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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°. 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 (Peighambardoust 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 isoascorbate, 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 aw 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.

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 (Muñoz-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):

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

$A_{control}$ 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 Pepmam 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 μL/min. Peptide separation was performed at 40 °C for all samples. Mobile phase buffer A was composed of water and 0.1% formic acid. Mobile phase B was composed of 20% acetonitrile and 0.1% formic acid. The samples were separated at 300 nL/min. Mobile phase B was increased from 4 to 45% in 60 min; from 45 to 90% for 1 min, followed by a 5 min wash at 90% B-phase and a final 15 min re-equilibration at 4% B-phase. Total chromatography time was 85 min. Identification of the peptide sequences of the NPN fractions of the different hams was performed at the Proteomics and Bioinformatics Unit of the University of Cordoba, Spain. MS2 spectra were found with SEQUEST HT against the UnitProtKB database.

The peptides of each 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 °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 overripeningor 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 °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. Overripening 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 °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

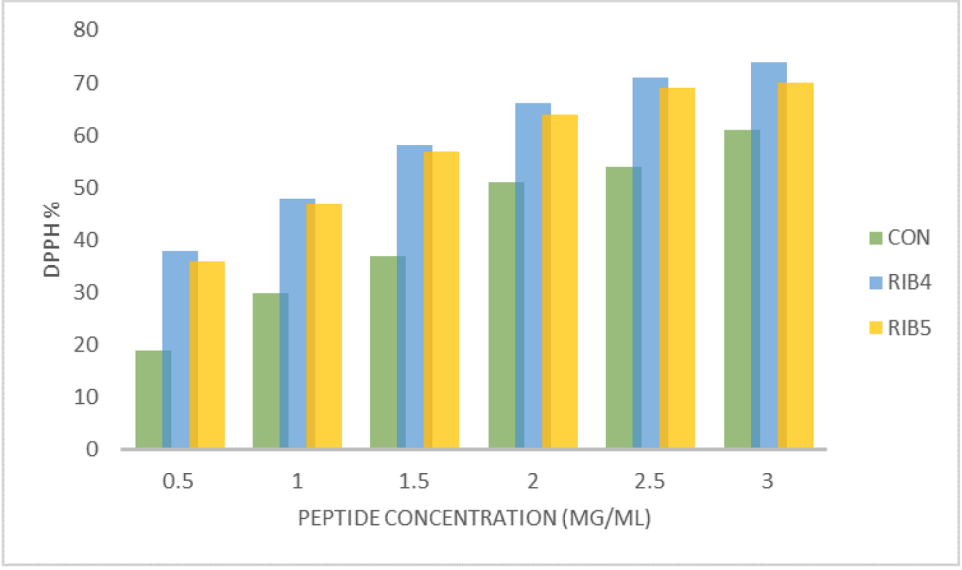

Fig. 1. Evolution of antioxidant activity as a function of peptide concentration in hams with different processing conditions.

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

Processing Conditions				p-value	
Formulation	CON	RIB4	RIB5	Processed	Temperature
DPPH(IC50)	1.76 ±0,241[a]	1.144 ±0,194[b]	1.172 ±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

