Meat Science 188 (2022) 108804

Contents lists available at ScienceDirect

Meat Science

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

Horse meat tenderization in relation to *post-mortem* evolution of the myofibrillar sub-proteome

Lorea R. Beldarrain [a,b], Enrique Sentandreu [b], Noelia Aldai [a], Miguel A. Sentandreu [b,*]

[a] Lactiker Research Group, Department of Pharmacy & Food Sciences, University of the Basque Country (UPV/EHU), 01006 Vitoria-Gasteiz, Spain
[b] Instituto de Agroquímica y Tecnología de Alimentos (IATA-CSIC), 46980 Paterna, Valencia, Spain

A R T I C L E I N F O

Keywords:
Foal meat
Equine
Tenderness
Proteolysis
OFFGEL
Mass spectrometry

A B S T R A C T

The ageing process after animal slaughter enhances tenderness and influences the value of meat. Horse meat is becoming more popular but lacks standardized ageing practices that should be supported by a better understanding of *post-mortem* muscle biochemistry. Steaks from *Longissimus Thoracis et Lumborum* (LTL) of eight Hispano-Bretón horses were aged for 0, 7, 14 and 21 days and myofibrillar proteins were resolved by one dimensional gel electrophoresis (1-DE). Ten protein bands were found to change ($p \leq 0.05$) among ageing periods. Most changes were observed between days 0 and 14, suggesting that tenderization occurred primary during the first two weeks. Liquid isoelectric focusing (OFFGEL) technology was applied to better resolve myofibrillar sub-proteome and evidenced fourteen protein bands that changed ($p \leq 0.05$) between 0 and 21 days. Three of them were protein fragments coming from troponins T and I and from creatine kinase. Identified molecules could be further studied as potential markers for horse meat tenderness.

1. Introduction

Over the last decades, the meat industry has applied *post-mortem* ageing practices to improve overall quality of traded meat and meat products (Warren & Kastner, 1992) as it is known that ageing enhances tenderness, aroma/flavour and colour properties of meat (Faustman & Cassens, 1990; Watanabe et al., 2015). Since these attributes exert great influence on the consumer acceptability of meat (Font-i-Furnols & Guerrero, 2014), the study of the ageing process became a major topic for researchers that mainly focused their efforts on texture and sensory evaluations of horse (Ruiz, 2018) and bovine (Moran et al., 2020) meats, as well as the study of the protein degradation of horse (Della Malva et al., 2019), bovine (Laville et al., 2009) and pig (Morzel et al., 2004) meats. Among existing practices, wet ageing is the most popular ageing alternative where meat portions (primals, sub-primals or steaks) are vacuum packaged and stored under refrigeration conditions (Kim et al., 2018). Independently of the strategy considered, the importance of this practice becomes evident since inconsistencies in meat texture ocurring during ageing are known to entail major economic losses (Ramanathan et al., 2020). However, it is necessary to point out that several other factors need to be considered when analysing meat texture. Indeed, irrespective of the ageing process, meat takes a “background toughness” as starting point. The background toughness is determined by both intrinsic (species-specific, breed, sex, age, muscle fibre characteristics, collagen amount) and extrinsic (animal feeding, handling, transport) animal factors (Ferguson & Warner, 2008; Koohmaraie & Geesink, 2006; Lepetit, 2007; Monson, Sañudo, & Sierra, 2005).

Both species-specific (Montowska & Pospiech, 2013) and muscle-specific (Moran et al., 2020) tenderization processes take place during ageing, which happen at different rates depending on genetic traits and individual experience of animals (Lana & Zolla, 2016; Lonergan, Zhang, & Lonergan, 2010). According to some authors, tenderization starts after animal death, sparking apoptosis followed by a multi-enzymatic cascade reactions causing degradation of proteins (Ouali et al., 2013). According to this, meat would be tenderized by the action of different proteases, mainly caspases, calpains, cathepsins and proteasome (Lana & Zolla, 2016; Sentandreu, Coulis, & Ouali, 2002). Understanding of protein degradation occurring in meat during ageing is conventionally approached through the study of the myofibrillar sub-proteome merging two dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) analysis (horse, Della Malva et al., 2019; bovine, Laville et al., 2009; pig, Morzel et al., 2004). Recently, liquid isoelectric focusing (OFFGEL) coupled to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis has been successfully implemented as a reliable alternative

* Corresponding author.
E-mail address: ciesen@iata.csic.es (M.A. Sentandreu).

https://doi.org/10.1016/j.meatsci.2022.108804
Received 13 January 2022; Received in revised form 7 March 2022; Accepted 8 March 2022
Available online 13 March 2022
0309-1740/© 2022 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

to study the myofibrillar sub-proteome in meat research (Beldarrain et al., 2018; Fuente-Garcia, Sentandreu, Aldai, Oliván, & Sentandreu, 2020). In any case, understanding of *post-mortem* myofibrillar protein degradation is essential to develop efficient ageing strategies (Kim et al., 2018). In this line, the search of protein biomarkers related to meat tenderness at early *post-mortem* period can help to predict meat quality as demonstrated in beef studies (Gagaoua et al., 2021; Ouali et al., 2013).

In the particular case of horse meat, there are no standardized ageing practices established yet. However, several studies addressed the effect of ageing time on instrumental texture (Beldarrain et al., 2021; Gomez & Lorenzo, 2012; Lorenzo & Gomez, 2012; Ruiz, 2018; Seong et al., 2016) and sensory properties (Beldarrain et al., 2020; Beldarrain, Moran, Sentandreu, Barron, & Aldai, 2022; Ruiz et al., 2019) of horse meat. Up to date only the studies of Della Malva et al. (2019, 2021) have approached this topic from a proteomic perspective. They studied meat from Heavy Draft horses aged up to two weeks using 2-DE/MS analysis. In this sense, the implementation of innovative proteomic methodologies to other breeds could imply further advancement in horse meat quality research, responding to current market demands in terms of increased horse meat consumption (Belaunzaran et al., 2015, 2017) and associated environmental benefits (Insausti et al., 2021).

The objective of this work was to study the evolution of myofibrillar sub-proteome of Hispano-Bretón horse meat during three weeks of ageing process. OFFGEL was used, for the first time, as an alternative to traditional 2-DE for protein fractionation of horse meat samples collected at different ageing times. Protein bands were further characterized by LC-MS/MS analysis to unveil changes in the myofibrillar proteome over time. The proposed proteomic approach could contribute to the development of optimized ageing practices by the horse meat industry and may favour the creation of new insights into the biochemistry of *post-mortem* horse muscle.

2. Materials and methods

2.1. Solvents and chemicals

Tris (tris-hydroxymethyl]-aminomethane), glycerol, urea, thiourea, dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), bromophenol blue and LC-MS grade solvents acetonitrile (ACN) and formic acid (FoA) were from Scharlab (Scharlab S.L., Barcelona, Spain). Water was of ultrapure grade from Millipore (EMD Millipore Co., Billerica, MA, USA). Sucrose, CHAPS (3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate) hydrate, protease inhibitor cocktail (P8340), sodium dodecyl sulfate (SDS), trichloroacetic acid (TCA), Coomassie Brilliant Blue G-250 and ammonium bicarbonate were from Sigma-Aldrich (Sigma-Aldrich Co., St. Louis, MO, USA). Bradford Protein Assay Kit and 30% Acrylamide/Bis solution were from Bio-Rad (Bio-Rad, Hercules, CA, USA). Modified trypsin was from Promega (Promega, Madison, WI, USA).

