Bioactive peptides generated in the processing of dry-cured ham

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Running title: **Bioactive peptides in dry-cured ham**

Abstract

Peptides and free amino acids are naturally generated in dry-cured ham as a consequence of proteolysis phenomenon exerted by muscle peptidases. The generation of bioactive peptides in different types of dry-cured ham produced in Spain, Italy and China is reviewed in this manuscript. Major muscle proteins are extensively hydrolysed firstly by endogenous endo-peptidases followed by the successive action of exo-peptidases, mainly, tri- and di-peptidylpeptidases, aminopeptidases and carboxypeptidases. Such proteolysis is very intense and consists of the generation of large amounts of free amino acids and a good number of peptides with different sequences and lengths, some of them exerting relevant bioactivities like angiotensin converting enzyme inhibitory activity, antioxidant activity, di-peptidylpeptidase IV inhibitory activity among other and *in vivo* antihypertensive, hypoglycemic or anti-inflammatory activity. This manuscript reviews the recent findings showing that dry-cured ham constitutes a good source of natural bioactive peptides that have potential benefit for human health.

Keywords: proteolysis, peptides, bioactive peptides, proteomics, enzymes, peptidases, exo-peptidases

1 Introduction

Dry-cured ham constitutes a high quality product elaborated through a traditional long procedure followed for centuries. It is typically produced in the Mediterranean area, like the Spanish Serrano and Iberian hams and Italian Parma ham, in China like the Jinhua and Xuanwei hams and certain states in the US with the Country style ham. Of course, there are differences in the quality due to the genetics of the hams used as raw material as well as processing conditions and length of the process depending on the area of origin (Bosse, Müller, Gibis et al., 2018). In fact, the price can be very expensive for those with long processing times, up to 2 or 3 years, and using hams from specific pigs (Toldrá, 1998).

Proteolysis is a relevant biochemical phenomena occurring during processing and responsible for the generation of large amounts of peptides and free amino acids (Rodríguez-Nuñez, Aristoy and Toldrá, 1995). Recent advances in both mass spectrometry instruments and experimental approaches in food processing have given the opportunity for developing correct peptides analysis resulting from unspecific hydrolysis. The application of peptidomics tools to complex processes like dry-curing has allowed the possibility to follow up proteolysis and identify those peptides of interest (Gallego, Mora & Toldrá, 2018a and 2018b). Furthermore, the use of peptidomic approach based on mass spectrometry detected differences between Spanish Teruel, Italian Parma and Belgian dry-cured hams due to the genetics and processing that differed between countries (Mora, Escudero & Toldrá, 2016) and the proteomic profile was also reported to differ in Jinhua ham between the traditional and modern processing (Zhou, Wu, Tang et al., 2019). Some of the generated peptides during the dry-curing process have been characterised and sequenced, and may be considered as

bioactive because they have special biological effects in human body with benefits for health. Such peptides may exert inhibition of the Angiotensin-I Converting Enzyme (ACE), inhibition of di-peptidylpeptidase activity (DPP IV), antioxidant activity, anti-inflamatory activity, or antimicrobial (Dellafiora, Paolella, Dall´asta et al., 2015). Bioactive peptides remain inactive while forming part of the parent muscle protein but they get active once released by muscle peptidases during either dry-curing process or gastrointestinal (GI) digestion (Toldrá, Reig, Aristoy & Mora, 2018). A scheme for the generation of bioactive peptides in dry-cured ham and its physiological effect is shown in Figure 1.

This manuscript is reviewing the recent studies reporting the generation of bioactive peptides in different types of dry-cured ham as well as its characterisation and identification, and the relevance of such generated peptides for health.

2. Processing of dry-cured ham and proteolysis

Pork muscle contains a variety of proteolytic enzymes, especially endo- and exo-peptidases, that play an important role in the proteolysis of myofibrillar and sarcoplasmic proteins during the dry-curing process. Proteolysis is initiated by endo-peptidases, like calpains in the first weeks of process and cathepsins, especially cathepsins B, H and L that are active even at 15 months of process, resulting in the breakdown of proteins into protein fragments and polypeptides. Such polypeptides can be further hydrolysed by exo-peptidases generating smaller peptides and free amino acids (Toldrá & Flores, 1998; Zhou and Zhao, 2007). The extent of proteolysis and the amount of generated bioactive peptides depend on multiple variables including the raw materials, the type and activity of muscle enzymes, processing conditions and the length

of processing (Pugliese, Sirtori, Sklerp et al., 2015; Zhu, Tian, Li et al., 2017; Zhou, Pan, Bai et al., 2019a). So, once dry-cured hams have been submitted to salting and post-salting stages, the temperature increases during drying and ripening, and the water activity decreases progressively while the amount of salt is quite high, above 4% in wet weight. The activity of peptidases is thus progressively reduced by the effect of lowering a_w and the inhibition by salt (Toldrá, Cerveró and Part, 1993; Zhao, Zhou, Wang et al., 2005; Zhang, Zhen, Zhang et al., 2010) and cathepsins B, H and L (Rico, Toldrá and Flores, 1993; Zhou, Wu, Tang et al., 2019) but some activity still remains during the full process for most muscle enzymes (Toldrá, 1998).

Major muscle peptidases are tri-peptidyl peptidases (TPP) and di-peptidyl peptidases (DPP) that are able to hydrolyse tri and dipeptides, respectively, from the N-terminal of protein fragments and polypeptides. DPP I, DPP II and TPP I are particularly active because their optimal activity is slightly acid, pH 5.5-6.5, near the pH 6.0-6.5 found in ham during processing (Sentandreu & Toldrá, 2001). DPP I and DPP II have been reported to hydrolyse dipeptides such as Ala-Gln, Arg-Gly, Asn-Pro, lle-Leu, Ala-Gly, Ser-Gly, Ser-Gln from the N-terminal while TPP I is able to hydrolyse tripeptides like Ile-Ile-Pro, Arg-Gly-Ala, Gly-Asn-Pro, Gly-Ala-Gly, Gly-Pro-Gly located at the N-terminal (Mora, Gallego, Escudero et al., 2015a). Other reported di and tripeptides released by exopeptidases in Spanish dry-cured ham are dipeptides Thr-Ser, Thr-Leu, Phe-Asp, Val-Lys, Ala-Thr and Gln-Thr, and tripeptides Ser-Arg-Glu, Thr-Val-Gln, Asn-Ala-Ser, Lys-Ile-Glu and Gly-Lys-Met (Gallego, Mora & Toldrá, 2019a). A total of 21 dipeptides and 12 tripeptides were reported after simulated GI digestion of Italian Parma ham (Paolella, Falavigna, Faccini et al., 2015).