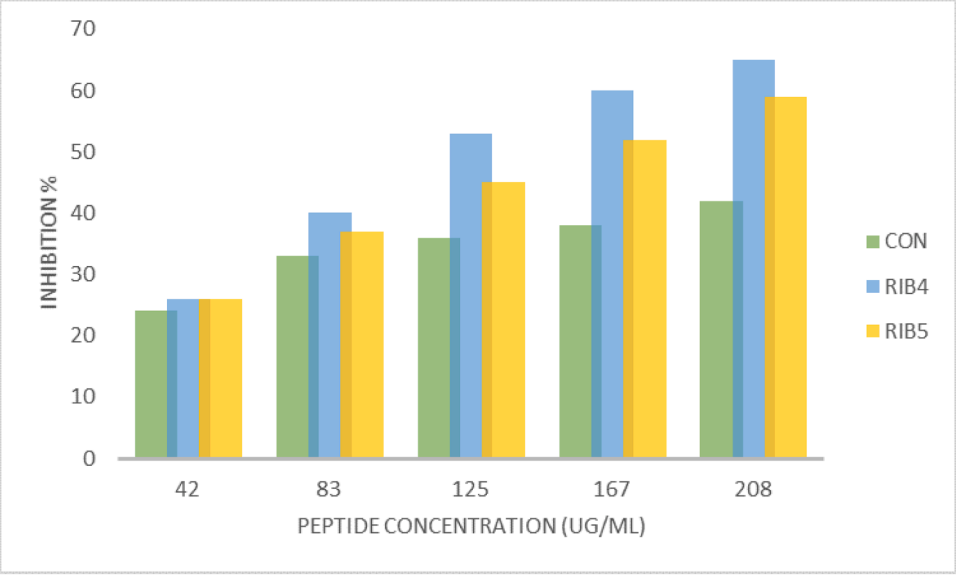

Fig. 2. Evolution of ACE-I activity as a function of peptide concentration in hams with different processing conditions.

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 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

Table 2
Effect of processing conditions on ACE-I during the final stage. Results are expressed in mg/mL as means values ± 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 $p \leq 0.05$).

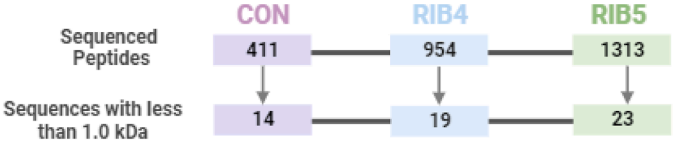

Fig. 3. Number of peptides identified per sample. Number of peptides identified with <1.0 kDa.

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 AEEEYPDL, 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 Zscored 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.

