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Application of 2-D DIGE to study the effect of ageing on horse meat myofibrillar sub-proteome

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ABSTRACT

Considering the high relevance of meat tenderness for consumer acceptability, the aim of this study was to investigate *post-mortem* changes in myofibrillar sub-proteome in steaks from *longissimus thoracis et lumborum* muscle of six Hispano-Bretón horses. Indeed, the ageing process that leads to meat tenderization has been scarcely studied in this species. Steaks ($n = 24$) were aged (4 °C) in the dark under vacuum for 0, 7, 14 and 21 days and the myofibrillar sub-proteome was extracted. Using 2-D DIGE minimal labelling, 35 spots that were differentially abundant between 0 and 21 days aged meat were detected. Of them, 24 were analysed by LC-MS/MS, identifying a total of 29 equine proteins. These were structural and metabolic proteins, and among them, four (Actin, Troponin T and Myosin binding proteins 1 and 2) were selected for Western blot analysis, reporting changes in their abundance after 0, 7, 14 and 21 days of ageing. Results revealed that they should be further studied as potential protein biomarkers of horse meat tenderization. Additionally, several protein fragments increased after ageing, as was the case of glyceraldehyde-3-phosphate dehydrogenase. Fragments of this protein were present in four protein spots, and their study could be useful for monitoring horse meat tenderization.

Significance: Tenderization during ageing has been widely studied in meat from several farm animal species; however, both research and standardized ageing practices are lacking for the particular case of horse meat. In this regard, this study presents novel proteomic findings related to *post-mortem* evolution of horse muscle proteins. Acquired knowledge would support the development and optimization of efficient ageing practices by horse meat industry.

1. Introduction

Ageing refers to the *post-mortem* intervention in which meat is stored under refrigeration (typically 0–4 °C) for a certain period in order to develop desired quality attributes (e.g., tenderness and flavour) while preventing spoilage. Wet ageing is the most commonly employed ageing method, in which meat primals or sub-primals are stored under refrigeration and vacuum, especially in order to improve tenderness [1]. The conversion of muscle to meat takes place during the ageing process, where the changes in the myofibrillar sub-proteome (primarily composed by structural proteins) play an essential role in the development of tenderness. Considering the high relevance of this attribute on consumer acceptability of meat [2], this biochemical process has traditionally attracted the attention of meat scientists during the last decades.

Muscle to meat conversion starts after animal slaughter with the *pre-rigor mortis* phase, in which programmed cell death or apoptosis is initiated by caspases due to absence of oxygen and nutrients in muscle [3]. The second step, the *rigor mortis* phase, is completed when the tissue reaches its maximum toughness. Finally, structural proteins from muscle fibers undergo degradation by the action of different endogenous proteases (calpains, cathepsins and proteasome), resulting in meat tenderization due to the decrease of the mechanical resistance of muscle [4,5]. Although tenderization during ageing has been widely studied in meat from most farm animal species and recommended ageing times have been established for different types of meat [1], standardized ageing practices for the particular case of horse meat are still lacking [6]. Considering that the contractile and metabolic properties of skeletal

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muscle are species- and breed-specific, further studies aiming at investigating muscle to meat conversion using adequate tools and analysis methods are needed to increase our understanding of *post-mortem* horse meat tenderization processes. Further knowledge about this process may contribute to the establishment of an optimized duration for the ageing process by the horse meat industry.

Proteomic methods have proven to be a reliable and powerful technology to study protein changes during ageing [7,8]. Particularly, separation of proteins by 1- or 2-DE and their subsequent identification by LC-MS/MS has been traditionally a key proteomic strategy, as evidenced in studies performed in beef [9,10], chicken [11], donkey [12] horse [13], lamb [14] and pork [15,16]. However, alternative strategies may be considered for this purpose, such as the use of 2-D DIGE. The latter reduces gel-to-gel variation by performing pre-electrophoretic minimal labelling and comparing two samples on the same gel based on an internal standard [17].

2-D DIGE analysis of the myofibrillar sub-proteome of meat has been performed only a few times [18–21] and has never been applied to horse meat. Overall, horse meat proteomic studies are scarce. Only two previous works have addressed the study of horse meat myofibrillar subproteome during ageing, using 2-DE [13] and liquid isoelectric focusing (OFFGEL) in combination with SDS-PAGE [22]. These studies highlighted that ageing affected horse meat proteolysis and tenderness. To this extent, our objective in the present study was to apply an integrative approach using 2-D DIGE in combination with LC-MS/MS for the first time in horse meat, for the elucidation of differences in the myofibrillar sub-proteome of Hispano-Bretón (HB) horse meat after ageing. The strategy was applied to meat from female and male horses, in order to elucidate protein changes common to both and then find general markers for the ageing process. Our previous studies pointed out that sex had not a significant effect on the evolution of HB muscle proteome *post-mortem* [21,22]. Acquired knowledge helps to gain new insights into the biochemistry of *post-mortem* horse muscle and further demonstrates the benefit of applying 2-D DIGE in meat science.

2. Materials and methods

2.1. Solvents and chemicals

Tris, urea, thiourea, DTT, EDTA and LC-MS grade solvents ACN and formic acid (FA) were from Scharlab (Scharlab S.L., Barcelona, Spain). Water was of ultrapure grade from Millipore (EMD Millipore Co., Billerica, MA, USA). Brij-35, sucrose, CHAPS hydrate, protease inhibitor cocktail (P8340), magnesium acetate, ammonium bicarbonate and RuBPS (ruthenium(*II*)tris bathophenanthroline disulfonate) were from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Bradford Protein Assay Kit was from Bio-Rad (Bio- Rad, Hercules, CA, USA) and modified trypsin from Promega (Promega, Madison, WI, USA). CyDyes, ampholytes and LMW molecular weight marker were from GE Healthcare (GE Healthcare, Munich, Germany). Serva Triple Colour Protein Standard III and IPG strips (pH 3–10) were from Serva (Serva, Heidelberg, Germany).

2.2. Animal handling and sampling

Six HB horses (three males and three females), a well-stablished breed in northern Spain, were reared as previously described [22]. Horses were slaughtered at 15–17 months of age in a commercial abattoir (average carcass weight of 251 ± 12.7 kg) following European Union regulations [23]. Two horses (female and male) were slaughtered per week during three consecutive weeks. After 48 h *post-mortem* at 4 °C (day 0), the whole right rib joint was removed from the carcass ($n = 6$) and transported to the laboratory under refrigerated conditions. *Longissimus thoracis et lumborum* (LTL) muscle was excised. The detailed lipid composition and characterization of several quality parameters from these samples has been previously reported [24]. After trimming adipose and connective tissues of muscle, four 1.5 cm thick steaks ($n = 24$) were cut, starting from the 5th rib. Samples were vacuum packed (99%) using an EVTGI-450 vacuum packaging machine (Irimar, Navarre, Spain) in polyethylene bags (120 μm 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 or 21 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 analysed. For instrumental texture analyses, maximum Warner-Bratzler shear force (WBSF) measurements were carried out as described in our previous work [24].

