Food Research International 154 (2022) 111020

Contents lists available at ScienceDirect

Food Research International

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

A proteomic approach for in-depth characterization and understanding the impact of immunocastration on dry-cured ham of male and female pigs

María López-Pedrouso [a], José M. Lorenzo [b,c], Leticia Pérez-Ciria [d], Guillermo Ripoll [e], María Ángeles Latorre [d], Daniel Franco [b,*]

[a] Departamento of Zooloxía, Xenética e Antropoloxia Física, Universidade de Santiago de Compostela, Santiago de Compostela 15872, Spain
[b] Centro Tecnolóxico da Carne, Rúa Galicia N° 4, Parque Tecnolóxico de Galicia, San Cibrao das Viñas, Ourense 32900, Spain
[c] Área de Tecnología de los Alimentos, Facultad de Ciencias de Ourense, Universidad de Vigo, 32004 Ourense, Spain
[d] Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, IA2-Universidad de Zaragoza, 50013 Zaragoza, Spain
[e] Centro de Investigación y Tecnología Agroalimentaria de Aragón, IA2-CITA, Campus de Aula Dei, 50009 Zaragoza, Spain

ARTICLE INFO

Keywords:
Animal welfare
Processed products
Protein modifications
Protein biomarkers
Myofibrillar proteins
Myosin heavy chain

ABSTRACT

Dry-cured ham is a high-quality product elaborated through a long and complex process. To ensure the success of the process, it is necessary to select the most suitable pork leg and one of the major factors is pig castration. Due to animal welfare, pig castration is becoming a paramount issue in recent years. The proteomic differences of dry-cured ham from immunecastrated pigs against entire females, as well as, between immunecastrated pigs and surgically castrated males were analysed.

The identification and quantification of proteins were carried out by sequential window acquisition of all theoretical mass spectra (SWATH-MS). A total of 249 proteins were identified across the samples of dry-cured ham, resulting in 17 and 37 differentially abundant proteins in the case of males and females, respectively. In the case of males, a high abundance of structural proteins in dry-cured ham from surgically castrated animals as well as a high abundance of trypsinogen and proteosome subunit C9-like protein with protease activity in samples from immunocastrated males suggests that immunocastration impact on myofibrils of dry-cured ham. Regarding females, the immunocastration provoked an increase of abundance in several structural proteins of the myosin heavy chain (MYH7, MYH7B and MYH4) and a decrease in others (ACTN2, TNNT3, MYL3 and TCAP) concerning entire. Overall, MYH4 and ACT were found to a greater degree in immunocastrated males and females indicating a potential for biomarkers.

1. Introduction

Dry-cured ham is a pork product with high added value and outstanding organoleptic characteristics. The process of production is very complex and time consuming increasing its cost During the dry-curing process, lipolysis and proteolysis reactions contributing to their textural and sensorial specific features (López-Pedrouso, Pérez-Santaescolástica, Franco, Carballo, Zapata, et al., 2019; Pérez-Santaescolástica et al., 2019). In this regard, subcutaneous and intramuscular fat are key components that contribute to volatile compounds from fatty acids (Timón et al., 2001). The formation mechanisms of bitterness and adhesiveness should be monitored through the processing parameters (Zhou et al., 2021) such as cathepsin B and B + L activity (Zhou, Wang, Tang, et al., 2019), protein modifications and water distribution (Zhou, Wang, Cai, et al., 2019). This explains the importance of high-quality raw meat to ensure the quality of final product. Genotype gender, rearing production systems (extensive and/or with various levels of intensification) and feed (acorn, pastures and/or feedstuffs) have been investigated to achieve the best quality standard (Díaz-Caro et al., 2019). Within gender factor, the effect of castration on quality attributes of dry-cured ham is still being tested but not so many studies have examined this issue.

Piglet castration is a widespread practice across countries in the EU. In the case of males, the main reasons are to reduce the occurrence of boar taint as well as to reduce aggressive and sexual behaviours. However, surgical castration without anaesthesia or analgesia should be abandoned in terms of animal welfare in agreement with European Commission (EC, 2019). In pork, the boar taint is an unpleasant odour

* Corresponding author.
E-mail address: danielfranco@ceteca.net (D. Franco).

https://doi.org/10.1016/j.foodres.2022.111020
Received 5 November 2021; Received in revised form 4 February 2022; Accepted 12 February 2022
Available online 17 February 2022

and taste provoked by an accumulation of androstenone and skatole in adult entire males. Androstenone is a testicular steroid produced by testicular Leydig cells in mature males and accumulated in adipose tissue. Meanwhile, skatole is a metabolite of tryptophan degradation which is partly metabolized in the liver. In the case of a high level of steroid hormones (including androstenone), the hepatic metabolism is reduced and accumulated in adipose tissue once again, causing pork consumers can detect the boar taint as an undesirable smell in the intramuscular fat (Čandek-Potokar et al., 2017). The immunocastration (a vaccination against Gonadotropin Releasing Hormone, GnRH) could be considered as an alternative method contributing to the best animal welfare. As a result of this anti-GnRH response, the gonadal steroid hormones are avoided, and consequently, reproductive and metabolic changes are induced (Werner et al., 2021). Regarding the meat industry, the pork ham of immunocastrated males is similar to entire males preventing boar taint perception. In comparison with entire and immunocastrated males, the surgically castrated males are the most appropriate for processing dry-cured ham, because the subcutaneous fat cover is thicker (Čandek-Potokar et al., 2020), but this procedure will not be permitted in recent years. These differences in fat content (backfat and intramuscular fat) are key for achieving a high-quality standard of dry-cured ham, juiciness and tenderness are closely linked to intramuscular fat.

Regarding gilts, it was demonstrated that about 30% of carcasses were not suitable for dry-cured ham due to the lack of fatness (Latorre et al., 2009). In contrast to males, the fat content of pork products elaborated from females varies greatly due to the estrus phase which reduces the feed intake and consequently the growth of gilts. On the contrary, immunocastration enhances fat retention and avoids undesirable pregnancies. Recently, it has been demonstrated that immunocastration of gilts increases the carcass fat thickness (Pérez-Ciria, Miana-Mena, Falceto, et al., 2021), the intramuscular fat, as well as, it modifies the fatty acid profile (higher content of saturated fatty acids and lower of polyunsaturated fatty acids) which are desirable for further processing of dry-cured ham (Pérez-Ciria, Miana-Mena, López-Mendoza, et al., 2021). However, the immunocastration of females has scarcely been used till now, despite resulting in an appropriate method to suppress the antral follicles and consequently inhibition of ovarian development (Mitjana et al., 2020).

This study will examine the differences of proteome between dry-cured hams from pigs raised by traditional practices (surgical castration in males and entire pig in females) versus immunocastrated animals. Proteomic changes from dry-cured ham related to castration in males and females will allow us to discover protein biomarkers in connection with animal welfare and to perform a comprehensive characterization of processing dry-cured ham. The search of protein biomarkers is particularly relevant for further meat classification and labelling to authenticate pork products.

2. Materials and methods

2.1. Animal husbandry

A total of 34 dry-cured hams from Duroc × (Landrace × Large White) pigs intended for the Spanish Protected Designation of Origin "Teruel ham" were used in the trial. Two trials were carried out:

Trial 1: Study of dry-cured ham from surgically castrated (SCM) and immunocastrated males (ICM). Fourteen hams belonged to males, being 7 from SCM and 7 from ICM. The surgical castration of males was done during the first week of life and the immunocastration was carried out by three doses of Improvac® (Zoetis) with approximately 25 kg (56 ± 3 d of age), 58 kg (101 ± 3 d of age) and 79 kg (122 ± 3 d of age) of body weight.

