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Journal Pre-proof

First insights about the underlying mechanisms of Martina Franca donkey meat tenderization during aging: A proteomic approach

Antonella della Malva[a,*] antonella.dellamalva@unifg.it, Mohammed Gagaoua[b,*] mohammed.gagaoua@teagasc.i; gmber2001@yahoo.fr, Antonella Santillo[a], Pasquale De Palo[c], Agostino Sevi[a], Marzia Albenzio[a]

[a]Department of Agriculture, Food, Natural Resources and Engineering (DAFNE), University of Foggia, Via Napoli, 25- 71121, Foggia, Italy.

[b]Food Quality and Sensory Science Department, Teagasc Food Research Centre, Ashtown, D15 KN3K, Dublin, Ireland

[c]Department of Veterinary Medicine, University "Aldo Moro" of Bari, S.P. per Casamassima, km 3, 70010 Valenzano, Bari, Italy

Corresponding authors.

Abstract

This study aimed to evaluate the effect of different aging times (1, 6 and 14 days) on the tenderization rate and protein changes in ten Martina Franca donkey striploins using a proteomic approach. During aging, a progressive decrease in share force, hardness, gumminess, and chewiness together with an increase in myofibril fragmentation index were observed. Proteolysis monitored by immunoblotting revealed a progressive degradation of desmin and fast troponin-T over time and an increase of their degradation products up to 14 days aging. Proteomics revealed by means of two-dimensional electrophoresis 37 protein spots corresponding to 15 proteins to change significantly by increasing aging time. These proteins belong to three pathways, these being "muscle contraction, structure, and associated proteins" (9 proteins); ii) "energy metabolism" (5 proteins); and iii) "chaperone" (1 protein). This study is the first to highlight the possible role of interconnected pathways in driving the final quality of donkey meat and predicting its texture.

Keywords

Donkey, meat aging, meat tenderness, proteomics, myofibrillar proteins, proteolysis, energy metabolism.

1. Introduction

Donkeys are unique livestock species, capable of living in harsh weather and environmental circumstances, such as high temperatures, limited rainfall and poor feed availability that are not in competition with human food (Camillo et al., 2019). In the last years, the rearing of donkey is

mainly linked to milk production, due to its nutritional composition that have certain similarities to that of human (Polidori & Vincenzetti et al., 2017). Conversely, donkey meat is very marginal in Europe, although it has been recognized as food with excellent nutritional characteristics for its high biological value in terms of proteins, vitamins and minerals, low intramuscular fat content, and high content of conjugated linoleic acid (Polidori et al., 2015; Marino et al., 2022). Martina Franca donkey (*Equus asinus*) is one of the autochthonous breeds of Southern Italy (Apulia region) known as a working animal in the past centuries. Although Martina Franca donkey meat is known to be healthy (Polidori et al., 2009; Polidori et al., 2015; De Palo et al., 2017) and tasty (Maggiolino et al., 2020), consumers are not familiar with consuming donkey meat.

Among the internal quality traits of meat, tenderness is one of the sensory properties that has been intensively studied as it is very important to consumers and known to drive their re-purchase decisions at the point of sale (Miller et al., 2001; Gagaoua et al., 2016). It is noteworthy that meat tenderness is a complex trait affected by multiple interrelated factors such as breed, slaughter age, sex, rearing practices and complex mechanisms related to muscle structure and its composition (Koohmaraie & Geesink, 2006; Gagaoua et al., 2018). Further, there are major influencing factors related to the extent and rate of tenderization, which include among others, the proteolytic events occurring on the post-mortem muscle and mediated by endogenous proteolytic systems during the aging period (Hopkins & Thompson, 2002; della Malva et al., 2019). Indeed, post-mortem aging is one of the widely used practices by meat industry to achieve a satisfactory increase of meat tenderness (Kim et al., 2018; Gagaoua et al., 2022).

During the last years, gel-based approaches combined with mass spectrometry (MS) have been widely applied to elucidate the in-depth dynamic biochemical changes taking place during the conversion of muscle into meat and the tenderization rate/process from different species such as beef (Marino et al., 2013; Gagaoua et al., 2020; 2021), pork (Di Luca et al., 2011; Sayd et al., 2006) and horse (della Malva et al., 2019; della Malva et al., 2022). This foodomics approach revealed that sophisticated mechanisms are involved (Ouali et al. 2013; Picard and Gagaoua, 2017). Furthermore, proteomics allowed researchers to discover several putative biomarkers of meat tenderness belonging to myriad and interconnected biological pathways such as energy metabolism, muscle structure, response to heat and oxidative stress, apoptosis signaling, proteolysis, protein binding and proteases (Picard and Gagaoua, 2020; Gagaoua et al., 2021). Thanks to the developments in the recent years of the bioinformatics tools (Gagaoua et al., 2021), a thorough understanding of the mechanisms was possible namely in revealing the extent of interconnectedness of the major pathways underpinning muscle to meat conversion. However and to the best of our

knowledge no proteomic study are yet available on donkey meat on which interest as a sustainable source of animal proteins is currently expressed by certain consumers. Therefore, there is a need, in the objective of developing high-quality products from autochthonous and sustainable breeds, to elucidate the underlying pathways in meat tenderization from donkey species. Accordingly, proteomics-based approaches can be a useful tool in investigating the proteolytic changes in the structural proteins and the biochemical processes affecting donkey post-mortem muscle and aged meat.

In this context, we aim by the present trial to study the tenderization rate and protein changes occurring during the aging of Martina Franca donkey meat using a gel-based proteomic approach and in-depth analyses of those protein changing using bioinformatics.

2. Materials and methods

2.1 Animals and meat sampling

Ten male Martina Franca donkeys reared on the same farm were used in the experiment. At about 18 months of age (average weight before slaughtering of 280-300 kg), the animals were slaughtered according to industrial routines used in Italy and following the European Union regulation rule n. 1099/2009. Before slaughter, all animals were fasting for 12 h with free access to water. Donkeys were stunned with a conventional captive bolt gun before exsanguination. Subsequently, carcasses were skinned and eviscerated and all the non-carcass component components (skin, head, feet, heart, lungs, liver, spleen, and the digestive tract) were removed.

The carcasses were not electrically stimulated and were transferred to a cold room at 2-4 °C and stored for 24 h according to standard commercial practices. The striploins (*Longissimus thoracis et lumborum* (LTL), an mixed oxido-glycolytic muscle) was excised at 24 h post-mortem from the both sides of the carcass from the 13th to the 18th rib (average weight of the LTL muscles 2.1 ± 0.15 kg), and subsequently transported under refrigerate conditions (4 °C) to the laboratory of the University of Foggia. Each LTL muscle, from both sides of carcasses, was divided into three equal-length sections (average weight 600 ± 25.59 g), vacuum packaged, and randomly assigned to aging time at 2°C for 1, 6 and 14 days (n=2 for each aging time, one from the left and one from the right). The cranial and caudal sections were randomized across aging times.

2.2. Estimation of *Warner-Bratzler shear force (WBSF) and texture profile analysis (TPA)*

Warner-Bratzler shear force (WBSF) and texture profile analysis (TPA) were tested on grill-cooked meat samples (2.0 cm of thickness) using an Instron 3343 universal testing machine equipped with a 500 N load cell (Instron Ltd., High Wycombe, United Kingdom) as previously described by Marino et al. (2015). Each steak sample was used to prepare 5 cores (1 cm^2 in cross-section) parallel to the longitudinal orientation of the muscle fiber. All the replicates were considered as an average and used for statistical analysis.

