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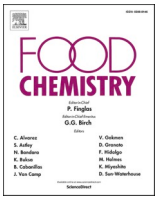

A proteomic approach to identify biomarkers of foal meat quality: A focus on tenderness, color and intramuscular fat traits

María López-Pedrouso [a], José M. Lorenzo [b,c], Aurora Cittadini [b,d], María V. Sarries [d], Mohammed Gagaoua [e], Daniel Franco [b,f,*]

[a] Department of Zoology, Genetics and Physical Anthropology, Universidade de Santiago de Comspostela, Santiago de Compostela, 15872, A Coruña, Spain
[b] Centro Tecnológico de la Carne de Galicia, Rúa Galicia Nº 4, Parque Tecnológico de Galicia, San Cibrao das Viñas, 32900 Ourense, Spain
[c] Área de Tecnología de los Alimentos, Facultad de Ciencias de Ourense, Universidad de Vigo, 32004 Ourense, Spain
[d] Instituto de Innovación y Sostenibilidad en la Cadena Agroalimentaria (IS-FOOD), Universidad Pública de Navarra (UPNA), Campus de Arrosadia, 31006 Pamplona, Spain
[e] PEGASE, INRAE, Institut Agro, 35590 Saint-Gilles, France
[f] Department of Chemical Engineering, Universidade de Santiago de Compostela, Campus Vida, 15782 Santiago de Compostela, Spain

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ABSTRACT

Foal meat is considered a healthy alternative to other meat sources and more environmentally sustainable. However, its quality is highly variable and there is lack of knowledge about the molecular mechanisms underlying its determination. Genotype and diet play a relevant role as the main factors that can allow a control of the final quality and the use of high-throughput analytical methods such as proteomics is a way to achieve this lofty goal. This research aimed to study-two breeds (Burguete and Jaca Navarra) supplemented with two different finishing diets: conventional concentrate and straw (C) *vs* silage and organic feed (S). The proteomic approach built a library of 294 proteins that were subjected to several statistical and bioinformatic analyses. Burguete breed finished with concentrate produced higher meat quality in terms of tenderness, intramuscular fat and color lightness mainly due to the high abundance of energy metabolic proteins. Tenderness was correlated to myofibrillar proteins (ACTA1, MYBPH, MYL1 and TNNC1) and energy metabolic proteins (ALDOA, CKM, TPI1 and PGMA2). Regarding color, the main pathways were energy metabolism, involving several glycolytic enzymes (ALDOA, PKM, PFKM and CKM). Oxidative stress and response to stress proteins (HSPA1A, SOD2 and PRDX2) were further involved in color variation. Moreover, we revealed that several proteins were related to the intramuscular fat accordingly to the breed. This study proposed several candidate protein biomarkers for foal meat quality that are worthy to evaluate in the future.

1. Introduction

Horse meat consumption is emerging as a healthy and nutritious alternative to traditional meat sources (beef or pork). Moreover, environmental and health concerns support the production of meat from pasture, such as horses raised under extensive livestock production systems. Nevertheless, horse meat is still not widely consumed in developed countries despite its excellent nutritional value characterized by a high content of protein, and iron as well as a low-fat and cholesterol amounts and a healthy fatty acid profile (Franco & Lorenzo, 2014; Franco et al., 2011). In the past, horse meat was not always fully accepted for social, historical, cultural and religious reasons as well as for the negative image acquired due to fraudulent practices, especially in beef adulteration. However, during the last decade changes were observed in consumers' attitudes towards this type of meat (Cittadini, Sarriés, Domínguez, Indurain, & Lorenzo, 2021). Indeed, according to The Food and Agriculture Organization (FAO), horsemeat production is slowly increasing, recording an increment of 7.57 % between 2010 and 2020 (FAOSTAT, 2021).

Generally speaking, horse meat is particularly considered a tough meat leading to a lower acceptability. Thus, any possibility to improve its tenderness should be a key driver for consumer-purchase choice and decision. Collagen and intramuscular fat are contributing factors to meat tenderness and textural properties. Collagen presents intermolecular

* Corresponding author at: Department of Chemical Engineering, Universidade de Santiago de Compostela, Campus Vida, 15782 Santiago de Compostela, Spain.
E-mail address: daniel.franco.ruiz@usc.es (D. Franco).