Trial 2: Proteomic study of dry-cured ham from entire (EF) and immunocastrated females (ICF). Twenty hams belonged to females, being 10 from EF and 10 from ICF. Immunocastration of females was carried out by two doses of Vacsincel® (Zoetis) with approximately 58 kg (102 ± 3 d of age) and 77 kg (122 ± 3 d of age) of body weight.

All pigs (females and males) were reared on the same farm, received the same management and feeding and were slaughtered on the same day (133 kg and 142 kg of average body weight for females and males, respectively) and in the same abattoir. At the slaughterhouse, the left ham was taken from each carcass, trimmed and individually weighed. A complete description of pig husbandry and experimental design can be obtained from (Pérez-Ciria, Miana-Mena, López-Mendoza, et al., 2021).

2.2. Dry-cured ham process

Upon arrival at the ham-curing facility, hams were classified according to the weight and then the residual blood was removed by a bleeding-massaging machine that presses the femoral artery. The phases of the dry-curing process were the following: i) salting; hams were put in stackable bins, coated with common salt and kept at 0–2 °C and 75–90% relative humidity for 0.8 days per kg of meat mass (around 10 days). A total of 2.5 g of nitrifying salt (a mixture of sodium chloride, maltodextrin, sodium ascorbate and potassium nitrate) per kg of meat mass was added to the common salt. ii) washing with water and molded. iii) resting; hams were hung in racks with hangers and stored from 3.5 to 5 °C and from 80 to 82 to 72–77% relative humidity during 90 days. iv) drying; the temperature was gradually increasing from 8 to 21 °C and the relative humidity reduced from 70 to 75 to 68–73% during 136 days. Finally, lard was applied manually to the muscular part of the hams to avoid over-drying. v) maturing; the temperature continued increasing from 25 to 28 °C and the relative humidity was maintained at 70–75% during 79 days. vi) aging; hams stayed in a natural dryer until reaching 32 °C for 256 days. The individual weight of each piece was recorded at the end of the process (around 20 months).

Once the dry-curing process was completed, dry-cured hams were manually boned, vacuum packaged and stored at 4 °C until the dissection. Two months later, the hams were dissected and the *quadriceps femoris* muscle of each one was taken for the proteomic study. These samples were lyophilized and stored at −80 °C until protein extraction.

2.3. Proteomic analysis

2.3.1. Protein extraction and digestion

Lyophilized sample (50 mg) of dry-cured ham was mixture in RIPA buffer consisting of 200 mmol/L Tris/HCl at pH 7.4, 130 mmol/L NaCl, 10% (v/v) glycerol, 0.1% (v/v) SDS, 1% (v/v) Triton X-100, 10 mmol/L $MgCl_2$ with anti-proteases and anti-phosphatases (Sigma-Aldrich, St. Louis, MO, USA). The resulting solution was centrifuged at 14,000g for 20 min in refrigerated conditions (4 °C). The final concentration of protein was evaluated using an RC-DC kit (Biorad Lab., Hercules, CA, USA) and aliquots containing 100 μg of protein were loaded on an SDS-PAGE for the only purpose of protein purification. The electrophoresis was only performed until the bromophenol reached the top of the resolving gel (10% SDS-PAGE) to concentrate the proteins in a gel single band. This gel band was cropped and divided into smaller pieces which were washed using Milli-Q water and 50 mM ammonium bicarbonate in 50% methanol followed by dehydration in acetonitrile (a vacuum centrifuge). The reduction of proteins was carried out using 10 mM DTT in 50 mM ammonium bicarbonate (60 °C, 30 min) and then alkylation using 55 mM iodoacetamide in 50 mM ammonium bicarbonate (room temperature, 30 min) in darkness. Finally, the digestion was performed by 20 ng/μL trypsin (Promega, Madison, USA) in 20 mM ammonium bicarbonate incubating at 37 °C for 16 h. For further analysis, the peptides were dissolved in 0.1% formic acid.

2.3.2. Generation of the reference spectral library

A mixture of each sample containing 4 μg of protein was used to obtain a representative solution. This final solution was analyzed by shotgun data-dependent acquisition (DDA) through micro-LC-MS/MS

system Ekspert nLC425 (Eksigen, Dublin, CA, USA) and YCM-TriartC18 column (150 μm × 0.3 mm, 12 nm, s-3 μm) (YMC CO, Japan). The flow rate was 5 μL/min using solvent A (water, 0.1% formic acid) and solvent B (ACN, 0.1% formic acid) in a linear gradient (of 5% to 95% B for 30 min, 5 min at 90% B and, finally, other 5 min at 5% B for column equilibration, for a total time of 40 min). Afterwards, the mass spectrometer used was a hybrid quadrupole-TOF mass spectrometer, model Triple TOF 6600 (SCIEX, Framingham, MA, USA) operating with a data-dependent acquisition system in positive ion mode. A 250 ms survey scan was performed from 400 to 1250 *m/z* followed by MS/MS experiments from 100 to 1500 *m/z* using 25 ms of acquisition time for a total cycle time of 2.8 s. The ProteinPilot software v.5.0.1. (SCIEX, Framingham, MA, USA) was used to search in the Uniprot Swiss-Prot database for *Sus scrofa* considering a false discovery rate (FDR) of 1% and proteins with a confidence score above 99%.

2.3.3. Quantification by SWATH and data analysis

The SWATH-MS was used by a data-independent acquisition (DIA) method. The samples were grouped to compare the protein quantifications using two technical replicates in each case. Another 4 μg of protein were subjected to LC as previously described. The mass spectrometer used a 50 ms survey scan and the MS/MS analysis an acquisition time of 50 ms in a total cycle time of 6.3 s. A cycle consisted of the acquisition of 65 scans per SWATH window of variable width (1 *m/z* overlap) covering the 400 to 1250 *m/z* mass range. The software PeakView v.2.2. (SCIEX, Framingham, MA, USA) was used for spectral alignment and targeted data extraction using the reference spectral library from the previous section. The following parameters were used to extract the proteins: ten peptides/protein and seven fragments/peptide excluded shared and modified peptides and FDR below 1%. The protein quantification was calculated by the peak area for the intensity of peptides.

2.3.4. Statistical and bioinformatic analysis

To assess statistical differences among groups, a Student's *t*-test was done at a level of confidence of 95%. The strength of differential protein abundance (DAP) between groups was assessed with fold change (*FC*) defined as $FC = Q1/Q2$ where Q1 and Q2 were the quantifications of the protein of groups 1 and 2, respectively. These DAPs were studied by heat map analysis and this hierarchical clustering of the proteins was generated by XLSTAT 2.01 (Addinsoft SARL, Paris, France) based on the protein quantifications, using Euclidean distances.

Finally, Gene Ontology (GO) enrichment and protein–protein interaction (PPI) were carried out using String 10.0. (Search Tool for the Retrieval of Interacting Genes/proteins) for understanding the molecular function of proteins.