The higher temperatures in the last phases of the processing of Jinhua ham were reported to contribute to a stronger proteolysis reflected in a large number of peptides

especially dipeptides with 23.59% of total relative peak areas and tripeptides with 48.28% (Zhu et al., 2017). Most abundant dipeptides with more than 1% relative peak areas were reported to be Val-Glu, Pro-Leu, Ala-His and Ala-Arg and tripeptides were Leu-Pro-Lys, Ser-Gly-Leu, Ala-Ala-Pro, Ser-Gly-Val and Leu-His-Ala (Zhu et al., 2017).

Other relevant muscle exo-peptidases are aminopeptidases and carboxypeptidases that release large amounts of free amino acids from the N and C terminal, respectively (Toldrá, Aristoy & Flores, 2000; Virgili, Saccani, Gabba et al., 2007). Major amino acids released from the N-terminal, mainly by alanyl and methionyl aminopeptidases, are Gly, Ser, Ala, Leu and Ile, while Leu, Tyr, Lys, Ala, Gly, Glu and Asp are released from the C-terminal by carboxypeptidases A and B (Mora et al., 2015a).

3. Identification of bioactive peptides in dry-cured ham

Bioactives peptides have been usually identified in dry-cured ham following empirical approaches (see figure 2). This involves the release of the peptides, their extraction and chromatographic purification, the screening of bioactivity in the collected fractions, further purification of peptides in the most active fractions, mass spectrometry identification of the sequences, synthesis of those more active peptides and confirmation with *in vitro* and *in vivo* assays (Sánchez-Rivera, Martínez-Maqueda, Cruz-Huerta, et al., 2014). This procedure may be quite troublesome so that time and costs can be reduced with a predictive strategy of bioactivity based on *in silico* analysis using bioinformatics tools and peptide databases. The steps for this *in silico* approach are shown in figure 3. It consists of selecting proteins of origin with known amino acid sequences, the hydrolysis with selected proteolytic enzymes and the bioactivity prediction based on the biochemical properties of the sequences and further information

158 provided in databases (Gu, Majumder & Wu, 2011; Lafarga, O'Connor & Hayes, 2014).
159 So, peptides with predicted bioactivity are then synthesised and assayed for *in vitro* and
160 *in vivo* bioactivity (Agyei, Ongkudon, Wei et al., 2016). Such *in vivo* activity are
161 necessary to validate the *in silico* approach and therefore to confirm the peptide as really
162 bioactive. For instance, peptide AAATP that was found in a ham extract showing ACE
163 inhibitory activity, was also selected based on the *in silico* analysis. Once synthetised,
164 peptide AAATP was administered to spontaneously hypertensive rats and found to exert
165 a very relevant decrease in the systolic blood pressure by −25.62±4.5 mm Hg after 8 h
166 administration (Escudero, Mora Fraser et al., 2013).
167 Several databases are available like BIOPEP, currently BIOPEP-UWM (Minkiewicz,
168 Iwaniak & Darewickz, 2019), which is widely used for identification of bioactive
169 peptides as well as for the *in silico* approach and bioactivity prediction (Minkiewicz,
170 Dziuba, & Michalska, 2011). Other structural and physico-chemical properties may be
171 characterised with models like quantitative structure–activity relationships (QSAR),
172 quantitative structure–property relationships (QSPR), and molecular docking
173 simulations (Agyei et al., 2016; Carrasco-Castilla, Hernández-Álvarez, Jiménez-
174 Martínez et al., 2012). For instance, they can be useful to understand molecular
175 mechanisms and ACE–peptide interactions (Pripp, Isaksson, Stepaniak & Sorhaug,
176 2004).
177 In summary, empirical and *in silico* approaches can be operated in parallel and can be
178 complementary since both determine the generated/predicted peptide and its activity in
179 a complex matrix like dry-cured ham. Furthermore, the identification of naturally
180 generated peptides has progressed rapidly thanks to the peptidomic approaches using
181 tandem mass spectrometry.
182

4. Characteristics of peptides exerting bioactivity

4.1. ACE-inhibitory activity

Angiotensin I-converting enzyme (ACE) is a chloride-activated zinc metallopeptidase able to cleave dipeptides from the C-terminal of peptides and is the enzyme responsible for the conversion of angiotensin I into the potent vasoconstrictor angiotensin II in the renin-angiotensin system and therefore, affecting the regulation of blood pressure. This enzyme is also able to degrade the vasodilative bradykinin in the kinin–kallikrein system. It has been reported that the last three positions at the C-terminal, such as aromatic, positively-charged, and basic amino acids, are very important for an effective ACE inhibition (Fernández, Benito, Martín et al., 2016; Gu et al., 2011).

Based on such characteristics, bioactive peptides constitute substances of interest for preventing cardiovascular diseases including hypertension (Gallego et al., 2018a). So, peptides able to inhibit the ACE enzyme were searched in Spanish ham (Escudero, Mora & Toldrá, 2014), Spanish Iberian (Mora, Escudero, Arihara & Toldrá, 2015), Italian Parma (Dellafiora, Paolella, Dall´Asta et al., 2015) and Chinese Xuanwei (Wang, Li, Li et al., 2018) dry-cured hams.

