mineral composition, free amino acid profile, instrumental texture, and sensory acceptability.

It is important to note that in all study trials, we used a control produced with the standard curing treatment. Therefore, the real challenge was to demonstrate that it was possible to achieve a 50% reduction in sodium without compromising the microbiological safety, quality, and sensory acceptability of the final product, with the additional aim of increasing the generation of bioactive peptides. This objective meant going beyond the reductions usually considered in the meat industry, providing evidence that cured ham with half the sodium can maintain technological and sensory characteristics comparable to the conventional product.

One of the main questions when drastically reducing the sodium content in cured hams is whether microbiological safety can be maintained. NaCl plays an essential role in decreasing water activity (a_w), creating a hostile environment for pathogens such as *Clostridium botulinum* (Sebranek & Bacus, 2007). In this study, even with a 50% reduction in sodium, the hams achieved a_w values <0.91 , which is below the risk threshold described in the literature (Affairs, 2018). This is consistent with the results of Blesa et al. (2008), who showed that hams cured with partial salt substitutions remained safe as long as the drying phase was prolonged and relative humidity and temperature parameters were controlled.

The lower NaCl content slowed dehydration, generating slightly higher a_w values, a phenomenon previously observed in reduced-salt Italian hams (Pinna et al., 2020). However, the values converged with the traditional controls, confirming that adjustments in curing time are sufficient to ensure safety. This aspect is key, as other salt reduction strategies, such as the application of high pressure (Duranton et al., 2012) or ultrasound (Gao et al., 2014), have proven effective in cooked products, but in long-cured hams, maturation management appears to be the most practical and realistic tool.

The proteolysis index was lower in batch 1 (around 28% sodium reduction), while batch 2 (around 50% sodium reduction) showed values comparable to the control, indicating that the medium salt grain compensated for the reduction by promoting proteolytic activity. This finding is explained by the inhibitory effect of NaCl on cathepsin and aminopeptidase activity, already documented by Zhao et

al. (2005) and confirmed in recent reviews on cured hams (Schivazappa & Virgili, 2020). In fact, studies on other meat products such as Galician pork shoulder (Lorenzo et al., 2015) showed that partial replacement of NaCl with K, Mg, or Ca salts intensified the release of free amino acids, a pattern similar to that observed in this study.

The FAAs detected in higher proportions included glycine, alanine, and serine (sweet flavors), as well as glutamate (umami). These amino acids play a key role in compensating for the reduced saltiness, as previously noted by Toldrá et al. 2000 and Virgili & Schivazappa. 2002. Fang & Zhu, 2025 demonstrated that in reduced-salt meat products, an increase in sweet and umami FAAs contributes to improving overall consumer acceptance. The fact that no excessive accumulation of bitter FAAs (e.g., histidine, arginine) was observed indicates that the proteolytic balance was maintained within a favorable range, avoiding flavor defects.

In addition, the increase in FAA may have biofunctional implications: glutamate, leucine, and proline are known to be part of sequences with antihypertensive and antioxidant potential (Escudero et al., 2013; Gallego et al., 2018; Heres et al., 2021). Therefore, reducing sodium not only does not compromise safety or flavor, but could increase the nutraceutical potential of ham.

The reduction in sodium resulted in a final sodium content of 1.2–1.5%. At the same time, a relative increase in potassium was observed, derived from the use of potassium nitrates and ion exchanges in a lower sodium environment. This result is positive from a nutritional point of view, as a higher intake of K helps to counteract the hypertensive effects of Na (Bidlas & Lambert, 2008).

This pattern coincides with that described in cured hams made with partial substitution of NaCl by KCl (Armenteros et al., 2012; Barat et al., 2013), where the final product had a more balanced mineral profile, with potentially beneficial implications for cardiovascular health.