2.3. Myofibrillar fragmentation index (MFI)

Myofibril fragmentation was performed according to Culler, Parrish, Smith and Cross (1978) with some modifications. Briefly, 4 g of muscle sample was homogenized with 40 mL of cold MFI buffer (100 mM KCl, 20 mM KH_2PO_4 [pH 7.0], 1 mM EGTA, 1 mM $MgCl_2$, and 1 mM NaN_3) using an Ultra-Turrax homogenizer (IKA T18 basic, Germany) at a speed of 22000 rpm. The mixture was centrifuged at 1000 *g* (Eppendorf 5810R, Eppendorf AG, Hamburg, Germany) for 15 min at 2°C, after which the pellet was resuspended in 20 mL of MFI buffer, vortexed and centrifuged again. Subsequently, the pellet was resuspended in 10 mL of buffer and filtered using a mesh screen (18 mesh) to remove fat and connective tissue. The filtrate was used to quantify the protein concentration using the biuret method using Bovine Serum Albumin (BSA) as a standard. The aliquots of the suspensions obtained were diluted with the same MFI buffer to a final protein concentration of 0.5 mg/mL. The myofibrils suspension was measured in duplicate immediately using a UV-Vis spectrophotometer (Biotek PowerWave XS2, Biotek Instruments, Inc. Highland Park, Winooski, Vermont, USA) at 540 nm. MFI was expressed as the absorbance multiplied by 200.

2.4. Total collagen content

The concentration of total hydroxyproline was determined using the method outlined by Hutson, Crawford, and Sorkness (2003) with slight modifications. Briefly, 0.2 g of meat was homogenized with 1 ml of 6 M HCl using an Ultra-Turrax homogenizer (IKA T18 basic, Germany). The homogenate was placed in a ventilated oven at 110°C for 18 hours by adding 3 ml of 6 M HCl for hydrolysis. Then, the tubes were cooled to room temperature, filtered with syringe filters (0.45 µm), and 4 mL of 6 M NaOH were added to each tube, then pH was brought to 9.5 ±1.0 with 6 M NaOH. Subsequently, 1 mL of this solution was placed into a chromatographic vial, where 50 µl of 2 mM sarcosine standard was added. Subsequent derivatization was performed as described by Henderson et al. (2000). An HPLC system (1260 Infinity series, Agilent Technologies) equipped with a binary pump with microvacuum degasser, an autosampler, a column compartment, a diode array detector

(model G1315C) and a fluorescence detector (model G1321B) was used for the quantification. The separation was performed using a Zorbax Eclipse-AAA column, 4.6 x 150 mm, 3.5 µm (Agilent Technologies). A fluorescence detector was used to determine the total hydroxyproline and sarcosine at 265 nm (excitation) and 305 nm (emission). Hydroxyproline standards of 25, 50, 100, 250, 500, 750, and 1000 µM in distilled water were prepared from a 2 mM stock solution. Then 50 mg of 2 mM sarcosine was added to each 1 mL sample of hydroxyproline standard. The amount of total collagen was calculated from the hydroxyproline concentration using a conversion factor of 7.14 (Stanton and Light, 1987) and expressed as µg/mg of fresh meat.

2.5 Water holding capacity (WHC)

Water holding capacity was measured as thawing loss according to Xia et al. (2012) with slight modifications. Briefly, thawing loss was determined on LTL steaks vacuum-packed in nylon/polyethylene bags and kept frozen at -20°C until analysis. Subsequently, 1 cm thick frozen steak was placed on plastic netting over a polystyrene tray and stored in a plastic bag for 48 h at 4°C. After storage, each meat sample was removed from the tray and the weight of the tray plus the juice was recorded. Thawing loss was expressed as a percentage of the initial weight of the meat:

Thawing loss (%) = [(Wt + j − Wt)/ (Wi + m − Wt)] × 100,

where Wt is the weight of the empty tray, Wt + j is the weight of the tray plus the juice, and Wi + m is the weight of the tray with meat.

2.6. *Gel-based proteomics on post-mortem muscle and aged meat*

2.6.1. *Protein extraction and mono-dimensional gel electrophoresis*

The extraction of both sarcoplasmic and myofibrillar proteins was performed according to the procedures described by Marino et al. (2013, 2014). Briefly, each muscle sample (2.5 g) was homogenized with 10 ml of 0.03 M cold phosphate buffer (pH 7) containing a protease inhibitors cocktail (Sigma-Aldrich, St. Louis, MO, USA) by using an Ultra Turrax homogenizer (IKA T18 basic, Germany) at 10000 rpm for 1 min. Homogenates were then centrifuged at 4 °C and 8000 *g* (Eppendorf 5810 R, Eppendorf AG, Hamburg, Germany) for 20 min, and then supernatants (sarcoplasmic protein extract) were collected and stored at -80°C. Subsequently, the resultant pellet (0.100 g) was resuspended in 1 ml of denaturing extraction buffer (8.3 M Urea, 2 M Thiourea, 64 mM dithiothreitol, 2% cholamidopropyl dimethyl hydroxypropane sulfonate CHAPS, 2% IGEPAL® CA-630 NP 40, 10 % glycerol and 20 mM Tris–HCl, pH 8) and incubated overnight on

an orbital shaker. Then, homogenates were centrifuged at 15000 *g* for 20 min at 10 °C, and supernatants (myofibrillar protein extract) were aliquoted and stored at -80°C.

The protein concentration in duplicate for both, sarcoplasmic and myofibrillar protein extracts, was determined with the 2-D Quant Kit assay (GE Healthcare). Absorbance was measured at 480 nm using a UV-Vis spectrophotometer (Biotek PowerWave XS2), with bovine serum albumin as a standard and the final concentration of samples was adjusted to 4 mg/mL.

Sarcoplasmic and myofibrillar proteins were resolved with an 8-18% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gradient gel using a Protean II xi system (Bio-Rad Laboratories). Coomassie Blue G-250 (Bio-Rad Laboratories) was used to visualize bands. Gels were destained, acquired by the ChemiDoc EQ system (Bio-Rad Laboratories) before their analyses with the Image Lab software (Bio-Rad Laboratories). The relative quantity of each individual protein band was expressed as a percentage of the total protein quantity of all bands in the lane.

2.6.2. Immunoblotting

Immunoblotting of desmin, troponin-T and calpastatin were performed on 10%, 12% and 8% acrylamide resolving gels, respectively. The myofibrillar fraction was used to determine desmin and troponin-T degradation, while the sarcoplasmic fraction to detect the calpastatin. Myofibrillar or sarcoplasmic reference samples were prepared by pooling equal parts of all respective extracts. The gels were loaded with 40 µg of protein/well and run at 100 V for 3 h with the Mini-Protean Tetra cell (Bio-Rad Laboratories). After run, gels were transferred to a nitrocellulose membrane (Mini format, 0.2 µm nitrocellulose, Bio-Rad Laboratories) by a semidry transfer method (Trans-Blot Turbo Transfer System, Bio-Rad Laboratories) for 7 min at 25V/2.5A. Membranes were blocked using 5% of BSA in a Tris-buffered saline solution containing 0.05% Tween-20 (TBS-Tween). Then, the membranes were incubated for 1 h at room temperature with the primary antibody (monoclonal anti-desmin, D1033, Sigma-Aldrich, St. Louis, MO; diluted 1:10,000; monoclonal anti-troponin-T produced in mouse (JLT-12; Sigma-Aldrich, St Louis, MO; diluted 1:40,000) and monoclonal anti-calpastatin (MA3-945, Thermo Scientific, Rockford, IL; diluted 1:5,000).