The ACE inhibitory activity of peptides from different types of dry-cured ham peptides is shown in Table 1. Relevant *in vitro* ACE inhibitory activities were reported for peptides Ala-Ala-Pro-Leu-Ala-Pro, Ile-Ala-Gly-Arg-Pro that had IC_{50} values of 14.38, 25.94 and 67.08 μM respectively (Escudero et al.. 2014). Furthermore, some peptides exerted additional activities to ACE like antioxidant or antiinflammatory as shown in Table 2. So, peptides Lys-Ala-Ala-Ala-Ala-Pro, Lys-Pro-Val-Ala-Ala-Pro, Lys-Ala-Ala-Ala-Thr-Pro and Thr-Gly-Leu-Lys-Pro had IC_{50} values for ACE inhibitory activity of 19.79, 12.37, 25.64 and 51.57 μM, respectively, and also exhibited antiinflammatory activity (Escudero et al., 2014). A particular case was found for

peptide Ala-Ala-Ala-Thr-Pro that had an *in vitro* IC_{50} of 100 µM for ACE inhibition and IC_{50} of 6.47 mM for DPP IV inhibition and showed good *in vivo* antihypertensive activity (Escudero, Mora, Fraser et a., 2013a).

Multifunctional activity has been previously reported for other food-derived bioactive peptides (Li & Aluko, 2010; Udenigwe & Aluko, 2012) that are more typically generated from proteins with high proportion of positively charged and hydrophobic residues (Rao, Sun, Liu et al., 2012). The molecular mechanism of action of multifunctional peptides may be characterized with bioinformatics tools once the active domain is identified (Lammi, Aiello, Boschin & Arnoldi, 2019). As shown in tables 1 and 2, myosin and titin are the main proteins of origin for most of the ACE inhibitory peptides which is quite relevant since they are the major proteins in muscle.

4.2. Antioxidant activity

Antioxidant peptides have the ability to reduce or prevent lipid and protein oxidation in dry-cured ham contributing to a better quality of the product. As shown in Tables 1 and 2, a good number of antioxidant peptides have been reported in Spanish dry-cured ham (Escudero, Aristoy, Nishimura et al., 2012; Escudero, Mora, Fraser et al., 2013a and 2013b; Mora, Escudero, Fraser et al., 2014; Gallego, Mora and Toldrá, 2018a), Chinese Jinhua ham (Zhu, Zhang, Zhou et al., 2013; Zhu, Zhang, Zhou and Xu, 2014) and Chinese Xuanwei ham (Xing, Hu, Ge et al., 2016). In general, molecular masses of peptides have been reported within the range 400-2000 Da and sequences lengths between 4 and 16 amino acids which are typical characteristics of antioxidant peptides (Liu, Xing, Fu et al., 2016). Assays for antioxidant activity determination included DPPH radical-scavenging activity, hydroxyl radical-scavenging activity, ABTS radical-scavenging activity, ferric-reducing antioxidant power, oxygen radical absorbance

capacity assay (ORAC), and lipid peroxidation inhibition activity in linoleic acid emulsion. Most active identified antioxidant peptides were Ala-Glu-Glu-Glu-Tyr-Pro-Asp-Leu (Gallego et al., 2018a) and Ser-Asn-Ala-Ala-Cys (Gallego, Mora & Toldrá, 2018b) in Spanish ham, Asp-Leu-Glu-Glu in Xuanwei ham (Xing et al., 2016), and Gly-Lys-Phe-Asn-Val (Zhu et al., 2013) and Phe-Leu-Lys-Met-Asn, Leu-Pro-Gly-Gly-Gly-His-Gly-Asp-Leu, Leu-Pro-Gly-Gly-Gly-Thr and Lys-Glu-Glu-Arg (Zhu et al., 2016) in Jinhua ham.

A comparison of the antioxidant profile of peptides extracted from Spanish Teruel, Italian Parma and Belgian dry-cured hams was performed after separation through size exclusion chromatography of the respective deproteinised extracts (Mora, Escudero & Toldrá, 2016). The results showed that all hams had a DPPH radical scavenging activity from 50% to 65% and a ferric-reducing antioxidant activity with a maximum of absorbance ranging from 1.21 to 1.28 units, although the elution area was wider for Spanish Teruel ham, between 200 and 250 mL, narrower for Parma ham, 205 to 225 mL and very narrow for Belgian ham, 210 to 200 mL, probably due to differences in the processing time and temperature conditions among other. Similarly, a comparison between Chinese Jinhua, Xuanwei and Rugao dry cured hams was performed. It was reported that peptides extracts from Xuanwei hams had higher DPPH radical scavenging activity than peptides from Jinhua and Rugao hams while peptides from Xuanwei hams had higher ferric reducing antioxidant power and oxygen radical absorbance capacity (ORAC) than the others (Zheng et al., 2018).

The stability of antioxidant peptides has also been reported. So, peptides Ser-Asn-Ala-Ala-Cys and Ala-Glu-Glu-Glu-Tyr-Pro-Asp-Leu derived from Spanish dry-cured ham showed excellent stability against heating up to 90ºC and presence of salt content up to 8% but the activity was drastically reduced after simulated GI digestion (Gallego et al.,

2018d and 2018e). Antioxidant peptides as an extract from Jinhua ham also showed stability against heating up to 60ºC and salt content up to 6%. However, disruption of the structure of peptides at high salt contents (> 8%) and consequent loss of antioxidant activity was reported (Zhu et al., 2014). Furthermore, such peptides were also affected by GI digestion, especially trypsin, that significantly reduced the antioxidant activity because the decline in surface hydrophobicity after free amino acids release.