2.3. Extraction of myofibrillar proteins

Protein extraction was carried out as previously described [25] with minor modifications. Briefly, a representative sample of 10 g of meat was retrieved from the center of the steak and homogenized with a meat grinder. From there, half a gram was weighted and solubilized in 5 mL extraction buffer (10 mM Tris buffer pH 7.5, 5 mM EDTA, 0.25 M sucrose, 0.01% Brij-35) containing 25 μL of protease inhibitors cocktail using an Ultra-Turrax Yellow Line Di 25 (IKA, Staufen, Germany). The homogenate was centrifuged at 2000*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 8.5, containing 7 M urea, 2 M thiourea, 5 mM magnesium acetate and 2% CHAPS, and centrifuged again. The supernatant (myofibrillar extract) was filtered through glass wool and pH was checked to be in the range 8.0–9.0 using pH strips. Total protein content was determined in triplicate using the Bradford Protein Assay Kit and samples were frozen at −80 °C until further use.

2.4. 2-D DIGE

Minimal labelling with CyDyes was performed to compare non-aged horse meat and horse meat aged for 21 d. Labelling was carried out according to published protocols [17] based on manufacturer recommendation (8 nmol dye/mg protein). Cy2 was used for the internal standard (a pool of all samples in the set), Cy3 and Cy5 for the single samples, including reverse labelling for all samples to avoid preferential labelling (for details about labelling scheme, see Supplemental Table 1). Molecular weight markers LMW and Serva Triple Colour Protein Standard III were additionally loaded on the gel.

2-D DIGE was performed as previously described [17]. In brief, 25 μg of two labelled samples (Cy3, Cy5) and 25 μg of an internal standard (Cy2) were diluted with rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 13 mM DTT, 1% ampholytes) and passively rehydrated into 11 cm long IPG strips with a linear gradient pH 3–10. First dimensional separation was performed in a Multiphor II electrophoresis system (GE Healthcare). After equilibration, the strip was transferred to the second dimensional SDS-PAGE gel (140 × 140 × 1.5 mm home-made gradient gel; T = 10–15%, C = 2.7%) and protein separation performed in a Hoefer SE600 system (Hoefer Scientific Instruments, San Francisco, CA, USA). Images were captured on a Typhoon RGB and evaluated with the DeCyder V6.0 software (both GE Healthcare). Selection criteria for spots defined as differentially regulated between non-aged and aged horse meat groups were: fold change ±1.5; $p \leq 0.05$.

2.5. In-gel trypsin digestion of protein spots

After image capturing, 2-D DIGE gels selected for spot picking were stained with an MS-compatible silver stain in order to obtain spot patterns visible to the eye [17]. Differentially abundant spots, determined as aforementioned, having enough intensity were excised manually. Then, spots were washed, destained [26] and digested with trypsin. For this, destained spots 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, liquid was transferred into a clean Eppendorf tube and the remaining peptides were recovered by adding ACN/0.1% FA (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% FA and transferred into glass vials.

2.6. Peptide sequence identification by LC-MS/MS

Tryptic digests were analysed by liquid chromatography performed on a Thermo Vanquish Horizon UHPLC system (Thermo Scientific, San Jose, CA, USA) with a quaternary pump, vacuum degasser and refrigerated autosampler hyphenated to a Thermo Orbitrap ID-X Tribrid mass spectrometer (ThermoFisher Scientific) loading a heated ESI probe operating in positive mode. Peptide separation was achieved on a 150 mm × 2.1 mm, 3 μm particle-size Luna Omega PS C18 column (Phenomenex Inc., Torrance, CA, USA) with the following experimental conditions: solvent A, water/FA (99.9:0.1); solvent B, ACN/FA (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, 10 μL. Autosampler and column temperatures were set at 10 and 23 °C, respectively.

General MS experimental conditions were the following: ion transfer temperature, 300 °C; higher-energy collisional dissociation energy for MS/MS analysis, 30%; spray voltage (static), 4.0 kV; heated ESI source, OFF; sheath gas, 50 (arbitrary units); auxiliary gas, 20 (arbitrary units); internal mass calibration source, ON; radiofrequency lens, 45%; orbitrap mass resolution for MS^1 and MS/MS analyses, 60,000 (FWHM); MS data acquisition, centroid. Analysis of samples multiplexed two scan events: full-MS analysis (MS^1, scan event 1) of intact peptide masses in the 400–2000 m/z range followed by a data-dependent MS/MS (scan event 2) experiment of most intense ions from event 1 considering +1 to +4 charges. The following dynamic parameters ruled scan events: intensity threshold for triggering MS/MS analysis, 2×10^4; exclusion duration, 15 s; exclusion mass tolerance (low and high), 10 ppm; repeat count for MS/MS of most intense ion, 2; repeat count duration, 15 s; quadrupole isolation window (m/z), 1.5; acquisition Gain Control for MS^1 and MS/MS analyses, standard and auto, respectively; number of micro scans for MS^1 and MS/MS analyses, 1; maximum injection time for MS^1 and MS/MS analyses, 120 ms. Control of the LC-MS system was featured by a PC loading Thermo Xcalibur v4.5 software (Thermo Scientific, San Jose, CA, USA).

Protein assignments were achieved through interrogation of MS/MS data against NCBIprot protein database (NCBIprot_20201130) using Mascot v2.8 search engine considering the following constraints: enzyme, trypsin; no fixed or variable modifications but "Error tolerant" option enabled; mass accuracy for MS^1 and MS/MS data, 5 ppm and 0.02 Da, respectively; taxonomy restriction parameter set to "Mammalia" option. 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 ($p < 0.05$).