Color, a critical parameter for ham acceptability, remained stable in sodium-reduced hams. The a^* values (redness) were not affected, indicating that nitrosylmyoglobin formation did not depend on NaCl content, but rather on the availability of residual nitrites and bacterial reducing activity (Honikel, 2008). This result is consistent with that described in Serrano and Parma hams with partial salt reductions, where the characteristic red color was maintained as long as the curing

process was sufficiently long (Costa-Corredor et al., 2009; Pinna et al., 2020). The slight decrease in L^* (lightness) in sodium-reduced hams may be due to higher residual moisture and the intensification of oxidation and Maillard reactions in advanced stages, a phenomenon also observed in Jinhua hams (Zhu et al., 2013).

In terms of texture, the reduction in sodium caused a decrease in cohesiveness and a slight increase in elasticity, attributable to the higher degree of proteolysis and lower osmotic force. Similar results have been described in other studies on reduced-salt hams (Harkouss et al., 2014; Lorenzo et al., 2015), as well as in cooked products where lower NaCl levels increase protein solubility and modify the myofibrillar network (Inguglia et al., 2017). However, the other parameters (hardness, chewiness) remained within acceptable values, demonstrating that the 50% reduction in sodium does not compromise the overall structure of the product.

Consumers rated the control hams and the reduced-sodium hams similarly, confirming that a 50% reduction is sensorially acceptable. This finding is in line with reports from other studies, where reductions of 25–30% in NaCl did not cause rejection (Armenteros et al., 2012; Muñoz-Rosique et al., 2022). The explanation lies in the fact that the higher concentration of sweet and umami FAAs compensates for the lower salty perception (Toldrá et al., 2000; Fang & Zhu, 2025). It is important to note that current consumption trends, increasingly oriented towards “healthy” and “clean label” products, facilitate the acceptance of less salty products (Taormina, 2010; Yin et al., 2023). In this regard, the 50% reduction in sodium in cured ham is in line with World Health Organization recommendations to reduce sodium intake to below 5 g/day.

Although a significant effect of salt grain size on initial NaCl absorption was expected, at the end of the process the results showed minimal differences between treatments. Aheto et al., 2019 demonstrated that reducing particle size accelerates salt diffusion and dissolution, but in long-maturing products these differences are diluted over time. Only slight variations in hardness and salting kinetics were observed, suggesting that this parameter is more relevant for optimizing process times than for our objective, which was to obtain faster salt diffusion to counteract the effect of sodium reduction.

In addition, the reduction of NaCl makes the process more dependent on environmental factors (temperature, humidity) and the action of residual nitrifying agents to ensure color and stability.

Finally, according to the EU Regulation 1924/2006 on nutrition claims, both reduced-sodium batches (28 and 50% less sodium) qualify for the claim “reduced sodium content”, since they exceed the minimum 25% reduction required compared with conventional dry-cured hams. However, the stricter claims “low sodium” or “sodium-free” are not applicable to this type of product due to its intrinsic composition.

In short, this work represents a step forward in the healthy reformulation of cured ham, showing that it is possible to significantly reduce sodium without compromising perceived quality or safety.

2.4.1. Bioactive Peptide Generation

After seeing that it was possible to reduce the sodium content in boneless cured hams up to 50% while maintaining safety, color, and sensory acceptance, we evaluated how these technological modifications influence the generation of bioactive peptides and their bioactivity *in vitro* and *in silico*. Until then, studies had shown that reducing NaCl intensifies proteolysis and increases the accumulation of free amino acids (Muñoz-Rosique et al., 2022; Pérez-Santaescolástica et al., 2018), suggesting greater functional potential. However, it remained to be seen whether sodium reduction actually favored the release of sequences with antihypertensive, antioxidant, antidiabetic, or antimicrobial activities, as previously described in cured hams (Escudero et al., 2013b; L. Fu et al., 2021; Gallego, Mora, & Toldrá, 2018b; Xu et al., 2025). This new study addressed this question by applying a comprehensive approach that included peptidomic analysis, *in vitro* ACE inhibition and antioxidant capacity assays, gastrointestinal digestion simulations, and *in silico* predictions of bioactivity and pharmacophore similarity.