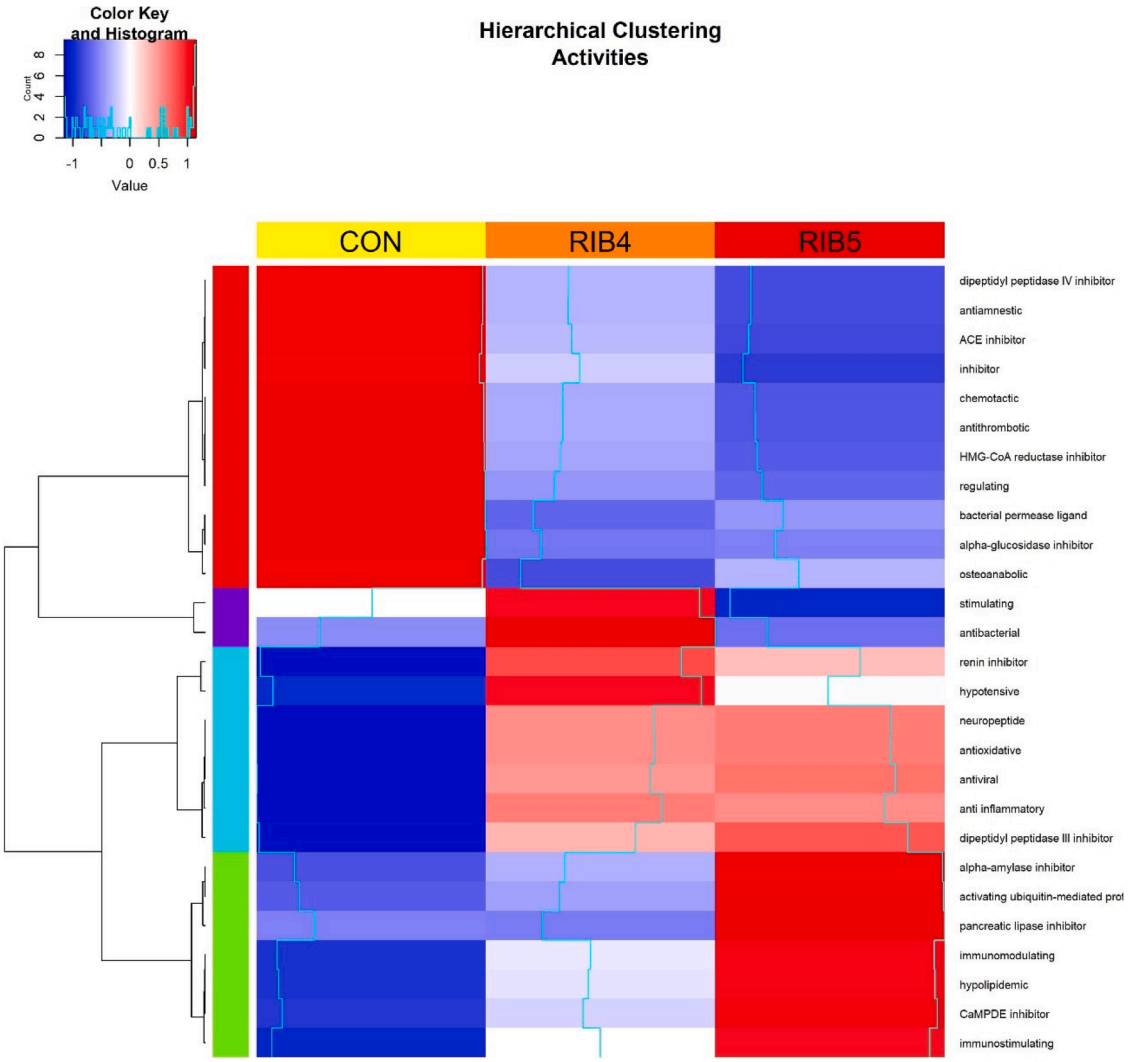

Fig. 4. Heatmap and dendrogram of bioactivities of the different ham samples studied. Quan-tification of bioactivity is regarding the mean. The grouping relationship between the groups of activities is defined.