2.7. Immunoblotting

Immunoblots were performed on myofibrillar extracts from all six animals of the experiment: non-aged and aged for 7, 14 and 21 d ($n = 24$). A total amount of 10 or 20 μg of extracts were loaded per lane, depending of the targeted protein, for optimal visualization of main protein and breakdown products, respectively (for details see the respective figure legends). The procedure was similar to previously published protocols [27]. Samples were separated in home-made 10–15% SDS-PAGE gradient gels under reducing and denaturing conditions and proteins then were semi-dry blotted onto NC (GE Healthcare). Protein patterns were stained with RuBPS for overall protein, scanned on the Typhoon RGB. Subsequent immunoprobing was performed with specific antibodies against ACTA1 (anti-Actin, polyclonal, produced in rabbit, num. A2066, Sigma-Aldrich), MYBPC1 (anti-MYBPC1, monoclonal, produced in rabbit, num. CSB-PA010297, Cusabio Technology, Houston, TX, USA), MYBPC2 (anti-MYBPC2, polyclonal, produced in rabbit, num. ARP42187_P050, Aviva Systems Biology, San Diego, CA, USA) and TNNT3 (anti-Troponin T, monoclonal, produced in mouse, num. T6277, Sigma-Aldrich), followed by detection with cross-absorbed anti-rabbit IgG-HRP (Novex, Life Technologies Corporation, Grand Island, NY, USA) or anti-mouse IgG-HRP (GE Healthcare, NA931), respectively. All antibodies were used in 1:5000 dilutions in the TBS/TTBS buffer system, with BSA as a blocking agent in all steps. The bands were detected by ECL (Clarity Western ECL, Bio-Rad) on a Vilber Lourmat FX system (Vilber-Lourmat, Eberhardzell, Germany) and band volume quantified using ImageJ software. Staining of overall protein with RuBPS was used as a loading control and for normalization. Molecular weight markers LMW and Serva Triple Colour Protein Standard III were additionally loaded on the gel.

2.8. Statistical analysis

IBM-SPSS Statistics Software (v. 26.0, IBM, Armonk, NY, USA) was applied for statistical analyses. The General Linear Model (GLM) of ANOVA was used on data from immunoblots for specific proteins (ACTA1, MYBPC1, MYBPC2, TNNT3) among ageing periods assayed (0, 7, 14 and 21 d). The model considered ageing time and the controlled distorting variation factor animal sex as fixed effects. 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. Significance was declared at $p \leq 0.05$.

2.9. Interaction analysis of differentially abundant proteins between non-aged and 21 d aged meats

Network analysis was performed with STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) v.11.5 freeware software (ELIXIR, Cambridgeshire, UK, https://string-db.org/) [28]. *Equus caballus* was selected as target organism for interrogations, and the network was constructed with a minimum interaction score confidence of 0.4 and four criteria for possible linkage: co-occurrence, experimental evidences, existing databases and text mining.

3. Results

3.1. 2-D DIGE of non-aged horse meat compared to horse meat aged for 21 days

2-D DIGE analysis of the myofibrillar sub-proteome of non-aged horse meat and horse meat aged for 21 d gave spot patterns of 619 matched spots. Of them, 35 spots were differentially abundant when applying the cut-off values established for the present experiment as described in section 2.4. A total of 29 proteins belonging to *Equus caballus* were successfully identified by LC-MS/MS in the 24 spots selected for identification (Fig. 1; Table 1). In some of the spots, more than one protein co-occurred. For that reason, those spots were excluded from discussion as the trends of the individual proteins cannot be elucidated (unless individual proteins were confirmed by immunoblotting). The selection criteria of spots for MS analysis were based on intensity, size, position, traceability in silver stained patterns and vicinity to others. Further details regarding peptide identification for each individual protein can be found in Supplemental Table 2.

Among changes observed in protein abundance (most of them in the alkali part of the gel, represented as green or red spots in Fig. 1A), nine spots were found to decrease after 21 d of ageing. These spots were identified as proteins MYBPC2, HSPBA1A, DLAT, ALB, DES, TNNT3, ALDOA, CKM, LDHA, MYOZ1 and HSPB1 (Green colour numbers in Table 1). In contrast, 15 spots increased with ageing. These were identified as an aggregate of ACTA1, fragments of MYBPC1, PYGM and MYBPC2, SDHA, ACTA1, a fragment of ENO3, PHB, NDUFS3 and fragments of ALDOA, ATP5PD and GAPDH (Red colour numbers in Table 1). From these latter, several were assumed to be protein fragments since they displayed a much lower apparent molecular weight than the theoretical one. Changes in abundance showed variable ratios between aged and non-aged samples (in both directions), reaching the highest difference in spot 22 (a fragment of glyceraldehyde-3-phosphate dehydrogenase), namely a fold change of 31 (Table 1). Most of the spots significantly more abundant in horse meat samples aged for 21 d showed molecular weights below 40 kDa.

Protein interaction analyses performed by STRING software were undertaken using the equivalent gene names gathered in Table 1, selecting "*Equus caballus*" as target species and resulting in the protein interaction network depicted in Fig. 2. It should be noted that this type of clustering included all differentially abundant proteins, not taking into account the direction or degree of fold change of each one. The obtained network was mainly divided in three smaller groups of proteins showing connections among each other. Overall, they consisted on proteins related to cell structure (ACTA1, DES, MYBPC1, MYBPC2, MYOZ, TNNT3), proteins involved in metabolic pathways (ALDOA, ATP5PD, CKM, DLAT, ENO3, GAPDH, LDHA, NDUFS2, PYGM, SDHA) and chaperones (HSPA1A, HSPB1). Two proteins, ALB and PHB, were also part of the network but did not belong to any of the groups.

Enrichment analysis of the network revealed that the enriched GO term for cellular component was "Supramolecular fiber", while the molecular process was "generation of precursor metabolites and energy", evidencing the relevance of proteins belonging to glycolysis or gluconeogenesis in the network. Detailed enrichment analysis can be found in Supplemental Table 3.

3.2. Immunoblotting

While 2-DE was performed only with samples from the starting (0 d) and end point (21 d) of the ageing period, for our immunoblotting experiments we included also samples of the time points 7 and 14 d. Selected proteins were further investigated with the help of specific antibodies and Western blot in both non-aged horse meat samples and samples aged for 7 to 21 d. This analysis was carried out for two main purposes: on the one hand, monitoring changes of proteins of interest in all the time course of the experiment (0, 7, 14 and 21 d) and, on the other, the confirmation of 2-D DIGE results by an alternative methodology.

3.2.1. Actin

A faint band of 137 kDa was observed when samples were tested against anti-Actin antibody, together with a more intense one at 38 kDa (Fig. 3). The former matched the molecular weight of spot 1 detected in 2-D DIGE (Fig. 1), and could presumably correspond to an Actin aggregate. This aggregate was faint in the blots under SDS-PAGE conditions (samples were boiled in the presence of DTT), but its abundance slightly increased when the heat treatment was omitted during the reduction step (data not shown). Under the Western blot conditions, no significant differences in abundance were detected for these two bands during the ageing process. However, both bands showed a trend to decrease as ageing time progressed. Further details and full Western blot images have been included in Supplemental Fig. 1.