2.2. Experimental design: Animal handling and sampling

Eight Hispano-Bretón horses (four females and four males), a well-stablished breed in northern Spain, were reared commercially under grazing conditions while suckling their mothers from birth until weaning (6–8 months). This breed is part of the genetic heritage and its conservation is key to maintain mountain ecosystems. Animals continued grazing (11–13 months of age) until they were moved to a feedlot and were finished on concentrate and straw *ad libitum*. Horses were slaughtered at 15–17 months of age in a commercial abattoir (average carcass weight of 246 ± 14.0 kg) following European Union regulations (Council regulation (EU), 2009). Two horses (female and male) were slaughtered per week during four consecutive weeks. After 48 h *post-mortem* at 4 °C (day 0), the whole right rib joint was removed from carcasses (n = 8) and transported to the laboratory under refrigerated conditions. *Longissimus thoracis et lumborum* (LTL) muscle was excised. After trimming adipose and connective tissues of the muscle, four 1.5 cm thick steaks (n = 32) were cut from it, starting from the 5th rib. Average pH value at day 0, measured using a portable pH meter (HI99163, Hanna Instruments, Smithfield, RI, USA) equipped with a penetrating glass electrode (FC232D, Hanna Instruments, USA), was 5.60 ± 0.09, meaning that a normal pH drop was assessed for all samples. Samples were vacuum packed (99%) using a EVTGI-450 vacuum packing machine (Irimar, Navarre, Spain) in polyethylene bags (120 μm and and oxygen permeability of 1 cc/m^2/day at 23 °C; Merkapack, Vitoria, Spain), randomly assigned to an ageing time of 0, 7, 14 and 21 days (d), and kept in a refrigerated room (4.0 ± 1.0 °C) without illumination. After reaching the corresponding ageing period, steaks were stored at −80 °C until analyzed.

The aforementioned procedure was considered as a full-randomized block design with slaughter day as a blocking factor and ageing time as split plot factor, where ageing levels were randomly allocated to different individual steaks obtained from each LTL. The experimental unit (LTL muscle) was considered as a plot and the steaks were the subplots (sampling units) in which ageing time (factor) was assessed. Animal sex and carcass weight were distorting variation sources controlled by the experimental design in order to minimize the residual variation.

2.3. Extraction of myofibrillar proteins

Protein extraction was carried out as described in Fuente-Garcia et al. (2020). Briefly, a representative sample of 10 g of meat was retrieved from the center of the steak, from which half a gram was cut in small cubes and weighted; then homogenized in 5 mL extraction buffer (10 mM Tris pH 7.6, 1 mM EDTA, 0.25 M sucrose) containing 25 μL of protease inhibitors cocktail using an Ultra-Turrax Yellow Line Di 25 (IKA-Werke, Staufen, Germany). The homogenate was centrifuged at 20000*g* for 20 min at 4 °C, the supernatant discarded and the precipitate was washed and centrifuged again. The resultant pellet was dissolved in 10 mM Tris buffer, pH 7.6, containing 7 M urea, 2 M thiourea and 2% CHAPS and centrifuged again. The supernatant (myofibrillar extract) was filtered through glass wool and total protein content was determined in triplicate according to Bradford assay using a Bio Rad commercial kit.

2.4. Protein OFFGEL fractionation

Myofibrillar extracts from non-aged and 21 d aged meat samples (n = 16) were fractionated according to their isoelectric point (pI) along 12 liquid fractions using 13 cm IPG strips with a linear gradient in the pH range 3–10 (GE healthcare, Uppsala, Sweden). A total of 3 mg protein per sample were fractionated in a Agilent 3100 OFFGEL fractionator (Agilent Technologies, Palo Alto, CA, USA) at a constant electric current of 50 mA for about 20 h. OFFGEL fractions were individually collected and stored at −20 °C until further analysis.

2.5. SDS-PAGE analysis

Sodium Dodecyl Sulphate-Polyacrilamide Gel Electrophoresis (SDS-PAGE) was used to resolve total myofibrillar extracts from 0, 7, 14 and 21 d aged meats (n = 32) in 12% polyacrylamide gels. In addition, OFFGEL fractions from non-aged and 21 d aged samples were resolved in 5–16% polyacrilamide gradient gels, previously cast using a Hoefer SG-100 gradient maker (Hoefer, San Francisco, CA, USA).

For both, total myofibrillar extracts and individual OFFGEL fractions, protein samples were gel loaded after being mixed (50:50, *v/v*) with a sample buffer solution (0.88 M Tris-HCl pH 6.8, 10% v/v glycerol, 2% *w/v* SDS, 0.2 M DTT and 0.04% bromophenol blue) and heated at 95 °C for 4 min to denature proteins. Two gels (1.5 mm × 8 cm × 9 cm) were simultaneously run in a Mighty Small II SE260 electrophoresis unit

(Hoefer, San Francisco, CA, USA) at a constant current of 50 mA. Subsequently, gels were fixed into 12% TCA for 1 h, washed twice with bidistilled water and stained overnight with colloidal Blue Coomassie (Candiano et al., 2015). Gels were destained with bidistilled water and digitalized using an Amersham ImageQuant 800 (GE healthcare, Uppsala, Sweden) biomolecular imager. Analyses were carried out in duplicate and samples were randomly assayed.

2.6. Image analysis

Gel images were processed and analyzed using ImageQuant TL 8.2 software (GE healthcare, Uppsala, Sweden). Intensity of bands was quantitatively determined using 1-DE gel analysis after rolling ball background subtraction with the radius set at 200 pixels. For an accurate correction of images, individual band intensities were normalized with respect to the total band volume per lane (for total myofibrillar extracts) or per gel (for individual OFFGEL fractions). Molecular weight of protein bands was determined by the software comparing migration results of sample proteins with those from the commercial Bio-Rad 1610317 broad-range molecular mass protein standards (Bio-Rad, Hercules, CA, USA).

2.7. Statistical analysis

Analyses were conducted using IBM-SPSS Statistics Software (v. 26.0, IBM, Armonk, NY, USA). Relative band intensities from image analysis were log transformed after checking normality and homoscedasticity. The General Linear Model (GLM) of ANOVA was considered to determine significant differences in the relative abundance of selected bands among ageing periods assayed: 0, 7, 14 and 21 d (total myofibrillar extracts) or between 0 and 21 d of ageing (individual OFFGEL fractions). The GLM included ageing time and the controlled distorting variation factor animal sex as fixed effects, and carcass weight as covariate. Slaughter day was also included as a random effect in the model (it was a simultaneous distorting factor of uncontrolled variation coming from at least individual animal, feeding, transport or slaughter conditions). Moreover, GLM included binary interactions ageing time*sex and ageing time*slaughter day. Fisher's Least Significance Difference test of estimated marginal means was used for pairwise comparisons among ageing time levels 0, 7, 14 and 21 d. Three significant figures were used to express the data and significance was declared at $p \leq 0.05$.

2.8. In-gel trypsin digestion of proteins bands

Protein bands showing significant differences among ageing times were excised from gels and digested with trypsin for further protein identification. Excised bands were cut into pieces and washed with 50 mM ammonium bicarbonate, then dehydrated with ACN and the remaining liquid removed using a Speed-Vac concentrator. Gel pieces were digested overnight at 37 °C with 15 μL of a 12.5 μg/mL trypsin solution in 50 mM ammonium bicarbonate (pH 8.5). After incubation, the liquid was transferred into a clean Eppendorf tube and the remaining peptides were recovered by adding ACN/0.1% TFA (50:50). This supernatant was combined with the previous one and the liquid phase was evaporated in a Speed-Vac. Samples were then acidified with 0.1% FoA and transferred into glass vials.

2.9. Peptide sequence identification by LC-MS/MS analysis

Tryptic digests were analyzed by liquid chromatography featured by a Thermo Surveyor Plus system (Thermo Sci., San Jose, CA, USA) with a quaternary pump, vacuum degasser and refrigerated autosampler coupled to a Thermo LCQ Advantage (Thermo Sci., San Jose, CA, USA) ion trap mass analyzer loading an electrospray ionization (ESI) probe operating in positive mode. Separation of peptides was performed through a 150 mm × 2.1 mm, 3 μm particle-size Luna Omega PS C18 column (Phenomenex Inc., Torrance, CA; USA) under the following separation conditions: solvent A, water/FoA (99.9:0.1); solvent B, ACN/FoA (99.9:0.1); separation gradient, initially 0% B, held for 2 min, linear 0–80% B in 23 min, 95% B in 0.1 min, held for 4.9 min for washing, 0% B in 0.1 min, and column equilibration for 14.9 min; total run time, 45 min; flow rate, 200 μL/min; injection volume, 25 μL. Column flow was conducted into the MS system during the 1–40 min time range diverting the rest of running time to waste. Autosampler and column temperatures were set at 10 and 23 °C, respectively.