3. Results and discussion

A prior study has noted that castration of males and females produce differences in raw meat quality, specifically in intramuscular fat and fatty acid profile (Pérez-Ciria, Miana-Mena, López-Mendoza, et al., 2021). Thus, it can be suggested that these variations could be associated with proteomic changes which might be affecting the further process of dry-cured ham elaboration. Indeed, the traditional manufacturing process of dry-cured ham including salting, post-salting, ripening and drying for a long time, that led to complex chemical reactions of proteins with sensorial and textural consequences (López-Pedrouso et al., 2018a; López-Pedrouso, Pérez-Santaescolástica, Franco, Carballo, Zapata, et al., 2019). This is supported by our data, as there were differences in dry-cured ham proteome from both comparisons (surgically castrated vs. immunocastrated males) and (entire vs. immunocastrated females). A total of 249 proteins were identified but only differentially abundant proteins (DAPs) were considered, resulting in 54 proteins with significant differences ($P < 0.05$). The 17 and 37 DAPs were detected for males and females, respectively (Tables 1 and 2). In the comparison with other studies conducted on fresh meat, a similar number of proteins were identified using SWATH-MS technology, despite the fact dry-cured ham samples poses some technical problems associated with high salinity and proteolysis degree (López-Pedrouso et al., 2019, 2021). Indeed, the degradation of proteins during the ripening of dry-cured ham led to polypeptides or amino acids that could be hindering the protein identification by MS, considering that only the fragments generated by trypsin could be used to search in the Uniprot Swiss-Prot database.

As expected, the proteomic differences generated by castration were more important in the female group (castrated vs. entire) than male ones (surgically vs. immuno), suggesting that castration itself had more effect than the castration method. However, the interpretation of these proteomic changes is a complex issue because they could be produced by either difference in protein expression from raw meat or by protein degradation during the process. However, these proteomic differences allow us to follow traceability and find out potential biomarkers of immunocastration. The following sections provide more details about these two comparisons.

3.1. Dry-cured ham proteome from surgically castrated (SCM) and immunocastrated (ICM) males

There were 17 DAPs ($p < 0.05$) unambiguously detected by LC-MS/MS between dry-cured ham from SCM and ICM, with 9 proteins with higher abundance in SMC samples and 8 proteins in ICM samples (Table 1). According to hierarchical clustering analysis (Fig. 1), samples of dry-cured ham from SCM and ICM were separated into two clusters except for one replica of SCM (SCM_5) which was included in the ICM cluster. This fact indicates a great consistency in our study, demonstrating that two different patterns of proteins in dry-cured ham were generated by two types of castration (surgical castration and immunocastration), which could lead to sensorial and textural differences. The

Table 1
Differentially abundant proteins (mean ± SE) in dry-cured ham from surgically castrated (SCM) and inmunocastrated (ICM) males. Proteins overexpressed in dry-cured ham from SCM and ICM are indicated by yellow and blue colour, respectively.

Gen name	Protein name	Molecular function	SCM	ICM	
UQCRC1	Ubiquinol-cytochrome c reductase core protein 1	Catalytic activity	285,389±21,652	232,760±12,817	1.23
HIBADH	3-hydroxyisobutyrate dehydrogenase, mitochondrial	Catalytic activity	74,808±4,855	59,536±4,416	1.26
-	Uncharacterized protein		1,127,250±54,296	887,075±64,009	1.27
GLO1	Lactoylglutathione lyase	Lyase activity	649,312±35,724	491,095±32,243	1.32
MYH4	Myosin-4	Structural molecule activity	2,819,634±304,096	2,081,714±182,791	1.35
IGFN1	Immunoglobulin like and fibronectin type III domain containing 1	Structural molecule activity	217,366±12,829	153,293±16,885	1.42
-	Uncharacterized protein		206,187±19,405	135,733±10,921	1.52
ACT	Actin, alpha skeletal muscle	Structural molecule activity	40,994,854±3,204,277	26,807,969±2,273,767	1.53
PDLIM5	PDZ and LIM domain protein 3	Receptor signaling complex scaffold activity	42,747±11,946	2,150±433	19.88
PRELP	Prolargin	Cytoskeletal anchoring activity	5,544±952	12,571±2,533	0.44
PSMB5	Proteasome subunit C9-like protein	Ubiquitin-specific protease activity	5,806±973	10,767±2,074	0.54
-	Uncharacterized protein		92,426±6,103	164,637±31,836	0.56
BRSK2	Non-specific serine/threonine protein kinase	Protein serine/threonine kinase activity	14,971±886	26,333±3,503	0.57
-	Uncharacterized protein		39,760±3,688	65,566±7,977	0.61
CP	Ceruloplasmin	Oxidoreductase activity	9,622±1,142	13,698±1,507	0.70
TTR	Transthyretin	Transporter activity	30,588±1,923	42,651±3,683	0.72
PRSS1	Trypsinogen	Serine-type peptidase activity	5,686,072±461,874	7,737,033±608,020	0.73

Table 2
Differentially abundant proteins (mean ± SE) in dry-cured ham from entire (EF) and inmunocastrated (ICF) females. Proteins overexpressed in dry-cured ham from EF and ICF are indicated by blue and yellow colour, respectively.

Gen name		Protein name	EF	ICF	
GDI1		Rab GDP dissociation inhibitor	75,233±3,539	65,829±2,119	1.14
ANXA2		Annexin	529,217±36,224	426,195±15,392	1.24
STIP1		Stress induced phosphoprotein 1	55,130±3,616	43,944±3,677	1.25
SOD1		Superoxide dismutase [Cu-Zn]	882,692±60,237	695,756±23,230	1.27
-		Uncharacterized protein	1,372,662±27,341	1,081,578±96,077	1.27
TCAP		Telethonin	89,948±6,373	69,574±4,220	1.29
FDPS		Farnesyl diphosphate synthase	34,327±2,691	26,043±1,294	1.32
APOA1BP		NAD(P)H-hydrate epimerase	83,091±7,327	60,034±5,132	1.38
ACY1		N-acyl-L-amino-acid amidohydrolase	173,088±10,887	125,296±14,197	1.38
GPDM		Glycerol-3-phosphate dehydrogenase [NAD(+)]	518,596±56,288	366,085±31,750	1.42
PPWD1		Peptidylprolyl isomerase	85,783±6,177	60,576±6,749	1.42
TNNT3		Fast skeletal muscle troponin C	984,506±85,978	669,197±42,637	1.47
MDH1		Malate dehydrogenase	1,510,903±170,285	978,018±79,129	1.54
ANXA11		Annexin	89,787±5,437	56,458±5,526	1.59
CMBL		Carboxymethylenebutenolidase homolog	83,522±7,977	51,401±6,313	1.62
MYL3		Myosin light chain 1	896,895±124,489	545,806±54,569	1.64
BIN2		Bridging integrator 1	133,765±8,930	81,281±7,828	1.65
DCN		Decorin	82,574±11,376	48,867±4,537	1.69
-		Uncharacterized protein	1,393,845±151,234	807,754±118,604	1.73
ACTN2		Actinin alpha 2	1,742,818±302,488	998,404±101,855	1.75
ERAP1		Aminopeptidase	33,081±3,731	17644±2339	1.87
GPI		Glucose-6-phosphate isomerase	3318886±635591	1,715,235±311,585	1.93
GSTP1		GST class-pi	181,637±29,580	87,786±12,863	2.07
-		Uncharacterized protein	7,378±1,169	3,528±383	2.09
PGAM2		Phosphoglycerate mutase	2,065,299±528,141	864,698±142,125	2.39
ENO3		2-phospho-D-glycerate hydro-lyase	46,216±11,906	18,042±1,879	2.56
CDADC1		CMP/dCMP-type deaminase domain-containing protein	14,681±3,859	4,996±583	2.94
HSP90AA1		Heat shock protein HSP 90-alpha	26,290±5,222	7,103±3,111	3.70
PITHD1		PITH domain containing 1	13,205±3,135	36,170±8,039	0.37
MYH7B		Myosin heavy chain 7B	3,915±699	9,468±1,560	0.41
MYH7		Myosin-7	1,447,318±317,978	2,656,050±377,357	0.54
MYH4		Myosin-4	5,042,623±1,033,282	9,113,677±1,354,660	0.55
MYLK2		Myosin light chain kinase 2, skeletal/cardiac muscle	96,248±16,329	145,757±8,634	0.66
-		Uncharacterized protein	394,727±40,346	565,664±65,008	0.70
CTSB		Cathepsin C	13,762±659	17,377±1,074	0.79