The membranes were subsequently washed 5 times using TBS-Tween before incubation for 1 hour at room temperature with the secondary antibody, goat anti-mouse-HRP (No 2554; Sigma-Aldrich) diluted 1:5,000, 1:30,000 and 1:10,000 for desmin, TnT and calpastatin, respectively. After 3 times wash for 10 min each, blots were detected using the Clarity Western ECLTM Substrate (Bio-Rad Laboratories). Images were acquired by the Chemi Doc EQ system using a Chemi-Hi

Sensitivity application and then analyzed with ImageLab software (Bio-Rad Laboratories) to determine the signal intensity of the intact and fragmented protein bands. Immunoreactive band intensities were compared with a pooled sample reference to normalize data and quantification.

2.6.3. Two-Dimensional Gel Electrophoresis (2DE) and image analysis

Two-dimensional gel electrophoresis (2DE) of the myofibrillar sub-proteome was conducted according to the procedure previously reported by Marino et al. (2015). The first dimension was performed using IPG dry strips of 17 cm pH 4–7 (Bio-Rad Laboratories) loaded with 300 µg of protein. Following rehydration (ready-Prep Rehydration/Sample Buffer, Bio-Rad Laboratories), the isoelectric focusing (IEF) was carried out at 20°C using a Protean IEF Cell (Bio-Rad Laboratories). After IEF, proteins were resolved on 8-18% SDS-PAGE gradient gel using Protean II xi system (Bio-Rad Laboratories). The gel images obtained using Chemi Doc EQ system (Bio-Rad Laboratories) were then analyzed with the PD-Quest 7.4.0 software (Bio-Rad Laboratories). All gel images were processed and analyzed under the same parameters and, after spot detection, automatically matched with the spots of a master gel (virtual gel comprehensive of all matched and unmatched spots of all 2DE images) used as a reference. Landmark spots were used to confirm spot matching across all gels and manual verification was used to screen out any dust artefacts or incorrectly identified spots. The relative volume of each spot in a gel was normalized as a percentage of the total volume of all spots detected.

2.6.4. In-gel digestion and identification of the proteins of interest by LC-MS/MS

The spots displaying statistically significant differences during all aging times were manually excised from the gels, destained with 50 mM NH_4HCO_3 and dried under vacuum. For each sample, 10 µL of sequencing grade modified trypsin (12.5 ng/mL, Promega) were added and digestion was carried out overnight at 37 °C. The next day, the peptides were extracted from the gel matrix by 3 changes (50 µL each) of 50% acetonitrile/0.1% formic acid.

Samples were dried under vacuum and suspended in 15 µL of 0.1% formic acid for LC-MS/MS analysis. This was conducted with a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) coupled online with a nano-HPLC Ultimate 3000 column (Dionex – Thermo Fisher Scientific). The samples were loaded into a homemade pico-frit column (75 µm I.D., 15 µm Tip, 100 mm, New Objective) packed with C18 material (Aeris peptide 3.6 µm XB-C18, Phenomenex) and peptides were separated using a linear gradient of ACN/0.1% FA (from 3% to 40% in 19 min), at a flow rate of 250 nL/min. The instrument operated in a data-dependent mode: a full MS scan at

60000 nominal resolution in the Orbitrap was followed by the acquisition of MS/MS spectra of the ten most abundant ions in the linear ion trap. The ion source capillary temperature was set at 200 °C and the spray voltage was optimized at 1.3 kV.

Raw data files were analyzed with the software package Proteome Discoverer 1.4 (Thermo Fisher Scientific) interfaced to a Mascot Search Engine server (version 2.2.4, Matrix Science), against the section *Equus caballus* of Uniprot databases (version Nov 2021, 52451 entries). Carbamidomethylation of Cys residues was set as static modification, while Met oxidation was set as variable modification. Precursor and fragment tolerance were set at 10 ppm and 0.6 Da, respectively. Data were filtered to keep into account only proteins identified with at least 3 unique peptides with high confidence (False Discovery Rate (FDR) < 0.01).

2.7. Bioinformatic approaches

Protein-protein interaction analysis of the 15 differentially abundant proteins during aging time (after 14 days of aging) was carried out using the open source STRING database v11.0 (Szklarczyk et al., 2019; https://string-db.org/). Considering the *Equus caballus* Gene Ontology (GO) limitation, the Uniprot ID genes were converted into the human orthologues EntrezGene ID following the procedure of Gagaoua et al. (2021) prior to analyses. This allowed us to obtain the most complete information on each gene name (protein). Confidence intervals was set to 0.400 (medium confidence) and false discovery rate (FRD) stringency was set to 1 (high percent) in order to obtain as many significant features as possible while incurring a relatively low proportion of false positives.

For Gene Ontology (GO) analyses, the Metascape® (https://metascape.org/, accessed on 18 February 2022) open-source tool was used to investigate the pathway and process enrichment analyses using the list of 15 differentially expressed proteins. As an additional process, Metascape® was further used to investigate the overlap in terms of GO terms using the differentially expressed proteins between 1 and 14 days of aging by means of hierarchical heat map clustering.

2.8. Statistical analysis

WBSF and texture traits, MFI, total collagen, WHC, SDS-PAGE and immunoblotting data were analyzed using the GLM procedure of the SAS statistical software (SAS Institute, 2013). Each individual donkey was the experimental unit (the variable LTL muscle from the right and the left sides, being irrelevant in the final model, was eliminated), the mathematical model included fixed effect of post-mortem aging and random residual error. All effects were tested for statistical

significance set at $p < 0.05$, and when significant effects were found ($p < 0.05$), Fisher's LSD test was used for comparison.

Principal component analysis (PCA) was applied to a matrix of 22 variables (WBSF, hardness, gumminess, chewiness, MFI, total collagen, WHC, and the 15 differentially expressed proteins using the PRINCOMP procedure of SAS to study the main tendencies in variation between donkey meat quality characteristics and differentially protein spots along aging process. The first two principal components were considered.

3. Results

3.1. Donkey meat quality traits

The results of the aging time (1, 6 and 14 days) effect on WBSF, TPA parameters, MFI, total collagen content and WHC of Martina Franca donkey meat are reported in **Table 1**. As expected, during aging a progressive and significant ($p < 0.01$) decrease of share force values was observed, showing the lowest values (average of 27.46 N) in donkey meat aged for 14 days. An effect of aging was also found for hardness ($p < 0.01$), gumminess ($p < 0.01$) and chewiness ($p < 0.01$) texture parameters. Particularly, hardness values decreased after 6 days of aging, while, a significant decrease was found after 14 days of aging for gumminess and chewiness. No significant differences were observed for cohesiveness and springiness (**Table 1**). Referring to the myofibril fragmentation index (MFI), a gradual increase ($p < 0.001$) was found during aging time. Conversely, a decrease ($p < 0.01$) was observed in the total collagen content during aging, with lowest values detected at 14 days. An effect of aging was found also for water holding capacity (WHC), which showed a decrease ($p < 0.05$) in meat aged 6 days and remaining constant thereafter.

3.2. Mono-dimensional SDS-PAGE electrophoresis of the sarcoplasmic and myofibrillar sub-proteomes

The SDS-PAGE gels of the sarcoplasmic and myofibrillar fractions from LTL muscle of Martina Franca donkey as affected by post-mortem aging (1, 6 and 14 days) are depicted in Fig.1.