4.3. Hypoglycemic activity

Several peptides isolated from dry-cured ham have shown inhibitory activity against DPP IV, in some cases in addition to other activities like ACE inhibition (see Tables 1 and 2). So, Ala-Ala-Ala-Thr-Pro showed an IC_{50} value of 6.47 mM, while that of Ala-Ala-Ala-Ala-Gly was 8.13 mM. Dipeptides Ala-Ala, Lys-Ala and Gly-Pro had IC_{50} values of 9.40 mM, 6.27 mM and 9.69 mM, respectively (Gallego, Aristoy & Toldrá, 2014). It must be mentioned that last dipeptide Gly-Pro is controversial since some authors confirm its DPP IV inhibitory activity (Lacroix & Li-Chan, 2012) while others not (Hatanaka, Inoue, Arima et al., 2012). A large value of IC_{50} of 493 mM was reported for carnosine, a natural dipeptide present in ham. However, its content in ham after 10 months of processing is quite high, around 56 mM, so that its inhibition rate would be about 13%. Other dipeptides Thr-Ser, Thr-Leu, Ala-Thr, Val-Lys, Gln-Thr generated from myosin heavy chain (Gallego et al., 2019a) have been described as DPP IV inhibitors (Lan, Ito, Ohno et al., 2015).

4.5. Anti-inflammatory activity

A recent study (Gallego, Mora & Toldrá, 2019b) assayed several peptides isolated from dry-cured ham as inhibitors of the platelet-activating factor-acetylhydrolase (PAF-AH)

283 that degrades oxidised phospholipids into pro-inflammatory lysophosphatidylcholine,
284 autotaxin (ATX) that hydrolyses lysophosphatidylcholine to generate lysophosphatidic
285 acid, and lipoxygenase (LOX) that generates reactive hydroperoxides and lipid
286 oxidative products from unsaturated fatty acids. The results showed 19 peptides able to
287 inhibit 1.28% to 26.06% of the PAF-AH activity, being peptide Phe-Asn-Met-Pro-Leu-
288 Thr-Ile-Arg-Ile-Thr-Pro-Gly-Ser-Lys-Ala the one with the highest inhibitory activity.
289 PAF-AH is an important enzyme for the prevention of inflammation and atherosclerotic
290 lesions (Wilensky et al., 2008). ATX is also implicated in inflammation (Gierse et al.,
291 2010) and 13 peptides were able to inhibit from 5.44% to 57.49% of its activity. Peptide
292 Pro-Ser-Asn-Pro-Pro showed the strongest inhibition, followed by Thr-Gly-Leu-Lys-
293 Pro and Lys-Ala-Ala-Ala-Ala-TPhr-.ro In the case of LOX, that can promote the
294 development of inflammation (Schurink, Van Berkel, Wichers, & Boeriu, 2006), 5
295 peptides showed up to 23.33% inhibitory activity for the peptide His-Cys-Asn-Lys-Tyr-
296 Arg- Ser-Glu-Met.
297 The assayed *in vitro* anti-inflammatory activities of the reported bioactive peptides from
298 dry-cured ham can be considered in general to be low even though the study of anti-
299 inflammatory peptides is quite complex mainly due to the importance of peptide
300 structure and the diversity and complexity of the inflammatory responses (Guha &
301 Majumder, 2018). Anyway, all these reported peptides with such bioactivity have also
302 exerted inhibitory activity against ACE (see Table 2) so that the global action may be a
303 benefit for cardiovascular health.
304
305 **5. Bioavailability and physiological effects of dry-cured ham bioactive peptides**
306 It is important to assess the bioavailability of bioactive peptides to be sure they can
307 remain active after GI digestion and can cross the intestinal membrane and reach the

bloodstream and target organs in active form and exert its bioactivity (Segura-Campos, Chel-Guerrero, Betancur-Ancona, & Hernandez-Escalante, 2011). So, simulated GI digestion with specific digestive enzymes at determined pH and temperature, has been assayed to evaluate the bioavailability of selected peptides from dry-cured ham followed by assays to verify its ability to cross the intestinal barrier. Several ACE inhibitory peptides identified in Spanish dry-cured hams were assayed using Caco2 cell monolayer for their ability of transport through the intestinal epithelium (Gallego, Grootaert, Mora et al, 2016). As can be observed in table 3, the three peptides were hydrolysed by brush border peptidases releasing a wide range of di- and tri-peptides. Only part of Lys-Pro-Val-Ala-Ala-Pro remained intact in the basal side after 60 min. Such small peptides are likely to be transported via intestinal peptide transporter T1 which keeps high bioavailability (Wang and Li, 2017). Blood plasma peptidases could also limit the *in vivo* health effects by hydrolysing bioactive peptides so that *in vivo* studies are also necessary to confirm full bioavailability (Bohn et al., 2017).

In vivo studies with spontaneously hypertensive rats have been conducted with dry-cured ham extracts and reported. This was the case of Spanish Iberian dry-cured ham extract that, after single oral administration to rats, exerted a significant decrease of 12 mm Hg in SBP after 8 h of ingestion returning to values similar to the control after 24 h of ingestion (Mora et al., 2015). More than 2000 sequences were identified in such extract being tripeptides Pro–Pro–Lys, Pro–Ala–Pro, and Ala–Ala–Pro, which are strong ACE inhibitors, those most abundant in such extract (Mora et al., 2015). Pure Ala-Ala-Ala-Thr-Pro pentapeptide, extracted from Spanish dry-cured ham, was also given as a single oral administration to spontaneously hypertensive rats and a relevant decrease in systolic blood pressure by 25.62±4.5 mm Hg was observed after 8 h of ingestion (Escudero et al., 2013a)

In the case of humans, an initial study on the effect of dry-cured ham consumption on cardiovascular health was conducted early this century in Spain when an epidemiologic cohort with 13293 university graduates was performed in order to determine the incidence of cardiovascular disease, hypertension and average yearly weight gain during a follow-up of 6 years. The results did not support any association between the consumption of cured ham and a higher risk of cardiovascular disease, hypertension or weight gain (Ruiz-Candela, Bes-Rastrollo, Zazpe et al., 2000). Other studies with human volunteers have followed. So, a two-arm, cross-over, randomised controlled trial involving 38 healthy subjects with pre-hypertension was performed. The results showed that daily consumption of 80 g of Spanish dry-cured ham did not affect the blood pressure and could be even beneficial for the lipid and glucose metabolism (Montoro-García, Zafrilla-Rentero, Celdrán de Haro et al., 2017). Other reported results on this study were that consumption of 80 daily g of dry-cured ham also impaired platelet and monocyte activation and the levels of plasmatic P-selectin, MCP-1 and interleukin 6 (Martínez-Sánchez, Minguela, Prieto-Merino et al., 2018).