3.2.2. Troponin T

Western blots from Troponin T (TNNT3) revealed six forms of intact

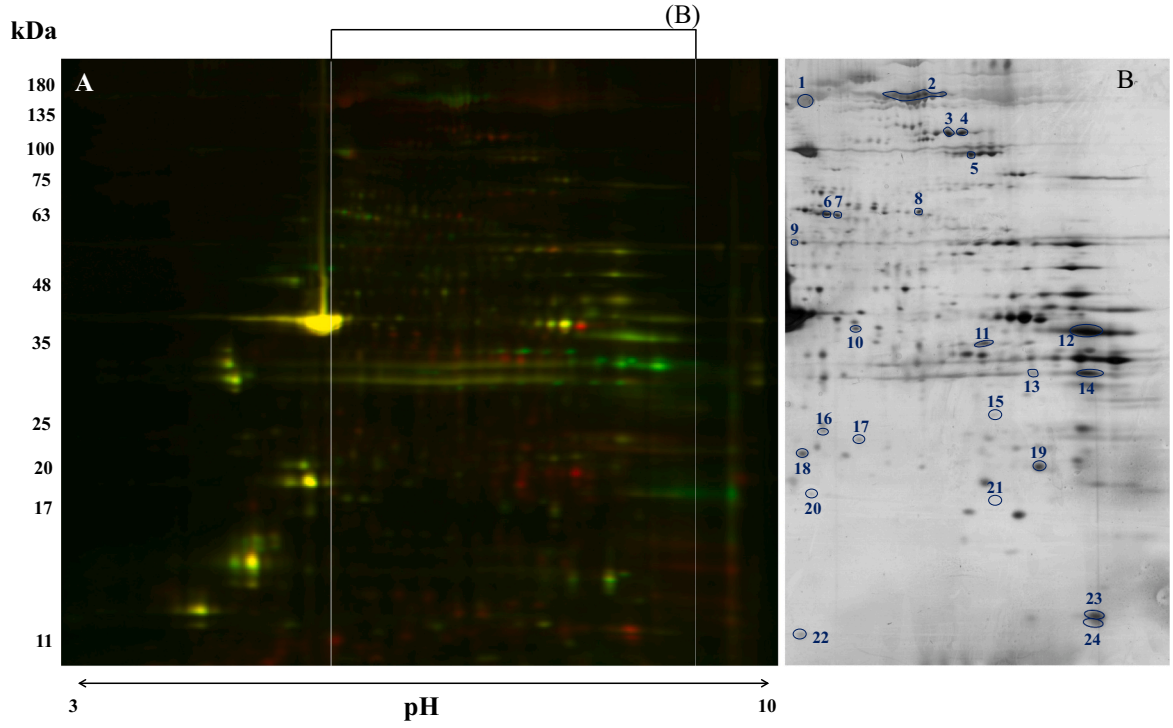

Fig. 1. Comparison of myofibrillar sub-proteomes from non-aged horse meat and horse meat aged for 21 days. **A**) 2-D DIGE of a representative non-aged sample (represented in green, Cy5) vs. sample aged for 21 days (represented in red, Cy3). Internal standard (pool Cy2) not shown. Overlapping spots in yellow. **B**) Image of the same gel as in A (box on top), post-stained with silver. Spots yielding a successful protein identification are encircled, numbers correspond to those of Table 1. 2-DE was made with IPG 3–10 and 10–15% SDS-PAGE gradient gels. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Table 1: LC-MS/MS protein identification and average ratio of spots 1–24 (depicted in Fig. 1) between non-aged and 21 days aged horse meat.

Spot[1] (in Fig. 1)	Accession number[2]	Protein(s) identified[2]	GENE	Theoretical[2]/ Apparent[3] pI	Theoretical[2]/ Apparent[3] Mr (Da)	Mascot score	Sequence coverage	Average ratio 21 d/0 d
1	NP_001304187.1	Actin, alpha 1, skeletal muscle, **aggregate**	ACTA1	5.23/5.7	42,000/140,000	138	10 %	3.46
2	XP_023505989.1	Myosin-binding protein C, fast-type	MYBPC2	6.37/6.3-7.2	132,620/133,000	283	7 %	0.45
3	XP_005606603.1	Myosin-binding protein C, slow-type isoform X2, **fragment**	MYBPC1	5.99/7.2	131,580/110,00	294	6 %	22.7
4	XP_005606603.1	Myosin-binding protein C, slow-type isoform X2, **fragment**	MYBPC1	5.99/7.3	131,580/110,00	412	8 %	7.37
5	NP_001138725.1	Glycogen phosphorylase, muscle form, **fragment**	PYGM	6.59/7.4	97,306/90,000	191	11 %	1.71
6	NP_001243852.1	Heat shock 70kDa protein	HSPA1A	5.55/5.8	70,113/65,000	646	27 %	0.51
	XP_001501871.2	Dihydrolipoyllysine-residue acetyltransferase component of	DLAT	7.89/5.8	68,829/65,000	369	14 %	
	4F5U_A	Chain A, Serum Albumin	ALB	5.60/5.8	66,420/65,000	136	6 %	
7	4F5U_A	Chain A, Serum Albumin	ALB	5.60/6.0	66,420/65,000	201	4 %	0.53
8	XP_023505989.1	Myosin-binding protein C, fast-type, **fragment**	MYBPC2	6.37/6.9	132,615/66,000	439	6 %	4.09
	ABD77320.1	Succinate dehydrogenase complex subunit A, partial	SDHA	6.08/6.9	60,051/66,000	155	2 %	
9	XP_001492052.2	Desmin	DES	5.22/5.5	53,432/56,000	522	49 %	0.27
10	NP_001304187.1	Actin, alpha 1, skeletal muscle	ACTA1	5.23/6.1	41,996/32,000	307	17 %	2.90
11	XP_023510446.1	Troponin T, fast skeletal muscle isoform X2	TNNT3	5.64/7.5	33,120/31,000	548	20 %	0.43
12	XP_003362760.1	Fructose-bisphosphate aldolase A isoform X2	ALDOA	8.30/8.2-9.1	39.433/32.000	1578	57 %	0.48
13	XP_001502572.1	Creatine kinase M-type	CKM	6.79/8.0	43,142/30,000	458	25 %	0.50
14	NP_001138352.1	L-lactate dehydrogenase A chain	LDHA	8.17/8.5	36,576/30,000	924	30%	0.55
	XP_001503948.1	Myozenin-1	MYOZ1	7.85/8.5	31,734/30,000	218	18 %	
	XP_023510446.1	Troponin T, fast skeletal muscle isoform X2	TNNT3	5.64/8.5	33,120/30,000	153	20 %	
15	NP_001254531.1	Beta-enolase, **fragment**	ENO3	8.05/7.6	47,023/28,000	52	2 %	23.4
16	XP_001502441.1	Prohibitin	PHB	5.57/5.8	29,816/27,000	301	34 %	3.03
17	XP_001491322.2	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3,	NDUFS3	7.03/6.2	30,116/27,000	302	31 %	2.42
18	XP_001504528.1	Heat shock protein beta-1	HSPB1	6.13/5.8	23,104/24,000	500	65 %	0.42
19	XP_003362760.1	Fructose-bisphosphate aldolase A isoform X2, **fragment**	ALDOA	8.30/8.0	39,433/24,000	290	17 %	14.9
20	XP_001496591.1	ATP synthase subunit mitochondrial isoform X2, **fragment**	ATP5PD	5.40/5.7	18.521/19.000	145	24 %	2.54
21	NP_001157328.1	Glyceraldehyde-3-phosphate dehydrogenase, **fragment**	GAPDH	8.22/7.6	35,801/20,000	127	10 %	18.0
22	NP_001157328.1	Glyceraldehyde-3-phosphate dehydrogenase, **fragment**	GAPDH	8.22/5.7	35,801/12,000	11	12 %	31.0
23	NP_001157328.1	Glyceraldehyde-3-phosphate dehydrogenase, **fragment**	GAPDH	8.22/8.5	35,801/13,500	114	10 %	16.1
24	NP_001157328.1	Glyceraldehyde-3-phosphate dehydrogenase, **fragment**	GAPDH	8.22/8.5	35,801/12,000	2201	12 %	29.1