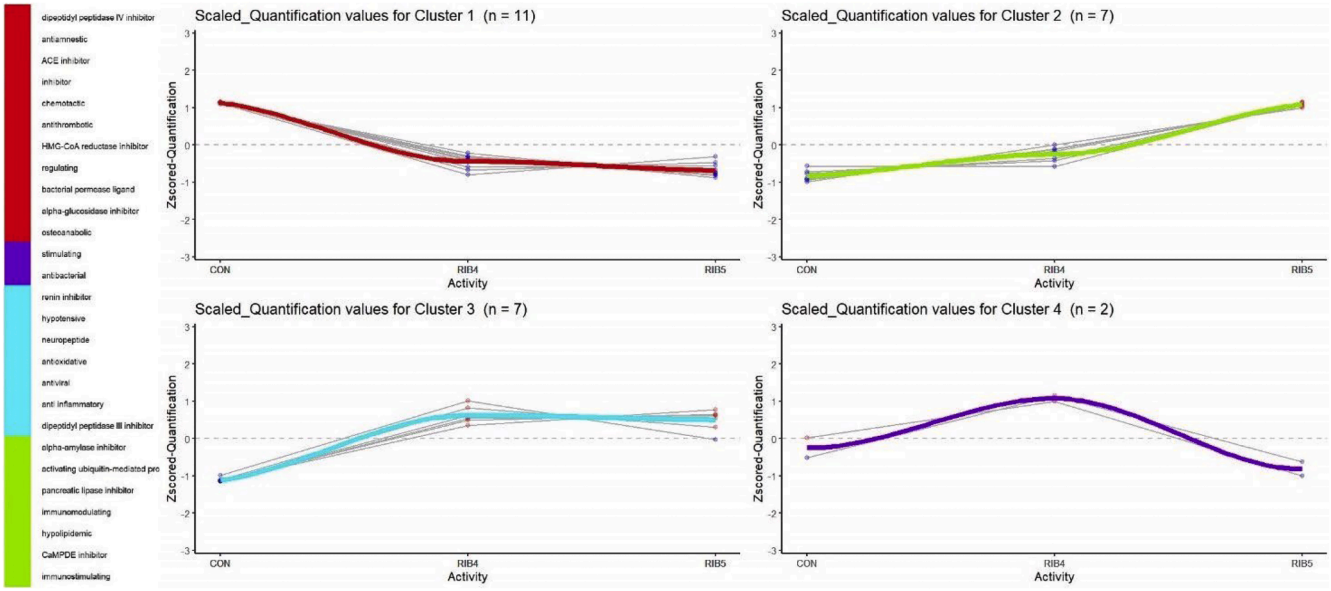

Fig. 5. Representation of the bioactivity in each sample for each cluster of activities.

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 eachsample. 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, antiamnestic, 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, antioxidative 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).

RIB5 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

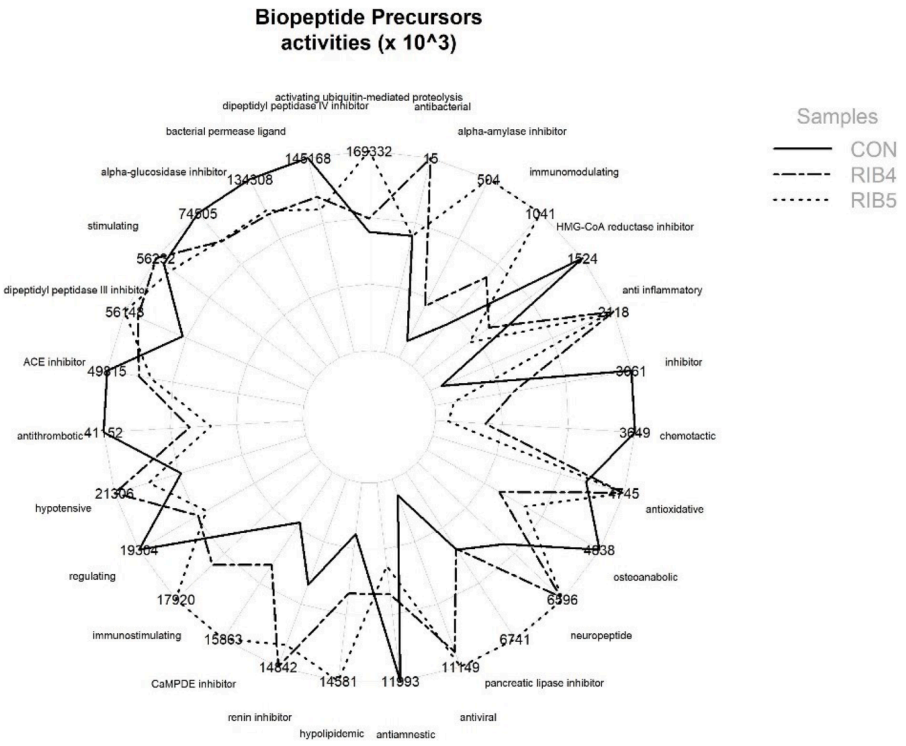

Fig. 6. Standardized quantification of peptide precursors (× 103) in different cured ham samples.