Instrumental MS settings considered were: capillary temperature, 300 °C; normalized collision-induced dissociation (CID) energy for MS/MS analysis, 35%; spray voltage, 4.0 kV; capillary voltage, 42.0 V; seath gas, 50 (arbitrary units); auxiliary gas, 20 (arbitrary units). The analysis combined two scan events: Full-MS analysis (scan event 1) of intact peptide masses in the 400–2000 m/z range followed by a data-dependent MS/MS (dd-MS^2, scan event 2) experiment of most intense ions from event 1 considering +1 to +4 charges with the following dynamic parameters: minimum MS/MS ion intensity threshold, 3×10^5; exclusion list, 25 masses including those from background provided by a blank injection; exclusion time, 3 min; exclusion mass width, 3 amu; repeat count for MS/MS of most intense ion, 2; repeat count duration, 0.3 min. Number of micro scans-maximum injection time was 1–200 ms for both scan events assayed. Control of the LC-MS system was featured by a PC loading Thermo Xcalibur v2.04 software (Thermo Scientific, San Jose, CA, USA).

Protein identification was carried out interrogating dd-MS^2 data against NCBIprot protein database using Mascot v2.7 search engine with the following settings: enzyme, trypsin; no fixed, or variable modifications but "Error tolerant" option enabled; mass accuracy set to 1.2 and 0.8 Da for MS^1 and MS/MS analyses, respectively; the option "Mammalia" was selected as taxonomy restriction parameter. Estimation of false positive rates by means of false discovery rate threshold 1% was achieved through the activation of the "Decoy" option. Only those assignments with high individual ion scores indicating identity on extensive homology were considered as reliable results ($p < 0.05$).

2.10. Functional and interaction analysis of differentially abundant proteins between non-aged and 21 d aged meats revealed by OFFGEL fractionation

A functional annotation of differential proteins found in OFFGEL fractions was performed using Gene Ontology (GO) slim terms through the AmiGO website (http://amigo.geneontology.org/amigo/). Protein networks were explored using STRING v.11.5 database (ELIXIR, Cambridgeshire, UK, https://string-db.org), selecting "*Equus caballus*" as target organism for interrogations, and they were constructed with a minimum interaction score confidence of 0.4 and four criteria for possible linkage: co-ocurrence, experimental evidences, existing databases and text mining.

3. Results and discussion

3.1. SDS-PAGE of myofibrillar extracts from 0, 7, 14 and 21 d aged horse meat

The myofibrillar protein distribution achieved in 12% SDS-PAGE gels from 0, 7, 14 and 21 d aged horse meat samples is illustrated in Fig. 1. Ten protein bands (A-J) were significantly affected by ageing time, and their LC-MS/MS identifications and relative image abundances are shown in Table 1. It should be noted that most of selected bands had more than one identified protein as consequence of limitations of 1-DE for resolving complex protein mixtures (Rabilloud et al., 2009). Still, tandem-MS allowed the simultaneous identification of those co-eluting proteins. Therefore, although in most cases there was not enough evidence to associate the observed patterns to a single protein, 1-DE enabled a visual exploration of changes occurring during the ageing

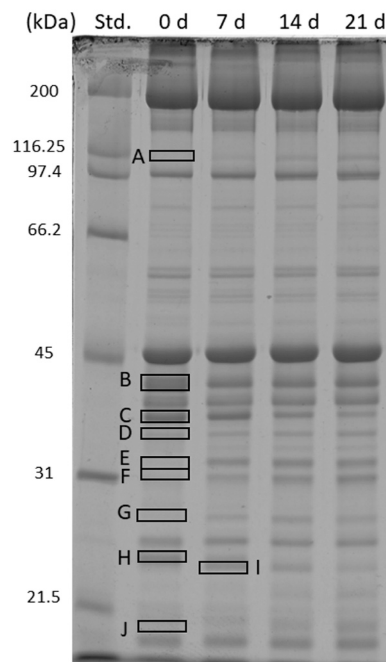

Fig. 1. 12% SDS-PAGE of myofibrillar extracts from 0, 7, 14 and 21 days aged horse meat samples. Std: Commercial molecular mass protein standard.

period, highlighting those proteins that were potentially involved. Furthermore, no significant effect of the interactions ageing time*sex or ageing time*slaughter day were found.

Theoretically, only structural proteins should be present in the myofibrillar sub-proteome, but previous studies performed in beef have demonstrated that changes in protein solubility may result in their different extractability patterns (Feng et al., 2020). This means that highly soluble proteins may appear in the myofibrillar fraction specially during the first days (0–5) of ageing, being this phenomenom attributed to *post-mortem* pH alterations, among others (Laville et al., 2009). In this regard, proteins related to energy metabolism, cellular processes or stress were also identified and included in the discussion, since although they are not targets in muscle architecture degradation, they could act as sentinels of horse meat ageing.

Most of changes in protein abundance were found in proteins with molecular weights below 45 kDa (Bands B-J, Fig. 1), as already observed by Della Malva et al. (2019) and Laville et al. (2009) in *post-mortem* proteomes of horse and bovine meats, respectively. The only exception was myosin-binding protein 1 fragment (band A; 115 kDa), its abundance increased by ageing (Table 1). This protein is one of the accessory components that regulates the rate, force and timing of muscle contraction in the cyclic interaction of actin and myosin (McNamara & Sadayappan, 2018) and achieving a high level of phosphorylation in *post-mortem* muscle compared to other structural proteins (Ren et al., 2019). The latter authors also reported that phosphorylation could be translated into a slower protein degradation as a way to protect muscle structure. To our knowledge, the increasing abundance of this protein fragment over *post-mortem* time has not been reported before. Indeed, detailed action mechanism and properties of this protein remain poorly characterized (McNamara & Sadayappan, 2018).

A single protein was identified in band I: myosin light chain 1/3. Its abundance decreased after two weeks of ageing and, then, remained constant. This is in accordance with what Anderson, Lonergan, and Lonergan (2012) hypothesized - myosin light chain 1/3 is released from the myofibrillar to the soluble fraction as consequence of *post-mortem* proteolysis, thus acting as an indicator of such a phenomenon. The same conclusion was reported by Della Malva et al. (2019) in meat from Italian Heavy Draft Horses after 14 d of ageing, clearly evidencing the usefulness of myosin light chain 1/3 as an indicator of horse meat proteolysis. Moreover, it could be also considered as a potential protein biomarker of meat tenderness as suggested by Gagaoua et al. (2021) in beef.

Among the remaining eight bands, some with increased abundance throughout ageing time (bands E, F and G) consisted of protein fragments generated by proteolytic activity. In such cases, proteins were correctly identified but showed a remarkable inconsistency between the theoretical and apparent molecular weights. Overall, from the selected bands (A-J, Fig. 1), five showed statistically significant differences between 0 and 7 days (bands B, E, F, G and H) and four of them between 7 and 14 days (bands C, H, I and J). However, none of the protein bands considered showed statistically significant differences between 14 and 21 days (Table 1). This is in line with changes of instrumental texture of horse meat recently observed by Beldarrain et al. (2021) in samples from the same study. Authors evidenced that 7 d aged steaks were significantly more tender than non-aged meats considering Warner-Bratzler shear force results achieved, while similar results were observed for 7 and 14 d aged steaks. In this sense, it was reported that the third week of ageing (21 d) did not significantly enhance meat tenderness, suggesting that horse meat tenderization mainly occurs during the first two weeks of ageing. Those findings were corroborated by the results of the present study considering the behaviour exhibited by bands A-J between 14 and 21 d of ageing.