quality of dry-cured ham is highly dependent on starting material used in processing. For instance, it has been reported that ICM were heavier as well as a clear tendency to accumulate more backfat thickness than SCM during the growth (Daza et al., 2016). The initial raw meat used in this study significant differences in intramuscular fat were detected for SCM (4.44%) vs. ICM (3.40%) (Pérez-Ciria, Miana-Mena, López-Mendoza, et al., 2021). Regarding the colour, meat from SCM showed a more intense chroma (Daza et al., 2016). On the contrary, the colours of these initial meat samples were not significantly different except for lightness which was higher in SCM than ICM (34.8 vs. 32.2, $P = 0.027$) (Pérez-Ciria, Miana-Mena, López-Mendoza, et al., 2021). It is reasonable to consider that these different attributes of raw meat could lead to differences in initial protein patterns from pork and different protein degradation during the process.

From these DAPs, several are related to structural and conformational functions such as actin (ACT), immunoglobulin-like and fibronectin type III domain containing 1 (IGFN1) and myosin-4 (MYH4) which were more abundant in SCM samples. This result may be explained by the fact that these structural proteins were more degraded during the processing in dry-cured ham from ICM. In the muscle tissue, the most abundant proteins are the myofibrillar proteins (60–70%). Among them, the most relevant are actin and myosin that form the myofibrils (Lana & Zolla, 2016). The structure of the actin-myosin complex is driven by the concomitant hydrolysis of adenosine triphosphate (ATP) for muscle contraction (Rayment et al., 1993). The degradation of actin and myosin is quite common, and it has a great technological impact. In living animals, the IGFN1 expression has also been correlated to the induction of muscle loss and it could be provoked by myostatin signalling from muscle atrophy animals. This suggests the crucial role of IGFN1 in skeletal muscle although more studies are needed to understand the molecular mechanism (Cracknell et al., 2020). Finally, PDZ and LIM domain protein 3 (PDLIM5) is a protein involved in cytoskeletal assembly due to its LIM domain-binding proteins in the vicinity of the cytoskeleton and its PDZ domain which can interact with actin-binding proteins. In addition, protein kinases can also interact with PDLIM5 (Passier et al., 2000). The higher presence of these structural proteins in SCM samples asserts that myofibrils and cytoskeleton are more preserved in SCM samples than in IMC samples. Before salt penetration, the calpain system could lead to differential muscle degradation during early postmortem. The calpains mainly degrade intermediate filament and structural proteins like desmin and titin, respectively without a major impact on myosin and actin. Indeed, this early proteolysis of structural proteins and cytoskeletal anchorage complexes by the calpain system is responsible for meat tenderness in fresh pork (Bhat et al., 2018). Due to the connection with this differential proteolysis in starting material, the effect of salt would differentially affect a wide variety of proteins including the myofibrillar with a sensorial and textural impact. It has been indicated that actin, heavy myosin (MHC) and desmin could be used as protein biomarkers of pastiness and salt level (Škrlep et al., 2011). In the same line, it has been proven that higher proteolysis of dry-cured ham provoked increased degradation of myofibrillar proteins such as myosin-1, actin and myosin-4 which could be used as biomarkers of proteolysis and adhesiveness of the final product (López-Pedrouso et al., 2018a). Other proteins involved in the metabolism such as ubiquinol-cytochrome *c* reductase core protein 1 (UQCRC1), 3-hydroxybutyrate dehydrogenase (HIBADH), lactoylglutathione lyase (GLO1) also were detected in higher abundance in dry-cured ham from the SCM group.

Most of the proteins with high abundance in dry-cured ham from ICM are proteins with enzymatic activity (Table 1). Because of the clear identification by LC-MS/MS, it is reasonable to believe that these proteins have not been very fragmented during the dry-cured ham process, and they could keep their biological activity longer than other more degraded proteins. For this reason, it is more relevant to know the molecular function of proteins in this case. Regarding our proteomic data, the trypsinogen was the most abundant protein with significant changes ($FC = 0.73$). Trypsinogen is commonly known as the inactive precursor of trypsin which is a member of the serine protease family. Its