2.4.1.1. *In vitro* analysis

The peptide extracts from the three batches showed strong ACE inhibitory activity, with inhibitions close to 90% at the highest concentrations. Although the differences between treatments were not statistically significant, a trend toward greater potency was observed in hams with up to 50% less sodium. This result is

consistent with the hypothesis that sodium reduction enhances proteolysis and the release of short ACE-inhibiting peptides (Mora et al., 2015; Heres et al., 2021).

The presence of peptide sequences such as KPVAAP or KAAAAP, derived from myosin and sarcoplasmic proteins, supports this trend (Escudero et al., 2014; Gallego et al., 2019). Previous studies on Iberian and Italian hams had shown that prolonged curing and reduced sodium content favored the generation of peptides such as Ala-Ala or Val-Pro, with efficacy comparable to antihypertensive milk peptides (M. Sentandreu & Toldrá, 2001; Udenigwe & Aluko, 2012).

The antioxidant capacity evaluated using DPPH confirmed that all boneless dry-cured ham batches are a significant source of antioxidant peptides, achieving inhibitions of 80–90% at high concentrations. Batches with less sodium showed significantly higher bioactivity ($p < 0.05$), especially those made with medium-grain salt. This effect can be attributed to the release of fragments rich in aromatic amino acids (Trp, Tyr, Phe), whose ability to donate protons or chelate pro-oxidant metals is well documented (Gallego et al., 2018; Harnedy et al., 2017; Zheng et al., 2016). The intensification of proteolysis under low salt conditions, also described by Muñoz-Rosique et al. 2023, reinforces the hypothesis that reducing sodium increases the antioxidant potential of cured ham. This finding is relevant in comparison with other protein sources: while in dairy products and legumes antioxidant peptides require controlled enzymatic hydrolysis (Peighambaroust et al., 2021), in ham they are generated naturally during maturation.

Peptidomic analysis identified more than 1,000 sequences < 1.5 kDa in the batches with lower sodium content, compared to slightly lower values in the control. This result confirms that sodium reduction promotes the release of short, bioavailable peptides, which is consistent with observations in other cured hams such as Panxian, where a higher proportion of small peptides associated with multifunctional bioactivity was detected (Li et al., 2022). The relevance of this finding lies in the fact that low molecular weight peptides are more likely to resist digestion and reach the systemic circulation (Escudero et al., 2014; Harnedy et al., 2017). In addition, sequences such as Ala-Ala, Pro-Val, and aromatic dipeptides have been consistently linked to antihypertensive, antioxidant, and cardioprotective effects (Heres et al., 2021; Gallego et al., 2018).

The gastrointestinal simulation confirmed the release of dipeptides such as EE, DG, VD, ES, and AA after digestion, with no significant variations between

batches except for increases in EE, ES, IE and VD in the control. Dipeptides are the most stable fragments and are most likely to be absorbed intact (Paolella et al., 2015; Escudero et al., 2014). The presence of EE and DG, previously linked to ACE inhibition and reduction of reactive oxygen species (Heres et al., 2023; Bailey et al., 2022), confirms that sodium reduction does not compromise the actual bioaccessibility of peptides and could even enhance it by promoting proteolysis.

2.5. BIOACTIVE PEPTIDES FROM DRY-CURED HAM: *IN VITRO* AND *IN VIVO* ANTIOXIDANT EFFECTS USING A *CAENORHABDITIS ELEGANS* MODEL.

After demonstrating that different technological curing strategies intensify proteolysis and promote the release of bioactive peptides with antihypertensive and antioxidant activities, both in *in vitro* analyses and *in silico* simulations, it was necessary to take a more decisive step: to validate the functionality of these peptides in a living organism.

Although cellular models such as *Caco-2* had confirmed the absence of cytotoxicity and the ability of peptides to reduce intracellular ROS generation (Gallego et al., 2020; Hu et al., 2023), it remained to be demonstrated whether these effects translate into a real improvement in oxidative stress resistance and longevity. In this context, the nematode *Caenorhabditis elegans*, a model widely used in studies of nutraceuticals and antioxidants (Mudd et al., 2022; Urbizo-Reyes et al., 2022), was used for the first time to evaluate the *in vivo* bioactivity of peptides derived from cured ham after simulated digestion.