For the sarcoplasmic proteins (Fig. 1A), the densitometry analysis evidenced that at the first day of aging, donkey muscle is characterized by 32 protein bands, while, after 14 days of aging only 24 protein bands can be observed. Among these protein bands and throughout post-mortem aging, a significant decrease in intensity was observed for several sarcoplasmic proteins, mainly glycogen phosphorylase b kinase (PHb, $p < 0.001$), creatine kinase M type (CKM, $p < 0.01$) and enolase

(ENO3 $p < 0.05$). For PHb, a sharp decline was found after 6 days of aging and, almost disappeared after 14 days. For CKM, a decrease in intensity was observed after 6 days of aging, while, ENO3 showed a decrease only after 14 days. Further significant aging effect was found for phosphoglucomutase (PGM1; $p < 0.05$) and phosphoglycerate mutase 1 (PGAM1; $p < 0.05$) proteins that seemed to increase in their abundance from 6 to 14 days of aging.

For the myofibrillar proteins (Fig. 1B), a gradual decrease in the abundance of troponin-T (TTNT3; $p < 0.01$) and desmin (DES; $p < 0.01$) was observed during aging time from day 1 to 14, while, changes in actin (ACTN1; $p < 0.05$) are observable after 6 days. Conversely, an increase of myosin light chain 2 (MYL2; $p < 0.01$) and myosin light chain 1 (MYL1; $p < 0.05$) can be observed after 6 and 14 days of aging, respectively. Additionally, the densitometry analysis revealed the appearance of new proteolytic fragments observable in 14 days muscle fraction around 110 kDa and 30 kDa.

3.3. Immunoblotting analyses on targeted myofibrillar proteins and calpastatin inhibitor

To further our understanding and confirm the post-mortem biochemical changes overserved above in the expression of specific and important proteins studied in the literature to play a role meat tenderization, we performed western blots for desmin (Fig. 2A), troponin-T (Fig. 2B) and calpastatin (Fig. 2C). The densitometry analyses of the revealed bands of each protein are given in Table 2.

For desmin, we revealed a significant and progressive decrease in the intensity of the 54 kDa intact form ($p < 0.001$) during aging, while, no significant differences were found for the 46 and 45 kDa isoforms among the 3 sampling times. The degradation of the 54 kDa intact band, was supported by the increase of its fragments: increase of the 40 kDa degradation product ($p < 0.001$) from 1 to 14 days of aging, and the appearance and increase in the abundance of the 37 kDa fragment ($p < 0.001$) from 6 days of aging.

For TNNT3, we observed the presence of 6 immunoreactive bands; 37, 36 and 34 kDa represent isoforms of the intact protein, while, the 33, 30 and 28 kDa bands are its degradation products. The degradation of Troponin-T in donkey meat occurred progressively by increasing the aging time (Table 2). A gradual decrease in the abundance of the 37 kDa ($p < 0.001$) intact isoform was observed along the aging, while, the 36 kDa band showed a decrease ($p < 0.01$) after 6 days and then remain stable. For the degradation products, a progressive increase of the 33 kDa ($p<0.01$) and

30 kDa ($p < 0.001$) polypeptides is observable during aging with greater intensity at 14 days of aging, while, the 28 kDa fragment seemed to increase after day 6 of aging.

For calpastatin, six bands with molecular weight ranging from 70 kDa to approximately 29 kDa were revealed (Fig. 2C). The densitometry analysis in Table 2 for this inhibitor of calpains highlighted an aging effect on the 70 kDa ($p < 0.001$), which was observed in donkey meat at 1 day post-mortem, while, it fully disappeared from 6 days of aging. No significant differences due to aging time were found for the 58 and 50 kDa bands, while, a decrease in the abundance of the 37 kDa ($p < 0.01$) band can be observed. Regarding the calpastatin breakdown pattern (30 kDa and 29 kDa bands), an increase was found during aging time, but with a different rate. More specifically, a progressive increase of the 29 kDa band ($p < 0.01$) during aging time was found, while, the estimated 30 kDa fragment of this calpain inhibitor appeared only after 14 days of aging time.

3.4. Two-dimensional electrophoresis (2DE) and identification of differentially abundant proteins by LC-MS/MS

The representative 2DE gel maps of the myofibrillar protein fractions of LTL muscle from Martina Franca donkey after 1, 6 and 14 days of aging are shown in Fig. 3A. As expected, an increase of spot numbers as a result of aging time was observed (191 at 1 day, 206 at 6 days and 249 after 14 days of aging, respectively). This demonstrates the significant impact of aging on the structural proteins due to proteolysis as a major phenomenon deeply connected with tenderization process.

A total of 37 protein spots, corresponding to 15 unique proteins, were found to be differentially expressed among the 3 aging times and were successfully identified, based on homology to *Equus caballus* (Table 3 and annotation of Fig. 3B). Protein spots differentially abundant at 14 days of aging belonged to three major biological pathways, these being i) muscle contraction, structure, and associated proteins (n = 28 protein spots; myosin-1 “MYH1”, myosin-2 “MYH2”, actin-alpha 1 “ACTA1”, myosin light chain, phosphorylatable “MYLPF”, myosin light chain 6B “MYL6B”, myosin light chain 1 “MYL1”, troponin C2, fast skeletal type “TNNC2”, tropomyosin 1 “TPM1”, tropomyosin 2 “TPM2”); ii) energy metabolism (n=6 protein spots; ATP synthase subunit d, mitochondrial “ATP5PD”, ubiquinol-cytochrome c reductase core protein 1 “UQCRC1”, cytochrome c oxidase polypeptide Va “COX5A”, glyceraldehyde-3-phosphate dehydrogenase “GAPDH”, creatine kinase “CKM”); and iii) chaperone (n= 3 protein spots; heat shock 27 kDa protein “HSPB1”). On the basis of protein spots abundances, 8 were down-regulated during aging time, while, 29 were up-regulated or appeared only after 14 days of aging. Among these, 2 protein

spots of tropomyosin (TPM1 and TPM2) and 6 spots of myosin light chain (MYLPF and MYL1) were highly expressed at day 1 of aging, while, after 14 days of aging, several fragments of structural proteins (n= 20) and also proteins involved in energy metabolisms and cell stress responses (n= 9) are hugely impacted and degraded.

To summarize the information obtained from this analysis, a principal component analysis (PCA) was performed to investigate the relationship between the meat quality parameters (WBSF, TPA, MFI, total collagen and WHC) and the protein spots with significant differential abundance ($p < 0.05$) during aging time (Fig. 3C). The first two principal components (PC) accounted for 90% of the total variance, with 85% explained by PC1 and only 5% by PC2.

The meat texture traits (WBSF, hardness, gumminess and chewiness), WHC and tropomyosin spots (TPM1 and TPM2) were the variables negatively related to the PC1, whereas MFI, myosin light chain isoforms, energy metabolism and protein spots related to cell stress were projected positively in the PC1. The score plot clearly differentiated the three aging times. Furthermore, the PCA analysis also showed that donkey meat aged 14 days was located in a zone of the plot characterized by higher MFI, together with higher contents of ACTA1, MYL1, MYL6B, MYH1, MYH2, MYLPF, TNNC2, ATP5PD, UQCRC1, COX5A, GAPDH, CKM and HSPB1.

3.5. Bioinformatics analysis of the differential proteins

The protein-protein interaction network among the 15 differentially abundant proteins (unique gene names) in the *Equus caballus* LTL muscle proteome is given in Fig. 4. Proteins are represented as network nodes, while the edges represent the functional associations between them. Two major interacting networks these being the striated muscle contraction/filament sliding (n= 9 proteins) and the ATP metabolic processes (n = 4 proteins) which are both linked with stress response protein HSPB1 were identified. The localization of the protein from muscle structure in the sarcomere and those related to energy metabolism mainly mitochondria, are highlighted in the Fig. 4. The biological and cluster analysis pathways on the differentially expressed proteins are reported in Fig. 5. The circos plot (Fig. 5A) revealed that only MLYLP is in commonly changing for the two aging times (1 and 14 days). The comparison of the significantly enriched GO terms by means of a heatmap on the protein lists of 1 and 14 days (Fig. 5B) revealed 6 enriched term clusters among which ”muscle organ development (GO:0007517)” and “muscle contraction (GO: 0006936)” are common. The “regulation of ATPase activity (GO:0043462)” term was specific to the proteome list aged meat for 1 day, while “ATP metabolic process (GO: 0046034)” term, “purine nucleotide metabolic process (GO:0009150)” and regulation of “I-kappaB kinase/NF-kappaB

signalling (GO:0043122)" were specific to meat aged for 14 days. The network association between the representative enrichment terms and their functional enrichment is further given in Fig. 5C, showing the extent of enrichment of the clusters contributing to each GO term.