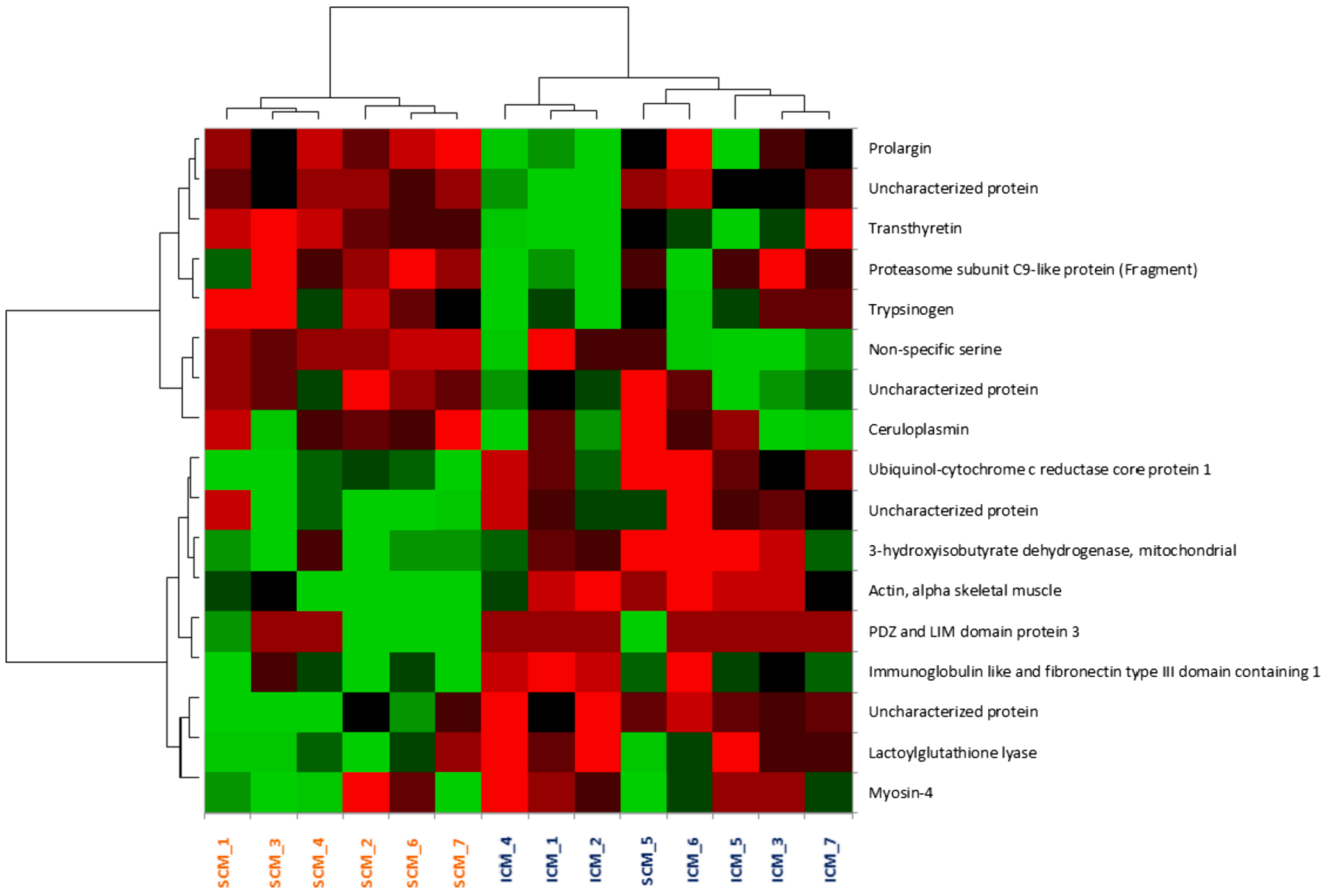

Fig. 1. Heat map analysis of differentially abundant proteins between dry-cured ham from surgically castrated (SCM) and inmunocastrated (ICM) males using seven replicates for each group. The bright green represents the higher abundance of proteins, and the bright red represents the low abundance of proteins.

activation is via hydrolysis of the peptide bond by the action of enteropeptidase, trypsinogen or trypsin resulting in different isoforms (Chen et al., 2013). On the other hand, the proteosome subunit C9-like protein (PSMB5) suffered a huge change between the samples of dry-cured ham from SCM to ICM (*FC* = 0.54). PSMB5 is a proteosome subunit which is a highly sophisticated protein complex responsible for the destruction of proteins (Tanaka, 2009). Those misfolded and damaged proteins, as well as, other proteins of critical cellular processes, are marked by ubiquitin molecules and then the proteosome carried out selective hydrolysis of them (Wu et al., 2020). In this sense, the high abundance of proteosome subunits and trypsin precursors indicated a higher degree of proteolysis in ICM samples.

Other proteins with transporter activity such as transthyretin (TTR) and Ceruloplasmin (CP) were detected in higher abundance probably indicating different biological pathways in raw meat. Transthyretin has two identical thyroid hormone binding sites as a transporter of thyroxine and retinol (vitamin A) (Schussler, 2000). This fact might explain differences in the growth and development of the pig body. Additionally, a non-specific serine/threonine protein kinase was found more abundant in dry-cured ham from ICM. This family of proteins is responsible to catalyze the addition of phosphate to target proteins in a complex role in regulating cellular signal transduction. Protein phosphorylation might produce physiological and biochemical changes in the living animal or during meat processing (Mato et al., 2019). Significant differences in protein phosphorylation led to differential cellular activity.

3.2. Dry-cured ham proteome from entire (EF) and immunocastrated (ICF) females

Considering the dry-cured ham from gilts, there were 35 DAPs ($p < 0.05$) between samples from EF and ICF groups, with 28 proteins more abundant in EF and 7 proteins more abundant in ICF (Table 2). As in the previous section, hierarchical clustering was carried out with these proteomic quantifications (Fig. 2). The proteomic analysis confirmed two clusters (EF vs. ICF of dry-cured ham) with a particular proteomic profile in each case, indicating that samples of dry-cured ham were affected by castration treatment.

Before the dry-cured ham processing, the starting raw material is different depending on the castration. Indeed, it has been reported that EF showed the leanest carcasses, and this lack of fatness level is the main cause of rejections at slaughterhouses. The initial raw meat used in this study significant differences in intramuscular fat were detected for entire (2.70%) vs. imnunocastrated (3.57%) gilts (Pérez-Ciria, Miana-Mena, López-Mendoza, et al., 2021). Regarding the colour, meat from ICF showed more intense redness (a*=4.21) than EF (Daza et al., 2016). However, there was no influence for immunocastration of our gilts on any meat-colour trait (Pérez-Ciria, Miana-Mena, López-Mendoza, et al., 2021). All these facts suggest that molecular events related to pork quality are being altered by castration. From proteomic data, it should be noted that the proteomic differences in females were greater than in the case of males because, in the case of males, two ways of castration were compared, meanwhile in the female group, immunocastration against entire pigs was compared.

The protein changes between entire and immunocastrated females according to STRING analysis included structural proteins and proteins related to glycolysis/gluconeogenesis among others (Fig. 3). This network contained 31 nodes (proteins) and 56 edges (interactions) using medium confidence (threshold of 0.04). There were only 9 proteins without interactions with the other proteins. The PPI enrichment p-value was lower than $1x10^{-6}$ and the average local clustering coefficient