3.2. SDS-PAGE of individual OFFGEL fractions from non-aged and 21 d aged horse meat

To overcome drawbacks caused by the limited resolving power of 1-DE and with the aim to analyse individual protein changes, OFFGEL fractionation was incorporated as a preliminary protein fractionation step coupled to SDS-PAGE in the analysis of non-aged and 21 d aged samples. These extreme ageing periods were chosen since the highest differences in protein abundance were observed when analysing total myofibrillar extracts by 1-DE. Since most protein bands showing significant changes in 1-DE analysis had molecular weights below 45 kDa, 5–16% polyacrylamide gradient gels were employed to analyse OFFGEL fractions, as they were the gels showing the highest resolving power for the aforementioned molecular weight range (data not shown).

Fig. 2 illustrates SDS-PAGE gels from OFFGEL fractions of non-aged (**2.a**) and 21 (**2.b**) d aged horse meat whereas Fig. 3 reports the relative quantification of signals, revealing the presence of 14 protein bands (A-N, **Figs. 2** and **3**) with significant abundance differences. No significant effect of the interactions ageing time*sex or ageing time*slaughter day were found. Table 2 shows LC-MS/MS identification of proteins from A-N bands, distributed through the 12 lanes depending on their pIs. It must be highlighted the feasibility of proteins to be isolated in two adjacent OFFGEL fractions since they were initially focused according to their pI, thus appearing at the same position in contiguous channels of SDS-PAGE gels (bands A, B, C and M, Fig. 2). In such cases, quantitative analysis merged individual measures from different bands corresponding to the same protein in a one single value (*i.e.*, A_1 and A_2 from Fig. 2**.a** and Table 2 is A in Fig. 3).

As expected, results from hyphenation of OFFGEL with SDS-PAGE fractionation favored the obtaining of protein bands with isolated species in most cases (Table 1 *vs* Table 2). Thus, discussion about functional analysis and protein interaction network of horse meat proteome elucidated will be mainly carried out according to qualitative (Table 2) and quantitative (Fig. 3) results.

3.3. Functional analysis of proteins

3.3.1. Structural and muscle contraction related proteins

Abundance of myosin light chain 1/3 (bands A_1 and A_2) was lower in meat aged for 21 d than in non-aged samples (Fig. 3) because of its *post-mortem* release from the myofibrillar to the soluble fraction (Anderson

Table 1
LC-MS/MS identification and relative image abundance (in arbitrary units) of proteins found in A-J bands depicted in Fig. 1 from total myofibrillar extracts of 0, 7, 14 and 21 days aged horse meat samples.

Band[1]	Protein identification [GENE][2]	Theoretical[2]/ Apparent[3] Mr (kDa)	Access Number[2]	Mascot score	Protein coverage	Mean 0 d[4]	Mean 7 d[4]	Mean 14 d[4]	Mean 21 d[4]	SEM	*P value*
A	Myosin-binding protein C1 slow type, [MYBPC1] **fragment**	129.06/115	XP_023487581.1	701	26	148^{b}	332^{ab}	614^{ab}	822^{a}	81	0.007
B	Tropomyosin 2, [TPM2] Fructose-bisphosphate aldolase A, [ALDOA]	39.43/41.38 32.82/41.38	XP_003362760.1 XP_003364171.1	945 391	76% 58%	10944^{a}	7461^{b}	7466^{b}	8261^{ab}	409	0.004
C	Glyceraldehyde-3-phosphate dehydrogenase, [GAPDH] F-actin-capping protein subunit alpha-2-like protein, [CAPZA2]	35.80/ 36.90 33.30/36.90	NP_001157328.1 AEB61386.1	187 79	36% 6%	6936^{a}	5261^{a}	2912^{b}	1765^{b}	391	<0.001
D	L-lactate dehydrogenase A chain, [LDHA] Malate dehydrogenase mitochondrial, [MDH2]	39.69/ 34.72 35.59/34.72	XP_023499905.1 NP_001182455.1	267 112	34% 18%	1015^{c}	1340^{bc}	1851^{ab}	2096^{a}	109	<0.001
E	Troponin T fast skeletal muscle, [TNNT3] **fragment** Voltage-dependent anion-selective channel protein 3, [VDAC3] ATP synthase subunit gamma mitochondrial, [ATP5F1C]	31.52/31.40 30.69/31.40 32.84/31.40	XP_023510451.1 NP_001296239.1 XP_001499911.3	236 128 90	42% 19% 25%	421^{b}	4131^{a}	4286^{a}	3864^{a}	248	<0.001
F	Carbonic anhydrase 3, [CA3] Troponin T fast skeletal muscle, [TNNT3] **fragment** Phosphoglycerate mutase 2, [PGAM2]	30.37/29.75 31.52/29.75 28.60/29.75	NP_001157426.1 XP_001502572.1 XP_001495686.1	183 177 89	30% 10% 17%	507^{c}	2965^{b}	3294^{ab}	4006^{a}	196	<0.001
G	Myosin light chain 3, [MYL3] Glyceraldehyde-3-phosphate dehydrogenase, [GAPDH] **fragment**	22.30/26.08 35.80/26.08	XP_001500321.1 NP_001157328.1	281 123	55% 24%	558^{b}	1504^{a}	1824^{a}	1732^{a}	100	<0.001
H	Troponin I fast skeletal muscle, [TNNI2] ATP synthase subunit O, mitocondrial, [ATP5PO]	21.38/22.54 24.14/22.54	XP_014685731.1 XP_001497214.1	87 69	18% 16%	4185^{a}	275^{b}	ND	ND	291	
I	Myosin light chain 1/3 skeletal muscle, [MYL1]	21.11/21.94	XP_008542802.1	495	79%	2957^{a}	4823^{a}	1732^{b}	1624^{b}	257	<0.001
J	Myosin regulatory light chain 2 skeletal muscle, [MYLPF] Troponin I fast skeletal muscle, [TNNI2]	19.03/18.12 21.38/18.12	XP_001496245.2 XP_014685731.1	218 46	40% 12%	596^{b}	810^{b}	1641^{a}	2296^{a}	135	<0.001

[SEM] Standard error of the mean.
[1] Colour indicates changes in the relative protein band quantification by ageing (green, increase; red, decrease).
[2] Protein, gene identification, theoretical Mr and accession number were from NCBIprot database interrogation (detailed in section 2.9). All of the identification correspond to *Equus caballus* or *Equus przewalskii*.
[3] Apparent Mr was calculated through band position in the gel using ImageQuant TL analysis.
[4] Means with different superscripts indicate statistically significant differences by Tukey's test ($p<0.05$).
[5] Results were estimated using the General Linear Model from ANOVA.

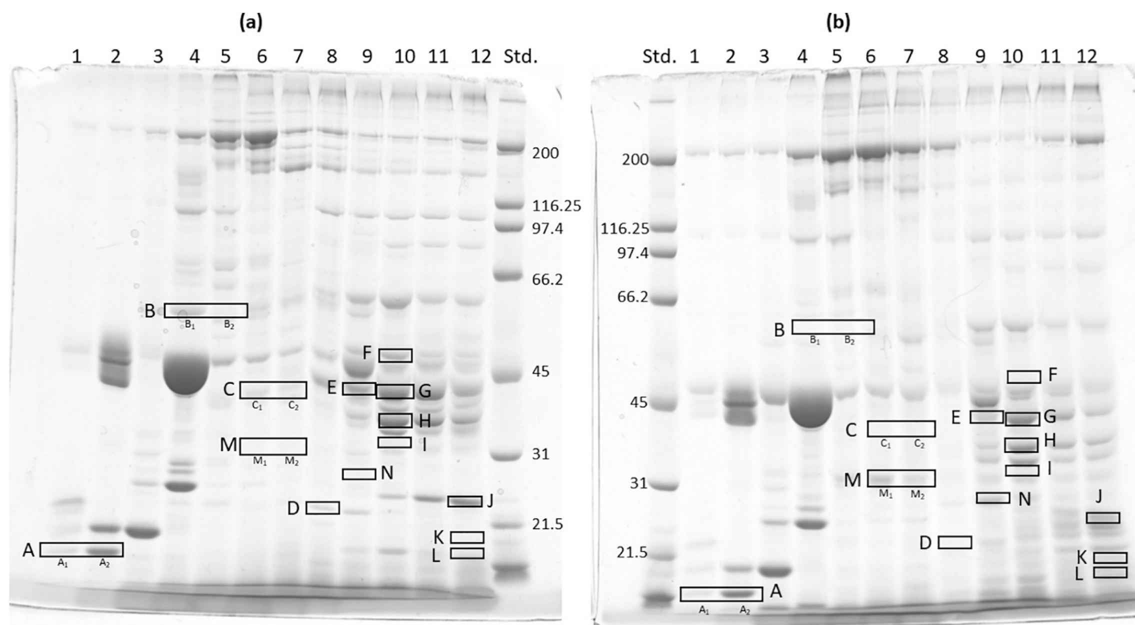

Fig. 2. 5–16% gradient SDS-PAGE gels of the 12 OFFGEL fractions from myofibrillar extracts of unaged **(a)** and 21 d aged **(b)** horse meat samples along the 3–10 pH range assayed. Std: Commercial molecular mass standard.