4. Discussion

4.1. Effect of aging time on Martina Franca donkey meat quality characteristics

Meat tenderness is a complex and multifactorial feature identified as one of the major challenges facing the red meat industry. In the present study, the increase in tenderness of Martina Franca donkey meat was observed during the aging period, as supported by the progressive decrease of all the texture traits we evaluated likely WBSF, hardness, gumminess and chewiness together with an increase of myofibrillar fragmentation index (a biochemical proxy of the extent of proteolysis and texture), especially in the first week of aging. Although the effect of aging time on bovine meat tenderization rate is well documented (Huff-Lonergan et al., 2010; Kim et al., 2018; Gagaoua et al., 2021; Sierra et al., 2021), to the best of our knowledge there exist only one study by Polidori et al. (2020) that reported in a short aging time the variation in donkey meat tenderness. In terms of WBSF values, our results at 6 days of aging are slightly lower than those found by the aforementioned authors on the same muscle (LTL) of crossbreed Martina Franca x Ragusana donkey slaughtered at a comparable age. In a recent study on horse meat aged up to 21 days, authors evidenced that horse meat tenderization occurs during the first two weeks of aging (Beldarrain et al., 2022) highlighting the importance of a prolonged aging time in equidae to achieve desirable tenderness of meat. However, it is worth noting that the WBSF results observed in our study are satisfactory in terms of tenderness being lower to the threshold of 40 N in the case of beef (Gagaoua et al. 2019; Holman and Hopkins, 2021).

In this study, the improvement of donkey meat tenderness during aging was also confirmed by the decrease and lower amounts of total collagen. The sensory tenderness could be also associated with the amount of connective tissue, although the weakening of myofibers and proteolysis during aging are the main factors that contribute to the tenderization rate (Purslow et al., 2018; Warner et al., 2021).

Nevertheless, it should be emphasized that the amount of collagen found in Martina Franca donkey meat were lower compared to that found in the LL muscle of other species such as beef (43.75- 69 µg/mg; Raes et al., 2003; Li et al., 2007), pork (31- 41 µg/mg; Wheeler et al., 2000; Therkildsen et al., 2002), lamb (57- 94 µg/mg; della Malva et al., 2017) and horse (46-50.4 µg/mg;

della Malva et al., 2022). However, these variation and their impact on final meat quality can depend on several other factors likely the cooking temperature and degree of cross-links among others (Purslow et al., 2018).

Water-holding capacity (WHC) is an important proxy measurement to estimate and assess juiciness, and consequently, essential to evaluate the sensory traits and the palatability of meat. It is well-known that the biochemical changes occurring during the post-mortem period in the skeletal muscle, such as proteolysis including the breakdown of collagen, could affect the drip loss amount in meat especially due to the protein denaturation as a consequence of pH decline (Kristensen & Purslow, 2001; Purslow et al. 2021). Accordingly, we retain that in our study, the highest WHC found in the first week of aging of donkey meat is mainly caused by greater ion-protein interaction and proteolysis leading to more fluid losses from muscle structure (Huff-Lonergan and Lonergan, 2005).

4.2. Effect of aging time on muscle proteome changes of Martina Franca donkey

The proteome analysis of Martina Franca donkey meat revealed changes of several protein patterns as a consequence of post-mortem aging. Particularly, a decrease in sarcoplasmic protein patterns (PHb, ENO3 and CKM) during aging time was observed through SDS-PAGE analysis as a result of both protein solubility changes due to denaturation processes and to the tenderization rate. Referring to PHb, the strongly decrease observed during aging confirms that monitoring changes of this glycolytic enzyme is a way to predict the potential of meat tenderness in agreement with a previous study on horse meat (della Malva et al., 2022). Furthermore, it should be noted that aging also affected the abundance of CKM in Martina Franca donkey meat after 6 days of aging. It is known that calpains and cathepsins gradually breakdown CKM during aging until complete inactivation (Purintrapiban, Wang, & Forsberg, 2001; Delbarre-Ladrat, Verrez-Bagnis, Noël, & Fleurence, 2004). Gagaoua et al. (2021) pointed out that CKM is one of the robust beef tenderness biomarkers in LTL muscle that has been consistently identified by proteomics. CKM, an enzyme used by striated muscles to generate ATP by creatine phosphate, is located in the M-band and binds to the central domains of both myomesin and M-protein. Thus, one could speculate that the rate of its fragmentation might by driving the rate of energy depletion and pH decline, hence explaining partly its association with several eating qualities such as water-holding capacity, drip loss, pH decline, color and tenderness (Yang et al., 2018; Gagaoua et al., 2020; Gagaoua et al., 2021; Sierra et al., 2021).

Regarding PGM1 and PGAM1, we observed an increase of about 33% and 62% from 1 to 14 days of aging, respectively. Studies on bovine and ovine meat (Anderson et al., 2014; Chen et al., 2018) reported that these enzymes were positively correlated with glycolytic rate. The changes in the abundances of PGM1 and PGAM1 found in donkey meat after 14 days of aging, confirm an involvement also of these sarcoplasmic proteins in the post-mortem tenderization processes. However, it is important to point out that, the post-mortem increase of these glycolytic enzymes could be due to aggregation processes during aging as previously reported by different studies on bovine meat (Bjarnadottìr et al., 2010; Laville et al., 2009). Moreover, the abundance can be also a consequence of better extractability of the proteins at 14 days, that can contribute to the variation. Therefore, such results need validation in terms of enzyme activity.

In terms of the changes in the myofibrillar protein profile of Martina Franca donkey meat during aging, our results revealed the degradation of different structural proteins known for being related to tenderization rate and degradation of myofibrils, including desmin, troponin-T and actin (Ouali et al., 2013; Lana & Zolla, 2016; Picard & Gagaoua, 2017; Gagaoua et al., 2020; 2021). The aging effect on donkey meat tenderness improvement and/or the degradation of key structural proteins is further supported by the appearance of major proteolytic fragments at 110 kDa and 30 kDa, recently highlighted of great importance by a targeted proteomic study (Gagaoua et al. 2020). Indeed, these fragments seemed to contain several proteins mostly dominated by those of structure that were shortlisted as biomarkers of several eating qualities of meat.

The changes in the degradation patterns of desmin, troponin-T and calpastatin followed by immunoblotting were confirmed to be of pivotal role in donkey post-mortem processes behind final meat quality development. The progressive degradation of the intact desmin (54 kDa), together with the increase of its degradation products found in donkey meat throughout aging confirms the role of proteolysis in LTL muscle as reported in other species (Marino et al., 2015; de Oliveira et al., 2019). It is also interesting to point out that a degraded form of desmin (37 kDa) appeared at 14 days highlighting the importance of prolonged aging time to increase the action of the endogenous muscle enzymes.