Another study with 100 young and healthy humans revealed that the daily consumption of 40 g of Spanish Iberian dry-cured ham, 100% acorn fed, did not affect blood pressure, total cholesterol content nor weight. Other results were a slight 5mg/dL of HDL cholesterol and a slight decrease of 10 mg/dL of LDL cholesterol and of tri-acylglycerols (Márquez-Contreras, Vázquez-Rico, Baldonedo-Suárez et al., 2018). Of course, *in vivo* studies with human volunteers are subject to inter-individual variability due to genetic factors, health status, diet, particular habits, etc. that can lead to different conclusions when compared with other clinical trials.

On the other hand, the IARC report by (IARC, 2015) stated cancer-related risks associated to the consumption of meat and processed meats. So, the report indicated

evidence of increased risk of colorectal, pancreatic, and prostate cancer associated to consumption of processed meat. The reported meat components potentially involved in carcinogenesis were haem iron, lipid oxidation products, heterocyclic aromatic amines (HAAs), polycyclic aromatic hydrocarbons (PAHs), N-nitroso compounds (NOCs) and the interactions between NOCs, haem iron and HAAs (IARC, 2015). Nevertheless, some of such reported meat components are not generally found, or found at very low levels, in dry-cured ham. This is the case of heterocyclic aromatic amines that are not generated in dry-cured ham because there is no cooking or intense thermal treatment, and the polycyclic aromatic hydrocarbons which are absent because dry-cured ham is not generally smoked (Flores, Mora, Reig et al., 2019). Nitrite acts as antioxidant during dry-cured ham processing and the formed nitric oxide also inhibits lipid oxidation (Kanner, 1994), and was also reported to reduce haem-induced lipid peroxidation in the colon (Chenni, Taché, Naud et al., 2013). Furthermore, the amounts of NOCs generated in dry-cured ham were reported to be very low. For instance, the sum of the mean content level of the major volatile nitrosamines N-nitrosodimethylamine and N-nitrosodiethylamine was reported to be below 4 µg/kg in 286 samples analysed throughout Europe (EFSA, 2017). So, the meat components potentially involved in carcinogenesis should mainly be the haem iron catalysing the endogenous formation of NOCs in the acidic environment of the stomach and some lipid oxidation products. In any case, the American Cancer Society also recommended a diet rich in plant foods, minimising the intake of processed meats, choosing fish, poultry, or beans instead of processed meat and red meat, and in case of eating red meat just choosing smaller portions of lean cuts (American Cancer Society, 2012).

6. Conclusions

The literature reports published in recent years confirm that dry-cured ham constitutes a very good source of bioactive peptides and a large number of them have been successfully identified. Such peptides showed relevant bioactivities like angiotensin converting enzyme inhibitory activity, antioxidant activity, di-peptidylpeptidase IV inhibitory activity among other. The natural generation of such bioactive peptides is a consequence on the intense proteolysis by muscle peptidases occurring during the processing of dry-cured ham. However, there is still little information on the quantitative amount of these peptides in the final product but it is rather difficult because of the large number of bioactive peptides, the low abundance each, and its presence within a complex matrix like dry-cured ham that makes difficult its extraction and analysis (Aroume, Froidevaux, Kapel et al., 2016; Mora, Gallego, Reig & Toldrá, 2017). The knowledge of quantitative amounts of bioactive peptides would help to better understand their bioavailability and their effects on consumers´ health. More clinical trials are also still needed.

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Conflicts of interest

All authors of this manuscript declare that they do not have any conflict of interest.

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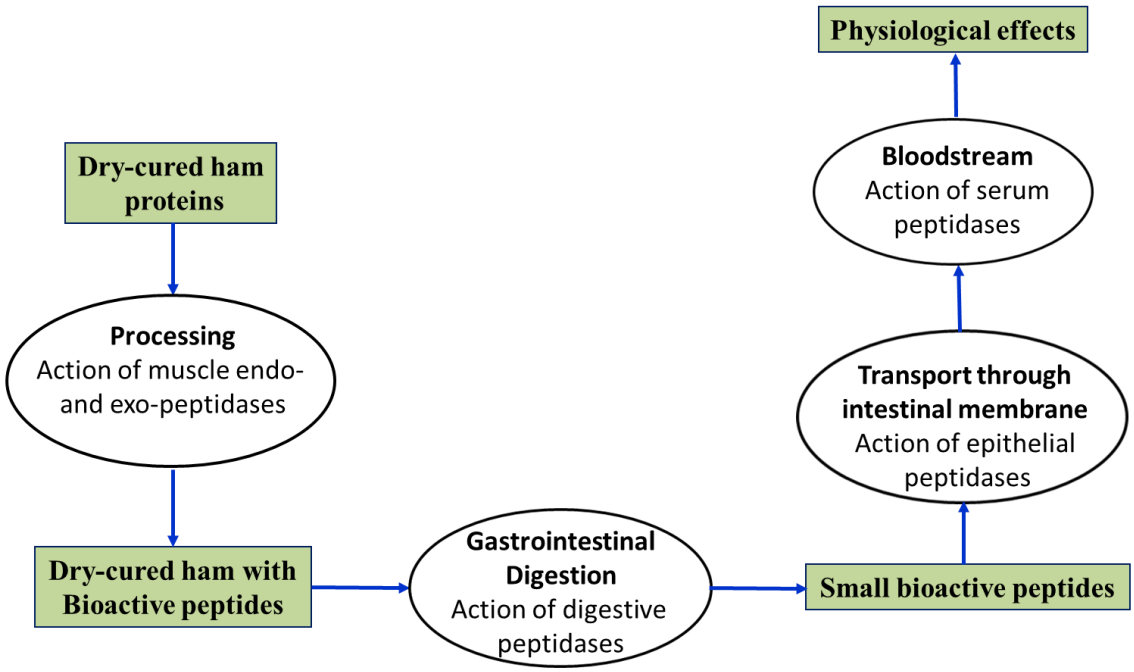
706 LEGENDS FOR THE FIGURES