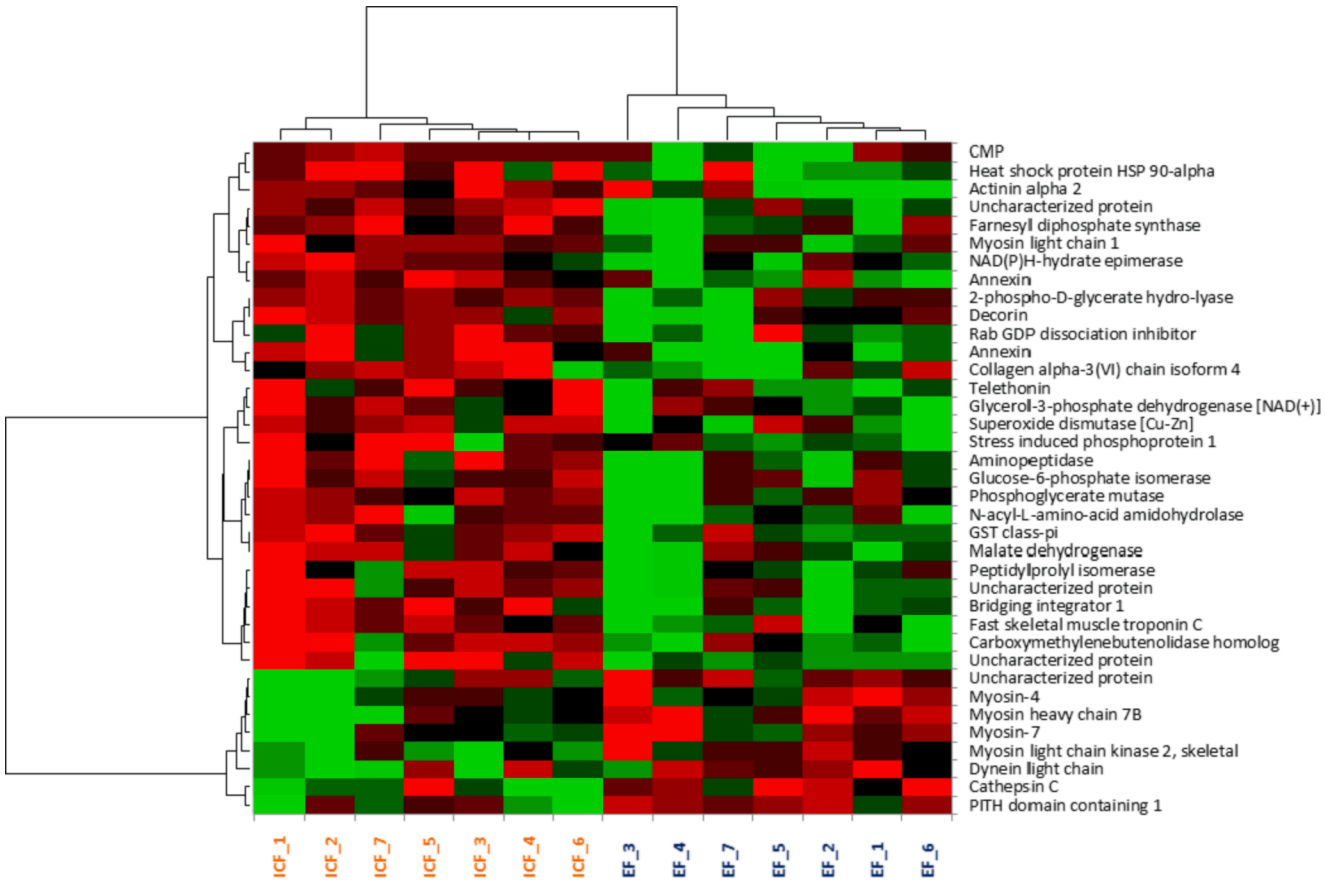

Fig. 2. Heat map analysis of differentially abundant proteins between dry-cured ham from entire (EF) and inmunocastrated (ICF) females using seven replicates for each group. The bright green represents the higher abundance of proteins and the bright red represents the low abundance of proteins.

was 0.486 which displayed a strong connection among the proteins. Mainly, two clusters are displayed in Fig. 3. One cluster includes structural proteins from muscle-related to sarcomere organization which were differentially altered by the castration effect. Castration of females significantly decreased the abundance of actinin alpha 2 (ACTN2), fast skeletal muscle troponin C (TNNT3), myosin light chain 1 (MYL3) and telethonin (TCAP); and increased the abundance of myosin-4 (MYH4), myosin-7 (MYH7), myosin heavy chain 7B (MYH7B) and myosin light chain kinase 2 (MYLK2) in dry-cured ham samples. Before the dry-cured ham process, molecular events relating to ageing meat are taking place as explained in the previous section. However, as a result of the salting and dry-curing process, an intense protein degradation could be produced, hindering these proteomic differences which would be present at the beginning. At this point of meat ageing, it has been demonstrated that protein degradation produced by the activity of endogenous muscle proteins has sensorial consequences. There are two types of enzymes: endopeptidases and exopeptidases according to the cleavage sites of proteins, which play a key role. The first ones (calpains and cathepsins) break down mainly the myofibrillar proteins that afterwards are more degraded by exopeptidases action. In addition, the dry-cured ham involves a high degree of proteolysis about 35% attributed to factors like raw materials, salting procedures and ripening process (López-Pedrouso et al., 2018b). The final result is a complex mixture of small peptides and amino acids providing specific organoleptic and textural parameters to the final product (Mora et al., 2009). Because of dry-cured ham process was carried out similar in both cases (EF and ICF), it is reasonable to assume that the proteomic differences are mainly due to differences in the proteome of fresh meat and their evolution with the manufacturing process. Our results may help to understand that protein degradation was different due to the castration of females suggesting these enzymes might be differentially acting in the two types of samples.

Myosin heavy chain and myosin light chain were differentially degraded in our study. In the early hours of the *postmortem* muscle, it has been described that both types of myosin chains could be fragmented by μ-calpain action. Moreover, slight variations of myosin degradation could drastically change the protein solubility and fiber shear strength (Huff Lonergan et al., 2010). Hence, it could be hypothesized that initial differences in pork leg quality caused by castration might be contributing to proteomic changes during the posterior manufacturing process. Indeed, it has been demonstrated that along with the ripening of the dry-cured ham process for 12 month-old, myofibrillar proteins (actin and myosin light chain) were gradually disappearing as a result of their hydrolyzation (Luccia et al., 2005; Mora et al., 2011).

In the present study, one isoform of myosin light chain (MYL3) was decreased and three isoforms of myosin heavy chain (MYH7, MYH7B and MYH4) were increased due to the immunocastration of females. Another protein related to muscle structure is the troponin T (TNNT) which interacts with thin filament proteins aiding muscle integrity. This is the reason that TNNT plays a role in the fragmentation of the myofibril contributing to characteristics dry-cured ham traits. Beyond the textural characteristics, sensorial differences could be provoked by this myofibrillar proteins fragmentation. Peptides and amino acids proved to be precursors of bitter, umami and sour compounds, and particularly, TNNT can inhibit the sour taste (Kęska & Stadnik, 2017).

Other metabolic proteins also were differentially abundant, which could result in suitable candidate biomarkers for immunocastration such as N-acyl-L-amino-acid amidohydrolase (ACY1), 2-phospho-D-glycerate hydro-lyase (ENO3), glucose-6-phosphate isomerase (GPI), rab GDP dissociation inhibitor (GPD2), malate dehydrogenase (MDH1) and

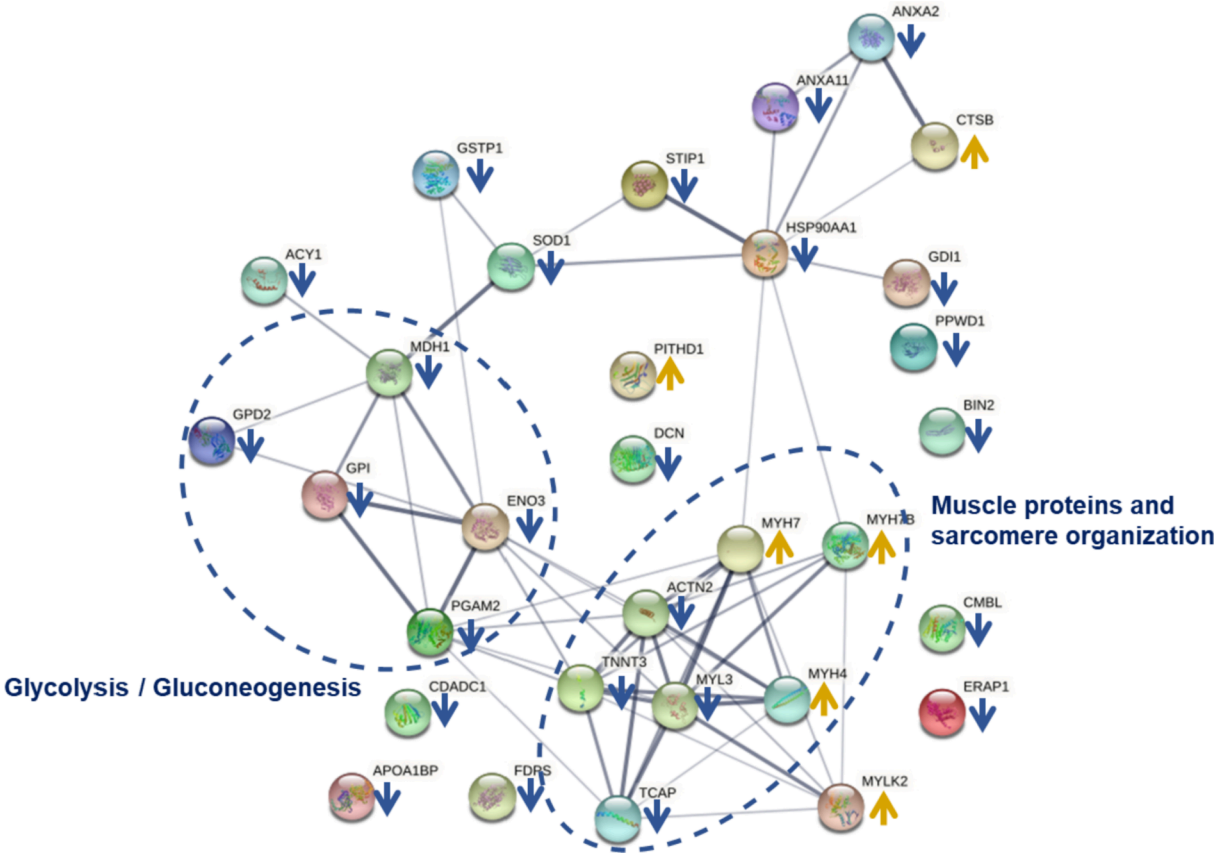

Fig. 3. Protein-protein interaction networks of differentially abundant proteins in dry-cured ham from entire females (EF) and immunocastrated females (ICF) according to STRING. The effect of immunocastration on the proteome of dry-cured ham is indicated by arrows. The increasing or decreasing abundance of these proteins is shown by blue or yellow arrows, respectively.