The degradation of intact troponin-T also confirmed the important role of this protein in explaining the post-mortem changes and involvement of known processes such as proteolysis. In fact, we observed a strong decrease of TNNT3 of about 72% from 1 to 14 days of aging, highlighting considerable role of proteolysis in Martina Franca donkey meat due to aging time in agreement to the available knowledge in other species. These results are also supported by the

higher content of TNNT3-derived fragments (30 and 28 kDa). Troponin T, identified as a putative biomarker of beef (Marino et al., 2015; Gagaoua et al., 2021) and horse tenderness (della Malva et al., 2019; Beldarrain et al., 2022), could be a candidate biomarker to characterize donkey meat tenderization. Taken all together, our results suggest that the post-mortem degradation of key proteins such as desmin and troponin-T, involved in the degradation of myofibrils, can be considered for validation as biomarkers of donkey meat tenderness.

Finally, the measurement of calpastatin expression patterns can be used to provide evidence of how active this endogenous cysteine inhibitor is abundant and how it can be used to monitor indirectly the calpain activity and consequently, the tenderization rate of meat. In the present study, the calpastatin immunodetection results evidenced that the degradation of intact calpastatin (115 kDa) occurred early post-mortem. It has been shown that the degradation of calpastatin can lead to the release of 5 degradation products at approximately 100, 80, 65, 54, 30, and 29 kDa (Crutzen et al., 2014; de Moura Souza et al., 2019; de Oliveira et al., 2019). The degradation of calpastatin, combined with the increase in intensity and appearance of breakdown fragments having molecular weights of 30 and 29 kDa, can partly explain the significant improvement in tenderness of Martina Franca donkey meat found in terms of texture traits. Additionally, these findings confirmed the effectiveness of endogenous proteolytic systems in degrading the skeletal muscle proteins during post-mortem aging of donkey meat.

4.3. Muscle proteome characterization of Martina Franca donkey meat during post-mortem aging

Proteomics is a valuable strategy that allows deciphering the biological mechanisms underpinning the tenderization process of Martina Franca donkey meat in the objective of managing/predicting the potential quality of the end-product as well as in identifying protein biomarkers of the desirable meat quality traits, namely tenderness. The investigation of the changes in the myofibrillar protein profile using proteomics, evidenced the effectiveness of aging, and revealed the involvement of several biological pathways to be mainly dominated by muscle structure and associated mechanisms.

Fifteen proteins (unique gene names) seemed in this study to contribute to the tenderization process in Martina Franca donkey meat during aging. In particular, the PCA analysis evidenced aging-specific proteins patterns, highlighting that at 1 day of aging donkey meat from the animals we analyzed was characterized by lower proteolysis as evidenced by the greater intensity of cytoskeletal protein spots (TPM1 and TPM2), together with the highest values of WBSF and texture profile traits. Conversely, more extensive post-mortem proteolysis, occurred in donkey meat aged

14 days, was confirmed by the highest values of MFI and changes in abundance of spots ascribed to myofibrillar fragments due to endogenous enzyme activity and sarcoplasmic proteins due to aggregation processes (Bjarnadottìr et al., 2010; Laville et al., 2009; della Malva et al., 2022).

Several pathways were involved, but mainly the striated muscle contraction/filament sliding pathway (ACTA1, MYL1, MYL6B, MYH1, MYH2, MYLPF, TNNC2, TPM1 and TPM2) followed by ATP metabolic processes (ATP5PD, UQCRC1, COX5A, GAPDH and CKM) and proteins involved in stress response with three isoforms of the small heat shock protein HSP27 known also as HSPB1. Interestingly, 14 of these putative protein biomarkers (ACTA1, TNNC2, TPM1, TPM2, MYL1, MYL6B, MYH1, MYH2, MYLPF, UQCRC1, ATP5PD, GAPDH, CKM and HSPB1) were identified in the integromics study of Gagaoua et al. (2021) as biomarkers of beef tenderness, while COX5A is identified, for the first time in this study, as a candidate biomarker of meat tenderness. This study is the first that used a proteomic approach to study the proteome changes in donkey meat during aging, hence evidencing the pathways underpinning the tenderization rate of donkey meat that seemed to be common with those of other species. From our results, myosin light chain, phosphorylatable (MLYL1) is the only protein common to 1 and 14 days aging periods suggesting that most of the proteins differentially expressed could be good candidates to explain the post-mortem changes as a consequence of aging.

Among the other myofibrillar proteins of interest, ACTA1 was identified as the protein most influenced by aging time due to the presence of 9 fragments in donkey meat after 14 days of aging. It is well known that actin is one of the most abundant proteins of the skeletal muscle, and the breakdown of the actomyosin complex, due to the action of endogenous enzymes, can cause an increase in tenderness. It was identified in the proteomic database of Gagaoua et al. (2021) as the top biomarker of beef tenderness and also as a common constitutive protein of the 30 and 110 kDa major proteolytic fragments appearing during meat aging (Gagaoua et al. 2020). ACTN1 is further suggested by Ouali et al. (2013) as a good marker of post-mortem apoptosis. The native form of ACTN1 was observed in line with our findings to decrease in abundance in the tender group by 2-DE and iTRAQ proteomic approaches in earlier studies (Kim et al., 2008, Laville et al., 2009, Bjarnadottir et al., 2010). Therefore, the presence of several fragments of ACTN1 in LTL muscle after 14 days of aging confirms the greater proteolysis in aged donkey meat and point out that actin-derived fragments are reliable biomarkers of tenderness and aging.

Different isoforms of myosin heavy chains, likely MHY1, MHY2 and the light chain MYL6B were found to be highly expressed in Martina Franca donkey meat after 14 days of aging

confirming the important role of myosin proteins in the degradation of skeletal muscle during the post-mortem period. These findings are in line with previous studies on beef (Ding et al., 2022; Gagaoua et al., 2020; 2021; Sierra et al., 2021; Zhu et al. 2021) and pork (Lametsch et al., 2003; Kim et al., 2013; López-Pedrouso et al. 2020).

Among the subunits of the troponin complex from the thin filament, the subunit C (Ca^{2}+ -binding protein C) is known to play a central regulatory role in muscle contraction and interacting pathways (Gomes et al. 2002). Therefore, the degradation of troponins points out that these interacting proteins might be damaged, hence leading to the breakdown of the thin filament position in the sarcomere. The appearance in Martina Franca donkey meat of one protein spot of TNNC2 after 14 days of aging confirmed the intense proteolysis in line with the texture traits data mainly WBSF. To the best of our knowledge, it is for the first time that the TNNC2 isoform was identified to be related with meat tenderness, but worthy to note that the TNNC1 is a known biomarker of beef tenderness (Gagaoua et al. 2021), suggesting it to be considered as a marker of the ongoing proteolysis in donkey meat.

Energy metabolism pathways, especially those related to mitochondria and involved in ATP metabolic processes, are strictly linked with post-mortem aging (Ouali et al. 2013; Purslow et al. 2021). Of the five protein spots identified (UQCRC1, GAPDH, CKM, ATP5PD and COX5A) in donkey meat after 14 days of aging, only UQCRC1, GAPDH and CKM have been previously identified as related to meat tenderization rate.

Creatine kinase M-type (CKM) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were identified in several studies as positively correlated to several meat quality traits (tenderness, WHC, pH and color) from different species (Lametsch et al., 2002; Marino et al., 2014; Gagaoua et al., 2021; della Malva et al., 2022). In the context of tenderness determination, these sarcoplasmic proteins could be linked to the greater abundance in muscles characterized by a high percentage of fast-twitch muscle fibers such as LTL muscle (Okumura et al., 2005), although muscle fiber typing is necessary in donkey meat to confirm this hypothesis.