[1] Colour indicates changes in relative protein spot quantification by ageing ($p < 0.05$) (green, decrease; red, increase).
[2] Protein identification, theoretical Mr. and accession number were from NCBIprot database interrogation (detailed in section 2.6). All identifications correspond to *Equus caballus*.
[3] Apparent pI and Mr. were calculated through spot position in the gel.

Troponin T with molecular weights above 32 kDa (bands 1–6; Fig. 4) in non-aged horse meat samples. These forms decreased significantly in abundance until practically disappearance from 0 to 7 d of ageing, with no additional changes afterwards. Band 5 still maintained a higher protein abundance after 7 d compared to the rest. The only exception in this trend through the time course was band 6, which also decreased in abundance, but between days 7 and 14 of the ageing process. Additionally, two fragments of Troponin T with molecular weights in the range of 25–29 kDa increased in abundance between non-aged and 7 d aged meat (bands 8 and 9), and maintained stable afterwards. Band 7, of 30 kDa, increased in abundance from 0 to 7 d of ageing, and then had a decreasing trend, although not significant. This could presumably also be a fragment of Troponin T generated during the first week of ageing that would be subsequently degraded into smaller fragments. Further details and full Western blot images have been included in Supplemental Fig. 2.

3.2.3. Fast and slow isoforms of Myosin binding protein

Both MYBPC1 and 2 were investigated by Western blot, showing a main protein band at around 130 kDa, likely the intact protein form. In both cases, this band had a decreasing trend from non-aged to 21 d aged meat, although no statistically significant differences were found. The fragmentation pattern of both isoforms was different during the ageing process (Fig. 5A and C). In the case of MYBPC2, a fragment of the protein was present from day 0 at around 74 kDa, but only clearly visible with longer blot exposure and higher protein load. This band 2 was presumably further degraded, giving rise to an Ab-reactive band 4 of around 67 kDa that increased in abundance after 14 d of ageing (Fig. 5A). In MYBPC1, a simpler degradation pattern was observed: a significant increase in abundance of a fragment of around 110 kDa during the first week of ageing, which kept stable until day 21. Additionally, fainter bands of lower molecular weights (<75 kDa) were observed in Western blots, however, experimental conditions did not allow to confirm their specificity to the antibody (data not shown). Further details and full Western blot images have been included in Supplemental Figs. 3 and 4.

4. Discussion

The present study demonstrated the applicability of 2-D DIGE to delve into proteomic differences between non-aged and aged meat. Previously published extraction and labelling protocols were valid for 2-D DIGE of meat samples [12,25], with two minor modifications. In the present work, magnesium acetate was added to the myofibrillar protein extraction buffer and in addition, particular attention was paid for the protein extracts to be in the pH of range of 8–9, as recommended by the dye manufacturer.

The present study focused on horse meat myofibrillar sub-proteome considering the scarce literature available [13,22]. We presented a reproducible gel pattern obtained from comparison between non-aged horse meat samples and horse meat samples aged for 21 d, revealing 619 matched spots and 35 spots showing differently abundant proteins. At this point, the utility of 2-D DIGE for minimization of intra-gel variation should be stressed, together with the lower number of gels needed to draw robust conclusions. Compared to other methodologies such as OFFGEL fractionation followed by SDS-PAGE, 2-D DIGE revealed over twice as many differences considering the same type of samples [22].

Unlike other meat studies that used the pH range 4–7 for mapping myofibrillar meat proteins [13,29,30], pH interval 3–10 yielded the best