phosphoglycerate mutase (PGAM2). However, there is little to discuss these proteins, because the origin of these differences is unclear and it could be caused by protein differentially expressed (Poklukar et al., 2021) or differentially fragmented (López-Pedrouso, Pérez-Santaescolástica, Franco, Carballo, Garcia-Perez, et al., 2019).

4. Conclusions

A comparison of dry-cured ham proteome using SWATH-MS allowed us to identify the differences induced by immunocastration against the traditional method (surgically castrated males and entire females), despite high salinity and proteolysis degree. From a proteomic approach, the impact of immunocastration was higher in females than in males as compared with entire females and surgically castrated males.

In the case of males, a higher abundance of structural proteins in dry-cured ham indicated that the myofibrils were more preserved in dry-cured ham from SCM. Supporting this finding, MYH4 and ACT proteins have been described as biomarkers of proteolysis and adhesiveness. On the other hand, the immunocastration of gilts caused an increase of abundance in several structural proteins of the myosin heavy-chain family (MYH7, MYH7B and MYH4) and a decrease in others (ACTN2, TNNT3, MYL3 and TCAP) concerning entire animals.

In both cases, consequently, they seem to be suitable biomarkers of castration, and particularly, MYH4 and ACT proteins. Further studies focused on pig welfare along with pork products quality are important for sustainable consumption and production as well as to meet consumer demands.

Funding

This research was funded by Agencia Estatal de Investigación —AEI— and Fondo Europeo de Desarrollo Regional —FEDER— (Project AGL2016-78532-R) and by FITE through the operative program "Construyendo Europa desde Aragón".

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.

Acknowledgements

José M. Lorenzo and Daniel Franco belong to the competitive reference research group, FunMeat (Axencia Galega de Innovación, GAIN-IN607A2019/01) and they are members of the HealthyMeat network, funded by CYTED (Ref. 119RT0568). Leticia Pérez-Ciria was supported by a fellowship from the Gobierno de Aragón in Spain.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.111020.

References

Bhat, Z. F., Morton, J. D., Mason, S. L., & Bekhit, A. E. D. A. (2018). Role of calpain system in meat tenderness: A review. *Food Science and Human Wellness, 7*(3), 196–204. https://doi.org/10.1016/j.fshw.2018.08.002

Čandek-Potokar, M., Škrlep, M., Kostyra, E., Żakowska-Biemans, S., Poklukar, K., Batorek-Lukač, N., … Stefanski, V. (2020). Quality of dry-cured ham from entire, surgically and immunocastrated males: Case study on Kraški Pršut. *Animals, 10*(2). https://doi.org/10.3390/ani10020239

Čandek-Potokar, M., Škrlep, M., & Zamaratskaia, G. (2017). Immunocastration as alternative to surgical castration in pigs. *Theriogenology*. https://doi.org/10.5772/intechopen.68650

Chen, J. M., Radisky, E. S., & Férec, C. (2013). Human trypsins. *Handbook of Proteolytic Enzymes, 3*(1984), 2600–2609. https://doi.org/10.1016/B978-0-12-382219-2.00576-7

Cracknell, T., Mannsverk, S., Nichols, A., Dowle, A., & Blanco, G. (2020). Proteomic resolution of IGFN1 complexes reveals a functional interaction with the actin nucleating protein COBL. *Experimental Cell Research, 395*(2), Article 112179. https://doi.org/10.1016/j.yexcr.2020.112179

Daza, A., Latorre, M. A., Olivares, A., & López Bote, C. J. (2016). The effects of male and female immunocastration on growth performances and carcass and meat quality of pigs intended for dry-cured ham production: A preliminary study. *Livestock Science, 190*(2016), 20–26. https://doi.org/10.1016/j.livsci.2016.05.014

Díaz-Caro, C., García-Torres, S., Elghannam, A., Tejerina, D., Mesias, F. J., & Ortiz, A. (2019). Is production system a relevant attribute in consumers' food preferences? The case of Iberian dry-cured ham in Spain. *Meat Science, 158*(July), Article 107908. https://doi.org/10.1016/j.meatsci.2019.107908

EC. (2019). Establishing on the production , the processing and the marketing of meat from uncastrated pigs or pigs vaccinated against boar taint (immunocastrated) (Issue March). https://doi.org/10.2875/541755.

Huff Lonergan, E., Zhang, W., & Lonergan, S. M. (2010). Biochemistry of postmortem muscle - Lessons on mechanisms of meat tenderization. *Meat Science, 86*(1), 184–195. https://doi.org/10.1016/j.meatsci.2010.05.004

Kęska, P., & Stadnik, J. (2017). Taste-active peptides and amino acids of pork meat as components of dry-cured meat products: An in-silico study. *Journal of Sensory Studies, 32*(6), 1–9. https://doi.org/10.1111/joss.12301

Lana, A., & Zolla, L. (2016). Proteolysis in meat tenderization from the point of view of each single protein: A proteomic perspective. *Journal of Proteomics, 147*, 85–97. https://doi.org/10.1016/j.jprot.2016.02.011

Latorre, M. A., Ripoll, G., García-Belenguer, E., & Ariño, L. (2009). The increase of slaughter weight in gilts as a strategy to optimize the production of Spanish high quality dry-cured ham. *Journal of Animal Science, 87*(4), 1464–1471. https://doi.org/10.2527/jas.2008-1362

López-Pedrouso, M., Pérez-Santaescolástica, C., Franco, D., Carballo, J., Garcia-Perez, J. V., Benedito, J., Zapata, C., & Lorenzo, J. M. (2019). Proteomic footprint of ultrasound intensification on sliced dry-cured ham subjected to mild thermal conditions. *Journal of Proteomics*, *193*(October 2018), 123–130. https://doi.org/10.1016/j.jprot.2018.10.002.

López-Pedrouso, M., Pérez-Santaescolástica, C., Franco, D., Carballo, J., Zapata, C., & Lorenzo, J. M. (2019). Molecular insight into taste and aroma of sliced dry-cured ham induced by protein degradation undergone high-pressure conditions. *Food Research International*, *122*(December 2018), 635–642. https://doi.org/10.1016/j.foodres.2019.01.037.

López-Pedrouso, M., Pérez-Santaescolástica, C., Franco, D., Fulladosa, E., Carballo, J., Zapata, C., & Lorenzo, J. M. (2018a). Comparative proteomic profiling of myofibrillar proteins in dry-cured ham with different proteolysis indices and adhesiveness. *Food Chemistry*, *244*(June 2017), 238–245. https://doi.org/10.1016/j.foodchem.2017.10.068.