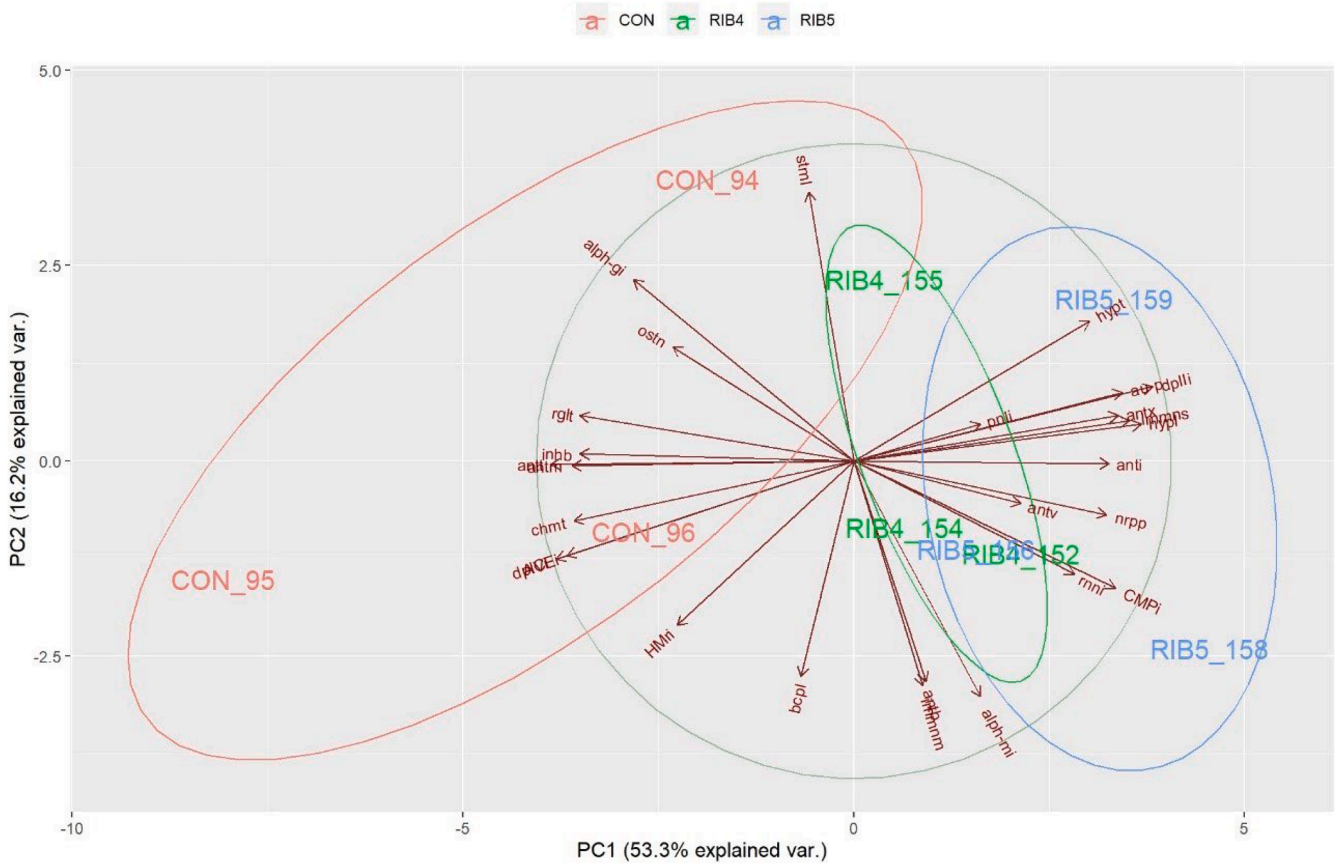

Fig. 7. Biplot of the first two principal components obtained from the PCA of the different bioactivities of ham produced under different processing conditions: ham produced in the traditional way (CON), ham subjected to overripening with (RIB5) or without (RIB4) temperature increase. To create the biplot, the loadings of the bioactivities and the PCA hams' scores have been standardised.

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, Inmunostimulating, Activating ubiquitin-mediated protein, Antioxidative, CaMPDE inhibitor, Anti-inflammatory, Neuropeptide, Hypotensive, Renin-inhibitor, Antiviral, Pancreatic lipase inhibitor, Alpha-amylase inhibitor, Antibacterial and Immunomodulating 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; Prevolnik et al., 2014; Rosell & Toldrá, 1998).