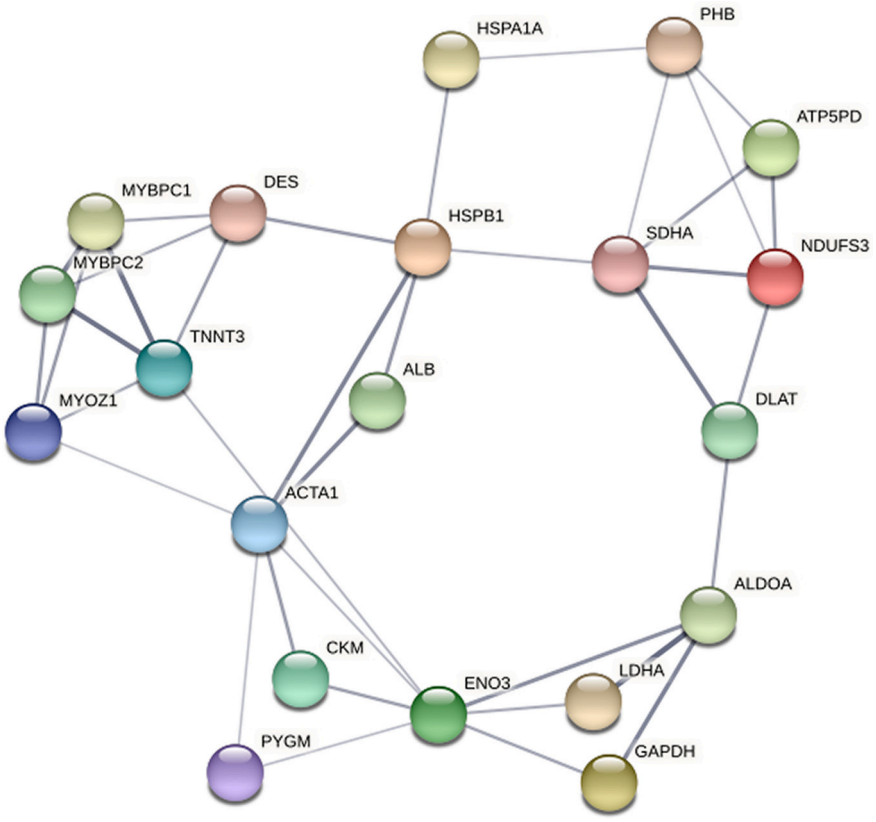

Fig. 2. Functional protein-protein association network of proteins listed in Table 1, created by STRING database search (medium confidence). Network nodes (circles) represent proteins, edges represent known or predicted functional associations and line thickness is an indicator for strength of the association. Full protein details are available in Table 1.

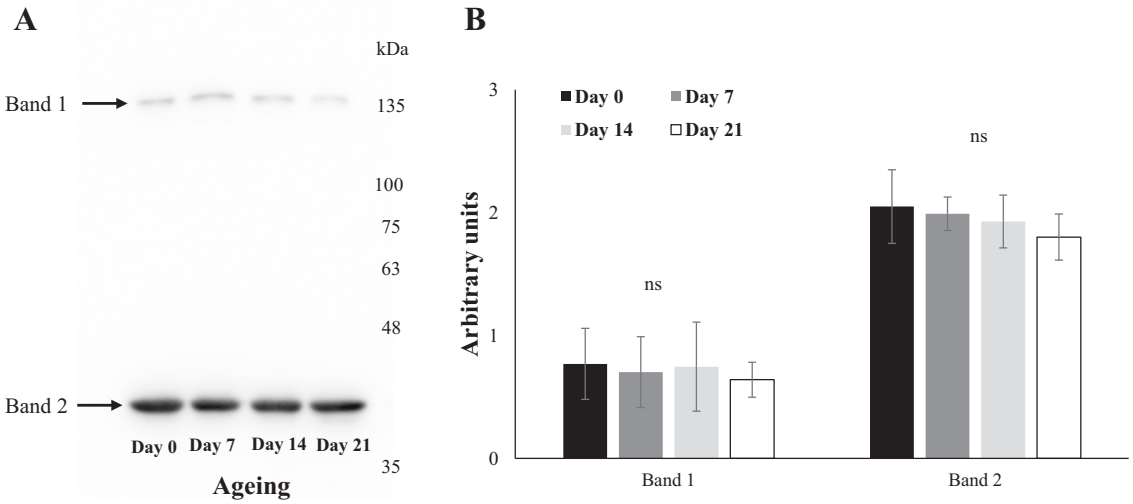

Fig. 3. Western blot of horse meat samples incubated with polyclonal anti-Actin antibody **A**) time course of ageing (0, 7, 14 and 21 days) using samples from one single animal. Per lane, 10 μg of protein were applied. **B**) Densitometry analysis of protein band intensities from Western blots for all 6 animals. Normalization of each band onto overall protein stain of the same lane was applied. Error bars indicate the standard error of the mean. Ns, non significant.

protein separation profile in the present study. This resulted in a higher number of changing protein spots in the alkaline region of the isoelectric focusing. Additionally, most of the protein spots detected by 2-D DIGE were visible after silver staining, except for some of the alkaline protein spots of low molecular weight that were lost, presumably during the staining procedure (Fig. 1A vs. 1B). Therefore, 2-D DIGE would be an advantageous alternative to study and compare the effect of ageing in meat from different breeds or muscles, where a higher inter-sample variability would be expected due to intrinsic proteome differences among breeds and muscles [31,32].

In order to better understand the impact of the differences observed in 2-D DIGE, the reader is referred to a previous study performed by our group [24], in which the degree of tenderness of meat from the LTL of 20 HB horses was characterized by WBSF measurements. Obtained results

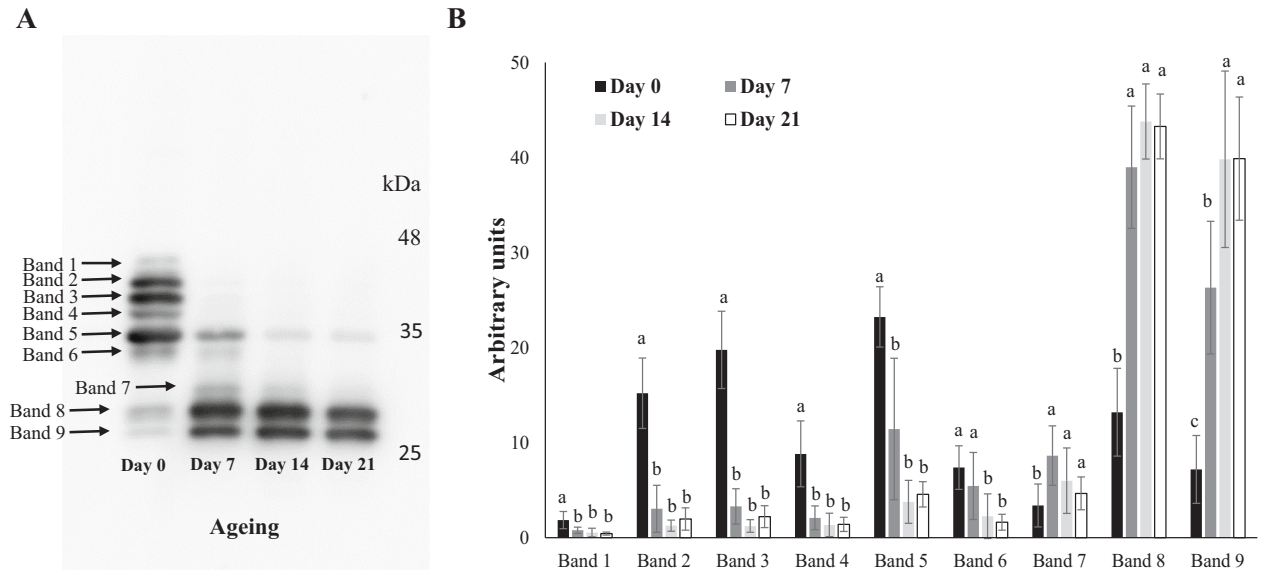

Fig. 4. **A**) Western blot of horse meat samples incubated with anti-Troponin T monoclonal antibody **A**) time course of ageing (0, 7, 14 and 21 days) taking samples from one single animal. Per lane, 10 μg of protein were applied. **B**) Densitometry analysis of protein bands from Western blot for all 6 animals. Normalization of each band onto overall protein stain of the same lane was applied. Error bars indicate the standard error of the mean and different superscripts indicate statistically significant differences among ageing days $p < 0.05$.