Aging was also found to impact several proteins involved in the mitochondrial oxidative phosphorylation processes (UQCRC1, ATP5PD, COX5A), evidencing the importance of this pathway as previously suggested (Sierra & Olivan, 2013; Grabez et al. 2015). UQCRC1 is a subunit of the cytochrome b-c1 complex involved in the regulation of energy metabolism and balance, which is also known as complex III of oxidative phosphorylation (Kunej et al., 2007). COX5A is the catalytic part of mitochondrial cytochrome-c oxidase, which catalyzes the production of ATP,

while ATP5PD catalyzes the rate-limiting step of ATP formation (Izquierdo, 2006). The release of cytochrome C from mitochondria into the cytoplasm is used as a hallmark of apoptosis onset (Sierra & Olivan, 2013; Ouali et al., 2013). Cytochrome C content in the mitochondria fraction rapidly decrease but activated caspase-3 and caspase-9 enzymes lead to the initial apoptosis phase, which may greatly influence meat tenderness (Wang et al., 2017). Further investigations are warranted on this pathway in donkey muscle to better understand the role of mitochondria and apoptosis in the determination of its final meat quality traits.

Although no data regarding the correlation of COX5A pattern to the meat tenderization process have been reported, ATP5PD has already been found to be related to the post-mortem changes in muscle but, only in beef (Gagaoua et al., 2021). In our study, the presence in donkey meat of these protein spots after 14 days of aging highlight the key role of mitochondrial apoptosis in the post-mortem processes and also suggest that these proteins can be further candidate biomarkers to explain or monitor meat tenderness.

In response to stress, cells rapidly produce a series of proteins known as heat-shock proteins (Lomiwes et al., 2014; Picard and Gagaoua, 2017, 2020). Among them, the small heat shock protein HSPB1 has been shown to interact with actin, suggesting that it could play a role in controlling actin filament dynamics and degradation (Ouali et al., 2013). However, the involvement of HSPB1 in the conversion of muscle to meat, as well as meat tenderness and other qualitative attributes, should be further investigated in the context of donkey muscle. In our study the greater abundance of HSPB1 at 14 days of aging in donkey meat highlight the possible role of such pathway in driving the final quality of donkey and its use as a predictor of donkey meat tenderness.

Differences in results using different protein separation methods (1-DE and 2-DE) is a common issue in proteomics. For example, 2-DE analysis has some limitations, such as the co-migration of certain proteins, and poor resolution and separation of basic and hydrophobic proteins. In addition, proteins in low abundance are often not revealed by conventional stains. Another issue of the 2-DE analysis is that, when applied to unfractionated complex samples that can be the case in analysis post-mortem muscle; it analyzes/shows the most abundant proteins only. To overcome these drawbacks, we suggest in-depth characterization using shotgun proteomics (Zhu et al. 2021)

5. Conclusion

The study of meat tenderization and underlying mechanisms behind the conversion of muscle into meat in Martina Franca donkey breed allowed to conclude that its meat can be judged as tender

after an aging time of 14 days. This study evidenced also the presence of known markers of tenderness likely the appearance after 14 days of aging of the major proteolytic fragments of 110 and 30 kDa on SDS-PAGE. This is accompanied by the appearance with aging of the degradative products of troponin-T and desmin. The proteomic approach applied for the first time on donkey meat by means of two-dimensional gels combined with LC-MS/MS confirmed the impact of aging on muscle structure and myofibrillar proteins. Other pathways, likely energy metabolism and the response to stress by the identification of HSP27 supports the known knowledge on meat tenderization. The putative biomarkers identified in this experiment would be considered for validation in the near future. The use of proteomics in the case of Martina Franca donkey breed opens new possibilities in deciphering the biological mechanisms underpinning the tenderization process of this species that is not extensively characterized. The results of this study will help also in predicting the potential quality of the end-product and in identifying protein biomarkers for monitoring the desirable meat quality traits in donkey.

Conflict of interest

The authors declare no conflict of interest.

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CRediT authorship contribution statement

Antonella della Malva: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Mohammed Gagaoua**: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Antonella Santillo:** Conceptualization, Funding acquisition, Investigation, Methodology, Writing – review & editing. **Pasquale De Palo:** Conceptualization, Funding acquisition, Investigation, Methodology, Writing – review & editing. **Agostino Sevi:** Conceptualization, Funding acquisition, Investigation, Methodology, Writing – review & editing. **Marzia Albenzio**: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing

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Figure 1. Representative SDS-PAGE profile of **A)** myofibrillar and **B)** sarcoplasmic fractions from *Longissimus lumborum* muscle of Martina Franca donkey after 1, 6 and 14 days (d) of aging. Abbreviations used: PHb= Phosphorylase b; PGM1= Phosphoglucomutase1; ENO3= beta-enolase; CKM= Creatine kinase M-type; PGAM= Phosphoglycerate mutase; ACTN= a-actinin; DES= desmin; TNNT3= Troponin-T; MYL1= myosin light chain 1; MYL2= myosin light chain 2. The blue and red arrow highlight the major proteolytic fragments appearing at 110 and 30 kDa respectively after 14 days of aging.

Figure 2. Representative immunoblots of **A)** desmin, **B)** troponin-T and **C)** calpastatin inhibitor performed on *Longissimus lumborum* muscle protein extracts from Martina Franca donkey after 1, 6 and 14 days (d) of aging.

Figure 3. Representative two-dimensional gel electrophoresis (2DE) and differentially expressed proteins. **A)** 2DE gels from the myofibrillar fraction of *Longissimus lumborum* muscle of Martina Franca donkey after 1, 6 and 14 days of aging. **B)** Representative 2DE map of the myofibrillar fraction highlighting the differentially expressed proteins (see **Table 3** for the identity of the proteins in each spot). **C**) Principal component analysis (PCA) highlighting the relationships between the meat quality traits (WBSF, TPA, MFI, total collagen and WHC) and the volume intensity of the differential protein spots at 1, 6 and 14 days of aging.

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Figure 4. Protein-protein interaction analysis of the differentially expressed proteins as affected by aging time. The proteins in the network are further explored in terms of the pathways were they are involved in terms of energy, mainly those playing a role in the oxidative pathway of energy production and the myofibrillar and structural protein as they are allocated in the sarcomere.

Figure 5. Biological pathway and process enrichment analysis using Metascape® on the proteins identified in the myofibrillar proteome extract to be changing during aging in Martina Franca donkey meat. **A)** Circos plot showing the common proteins between 1 and 14 days of aging. **B)** Heatmap based on significantly enriched Gene Ontology (GO) terms using the protein lists of 1 and 14 days of aging. Gray indicate a lack of significance in that GO term in the corresponding aging time. **C)** Cluster process enrichment analysis based on the proteins present in each of six clusters colored by cluster ID, where nodes that share the same cluster are typically close to each other.

Table 1. Warner Bratzler share force (WBSF), texture profile analysis (TPA), myofibrillar fragmentation index (MFI), total collagen, and water holding capacity (WHC) of *Longissimus lumborum* muscle from Martina Franca donkey after 1, 6 and 14 days of aging (means ± SE).

Variables	**Aging time**			**SEM**	**Effect, P**
	d1	**d6**	**d14**		
WBSF (N/cm^2)	44.64 a	39.11 b	27.46 c	1.68	**
Hardness (N)	75.53 a	51.14 b	45.78 b	5.96	**
Cohesiveness	0.14	0.15	0.15	0.01	NS
Springiness (mm)	7.89	7.84	7.08	0.28	NS
Gumminess (N)	8.61 a	7.88 a	6.78 b	0.32	**
Chewiness (N x mm)	67.95 a	61.72 a	47.95 b	2.88	**
MFI	52.35 b	82.62 a	104.47 a	8.89	**
Total collagen (μg/mg)	34.44 a	33.37 ab	28.77 b	1.58	**
WHC (%)	6.68 a	4.22 b	3.54 b	0.81	*

NS= not significant; *= P<0.05; **=P<0.01; ***=P<0.001.