This study used peptide fractions obtained from boneless cured hams with a 50% reduction in sodium, developed as part of the previous study in this thesis. These fractions, released after simulated gastrointestinal digestion, form the experimental basis for evaluating the antioxidant effects both *in vitro* and in an *in vivo* model of *Caenorhabditis elegans*. Thus, this chapter is not an isolated study, but rather a direct continuation of the previous work, where the technological process was optimized to maximize the generation of bioactive peptides in sodium-reduced ham.

2.5.1. Antioxidant potential after simulated gastrointestinal digestion

The antioxidant potential of cured ham peptides was evaluated using, on one hand, fractions subjected to simulated gastrointestinal digestion to generate digested ham peptides (HDP) and, on the other hand, fractions derived from undigested peptides (HPF) to determine the effect of digestion on antioxidant capacity.

The results of chemical assays (ABTS and DPPH) showed that peptides derived from cured ham after gastrointestinal digestion exhibit significantly higher antioxidant activity than undigested peptide fractions. This increase can be explained by the fact that digestion promotes the release of short, electrically charged fragments with a greater capacity to donate electrons or protons, which increases their neutralizing action on free radicals (Hernández-Ledesma et al., 2007; L. Zhu et al., 2008). This behavior has also been described in other protein matrices. In fish hydrolysates (Abachi et al., 2019; Y. Wu et al., 2022) and in cereals such as corn and rice, enzymatic hydrolysis has been shown to release short peptides with a higher antioxidant effect than intact proteins (Cai et al., 2022; Ren et al., 2018). Therefore, the pattern observed in cured ham is part of a general trend.

2.5.2. Cytotoxicity and bioactivity in intestinal cells *Caco-2*

The use of human intestinal *Caco-2* cells as a model provided key information on the safety and functionality of the peptides. To evaluate the cytotoxicity of the ham-derived peptides, two types of extracts were tested: the ham peptide fraction (HPF) and the ham digested peptides (HDP) obtained after simulated gastrointestinal digestion of the sodium-reduced dry-cured hams. *Caco-2* intestinal cells were exposed to increasing concentrations (0.25–2.5 mg/mL) of HDP to determine their effect on cell viability using the MTT assay. No cytotoxic effects were detected until relatively high concentrations (2.5 mg/mL), which is consistent with previous results obtained in milk protein hydrolysates (Hernández-Ledesma et al., 2007) and vegetable protein hydrolysates (Liang et al., 2018; Q. Zhang et al., 2019). The absence of negative effects on cell viability allows these peptides to be considered safe candidates for the development of nutraceuticals.

The ham digested peptides (HDP) fractions obtained were also used to investigate the intracellular antioxidant activity in Caco-2 cells. Cells were treated with different concentrations of HDP (0.5, 1.0, and 1.5 mg/mL) and subjected to oxidative stress. Two complementary assays were performed: DHE, which specifically detects superoxide anions, and DCFDA, which measures general intracellular ROS levels. In both tests, pre-incubation with HDP significantly reduced ROS accumulation in a dose-dependent manner, with the strongest effects observed at 1.0 and 1.5 mg/mL ($p < 0.05$). These results demonstrate that the digested peptide fractions from sodium-reduced hams are capable not only of scavenging free radicals in chemical assays, but also of modulating antioxidant defenses at the cellular level. This dual action supports their potential role in protecting intestinal cells against oxidative stress. These data are consistent with those observed in Jinhua ham hydrolysates, which also showed a reduction in ROS in cells exposed to oxidative stress (Hu et al., 2016). Similarly, seed and legume extracts have been shown to modulate cellular antioxidant defenses and attenuate oxidative damage (Urbizo-Reyes et al., 2022).