707 Figure 1.- Scheme of generation of bioactive peptides in dry-cured ham



708 Figure 1

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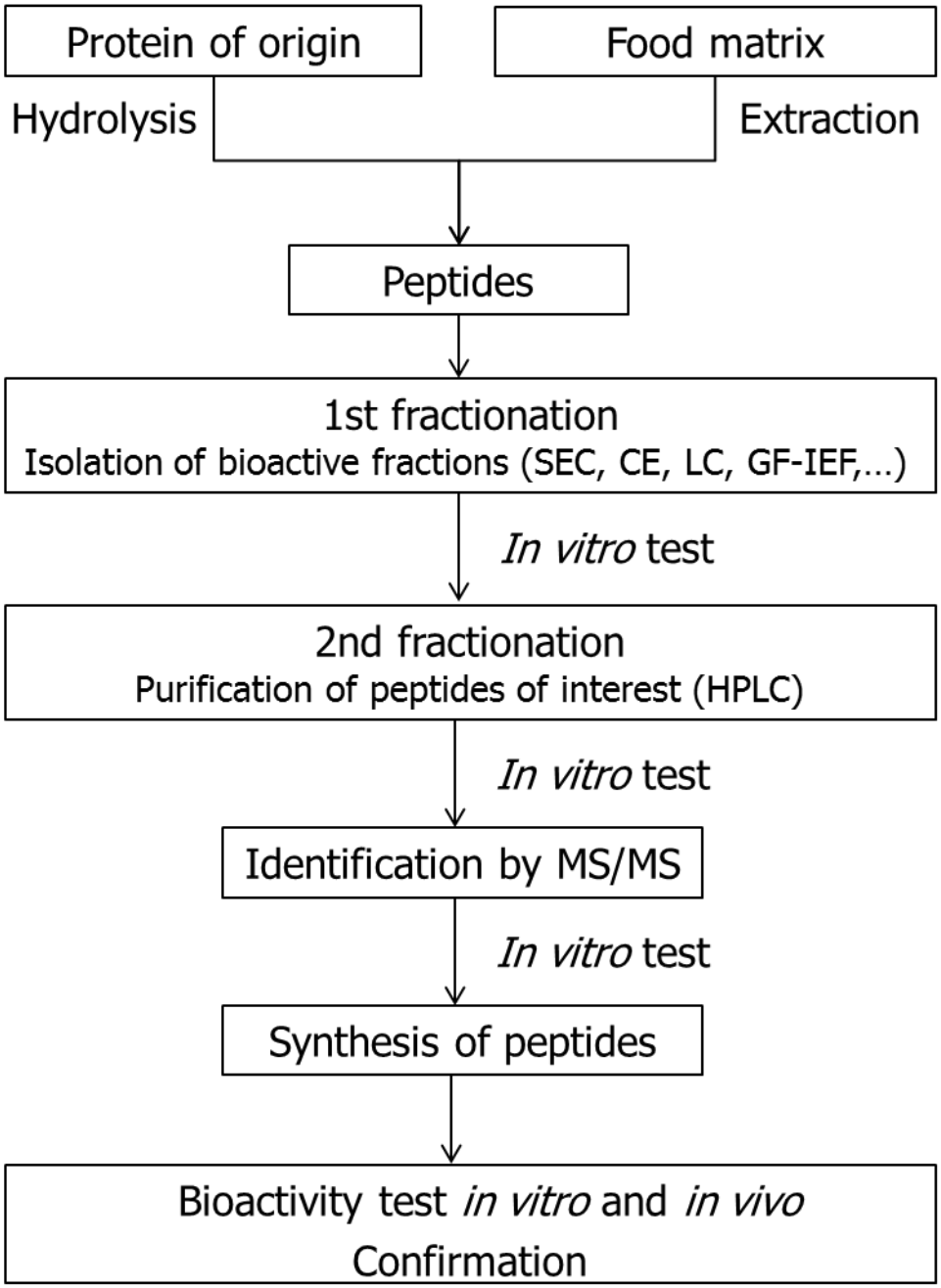
711 Figure 2. Scheme of the traditional empirical procedure for the identification and
712 confirmation of bioactive peptides from food matrices. SEC: size-exclusion
713 chromatography; CE: capillary electrophoresis; LC: liquid chromatography; IEF:
714 isolectric focusing; HPLC: high performance liquid chromatography; MS/MS: mass
715 spectrometry in tandem. Reproduced from Mora et al. (2018).



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718 Figure 3. Main steps of in silico approaches and open access databases for the selection
719 of the protein, hydrolysis simulation and bioactivity prediction. Reproduced from Mora
720 et al. (2018).