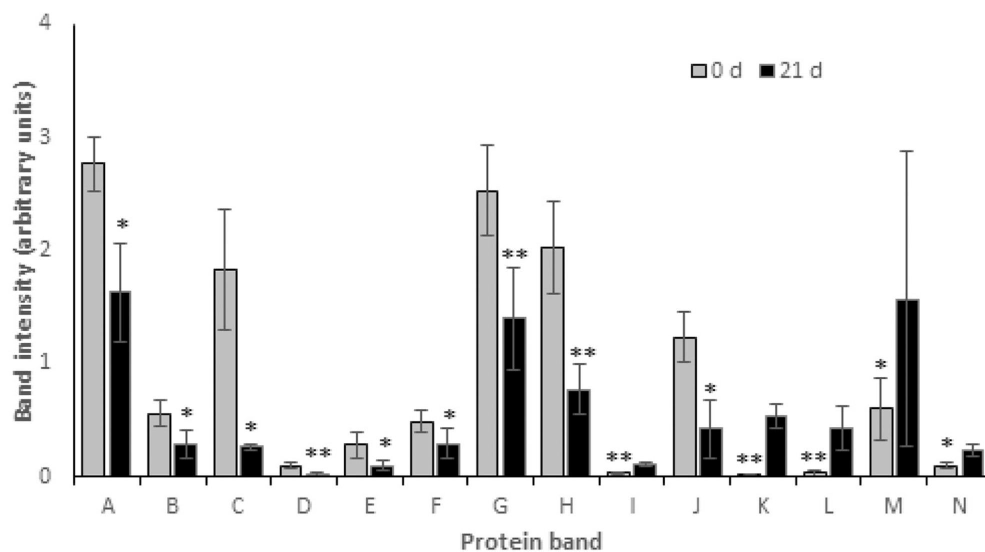

Fig. 3. Quantification by gel image analysis of SDS-PAGE protein bands A-N obtained after OFFGEL fractionation (Fig. 2; Table 2) corresponding to unaged (■) and 21 d aged (■) horse meat samples. Error bars indicate the standard deviation of the means for each group. *$p < 0.05$; **$P \leq 0.01$. Identification of protein bands detailed in Table 2.

et al., 2012). Similarly, abundance of tubulin alpha-4a chain (bands B_1 and B_2), constituent of microtubules and by extension, of centrosome, decreased by ageing (Fig. 3). It plays important role in cellular processes as intracellular tracking, cell division and maintenance of cellular architecture (Becker, Leone, & Engel, 2020). This protein has already been linked to tenderization in beef (Huang et al., 2020) and has a muscle-specific behaviour (Picard et al., 2018). According to present results, it may also be linked to tenderization in horse meat (Fig. 3).

Bands C_1 and C_2 also decreased with ageing. They were identified as troponin T, the tropomyosin-binding subunit of the troponin complex that is constituent of myofibrils. In addition, its degradation product of about 31 kDa was identified in bands M_1 and M_2 (Table 2). As expected, these increased remarkably as the ageing period advanced (Fig. 3). It has been recognized during years that the degradation of troponin T releasing polypeptides migrating at approximately 30 kDa are strongly related to beef (Lonergan et al., 2010) and horse meat (Della Malva et al., 2019) tenderization, as also observed in the present study (Fig. 2). Therefore, it is reasonable to think that troponin T, defined as good biomarker of tenderness in beef (Lana & Zolla, 2016), could also be investigated as an interesting descriptor of horse meat tenderness.

Troponin I has also been proposed, by several authors, as a biomarker of beef tenderness, although to a lesser extent than T subunit (Gagaoua et al., 2021). In this study, troponin I was identified in band J, and two fragments around 19 and 16 kDa were identified in bands K and L, respectively (Table 2). Again, abundance of intact troponin I decreased during ageing (Fig. 3) while its related fragments noticeably increased. Same evolution of intact troponin I during ageing with the inherent increase of the 19 kDa fragment was previously observed by

Table 2
LC-MS/MS protein identification of A-N protein bands from Fig. 2 obtained after OFFGEL fractionation of myofibrillar extracts at 0 and 21 days of ageing.

Band	Protein identification/[GENE[a]]	Theoretical[a]/ apparent[b] Mr (kDa)	Theoretical/ Apparent pI	Access number[a]	Mascot score	Protein coverage	Biological process[c]
A_1	Myosin light chain 1/3 skeletal muscle, [MYL1]	16.68/17	4.62/ (3.59–4.08)	XP_008542803.1	106	47%	Structure
A_2	Myosin light chain 1/3 skeletal muscle, [MYL1]	16.67/17	4.62/ (4.08–4.56)	XP_008542803.1	71	9%	Structure
B_1	Tubulin alpha-4A chain, [TUBB4A], *Bos mutus*	49.55/63	4.78/(5.05–5.53)	XP_001491960.2	52	9%	Structure
B_2	Tubulin alpha-4A chain, [TUBB4A], *B. mutus*	49.55/63	4.78/(5.53–6.02)	XP_001491960.2	102	12%	Structure
C_1	Troponin T fast skeletal, [TNNT3]	37.67/38	6.13/(6.02–6.50)	XP_014685676.1	93	10%	Muscle contraction
C_2	Troponin T fast skeletal, [TNNT3]	37.67/38	6.13 /(6.50–6.98)	XP_014685676.1	59	9%	Muscle contraction
D	Alpha-crystalin B chain, [CRYAB], *B. mutus*	20.02/23	6.76/(6.98–7.46)	XP_001501829.1	89	18%	Heat stress chaperones
E	Creatine kinase M type, [CKM]	43.16/43	6.79/(7.46–7.94)	XP_0015025572.1	174	17%	Energy metabolism
F	Beta enolase, [ENO3]	47.02/47	8.05/(7.94–8.42)	NP_001254531.1	139	18%	Energy metabolism
G	Fructose-bisphosphate aldolase A chain, [ALDOA]	39.43/42	8.30/(7.94–8.42)	XP_003362760.1	120	13%	Energy metabolism
	Glyceraldehyde-3-phosphate dehydrogenase, [GAPDH]	35.80/42	8.22/(7.94–8.42)	NP_001157328.1	66	11%	Energy metabolism
H	Glyceraldehyde-3-phosphate dehydrogenase, [GAPDH]	35.80/37	8.22/(7.94–8.42)	NP_001157328.1	83	12%	Energy metabolism
I	L-lactate dehydrogenase A chain, [LDHA]	36.57/32	8.17/(7.94–8.42)	NP_001138352.1	125	18%	Energy metabolism
J	Troponin I fast skeletal muscle, [TNNI2]	21.38/23	8.86/(8.9–9.38)	XP_014685731.1	141	9%	Muscle contraction
K	Troponin I fast skeletal muscle, [TNNI2] **fragment**	21.38/19	8.86/(8.9–9.38)	XP_014685731.1	147	9%	Muscle contraction
L	Troponin I fast skeletal muscle, [TNNI2] **fragment**	21.38/16	8.86/(8.9–9.38)	XP_014685731.1	128	17%	Muscle contraction
M_1	Troponin T fast skeletal, [TNNT3] **fragment**	37.67/31	6.13/(6.02–6.50)	XP_0145585229.2	66	11%	Muscle contraction
M_2	Troponin T fast skeletal, [TNNT3] **fragment**	37.67/31	6.13 /(6.50–6.98)	XP_0145585229.2	74	11%	Muscle contraction
N	Creatine kinase M type, [CKM] **fragment**	43.16/27	6.79/(7.46–7.94)	XP_0015025572.1	98	9%	Energy metabolism

[a] Protein and gene identification and theoretical Mr. were from NCBInr database interrogation. All of the identification correspond to *Equus caballus* or *Equus przewalskii* unless otherwise indicated.
[b] Apparent Mr. was calculated through band position in the gel using ImageQuant TL.
[c] Proteins were categorized according to their biological pathways using Gene Ontology (GO) slim terms.