Overall, the evidence indicates that cured ham peptides are not only safe for the human intestinal epithelium, but also act as protectors against intracellular oxidative stress, which is essential for their consideration as functional ingredients.

2.5.3. Validation in *Caenorhabditis elegans*

The use of *C. elegans* was a decisive step in transferring the findings to the *in vivo* context. This nematode is a well-established model in antioxidant and nutraceutical studies due to its similarity in genetic pathways to mammals (An & Blackwell, 2003; Inoue et al., 2005; Oliveira et al., 2009). Supplementation with ham digested peptides (HDP) significantly improved survival under conditions of oxidative stress, both acute (exposure to t-BOOH) and chronic (exposure to paraquat).

For the acute oxidative stress assays, we used ham digested peptides (HDP) obtained after simulated gastrointestinal digestion of boneless dry-cured hams with 50% sodium reduction. *C. elegans* nematodes at the L4 stage were pre-incubated for 24 or 48 h with HDP at different concentrations (0.25–2.0 mg/mL) and

subsequently exposed to tert-butyl hydroperoxide (t-BOOH) to induce acute oxidative stress. The results demonstrated that pre-treatment with HDP significantly increased nematode survival in a dose- and time-dependent manner compared to the control group without peptides. The best protective effect was observed at 1.0 and 1.5 mg/mL, where mean survival increased from 390 min in controls to 600–630 min in treated worms. These findings confirm that peptides derived from sodium-reduced ham digestion enhance resistance to acute oxidative stress by activating endogenous defense mechanisms.

The same fractions were used to evaluate the effect of chronic oxidative stress. L4-stage *C. elegans* were pre-incubated with HDP (0.25–2.0 mg/mL) for 24 or 48 h and then exposed to paraquat, a mitochondrial toxicant that generates reactive oxygen species. Nematodes pretreated with HDP showed significantly greater longevity than controls. The most consistent results were again found at 1.0–1.5 mg/mL, where average survival increased from 3 days in controls to 4.5–5 days in treated worms ($p < 0.05$). No additional benefits were observed at the highest peptide concentrations. These results indicate that these peptides also provide sustained protection under chronic oxidative stress conditions, likely through the modulation of intracellular antioxidant enzymes.

The increase in survival in nematodes treated with peptides is comparable to that observed in studies with plant extracts such as açai (Bonomo et al., 2014), with peptides derived from ginseng (Q. Wang et al., 2016), or with marine protein hydrolysates (Yu et al., 2020). In all cases, the organisms showed greater resistance to oxidative stress, confirming that the antioxidant action of peptides goes beyond the direct neutralization of radicals and is linked to endogenous regulatory mechanisms.

2.5.3.1. *Reduction of ROS and activation of gene overexpression pathways*

Analysis of intracellular ROS levels in *C. elegans* confirmed a significant reduction following treatment with the peptides, accompanied by overexpression of antioxidant genes such as *GST-4*, *GST-10*, *SOD-3*, and *SKN-1*. The latter, homologous to the Nrf2 factor in mammals, plays a central role in antioxidant defense and longevity extension (An & Blackwell, 2003; Inoue et al., 2005). The activation of SKN-1 has also been described in nematodes supplemented with

antioxidant peptides from sesame and rice (Wang et al., 2016; Cai et al., 2022), reinforcing the hypothesis of a conserved mechanism. The increased expression of SOD-3, a key enzyme in the elimination of superoxide radicals, supports the idea that peptides do not act solely as direct antioxidants, but rather induce the expression of endogenous defenses. This type of mechanism has also been observed in edible insect extracts, where supplementation prolonged the lifespan of *C. elegans* under conditions of oxidative stress (Mudd & Liceaga, 2022). The partial activation of DAF-16, linked to the insulin/IGF-1 signaling pathway, suggests an indirect effect on longevity, possibly mediated by interactions between the SKN-1 and DAF-16 pathways (Back et al., 2010; Chávez et al., 2007). This finding connects cured ham peptides with conserved molecular mechanisms of healthy aging.