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
Antiamnestic	−0.234	−0.007
Chemotactic	−0.232	−0.090
Immunostimulating	0.229	0.061
Regulating	−0.228	0.068
Inhibitor	−0.228	0.010
AU-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-CoAr 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

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 & Toldrá, 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 DEDETTAL, 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 APAPAPPKE sequence identified in these hams contains three hydrophobic AAs at the C-terminus that confer ACE-I activity. This

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

N	Peptide Seq	Exp. Mass	Protein Source	Protein Accession
1	FDFIHL	791,409	tRNA methyltransferase	I3LG35
2	AGPSIVHR	836,474	Actin, alpha skeletal muscle	P68137
3	FAGDDAPR	848,390	Actin, alpha skeletal muscle	Q6QAQ1; P68137; I3LVD5
4	KPTDKHK	853,489	Creatine kinase M-type	Q5XLD3
5	APAPAPPKE	877,478	Uncharacterized protein	A0A286ZVM3
6	KEDTEKE	878,410	Uncharacterized protein	A0A287BG25; A0A287AGJ6
7	SDEEVEH	886,342	Troponin T fast skeletal muscle type	A0A287AFK2; A0A287A421; Q75NH2
8	DSGDGVTHN	901,365	Actin, alpha skeletal muscle	P68137
9	ADAEAGGGKK	903,453	Myosin-2	F1SS65
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	LEEETKAK	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, FAGDDDAPR, APAPAPPKE, DSGDGVTHN, ADAEAGGGKKK, DGIIDKED, ADGGGDDISVK, 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	Q6QAQ1; P68137; I3LVD5
2	SPEKPPE	825,399	SAM and SH3 domain containing 1	A0A286ZVI6
3	GAGPALEAF	832,420	URB1 ribosome biogenesis 1 homolog	A0A286ZYB9
4	VGDEAQSK	833,400	Actin, alpha skeletal muscle /Actin, cytoplasmic 1 / Actin gamma 1	Q6QAQ1; 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	Q6QAQ1; P68137; I3LVD5
7	GTDADKEH	872,374	L-lactate dehydrogenase	A0A286ZXT7
8	MDAIKKK	875,502	Uncharacterized protein / Tropomyosin alpha-1 chain	A0A286ZRE1; 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	NGSGLVKAGF	949,510	Actin, alpha skeletal muscle	P68137
13	ISQQISGGK	959,516	Calcium binding protein 2	I3L7T1
14	AGFAGDDAPR	976,448	Actin, alpha skeletal muscle / Actin, cytoplasmic 1 / Actin gamma 1	Q6QAQ1; P68137; I3LVD5
15	DGGGDISVKE	976,458	Fast skeletal muscle troponin C	A1XQV5
16	AKEWGYAD	981,431	Carbonic anhydrase 3	Q5S1S4
17	SEDKLRDK	990,521	Troponin T fast skeletal muscle	A0A287AFK2; A0A287A421; Q75NH2
18	YETDAIQR	995,479	Myosin-2 / Myosin-4 / Myosin-7	Q9TV62; F1SS64; F1SS65; A0A287AGU3
19	VLDSGDGVTH	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).

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	Q6QAQ1; P68137
2	MEAAHSK	815,372	Calcium-transporting ATPase	A0A287APK5
3	VGDEAQSK	833,400	Actin, alpha skeletal muscle / Actin, cytoplasmic 1	Q6QAQ1; P68137
4	VGGASLKPE	857,473	Triosephosphate isomerase	A0A286ZRV2
5	VIISAPSAD	872,472	Glyceraldehyde-3-phosphate dehydrogenase	A0A287BG23
6	MDAIKKK	875,502	Uncharacterized protein / Tropomyosin alpha-1 chain	A0A286ZRE1; A0A287AN33
7	DSGDGVTHN	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	F1SS62; Q9TV62; F1SS65; A0A287AGU3
10	GMESAGIHE	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	F1SS62; 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	Q6QAQ1; P68137
17	LGTDADKEH	985,459	L-lactate dehydrogenase	A0A286ZXT7
18	NNLGELINT	987,511	Triosephosphate isomerase	A0A286ZRV2
19	LYEQHLGK	987,526	Myosin-4 / Myosin-2	Q9TV62; F1SS65
20	RQQEGESR	989,476	L-lactate dehydrogenase / L-lactate dehydrogenase	F1SR05; A0A286ZXT7
21	YETDAIQR	995,479	Myosin-4 / Myosin-2 / Myosin heavy chain 8 / Myosin-7	F1SS62; Q9TV62; F1SS65; A0A287AGU3
22	YVGDEAQSK	996,463	Actin, alpha skeletal muscle / Actin, cytoplasmic 1	Q6QAQ1; 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, VGDEAQSK, VGGASLKPE, VIISAPSAD, VIISAPSAD, MDAIKKK, DSGDGVTHN, ISAPSADAPM, ADQLTEEQ, AGFAGDDDAPR, LGTDADKEH, YETDAIQR, YVGDEAQSK) 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 overripened 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