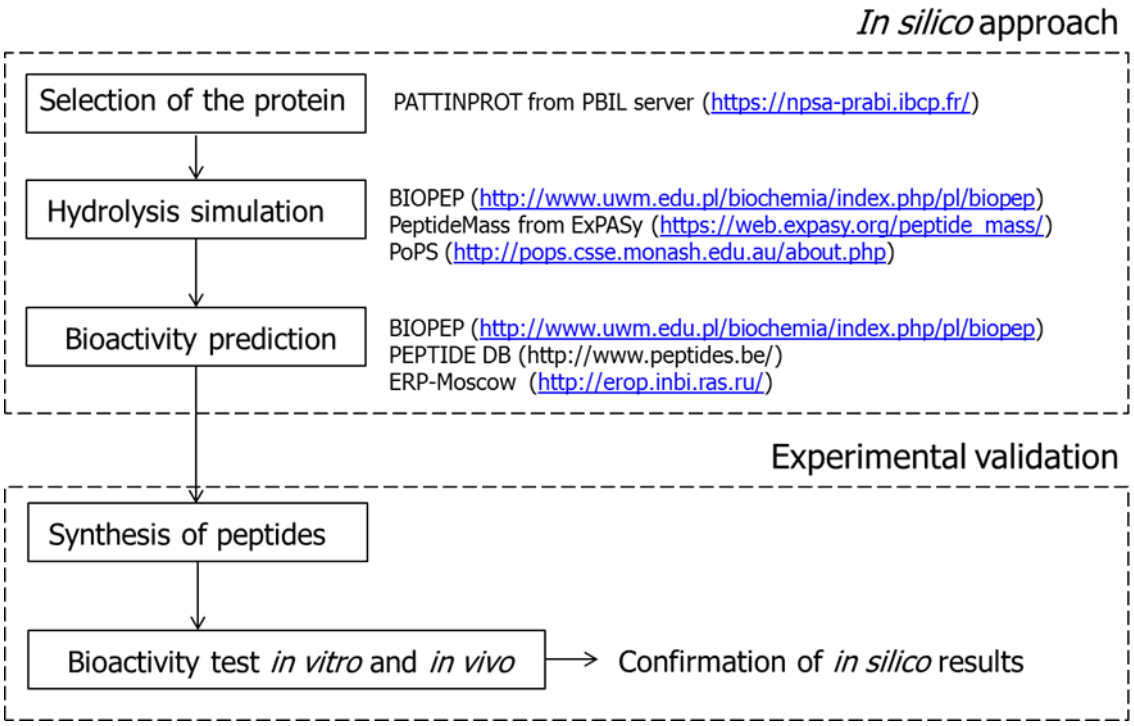

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Table 1.- Peptides identified in different types of dry-cured ham with indication of respective proteins of origin and bioactivity.

Peptide sequence	Protein of origin	Dry-cured ham	Bioactivity	Values of bioactivity*	Reference
AAPLAP	Myosin XV	Spanish Teruel	ACE inhibitory	IC_{50} = 14.38 µM	Escudero et al., 2014
AMNPP	Myosin 3	Spanish Teruel	ACE inhibitory	IC_{50} = 304.5 µM	Escudero et al., 2014
ASGPINFT	Myosin regulatory light chain 2	Spanish	ACE inhibitory	IC_{50} = 975 µM	Escudero et al.,2013a
DLEE	—	Chinese Xuanwei	Antioxidant	DPPH: 74.4% at 0.5 mg/mL	Xing et al., 2016
DVITGA	Myosin light chain	Spanish	ACE inhibitory	IC_{50} = 900 µM	Escudero et al.,2013a
FLKMN	—	Chinese Jinhua	Antioxidant	DPPH: 65% at 1 mg/mL, OH-:60% at 1 mg/mL	Zhu et al., 2016
GGVPGG	Elastin	Spanish	ACE inhibitory	79.90 at 1mM	Gallego et al., 2019
GKFNV	—	Chinese Jinhua	Antioxidant	DPPH: 92.7% at 1 mg/mL	Zhu et al., 2013, 2016
GLAGA	Collagen VII	Spanish	Antioxidant	RP: 0.5 AU at 1 mg/mL	Escudero et al.,2013b
GVVPL	—	Italian Parma	ACE inhibitory	IC_{50} = 956 µM	Dellafiora et al., 2015
IAGRP	Titin	Spanish Teruel	ACE inhibitory	IC_{50} = 25.94 µM	Escudero et al., 2014
IKLPP	Myosin IXb	Spanish Teruel	ACE inhibitory	IC_{50} = 193.9 µM	Escudero et al., 2014
KPGRP	Titin	Spanish Teruel	ACE inhibitory	IC_{50} = 67.08 µM	Escudero et al., 2014
KVLPG	Phosphoglycerate kinase 1	Spanish Teruel	ACE inhibitory	IC_{50} = 265.44 µM	Escudero et al., 2014
LGL	—	Italian Parma	ACE inhibitory	IC_{50} = 145 µM	Dellafiora et al., 2015
LPGGGHGDL	—	Chinese Jinhua	Antioxidant	OH^-: 85% at 1 mg/mL	Zhu et al., 2016
LPGGGT	—	Chinese Jinhua	Antioxidant	DPPH: 65% at 1 mg/mL, OH-:60% at 1 mg/mL	Zhu et al., 2016
PAPPK	Myosin light chain 1/3	Spanish Teruel	ACE inhibitory	IC_{50} = 199.58 µM	Escudero et al., 2014
RHGYM	Dynein heavy chain	Spanish	Antilisterial	MIC = 6.25 mM	Castellano et al., 2016
SAGNPN	Integrin α-3	Spanish	Antioxidant	DPPH: 50% at 1.5 mg/mL	Escudero et al.,2013b
AEEEYPDL	Creatine kinase	Spanish	Antioxidant	DPPH: 63.6% at 3 mg/mL, RP: 0.5 AU at 1 mg/mL ORAC: 960.04 nmol TE/mg, ABTS: 1474.08 nmol TEAC/mg	Mora et al., 2014 Gallego et al., 2018d
SNAAC	Myosin heavy chain	Spanish	Antioxidant	DPPH: 95.7% at 3 mg/mL, RP: 1.7 AU at 1 mg/mL ORAC: 2737.4 nmol TE/mg, ABTS: 3097.04 nmol TEAC/mg	Mora et al., 2014 Gallego et al., 2018e

AAAAG	Histone-lysine N-methyltransferase	Spanish	DPP IV inhibitory	IC_{50} = 8.13 mM	Gallego et al., 2014
AA	—	Spanish	DPP IV inhibitory	IC_{50} = 9.40 mM	Gallego et al., 2014
KA	—	Spanish	DPP IV inhibitory	IC_{50} = 6.27 mM	Gallego et al., 2014
GP	—	Spanish	DPP IV inhibitory	IC_{50} = 9.69 mM	Gallego et al., 2014
SFVTT	—	Italian Parma	ACE inhibitory	IC_{50} = 395 μM	Dellafiora et al., 2015

* IC_{50} value is the peptide concentration that inhibits 50% of activity. Antioxidant activity measured by DPPH radical scavenging assay (DPPH), ferric-reducing power (RP), and hydroxyl radical

scavenging (OH^-). MIC is the minimum concentration of peptide that inhibits the visible growth of bacteria.