Although the results are promising, it is important to highlight the limitations. The *C. elegans* model, while useful and highly conserved, does not reproduce the full physiological complexity of mammals. It is essential to validate these effects in higher models and, above all, in clinical trials with consumers.

Similarly, the possible synergistic effects of cured ham peptides with other nutrients, as well as their stability in complex matrices, must be evaluated. The multifunctionality observed (antioxidant, antihypertensive, antidiabetic, antimicrobial) suggests great potential, but also the need to explore interactions that may modulate bioavailability or final efficacy (Gallego et al., 2019; Heres et al., 2022).

VI – CONCLUSIONS

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This doctoral thesis demonstrates that, by optimizing curing strategies (sodium reduction, nitrite and nitrate modulation, increased maturation time [overripening], and temperature control), it is possible to obtain boneless cured ham with an acceptable sensory profile, lower sodium content, and, at the same time, a high potential for bioactive peptides with antioxidant, antihypertensive, and antidiabetic functions, making this food an ideal candidate to be considered a functional food.

The conclusions presented below respond to all the objectives set for the development of the thesis:

FIRST. The review of the state of the art confirmed that cured ham is a relevant source of bioactive peptides (antioxidants, anti-ACE, antidiabetics, among others) and highlighted the need to move from *in vitro* trials and *in silico* predictions to *in vivo* validations, establishing the conceptual framework and gaps that motivate the experimental plan of the thesis.

SECOND. The application of overripening in boneless, reduced-salt Iberian ham intensified proteolysis and promoted a significant increase in the concentration of free amino acids, without producing a negative impact on the sensory quality of the product. This behaviour indicates that overripening acts as an effective technological tool to stimulate the release of bioactive peptides, even in matrices with lower sodium content, where salt reduction could limit hydrolysis.

THIRD. The combination of overripening and temperature modulated both the diversity and peptide functionality of reduced-salt boneless Iberian hams: at 30°C, antioxidant activity and ACE inhibition was maximized, while at 36°C, a greater number of peptides were detected without a proportional increase in

bioactivity. Peptide precursors with the potential to generate bioactive di- and tripeptides of interest (such as AA, VP, EE, DG, and VD) after digestion were also detected.

FOURTH. The reduction of nitrifying salts in boneless cured ham was shown to be technologically feasible, allowing a significant decrease in nitrite and nitrate content without compromising the microbiological safety or sensory acceptance of the product. The reduction of these salts modified the dynamics of proteolysis and the free amino acid profile, indicating a direct effect on enzymatic processes during curing. Likewise, specific changes were observed in color stability (particularly in the a^* parameter) and in the formation of volatile compounds, both indicators of the influence of nitrifying agents on final quality. Overall, the results demonstrate that it is possible to produce healthier cured hams with a lower nitrite additive load, while maintaining the safety, quality and sensory properties.

FIFTH. Reference to hams obtained under different nitrite salt treatments, the integration of *in vitro* and *in silico* approaches demonstrated that curing conditions have a decisive influence on the generation and physiological fate of bioactive peptides. *In vitro* assays confirmed notable activities, especially antioxidant and anti-ACE, while *in silico* gastrointestinal digestion simulations predicted the release of di- and tripeptides with a high probability of intestinal absorption. This prediction was corroborated by the actual quantification of selected peptides, which allowed their effective presence to be associated with measured bioactivity. Additionally, the pharmacological similarity assay reinforced the potential of sequences such as EE, DG, VD, and AA by demonstrating structural similarity with compounds known to act on cardiovascular and metabolic targets. Taken together, these results consolidate a dual approach that not only identifies bioactive peptides in cured ham but also validates their functional relevance and potential as nutraceutical ingredients.