Sierra et al. (2012) in beef, suggesting that degradation of troponin I in the first 24 h *post-mortem* could be indicative of the tenderization rate in beef. In our case, further research is necessary to corroborate this assumption in horse meat.

3.3.2. Chaperones

Band D comprised alpha-crystalin B chain, a small Heat Shock protein (HSP) that binds to myofibrils and protects skeletal muscle from protein degradation of protein complexes. HSPs are produced after animal bleeding as a mechanism to preserve cell functions (Ouali et al., 2013), but decreasing abundance of these particular proteins has been described by Ma and Kim (2020) during beef ageing process. Same authors suggested that such decrease could be related to the evolution of tenderness, claiming that small HSPs have no longer the ability to prevent damage of muscle structure (Cramer, Penick, Waddell, Bidwell, & Kim, 2018). In the present study, we found that alpha-crystalin B chain was less abundant at 21 d of ageing compared to non-aged horse meat (Fig. 3).

3.3.3. Proteins related to energy metabolism

Creatine kinase is one of the most cited tenderness biomarker in the literature, especially in LTL muscle (Gagaoua et al., 2021). In our case, this protein was identified in band E. This enzyme is involved in the initial *post-mortem* energy metabolism before glycolysis. It has been reported that it is progressively degraded during meat ageing by calpains (Purintrapiban, Wang, & Forsberg, 2001) and cathepsins (Delbarre-Ladrat, Verrez-Bagnis, Noël, & Fleurence, 2004) until its complete inactivation. This seemed to be the case in *post-mortem* horse muscle since abundance of band E decreased over ageing, while an important increase was observed for the 27 kDa creatine kinase fragment (band N in Table 2 and Fig. 3).

Abundance of bands F, G and H decreased from non-aged to 21 d aged horse meat samples, being identified as the glycolytic enzymes beta-enolase, fructose-bisphosphate aldolase A, and glyceraldehyde-3-phosphate dehydrogenase, respectively. The decreasing trend of these enzymes has been previously observed after 14 d of ageing of horse meat (Della Malva et al., 2021). Glycolysis is an important biochemical pathway affecting *post-mortem* meat quality since different metabolic kinetics and degrees of glycolysis can modify lactic acid accumulation rates and, in consequence, pH decline affecting the activity of proteolytic enzymes (Ferguson & Gerrard, 2014). It seems clear that *post-mortem* evolution of glycolytic enzymes may affect meat tenderization. However, since they are not an intrinsic component of the muscle structure, their connection to tenderness is not direct, giving rise to inconsistent results in the literature (Marino et al., 2014).

According to Ouali et al. (2013), immediately after entering into the anoxia state, the concentration of glycolytic enzymes increases as a mechanism to provide energy, but later on, they have been reported to be target of proteolytic degradation. As an example, in beef, *post-mortem* degradation of beta-enolase, fructose-bisphosphate aldolase A, and glyceraldehyde-3-phosphate dehydrogenase have been reported (Laville et al., 2009; Marino et al., 2014). The decrease in abundance of these proteins could also indicate the slowdown of glycolytic activities or further changes in their solubility during the ageing process. In relation to this, it has been recently suggested that the *post-mortem* oxidative damage of glycolytic enzymes greatly affects meat tenderization rate (Malheiros et al., 2019).

Band I also was identified as a glycolytic enzyme, lactate dehydrogenase. However, its abundance increased after three weeks of ageing. This enzyme catalyses the last step of glycolysis, and has been studied with particular interest during the ageing process, due to its role in *post-mortem* meat colour stability by regulating the generation of NADH, that reduces brown metmyoglobin (Kim et al., 2009). The higher abundance of lactate dehydrogenase A chain in 21 d aged horse meat may reflect a higher lactate dehydrogenase activity or, as mentioned for the other glycolytic enzymes, a loss in solubility of this protein as the ageing process advances. Further research is required to understand the relation of glycolytic enzymes with meat tenderization in *post-mortem* muscle.

3.4. Protein-protein interactions of differentially abundant proteins

Considering proteins that significantly changed in abundance between non-aged and 21 d of ageing (Fig. 3), a horse meat interaction network was constructed to unveil the interconnection of the biological pathways involved (Fig. 4). Proteins were mainly clustered into two categories: proteins involved in energy metabolism (in the lower part) and proteins involved in muscle structure and contraction (in the upper part), while creatine kinase remained connected to the centre of the

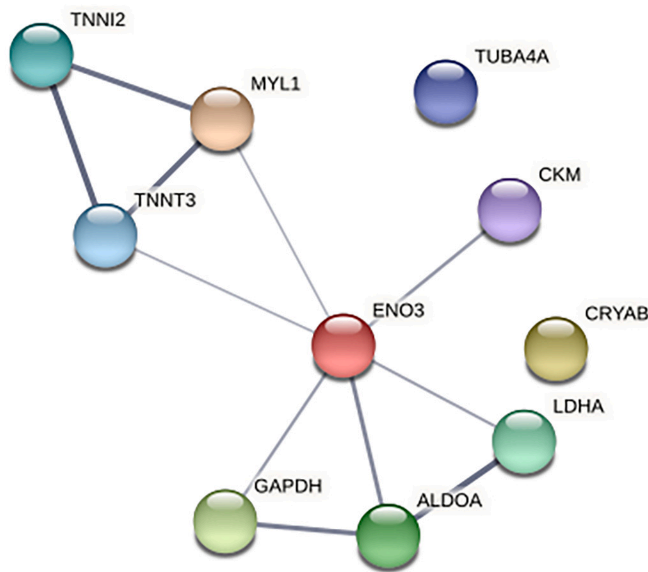

Fig. 4. Protein-protein interaction analysis of differentially abundant proteins found in non-aged and 21 d aged horse meat samples. Network nodes (circles) represent proteins, edges represent known or predicted functional associations and line thickness is an indicator for the strength of the association. Full protein details available in Table 2.

network linked to beta-enolase.

Interrelation of proteins from the glycolytic metabolism was reasonable since fructose bisphosphate aldolase A is a metabolic enzyme of the first stage of glycolysis. On the other hand, beta-enolase, glyceraldehyde-3-phosphate dehydrogenase and L-lactate dehydrogenase are enzymes belonging to the second phase of glycolysis, ensuring the conversion of 2 triose-phosphate to pyruvate and then, to lactate (Ouali et al., 2013). This node of four proteins was connected *via* beta-enolase to creatine kinase because the latter is also a key protein for energy transduction. Regarding the upper node, troponins I and T belonging to the troponin complex (central regulatory protein of muscle contraction) are connected to myosin light chain 1/3, which is part of the motor domain of myosin that protrudes off the surface to interact with actin in muscle contraction events. Interestingly, upper and lower nodes are connected by beta-enolase. This finding was logical since even if the main role of this cytosolic enzyme is the conversion of 2-phosphoglycerate into phosphoenolpyruvate during glycolysis, playing an important role in pH decline and *post-mortem* metabolism, it also regulates skeletal muscle structure regeneration (Merkulova et al., 2000). It also executes a cellular stress response to the deprivation of both oxygen supply and glucose levels *post-mortem*.