López-Pedrouso, M., Pérez-Santaescolástica, C., Franco, D., Fulladosa, E., Carballo, J., Zapata, C., & Lorenzo, J. M. (2018b). Comparative proteomic profiling of myofibrillar proteins in dry-cured ham with different proteolysis indices and adhesiveness. *Food Chemistry*, *244*(June 2017), 238–245. https://doi.org/10.1016/j.foodchem.2017.10.068.

López-Pedrouso, M., Franco, D., Serrano, M. P., Maggiolino, A., Landete-Castillejos, T., De Palo, P., & Lorenzo, J. M. (2019c). A proteomic-based approach for the search of biomarkers in Iberian wild deer (Cervus elaphus) as indicators of meat quality. *Journal of Proteomics, 205*(March), Article 103422. https://doi.org/10.1016/j.jprot.2019.103422

López-Pedrouso, M., Lorenzo, J. M., Di Stasio, L., Brugiapaglia, A., & Franco, D. (2021). Quantitative proteomic analysis of beef tenderness of Piemontese young bulls by SWATH-MS. *Food Chemistry, 356*(February). https://doi.org/10.1016/j.foodchem.2021.129711

Luccia, A. D., Picariello, G., Cacace, G., Scaloni, A., Faccia, M., Liuzzi, V., … Musso, S. S. (2005). Proteomic analysis of water soluble and myofibrillar protein changes occurring in dry-cured hams. *Meat Science, 69*(3), 479–491. https://doi.org/10.1016/j.meatsci.2004.10.004

Mato, A., Rodríguez-Vázquez, R., López-Pedrouso, M., Bravo, S., Franco, D., & Zapata, C. (2019). The first evidence of global meat phosphoproteome changes in response to pre-slaughter stress. *BMC Genomics, 20*(1), 1–15. https://doi.org/10.1186/s12864-019-5943-3

Mitjana, O., Bonastre, C., Teresa, M., Garza, L., Moreno, B., & Victoria, M. (2020). Immuno-castration of female and male pigs with anti-gonadotrophin releasing hormone vaccine : Morphometric, histopathological and functional studies of the reproductive system. *Animal Reproduction Science, 221*(September), 1–10. https://doi.org/10.1016/j.anireprosci.2020.106599

Mora, L., Sentandreu, M. A., Koistinen, K. M., Fraser, P. D., Toldrá, F., & Bramley, P. M. (2009). Naturally generated small peptides derived from myofibrillar proteins in serrano dry-cured ham. *Journal of Agricultural and Food Chemistry, 57*(8), 3228–3234. https://doi.org/10.1021/jf803480v

Mora, L., Sentandreu, M. A., & Toldrá, F. (2011). Intense degradation of myosin light chain isoforms in Spanish dry-cured ham. *Journal of Agricultural and Food Chemistry, 59*(8), 3884–3892. https://doi.org/10.1021/jf104070q

Passier, R., Richardson, J. A., & Olson, E. N. (2000). Oracle, a novel PDZ-LIM domain protein expressed in heart and skeletal muscle. *Mechanisms of Development, 92*(2), 277–284. https://doi.org/10.1016/S0925-4773(99)00330-5

Pérez-Ciria, L., Miana-Mena, F. J., Falceto, M. V., Mitjana, O., & Latorre, M. A. (2021). Effect of immunocastration and diet on growth performance, serum metabolites and sex hormones, reproductive organ development and carcass quality of heavy gilts. *Animals, 11*(7). https://doi.org/10.3390/ani11071900

Pérez-Ciria, L., Miana-Mena, F. J., López-Mendoza, M. C., Álvarez-Rodríguez, J., & Latorre, M. A. (2021). Influence of immunocastration and diet on meat and fat quality of heavy female and male pigs. *Animals, 11*(12), 1–19. https://doi.org/10.3390/ani11123355

Pérez-Santaescolástica, C., Fraeye, I., Barba, F. J., Gómez, B., Tomasevic, I., Romero, A., Moreno, A., Toldrá, F., & Lorenzo, J. M. (2019). Application of non-invasive technologies in dry-cured ham: An overview. *Trends in Food Science and Technology*, *86*(November 2018), 360–374. https://doi.org/10.1016/j.tifs.2019.02.011.

Poklukar, K., Čandek-potokar, M., Vrecl, M., Batorek-lukač, N., Fazarinc, G., Kress, K., … Škrlep, M. (2021). Adipose tissue gene expression of entire male, immunocastrated and surgically castrated pigs. *International Journal of Molecular Sciences, 22*(4), 1–17. https://doi.org/10.3390/ijms22041768

Rayment, I., Holden, H. M., Whittaker, M., Yohn, C. B., Lorenz, M., Holmes, K. C., & Milligan, R. A. (1993). Structure of the actin-myosin complex and its implications for muscle contraction. *Science, 261*(5117), 58–65. https://doi.org/10.1126/science.8316858

Schussler, G. C. (2000). *The thyroxine-binding proteins., 10*(2), 141–149.

Škrlep, M., Čandek-Potokar, M., Mandelc, S., Javornik, B., Gou, P., Chambon, C., & Santé-Lhoutellier, V. (2011). Proteomic profile of dry-cured ham relative to PRKAG3 or CAST genotype, level of salt and pastiness. *Meat Science, 88*(4), 657–667. https://doi.org/10.1016/j.meatsci.2011.02.025

Tanaka, K. (2009). The proteasome: Overview of structure and functions. *Proceedings of the Japan Academy Series B: Physical and Biological Sciences, 85*(1), 12–36. https://doi.org/10.2183/pjab.85.12

Timón, M. L., Ventanas, J., Carrapiso, A. I., Jurado, A., & García, C. (2001). Subcutaneous and intermuscular fat characterisation of dry-cured Iberian hams. *Meat Science, 58*(1), 85–91. https://doi.org/10.1016/S0309-1740(00)00136-4

Werner, D., Baldinger, L., Bussemas, R., Büttner, S., Weißmann, F., Ciulu, M., … Mörlein, D. (2021). Early immunocastration of pigs: From farming to meat quality. *Animals, 11*(2), 1–15. https://doi.org/10.3390/ani11020298

Wu, T., Yoon, H., Xiong, Y., Dixon-Clarke, S. E., Nowak, R. P., & Fischer, E. S. (2020). Targeted protein degradation as a powerful research tool in basic biology and drug target discovery. *Nature Structural and Molecular Biology, 27*(7), 605–614. https://doi.org/10.1038/s41594-020-0438-0

Zhou, C. Y., Pan, D. D., Cao, J. X., & Zhou, G. H. (2021). A comprehensive review on molecular mechanism of defective dry-cured ham with excessive pastiness, adhesiveness, and bitterness by proteomics insights. *Comprehensive Reviews in Food Science and Food Safety, 20*(4), 3838–3857. https://doi.org/10.1111/1541-4337.12779

Zhou, C. Y., Wang, C., Cai, J. H., Bai, Y., Yu, X. B., Li, C. B., … Cao, J. X. (2019). Evaluating the effect of protein modifications and water distribution on bitterness and adhesiveness of Jinhua ham. *Food Chemistry, 293*(January), 103–111. https://doi.org/10.1016/j.foodchem.2019.04.095

Zhou, C. Y., Wang, C., Tang, C. B., Dai, C., Bai, Y., Yu, X. B., … Cao, J. X. (2019). Label-free proteomics reveals the mechanism of bitterness and adhesiveness in Jinhua ham. *Food Chemistry, 297*(June), Article 125012. https://doi.org/10.1016/j.foodchem.2019.125012