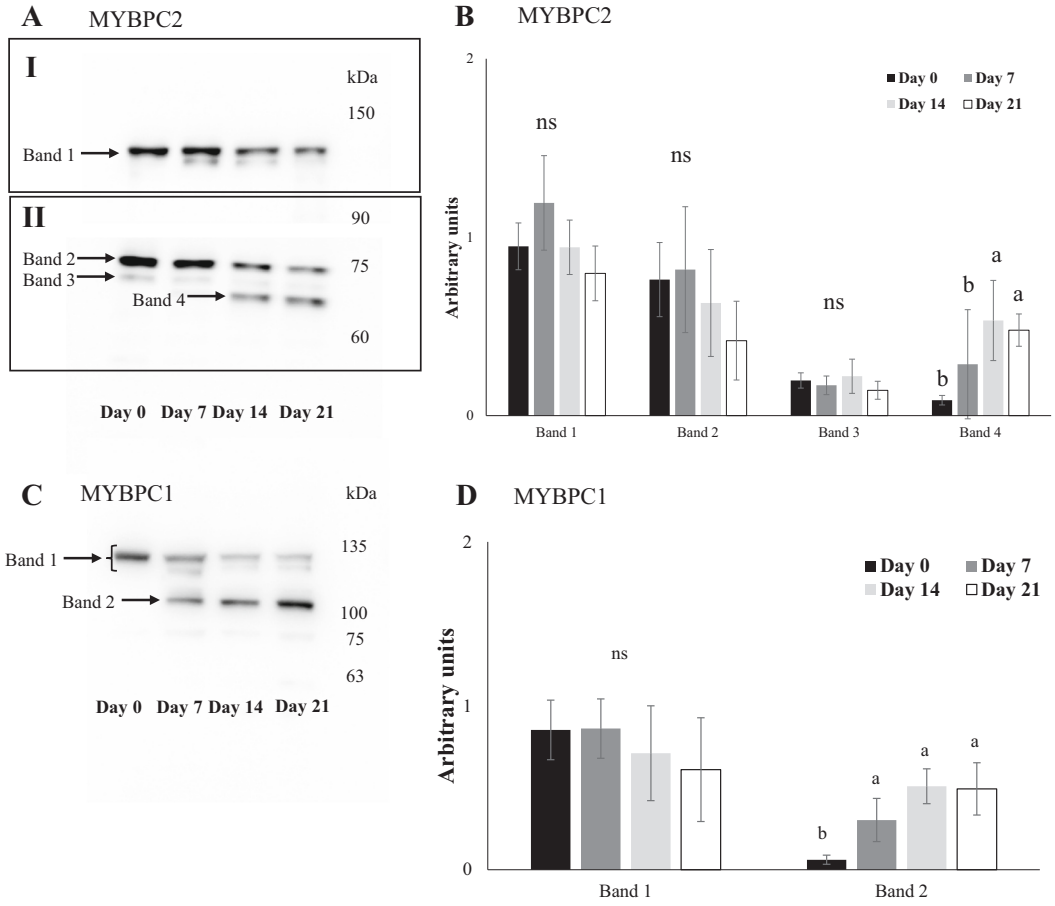

Fig. 5. Western blots of horse meat samples incubated with anti Myosin-binding protein 2 (**A**) and 1(**C**) antibodies, respectively. Time courses of ageing (0, 7, 14 and 21 days) taking samples taken from one single animal. In **A.I**, 10 micrograms of sample were loaded, while in **A.II** and **C**, 20 micrograms were loaded. Densitometry evaluation of protein bands from Myosin-binding proteins 1(**D**) and 2 (**B**) Western blot for all 6 animals. Normalization of each band onto overall protein stain of the same lane was applied. Error bars indicate the standard error of the mean and different superscripts indicate statistically significant differences among ageing days $p < 0.05$. Ns, non significant.

showed that toughness decreased significantly from non-aged meat to meat aged for 14 days, while from 14 to 21 days, a decreasing but not significant trend was observed [24]. In the particular case of the six animals employed in the present study, the same pattern was observed: values of 70.0 ± 3.08 N were obtained for non-aged meat, while meat aged during 7, 14 and 21 days obtained values of 42.5 ± 4.75, 35.3 ± 2.67 and 30.5 ± 2.56 N, respectively. Considering that tenderization takes place through hydrolysis of myofibrillar proteins from muscle fibers [4,5], the comparison of 0 and 21 d proteome was of greatest interest and therefore topic for 2-D DIGE investigation. In this setup, proteins identified to change in abundance between non-aged and 21 d aged horse meat (Table 1) were, in a significant proportion, proteins related to muscle structure. In this regard, four of these proteins were chosen to be further investigated by Western blot. Indeed, after evaluating the whole 2-D DIGE map, we made that selection considering those candidates that can become potential markers of the ageing process in horse meat. Considering the analytical features of Western blot, including also samples from the other two time points to investigate pattern changes of these four proteins over the whole ageing process (0, 7, 14 and 21 days) seemed of interest. No significant effect of animal sex or slaughter day was found for any of the proteins when statistically evaluated, confirming our previous observations [22]. Actin (ACTA1), the main constituent of thin filaments, was one of the selected proteins. This protein was identified in spot 1 as an aggregate (or oligomer) of an apparent molecular weight of 140 kDa, also being present in Western blot (Fig. 1 and Fig. 3**,** band 1). The formation of covalently linked Actin dimers and higher oligomers by the slow oxidation of sulfhydryls described previously [33] could explain the presence of this Actin aggregate with variable intensity depending on the reducing conditions (with/without heating treatment). In 2-D DIGE analysis, a higher abundance of the aggregate was observed on aged meat. Higher abundance of this aggregate could be related to the increased amount of oxidized proteins in aged meat, as a consequence of the presence of reactive oxygen species [34]. Indeed, oxidation of meat proteins during ageing has been reported [35]. Differences of the actin pattern between 2-DE (2-D DIGE) and SDS-PAGE (Western blot) are likely due to the different methodological conditions (extended presence of urea/thiourea and focusing to pI during IPG step; reductive treatment in SDS-PAGE).