Finally, alpha-crystallin and tubulin-4-a chain remained outside of the protein network (Fig. 4), with no interaction between these two proteins and the others. In the case of the HSP, it is associated with the protection of actin filaments in response to stressors (Dimauro, Antonioni, Mercatelli, & Caporossi, 2017) having anti-apoptotic functions. Tubulin-4-a, despite being a structural protein, it is part of another muscle component, the microtubules (Becker et al., 2020).

4. Conclusions

As previously demonstrated in beef, this research revealed how OFFGEL technology can be successfully implemented as a previous step to 1-DE in the study of horse myofibrillar proteins. For the first time, the abundance of several proteins has been reported to change during *post-mortem* horse meat ageing, opening a way for the further study of these proteins as potential biomarkers of horse meat tenderness. Abundances of myosin light chain 1, myosin binding protein C fragment and troponins T and I (and their fragments) significantly changed during ageing, suggesting their role in the development of horse meat tenderness as already proposed in other animal species.

During ageing process, most protein changes found between 0 and 14 d were in accordance with previous instrumental tenderness data, highlighting that, under our conditions, horse meat tenderization primarily occurred during the first two weeks of ageing. Overall, the present proteomic study constitutes a step forward in the understanding of the molecular mechanism of horse meat tenderization.

Author contributions

Conceptualization, L.R.B and M.A.S.; methodology, L.R.B., E.S. and M.A.S.; investigation, L.R.B., N.A. and M.A.S.; data curation, N.A.; writing—original draft preparation, L.R.B.; writing—review and editing, L.R.B., E.S., N.A., and M.A.S.; supervision, N.A. and M.A.S.; project administration, M.A.S.; funding acquisition, M.A.S. All authors have read and agreed to the published version of the manuscript.

L.R.B.: Lorea R. Beldarrain.
M.A.S.: Miguel A. Sentandreu.
N.A.: Noelia Aldai.
E.S.: Enrique Sentandreu.

Declaration of Competing Interest

Authors declare no conflict of interest.

Acknowledgements

Department of Economic Development and Infrastructures of the Basque Government is acknowledged for the doctoral fellowship of L.R. Beldarrain. Lactiker Research Group (Basque Government IT944-16) funded this work. Funding and contract of E. Sentandreu from project RTI2018-096162-R-C22 (Spanish Agencia Estatal de Investigación) are also fully acknowledged.

References

Anderson, M. J., Lonergan, S. M., & Lonergan, E. H. (2012). Myosin light chain 1 release from myofibrillar fraction during postmortem aging is a potential indicator of proteolysis and tenderness of beef. *Meat Science, 90*, 345–351.

Becker, R., Leone, M., & Engel, F. B. (2020). Microtubule organization in striated muscle cells. *Cells, 9*. Article 1395.

Belaunzaran, X., Bessa, R. J. B., Lavín, P., Mantecón, A. R., Kramer, J. K. G., & Aldai, N. (2015). Horse-meat for human consumption - current research and future opportunities. *Meat Science, 108*, 74–81.

Belaunzaran, X., Lavín, P., Barrón, L. J. R., Mantecón, A. R., Kramer, J. K. G., & Aldai, N. (2017). An assessment of the fatty acid composition of horse-meat available at the retail level in northern Spain. *Meat Science, 124*, 39–47.

Beldarrain, L. R., Aldai, N., Picard, B., Sentandreu, E., Navarro, J. L., & Sentandreu, M. A. (2018). Use of liquid isoelectric focusing (OFFGEL) on the discovery of meat tenderness biomarkers. *Journal of Proteomics, 183*, 25–33.

Beldarrain, L. R., Etaio, I., Morán, L., Sentandreu, M. A., Barrón, L. J. R., & Aldai, N. (2020). Effect of ageing time on consumer preference and sensory description of foal meat. *Food Research International, 129*, Article 108871.

Beldarrain, L. R., Moran, L., Sentandreu, M. A., Barron, L. J. R., & Aldai, N. (2022). Effect of ageing time on the volatile compounds from cooked horse meat. *Meat Science, 184*. Article 108692.

Beldarrain, L. R., Moran, L., Sentandreu, M. A., Insausti, K., Barron, L. J. R., & Aldai, N. (2021). Muscle and subcutaneous fatty acid composition and the evaluation of ageing time on meat quality parameters of Hispano-Bretón horse breed. *Animals, 11*, 1241.

Candiano, G., Bruschi, M., Musante, L., Santucci, I., Ghiggeri, G. M., Camemoll, B., … Righetti, P. G. (2015). Blue silver: A very sensitive colloidal Coomassie G-250 staining from proteome analysis. *Electrophoresis, 25*, 1327–1333.

Council regulation (EU). (2009). Council regulation (EU) no 1099/2009 of 24 September 2009 on the protection of animals at the time of killing. *Official Journal of European Union, L, 303*, 1–30.

Cramer, T., Penick, Waddell, Bidwell, & Kim. (2018). A new insight into meat toughness of callipyge lamb loins-the relevance of anti-apoptotic systems to decreased proteolysis. *Journal of Animal Science, 79*, 398–412.

Delbarre-Ladrat, C., Verrez-Bagnis, V., Noël, J., & Fleurence, J. (2004). Relative contribution of calpain and cathepsins to protein degradation in muscle of sea bass (*Dicentrarchus labrax L.*). *Food Chemistry, 88*, 389–395.

Della Malva, A., de Palo, P., Lorenzo, J. M., Maggiolino, A., Albenzio, M., & Marino, R. (2019). Application of proteomic to investigate the post-mortem tenderization rate of different horse muscles. *Meat Science, 157*, Article 107885.

Della Malva, A., Maggiolino, A., De Palo, P., Albenzio, M., Lorenzo, J. M., Sevi, A., & Marino, R. (2021). Proteomic analysis to understand the relationship between the sarcoplasmic protein patterns and meat organoleptic characteristics in different horse muscles during aging. *Meat Science, 184*, Article 108686.

Dimauro, I., Antonioni, A., Mercatelli, N., & Caporossi, D. (2017). The role of αB-crystallin in skeletal and cardiac muscle tissues. *Cell Stress & Chaperones, 23*, 491–505.

Faustman, C., & Cassens, R. G. (1990). The biochemical basis for discoloration in fresh meat: A review. *Journal of Muscle Foods, 1*, 217–243.

Feng, Y. H., Zhang, S. S., Sun, B. Z., Xie, P., Wen, K. X., & Xu, C. C. (2020). Changes in physical meat traits, protein solubility, and the microstructure of different beef muscles during post-mortem aging. *Foods, 9*, 806.

Ferguson, D. M., & Gerrard, D. E. (2014). Regulation of post-mortem glycolysis in ruminant muscle. *Animal Production Science, 54*, 464–481.

Ferguson, D. M., & Warner, R. D. (2008). Have we underestimated the impact of pre-slaughter on meat quality in ruminants? *Meat Science, 80*, 12–19.

Font-i-Furnols, M., & Guerrero, L. (2014). Consumer preference, behavior and perception about meat and meat products: An overview. *Meat Science, 98*, 361–371.

Fuente-Garcia, C., Sentandreu, E., Aldai, N., Oliván, M., & Sentandreu, M. A. (2020). Characterization of the myofibrillar proteome as a way to better understand differences in bovine meats having different ultimate pH values. *Proteomics, 20*. Article 2000012.

Gagaoua, M., Terlouw, E. M. C., Mullen, A. M., Franco, D., Warner, R. D., Lorenzo, J. M., Purslow, P. P., Gerrard, D., Hopkins, D. L., Troy, D., & Picard, B. (2021). Molecular signatures of beef tenderness: Underlying mechanisms based on integromics of protein biomarkers from multi-platform proteomics studies. *Meat Science, 12*. Article 108311.

Gomez, M., & Lorenzo, J. M. (2012). Effect of packaging conditions on shelf-life of fresh foal meat. *Meat Science, 91*, 513–520.

Huang, C., Hou, C., Ijaz, M., Yan, T., Li, X., & Zhang, D. (2020). Proteomics discovery of protein biomarkers linked to meat quality traits in post-mortem muscles: Current trends and future prospects: A review. *Trends in Food Science & Technology, 105*, 416–432.