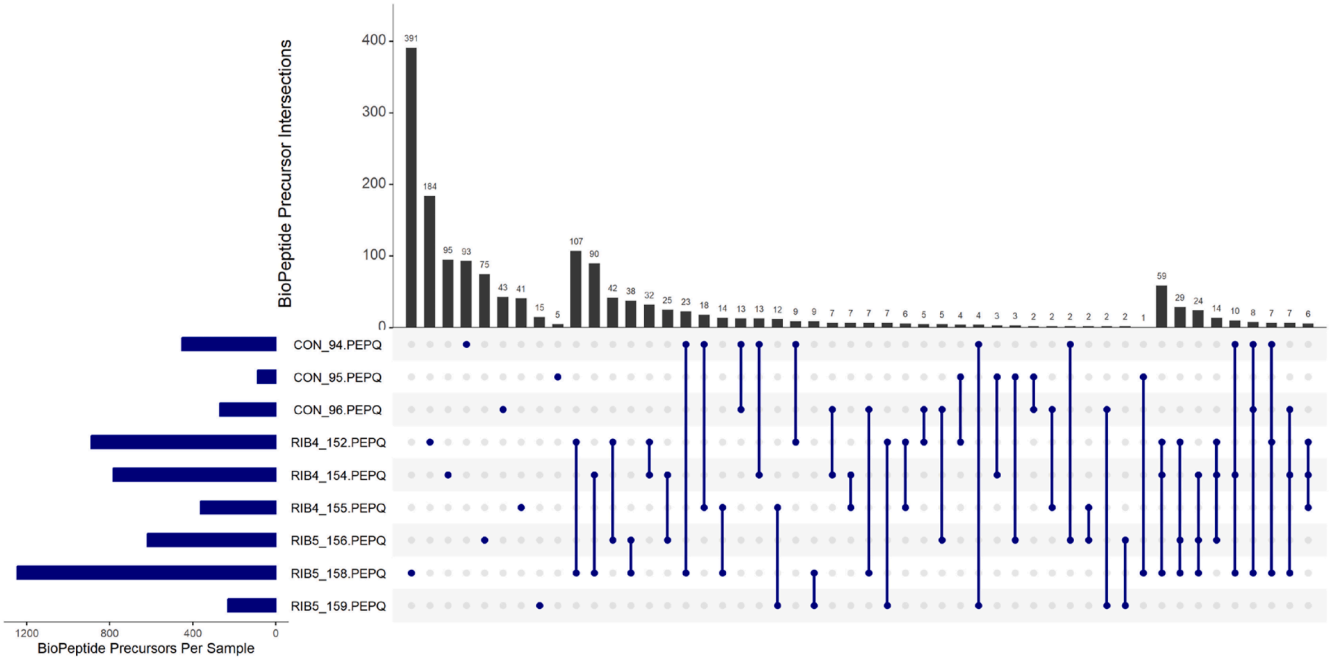

Fig. 8. Graph of intersections of peptides with fragments of sequences with bioactivity identified in the different extracts of the hams studied.

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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