The decreasing trend in abundance of monomeric ACTA1 (Fig. 3B; band 2) during ageing was in line with another study reporting that the degradation of ACTA1 is not significant in *longissimus lumborum* horse muscle during ageing [13]. However, it is reported that this degradation can give rise to fragments of 32 and 30 kDa after ageing of pork [36] and beef [9,37], respectively. Similarly, the generation of the 32 kDa Actin fragment in dromedary meat up to 24 h *post-mortem* was recently observed [38], suggesting that this Actin degradation pattern would occur for all animal species. The amount of this 32 kDa fragment still observed in horse meat at 21 d *post-mortem* would correspond to a minor amount that had not been further degraded to smaller fragments [39]. Previous experiments demonstrated that caspase 3 action would be the responsible of the generation of the 32 kDa Actin fragment soon after slaughter [40].

Troponin T (TNNT3), also in the thin filaments, is part of the protein complex that regulates muscle contraction. According to literature, different degradation degrees are observed for the different isoforms of this protein during ageing, indicating that those isoforms show different susceptibility to proteolysis [41]. In this line, further investigations of the two fragments identified here by 2-D DIGE (Fig. 1**,** spots 11 and 14) gave rise to a Western blot pattern with six reactive bands for TNNT3 in the molecular weight range 32–40 kDa (Fig. 4), showing different extents of degradation. The presence of six isoforms of TNNT3 in horse meat seemed logical, since up to eight isoforms of TNNT3 formed by alternative splicing were identified in beef within a molecular weight range of 29–33 kDa [42]. These authors reported that all of the isoforms of TNNT3 decreased during beef ageing. In line with this, degradation of TNNT3 has also been reported during horse meat ageing [13,22], showing that TNNT3 isoforms and degradation products could also be appropriate markers of the time course of ageing in horse meat. However, a deeper investigation of these protein species would be required, including MS characterization to elucidate their peptide composition.

Concerning thick filaments, two proteins having a relevant role in muscle functions are anchored to them. These proteins are MYBPC1 and 2, and were chosen for an in-depth monitoring by Western blot through observation of their degradation and concomitant increase of fragments in 2-D DIGE (Fig. 1, spots 2, 3 and 4). Additionally, MYBPC1 and 2 have been previously reported to be related to beef ageing as part of a 110 kDa proteolytic fragment which increased during the process [9]. The mentioned fragment was also detected in previous horse meat ageing studies [22], but no monitoring during the ageing process had been reported before. In this regard, the present study revealed that while MYBPC1 degradation primarily gave rise to a protein fragment of 110 kDa after 7 d of ageing (Fig. 5C, band 2) represented as 2 spots in 2-D DIGE (Fig. 1, spots 3 and 4). On the other hand, MYBPC2 degraded to one fragment of around 67 kDa after 14 d (Fig. 5A, band 4). In this regard, both fragments could be potentially used as markers of the ageing time.

2-D DIGE elucidated also several other changes between non-aged and 21 d aged meat that served to confirm, for the particular case of horse meat, results reported for other types of meat. To this extent, Desmin (DES), as an integrant of intermediate filaments, has been reported to undergo *post-mortem* proteolysis, being related to tenderization in beef [43]. Indeed, DES is a known substrate of μ-calpain [44] and its limited degradation has been related to a loss in water holding capacity in pork [45] and poultry [46]. Degradation of DES was observed in the present study (spot 9) despite that other horse meat studies did not observe this degradation in *Longissimus lumborum* muscle during ageing [13]. Further research would be required to elucidate the time course of DES degradation during ageing and its implications on the quality of meat.

It is worth highlighting that beyond structural proteins discussed above, an important number of enzymes from energy metabolism pathways showing different abundances over ageing was also observed. In fact, ALDOA, ATP5PD, CKM, DLAT, ENO3, GAPDH, LDHA, NDUFS2, PYGM and SDHA constituted the biggest cluster from the protein network (Fig. 2). The presence of metabolic, water-soluble, proteins in the myofibrillar sub-proteome has been reported to be a consequence of the decrease in their solubility due to *post-mortem* conditions (i.e., pH and temperature drop) [47,48]. This phenomenon has been observed especially at early *post-mortem* times (0–5 d) [37]. Moreover, most of these proteins showed a decreasing abundance of their intact form (ALDOA, CKM) and/or an increasing abundance of their fragments (ATP5PD, ENO3, GAPDH, PYGM) after 21 d of ageing (Table 1). This would indicate that even if they are not part of muscle architecture, they would also undergo proteolytic degradation during ageing as already observed in beef [49]. In this regard, four spots were identified as GAPDH fragments (spots 21–24), whose peptide hits detected by MS would indicate that they correspond to C-terminus (spots 21 and 22) and N-terminus (spots 23 and 24) fragments (Supplemental Fig. 5) of the protein. GAPDH fragments have already been reported to appear during horse meat ageing [22,50] and may also be appropriate to monitor the process. The rest of the identified metabolic enzymes co-occurred in a single spot with other proteins and thus, their independent trends could not be elucidated (DLAT, LDHA, SDHA). In the case of NDUFS2, its abundance increased after ageing.

Beside the two big functional groups (structure and energy metabolism), other protein types were also found to change in abundance after ageing. The decrease in abundance of a chaperone protein (HSPB1) was observed in spot 18. The family of Heat Shock proteins increase in abundance at early *post-mortem* times in response to cell damage [40]. However, in extended ageing, they undergo proteolysis. Therefore, their degradation would indicate a development of ageing. Additionally,

Albumin (ALB, spot 7), which is not usually reported in muscle tissue, was found to decrease in abundance after 21 d of ageing, contrary to PHB.

5. Conclusion

This work opened a way for the application of 2-D DIGE on the study of myofibrillar meat sub-proteome with the aim to investigate the ageing process. Previous proteomic findings regarding the biochemistry of horse meat ageing have been confirmed, while some novel findings were incorporated. Several proteins reported to change in abundance during ageing should be further studied as protein biomarker candidates of horse meat tenderization (TNNT3, MYBPC1, MYBPC2, ACTA1, GAPDH). This would help to reach a standardized horse meat quality by meat industry. In the future, meat from other horse breeds and muscles should be investigated, and also 2-D DIGE should be included as a suitable methodology to achieve this goal.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2022.104770.

Declaration of Competing Interest

Authors declare no conflict of interest.

Data availability

No

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