Table 2. Relative abundance (%) of desmin, troponin T and calpastatin quantified from immunoblotting membranes on the *Longissimus lumborum* muscle samples taken from Martina Franca donkey after 1, 6 and 14 days of aging (means ± SEM).

Targeted proteins	**Aging time**			**SEM**	**Effect, P**
	d1	**d6**	**d14**		
Desmin					

myofibrillar fraction					
54 kDa	64.63 a	56.72 b	41.49 c	1.21	***
46 kDa	20.15	21.10	21.09	0.47	NS
45 kDa	10.90	10.18	10.10	0.54	NS
40 kDa	4.31 c	9.23 b	13.29 a	1.05	***
37 kDa	ND	2.76 b	14.02 a	0.87	***
Troponin T					
myofibrillar fraction					
37 kDa	25.51 a	19.81 b	3.15 c	1.72	***
36 kDa	22.80 a	12.25 b	10.31 b	0.87	**
34 kDa	23.05	25.95	25.85	1.02	NS
33 kDa	5.51 c	10.05 b	13.05 a	0.62	**
30 kDa	7.59 c	13.26 b	20.09 a	0.74	***
28 kDa	15.53 b	18.68 b	27.54 a	1.29	***
Calpastatin					
sarcoplasmic fraction					
70 kDa	2.50	ND	ND	0.01	***
58 kDa	7.18	8.62	8.61	0.64	NS
50 kDa	15.85	16.57	16.17	0.32	NS
37 kDa	58.61 a	47.23 b	22.29 c	0.98	**
30 kDa	ND	ND	22.06	0.02	***
29 kDa	15.85 c	27.57 b	30.86 a	0.96	**

ND= not detected; NS= not significant; *=P<0.05; **=P<0.01; ***=P<0.001. a, b, c= P< 0.05 in the row (aging effect).

Table 3. Identified proteins from 2DE myofibrillar fraction of *Longissimus thoracis* muscle of Martina Franca donkey meat as affected by aging time.

Spot number	Identified protein	Gene name	Uniprot ID	MW	pI	Matched peptides	Sequence Coverage (%)	Score	Up at
975	Myosin regulatory light chain 2, skeletal muscle isoform	MYLPF	F6RW00	19.03	4.92	8	41.76	4086.57	day 1
1182	Myosin regulatory light chain 2, skeletal muscle isoform	MYLPF	F6RW00	19.03	4.92	19	88.82	9225.33	day 1
1208	Tropomyosin 2	TPM2	A0A3Q2H546	32.82	4.70	25	62.68	5176.64	day 1
1265	Myosin regulatory light chain 2, skeletal muscle isoform	MYLPF	F6RW00	19.03	4.92	12	62.94	6163.72	day 1
1292	Tropomyosin alpha-1 chain	TPM1	A0A3Q2I808	28.55	4.74	17	52.82	4335.01	day 1
1307	Myosin regulatory light chain 2, skeletal muscle isoform	MYLPF	F6RW00	19.03	4.92	18	91.18	14256.95	day 1

1865	Myosin light chain 1/3, skeletal muscle isoform	MYL1	A0A5F5PWZ5	16.67	4.68	8	58.00	5754.81	day 1
2105	Myosin light chain 1/3, skeletal muscle isoform	MYL1	F6ZGD1	20.85	5.10	10	54.40	3011.66	day 1
2291	Myosin regulatory light chain 2, skeletal muscle isoform	MYLPF	F6RW00	19.03	4.92	8	45.29	3486.42	day 14
3488	Myosin regulatory light chain 2, skeletal muscle isoform	MYLPF	F6RW00	19.03	4.92	6	42.94	457.37	day 14
3494	Actin, alpha skeletal muscle	ACTA1	F7CZ92	42.02	5.39	15	49.07	1190.72	day 14
3510	Actin, alpha skeletal muscle	ACTA1	F7CZ92	42.02	5.39	16	55.17	1926.59	day 14
3522	Actin, alpha skeletal muscle	ACTA1	F7CZ92	42.02	5.39	15	49.07	1559.67	day 14
3558	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	F6YV40	35.80	8.13	4	23.12	855.77	day 14
3609	Actin, alpha skeletal muscle	ACTA1	F7CZ92	42.00	5.39	24	72.15	7908.06	day 14
3629	Actin, alpha skeletal muscle	ACTA1	F7CZ92	42.02	5.39	22	73.21	3607.64	day 14
3698	Troponin C, skeletal muscle	TNNC2	F6W0D2	18.08	4.20	16	95.00	13057.62	day 14
3702	Myosin-1	MYH1	A0A3Q2GYL4	222.63	5.82	38	19.98	4049.6	day 14
3701	Myosin-1	MYH1	A0A3Q2GYL4	222.63	5.82	59	29.43	6168.1	day 14
4019	Myosin-2	MYH2	F6QLB5	222.50	5.86	28	13.22	1658.7	day 14
4037	Myosin-2	MYH2	F6QLB5	222.45	5.86	26	11.31	1703.9	day 14
4298	Cytochrome b-c1 complex subunit 1, mitochondrial	UQCRC1	UQCRC1	52.64	6.10	13	27.92	1052.13	day 14
5781	Actin, alpha skeletal muscle	ACTA1	F7CZ92	42.02	5.39	10	28.38	514.78	day 14
5868	Heat shock protein beta-1	HSPB1	F7E3Y7	23.10	6.55	9	32.54	1504.60	day 14
5872	Actin, alpha skeletal muscle	ACTA1	F7CZ92	42.02	5.39	19	66.58	3401.70	day 14
5889	Heat shock protein beta-1	HSPB1	F7E3Y7	23.10	6.55	10	32.54	1920.59	day 14
6102	Actin, alpha skeletal muscle	ACTA1	F7CZ92	42.02	5.39	18	58.36	3158.25	day 14
6557	Myosin light chain 6B	MYL6B	F6QBL3	22.91	5.53	7	23.56	561.08	day 14
6988	Cytochrome c oxidase subunit 5A, mitochondrial	COX5A	F7CZM1	16.72	7.42	4	21.05	311.63	day 14
7101	Myosin-1	MYH1	A0A3Q2GYL4	222.63	5.82	17	7.54	1382.08	day 14

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7105	Myosin-2	MYH2	F6QLB5	222.50	5.86	18	7.75	997.99	day 14
7174	ATP synthase subunit d, mitochondrial	ATP5PD	A0A5F5Q2Y2	15.78	8.05	4	29.20	243.95	day 14
7279	Heat shock protein beta-1	HSPB1	F7E3Y7	23.10	6.55	7	28.71	1487.24	day 14
7309	Actin, alpha skeletal muscle	ACTA1	F7CZ92	42.02	5.39	15	44.56	1381.00	day 14
8307	Creatine kinase M-type	CKM	F7BR99	43.14	7.28	17	44.36	2057.36	day 14
8489	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	F6YV40	35.80	8.13	14	45.35	3881.53	day 14
9306	Myosin-2	MYH2	F6QLB5	222.45	5.86	19	7.54	1297.05	day 14

Highlights

- Significant impact of aging on texture traits of donkey meat
- Martina Franca donkey meat can be judged as tender after 14 days of aging
- Tenderization of donkey meat is supported by the appearance of TNNT3 and desmin fragments
- First proteomic study on donkey meat aiming to discover protein biomarkers
- Donkey meat tenderization is a consequence of interconnected pathways
- Changes in 15 proteins during the aging process of donkey meat

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