SIXTH. Reducing the sodium content in boneless cured ham by up to 50% was technologically feasible, resulting in hams with up to 50% less sodium while maintaining adequate physicochemical profiles, microbiological safety, and

sensory acceptance of the product. Despite the significant reduction in salt content, the hams retained color, texture, and mineral composition parameters within ranges comparable to traditional controls, without compromising their overall quality. The curing strategy effectively achieved the objective of sodium reduction, obtaining hams with up to 50% less sodium than conventional products. Batch 1 reached 1.69 g Na/100 g (28% less sodium), while Batch 2 achieved 1.19 g Na/100 g (64% less sodium), confirming the successful development of lower-sodium dry-cured hams without compromising product quality or bioactive peptide formation.

SEVENTH. The reduction of up to 50% of sodium in boneless cured ham did not compromise proteolysis or peptide release but rather favored the formation of low molecular weight peptides with recognized bioactivity. *In vitro* analyses confirmed an increase in antioxidant activity. Numerous peptide precursors were also identified which, after simulated *in vitro* gastrointestinal digestion, released dipeptides and tripeptides of functional interest. Actual quantification of selected sequences supported these observations, confirming the effective presence of bioactive peptides in the matrix. Taken together, these results demonstrate that reducing sodium by up to 50% in cured ham is not only compatible with technological and sensory quality but also enhances its role as a source of bioactive peptides with nutraceutical potential.

EIGHTH. Validation in *Caenorhabditis elegans* confirmed that the digested peptides derived from boneless, reduced sodium cured ham exert antioxidant effects *in vivo*, resulting in greater resistance to oxidative stress, a significant reduction in intracellular ROS, and increased longevity. At the molecular level, the induction of defensive pathways associated with the overexpression of key genes such as *GST-4*, *GST-10*, and *SKN-1* was observed, consolidating the role of these peptides as modulators of the endogenous antioxidant response. These findings represent a decisive step forward, as they provide real physiological evidence of the functionality of the peptides previously identified through *in vitro* and *in silico* analyses. Overall, the study demonstrates that it is possible to transform traditional cured ham into a healthier food, with lower sodium content but higher concentration and effectiveness of bioactive peptides, making it an ideal candidate for consideration as a functional food.

VII – LIMITATIONS AND FUTURE LINES OF RESEARCH

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Limitations

The development of this doctoral thesis has demonstrated that dry-cured ham can evolve from a traditional product with a high salt content to a food with functional properties derived from its richness in bioactive peptides. However, like all experimental work, it has a number of limitations that must be considered when interpreting the results, as well as multiple opportunities for improvement that pave the way for future research.

Although very solid results have been obtained *in vitro* and, in the *C.elegans* model, no mammalian trials or clinical studies in humans have been conducted yet, so the translation of the bioactive effects of ham-derived peptides to the end consumer is not guaranteed.

Despite the identification of peptides by LC-MS/MS, their absolute quantification, and the establishment of a clear dose–bioactivity relationship, remain unresolved challenges. It is also necessary to further explore the possible synergistic or antagonistic interactions between peptides, both within the food matrix and after digestion, as these could substantially modulate the functional outcomes.

Future lines of research

Future work should aim to confirm the physiological relevance of ham-derived peptides in mammalian models, assessing their bioavailability, pharmacokinetics, effective doses and safety. Subsequently, it will be necessary to carry out controlled clinical studies in humans to validate their impact on cardiovascular, metabolic and oxidative stress biomarkers, consolidating their role as functional ingredients or nutraceuticals.

From a technological perspective, it is relevant to explore targeted strategies to intensify peptide release, beyond extended maturation or temperature modulation. This could include:

The application of emerging technologies such as high hydrostatic pressure or ultrasound to promote proteolysis, and the use of starter cultures adapted to ham fermentation, capable of directing enzymatic activity and enhancing the generation of peptides with proven bioactivity, without altering product safety or sensory acceptance.

Finally, given the large peptide diversity identified, future research should focus on the absolute quantification of key sequences (e.g., di- and tri-peptides with anti-ACE or antioxidant properties), the establishment of structure–activity relationships, and the validation of specific biomarkers of intake and efficacy in humans. Such work will be decisive to consolidate the positioning of reduced-sodium cured ham as a functional meat product with real health-promoting potential.

VIII – BIBLIOGRAPHIC REFERENCES

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