Insausti, K., Beldarrain, L. R., Lavín, P., Aldai, N., Mantecón, A., Sáez, J. L., & Canals, R. M. (2021). Horse meat production in northern Spain: Ecosystem services and sustainability in high nature value farmland. *Animal Frontiers, 11*, 48–54.

Kim, Y. H., Keeton, J. T., Smith, S. B., Berghman, L. R., & Savell, J. W. (2009). Role of lactate dehydrogenase in metmyoglobin reduction and color stability of different bovine muscles. *Meat Science, 83*, 376–382.

Kim, Y. H. B., Ma, D., Setyabrata, D., Farouk, M. M., Lonergan, S. M., Hyff-Lonergan, E., & Hunt, M. C. (2018). Understanding post-mortem biochemical processes and post-harvest ageing factors to develop novel smart-aging strategies. *Meat Science, 144*, 74–90.

Koohmaraie, M., & Geesink, G. H. (2006). Contribution of postmortem muscle biochemistry to the delivery of consistent meat quality with particular focus on the calpain system. *Meat Science, 74*, 34–43.

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.

Laville, E., Sayd, T., Morzel, M., Blinet, S., Chambon, C., Lepetit, J., Renand, G., & Hocquette, J. F. (2009). Proteome changes during meat aging in tough and tender beef suggest the importance of apoptosis and protein solubility for beef aging and tenderization. *Journal of Agricultural and Food Chemistry, 57*, 10755–10764.

Lepetit, J. A. (2007). A theoretical approach of the relationships between collagen content, collagen cross-links and meat tenderness. *Meat Science, 76*, 147–159.

Lonergan, E. H., Zhang, W., & Lonergan, S. (2010). Biochemistry of post-mortem muscle. Lessons on mechanisms of meat tenderization. *Meat Science, 86*, 184–195.

Lorenzo, J. M., & Gomez, M. (2012). Shelf life of fresh foal meat under MAP, overwrap and vacuum packaging conditions. *Meat Science, 92*, 610–618.

Ma, D., & Kim, D. M. Y. (2020). Proteolytic changes of myofibrillar and small heat shock proteins in different bovine muscles during aging: Their relevance to tenderness and waterholding capacity. *Meat Science, 163*. Article 109090.

Malheiros, J. M., Braga, C. P., Grove, R. A., Ribeiro, F. A., Calkins, C. R., Adamec, J., & Chardulo, L. A. L. (2019). Influence of oxidative damage to proteins on meat tenderness using a proteomic approach. *Meat Science, 148*, 64–71.

Marino, R., Albenzio, M., Della Malva, A., Caroprese, M., Santillo, A., & Sevi.. (2014). Changes in meat quality traits and sarcoplasmic proteins during aging in three different cattle breeds. *Meat Science, 98*, 178–186.

McNamara, J. W., & Sadayappan, S. (2018). Skeletal myosin binding protein-C: An increasingly important regulator of striated muscle physiology. *Archives of Biochemistry and Biophysics, 660*, 121–128.

Merkulova, T., Dehaupas, M., Nevers, M. C., Créminon, C., Alameddine, H., & Keller, A. (2000). Differential modulation of alpha, beta and gamma enolase isoforms in regenerating mouse skeletal muscle. *European Journal of Biochemistry, 267*, 3735–3743.

Monson, F., Sañudo, C., & Sierra, I. (2005). Influence of breed and ageing time on the sensory meat quality traits and consumer acceptability in intensively reared beef. *Meat Science, 71*, 471–479.

Montowska, M., & Pospiech, E. (2013). Species-specific expression of various proteins in meat tissue: Proteomic analysis of raw and cooked meat and meat products made from beef, pork and selected poultry species. *Food Chemistry, 136*, 1461–1469.

Moran, L., Barron, L. J. R., Wilson, S. S., O'Sullivan, M. G., Kerry, J. P., Prendiville, R., & Moloney, A. P. (2020). Effect of pelvic suspension and post-mortem ageing on the quality of three muscles from Holstein Friesian bulls and steers. *Journal of the Science of Food and Agriculture, 101*, 1892–1900.

Morzel, M., Chambon, C., Hamelin, M., Santé-Lhoutellier, V., Sayd, T., & Monin, G. (2004). Proteome changes during pork meat ageing following use of two different pre-slaughter handling procedures. *Meat Science, 67*, 689–696.

Ouali, A., Gagaoua, M., Boudida, Y., Becila, S., Boudjellal, A., Herrea-Mendez, C. H., & Sentandreu, M. A. (2013). Biomarkers of meat tenderness: Present knowledge and perspectives in regards to our current understanding of the mechanisms involved. *Meat Science, 95*, 854–870.

Picard, B., Gagaoua, M., Al-Jammas, M., Koning, L. D., Valais, A., & Bonnet, M. (2018). Beef tenderness and intramuscular fat proteomic biomarkers: Muscle type effect. *PeerJ, 6*, Article e4891.

Purintrapiban, J., Wang, M. C., & Forsberg, E. (2001). Identification of glycogen phosphorylase and creatine kinase as calpain substrates in skeletal muscle. *International Journal of Biochemistry & Cell Biology, 33*, 531–540.

Rabilloud, T., Vaezzadeh, A. R., Potier, N., Lelong, C., Leize-Wagner, E., & Chevallet, M. (2009). Power and limitations of electrophoretic separations in proteomics strategies. *Mass Spectrometry Reviews, 28*, 816–843.

Ramanathan, R., Mafi, G., Yoder, L., Perry, M., Pfeiffer, M., VanOverbeke, D. L., & Maheswarappa, N. B. (2020). Biochemical changes of postmortem meat during the aging process and strategies to improve the meat quality. In K. B. Ashim, & K. M. Prabhat (Eds.), *Meat quality analysis* (pp. 67–80). Cambridge, UK: Academic Press.

Ren, C., Hou, C., Li, Z., Li, X., Bai, Y., & Zhang, D. (2019). Effects of temperature on protein phosphorylation in postmortem muscle. *Journal of the Science of Food and Agriculture, 100*, 551–559.

Ruiz, M. (2018). *Effect of Production Factors on Foal Carcass and Meat Quality. Consumers Preferences. Colour and Texture Evolution of Foal Meat during the Storage Time as Quality Attributes, from Animals Reared under Sustainable Conditions*. Navarre, Spain: Public University of Navarre. Ph.D. Thesis.

Ruiz, M., Insausti, K., Beriain, M. J., Crecente, S., Lorenzo, J. M., & Sarriés, M. V. (2019). Effect of slaughter age and finishing diet on sensory evaluation and consumers' preference of foal meat. *Spanish Journal of Agricultural Research, 17*. Article e0609.

Sentandreu, M. A., Coulis, G., & Ouali, A. (2002). Role of muscle endopeptidases and their inhibitors in meat tenderness. *Trends in Food Science & Technology, 13*, 400–421.

Seong, P., Park, K. M., Cho, S., Kang, G. H., Chae, H. S., Park, B. Y., & Ba, H. V. (2016). Effect of cut type and post mortem ageing on technological quality,textural profile and sensory characteristics of foal meat. *Animal Production Science, 56*, 1551–1559.

Sierra, V., Fernández-Suarez, V., Castro, P., Osoro, K., Vega-Naredo, I., García-Macía, M., Rodríguez-Colunga, P., Coto-Montes, A., & Oliván, M. (2012). Identification of biomarkers of meat tenderisation and its use for early classification of Asturian beef into fast and late tenderising meat. *Journal of the Science of Food and Agriculture, 92*, 2727–2740.

Warren, K. E., & Kastner, C. L. (1992). A comparison of dry-aged and vacuum-aged beef strip loins. *Journal of Muscle Foods, 3*, 151–157.

Watanabe, A., Kamada, G., Imanari, M., Shiba, M., Yonai, M., & Muramoto, T. (2015). Effect of ageing on volatile compounds in cooked beef. *Meat Science, 107*, 12–19.