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Table 2.- Multifuntional peptides identified in different types of dry-cured ham with indication of respective proteins of origin and bioactivity.

Peptide sequence	**Protein of origin**	**Dry-cured ham**	**Bioactivity**	**Values of bioactivity***	**Reference**
AAATP	Allantoicase	Spanish	ACE inhibitory, Antihypertensive	IC_{50} = 100 µM, SBP: -25.62 mmHg	Escudero et al.,2013a
			DPP IV inhibitory	IC_{50} = 6.47 mM	Gallego et al., 2014
FNMPLTIRITPGSKA	LIM domain-binding protein 3	Spanish	Anti-inflammatory	PAF-AH: 26.06 % at 1mM	Gallego et al., 2019
			ACE inhibitory	68.34% at 1mM	"
HCNKKYRSEM	Dynein heavy chain	Spanish	Antilisterial	MIC = 50 mM	Castellano et al., 2016
			Anti-inflammatory	LOX: 23.33% at 1mM	Gallego et al., 2019
			Antioxidant	ORAC: 1767.56nmol TE/mg	"
			ACE inhibitory	99.34% at 1 mM	"
KAAAAP	Myosin light chain 3	Spanish Teruel	ACE inhibitory	IC_{50} = 19.79 µM	Escudero et al., 2014
			Anti-inflammatory	PAF-AH: 14.50% at 1mM	Gallego et al., 2019
KAAAATP	PR domain zinc finger protein 2	Spanish Teruel	ACE inhibitory	IC_{50} = 25.64 µM	Escudero et al., 2014
			Anti-inflammatory	PAF-AH: 13.73% at 1mM, ATX: 43.53% at 1mM	Gallego et al., 2019
KPVAAP	Myosin XV	Spanish Teruel	ACE inhibitory	IC_{50} = 12.37 µM	Escudero et al., 2014
			Anti-inflammatory	PAF-AH: 13.61% at 1mM, LOX: 5.13% at 1mM	Gallego et al., 2019
MDPKYR	Titin	Spanish	Antilisterial	MIC = 50 mM	Castellano et al., 2016

			Anti-inflammatory	PAF-AH: 13.48% at 1mM, ATX: 14.51% at 1mM	Gallego et al., 2019
			Antioxidant	ORAC: 3087.5 nmol TE/mg, ABTS: 5444.3 nmol TEAC/mg	"
			ACE inhibitory	60.64% at 1mM	"
PSNPP	Titin	Spanish Teruel	ACE inhibitory	IC_{50} = 192.77 µM	Escudero et al., 2014
			Anti-inflammatory	ATX: 57.49% at 1mM	Gallego et al., 2019
TGLKP	Aspartate aminotransferase	Spanish Teruel	ACE inhibitory	IC_{50} = 51.57 µM	Escudero et al., 2014
			Anti-inflammatory	ATX: 43.06% at 1mM	Gallego et al., 2019
TKYRVP	Titin	Spanish	Anti-inflammatory	PAF-AH: 11.04% at 1mM, ATX: 22.47% at 1mM	Gallego et al., 2019
			Antioxidant	ORAC: 2886.8 nmol TE/mg, ABTS: 6987.8 nmol TEAC/mg	"
			ACE inhibitory	80.85% at 1mM	"
TSNRYHSYPWG	Serine/threonine-protein kinase	Spanish	Anti-inflammatory	PAF-AH: 16.30 % at 1mM, ATX:18.93% at 1mM	Gallego et al., 2019
			Antioxidant	ABTS: 3036.03 nmol TEAC/mg	"
			ACE inhibitory	71.62% at 1mM	"

* IC_{50} value is the peptide concentration that inhibits 50% of activity. SBP means the maximum decrease in systolic blood pressure after administration of the peptide to spontaneously hypertensive rats.

Antioxidant activity measured by DPPH radical scavenging assay (DPPH), ferric-reducing power (RP), oxygen radical absorbance capacity assay (ORAC), and ABTS radical-scavenging activity (ABTS).

Anti-inflammatory activity measured by platelet-activating factor-acetylhydrolase inhibition (PAF-AH), lipoxygenase inhibition (LOX), and autotaxin inhibition (ATX). MIC: minimum concentration of peptide that inhibits the visible growth of bacteria

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Table 3. Transport through Caco-2 cell monolayers of three ACE inhhibitory peptides derived from Spanish dry-cured ham. Adapted from Gallego M, Grootaert C, Mora L, Aristoy MC, Van Camp J, Toldrá F: Transepithelial transport of dry-cured ham peptides with ACE inhibitory activity through a Caco-2 cell monolayer. J Funct. Foods 2016, 21:388-395 with permission from Elsevier.

Precursor peptide	Peptide fragments[a]	IC_{50} (μM)	Monoisotopic mass (Da)[b]	Apical – times (min)[c]				Basal – times (min)[d]		
				0	15	30	60	15	30	60
AAATP		100	429,22	x	x					
	AATP	300,74	358,19		x	x	x			
	AAAT	513,65	332,17						x	x
	ATP	406,56	287,15		x			x	x	x
	AAA	111,47	231,12						x	
AAPLAP		14,38	538,31	x	x	x	x			
	PLAP	76,5	396,24		x	x	x			
	APLA	> 1000	370,44					x	x	
	AAPL	> 1000	370,22					x	x	
	PL	337,32	228,15					x	x	x
	LA	310	202,13							x
KPVAAP		12,37	581,35	x	x	x	x	x	x	x
	VAAP	16,75	356,21			x		x	x	
	KPV	> 1000	342,23			x		x	x	
	KP	22	243,16		x		x			
	VA	607,96	188,12		x	x	x	x	x	x
	AP	230	186,10		x	x	x			x

[a] Fragments derived from the degradation of the precursor peptide detected by using MALDI-ToF/ToF MS.

[b] Monoisotopic molecular mass in Daltons of the matched peptide.

[c] Peptides detected in the apical compartment at different transport times.

[d] Peptides detected in the basal compartment at different transport times.

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