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crosslinks which provide structure and strength, as well as, to a lesser extent, elastin and reticulin (Roy, Walker, Rahman, Bruce, & McMullen, 2018). Furthermore, the sarcomere shortening during the ageing process and the proteolysis of myofibrillar proteins affect meat toughness (Ertbjerg & Puolanne, 2017). In *pre-rigor mortis*, there is no supply of oxygen, but the muscle tissue remains unaffected. Afterwards, during the *rigor mortis* phase there is a lack of the production of ATP and phosphocreatine favoring a high rigidity. Finally, the *postmortem* phase is related to the proteolytic activity of enzymes such as calpains, cathepsins, proteasome and caspases systems (Lana & Zolla, 2016; Ouali et al., 2006). In addition, oxidative stress is also a pivotal pathway that has been used as an indicator of structural protein degradation during the *postmortem* phase and it is consequently associated with beef tenderness (Gagaoua, Terlouw, et al., 2021; Malheiros et al., 2019). Another peculiar feature of the horse meat is its dark color with a bluish sheen produced by the high content of myoglobin. Myoglobin is a sarcoplasmic heme protein, mainly responsible for meat color depending on its oxidation state (Purslow, Gagaoua, & Warner, 2021). In this sense, the myoglobin has a high ability to combine red oxymyoglobin with oxygen-generating brown metmyoglobin.

Proteomics is a powerful tool that enables in-depth characterization of the molecular pathways involved in meat color variation as well as for the discovery of protein biomarkers. The relationship between proteomic changes and tenderness has been widely studied in beef (Gagaoua, Terlouw, et al., 2021; Zhao et al., 2014) and pork (Huang, Larsen, Palmisano, Dai, & Lametsch, 2014; López-Pedrouso, Lorenzo, Gagaoua, & Franco, 2020). Indeed, protein biomarkers linked to meat quality have been searched in beef and pork and relevant biological pathways such as muscle contraction, metabolism, response to stress, oxidation, proteolysis and apoptosis among other pathways were found to be involved in the tenderness of meat. On the other hand, in horse, few studies have been reported using proteomics to investigate the variation in its meat quality (Beldarrain, Sentandreu, Aldai, & Sentandreu, 2022; della Malva et al., 2021). Therefore, this work aimed to understand the molecular mechanisms of tenderness, color and intramuscular fat in foal meat and to identify candidate protein biomarkers related to these relevant quality traits. This information will bring us new insights for management decisions to ensure a high and consistent foal meat quality.

2. Materials and methods

2.1. Rearing and slaughtering of the foals

Twenty male foals, ten from Jaca Navarra and ten from Burguete were used in this trial. The horses were from local farms after a weaning period and reared under a semi-extensive livestock system. From 12 to 17 months of age, foals were fed at pasture and then kept indoors on an experimental farm of the Institute for Agri-food and Technology and Infrastructures of Navarre (Roncesvalles, Navarra, Spain). Finally, each genotype was divided into two equal parts and finished during 3–4 months with two different diets. Ten male foals (5 of Jaca Navarra and 5 of Burguete breed) were fattened with conventional concentrates (starter and finisher ones) and straw (*ad libitum*) (C). The other group of ten male foals (5 of Jaca Navarra and 5 of Burguete breed) were finished with silage (produced by local farmers) and an organic fodder (with certification of UE/no UE Organic Agriculture), where silage represented the majority component of the diet (approximately 60 % of silage and 40 % of organic feed) (S). Additional details were previously reported (Cittadini, Sarriés, Domínguez, Pateiro, & Lorenzo, 2022).

Briefly, foals at 21 months of age were transported to the slaughterhouse (Protectora de carne S.L., Salinas de Pamplona, Navarra, Spain) the day before the sacrifice following the European regulations (European Commission, 2005) and the conditions described in a recent work (Cittadini et al., 2022). The foals were stunned in the frontal region of the head with a captive bolt, slaughtered and dressed according to the Council Regulation 1099/2009 (European Commission, 2009). Then, the carcasses were chilled for 24 h in a conventional room at 0 °C and the following day, the half-carcasses were transported under refrigeration to Cárnicas Mutiloa (Sangüesa, Navarra, Spain) and stored in a cooling chamber at 0 °C, for four days in line with the local procedure. Subsequently, the *longissimus thoracis and lumborum* (LTL) muscle from each half-carcass was removed from the sixth to the thirteenth ribs and cut into 25 mm thick steaks, vacuum-packed and stored at −20 °C until physicochemical and proteomic analyses.

2.2. Foal meat quality

Chemical composition (moisture, intramuscular fat, protein, ash, iron content and cholesterol), pH, color parameters [lightness (L*), redness (a*), yellowness (b*), chroma (c*), hue (h*), myoglobin (Mb), metmyoglobin (MMb) and oxymyoglobin (OMb)], cooking loss and shear force were assessed following the protocols detailed in Cittadini et al. (2022) and Pateiro et al. (2013).

2.3. Proteomic analysis

2.3.1. Protein extraction and trypsin digestion

Lyophilized horse meat powder (50 mg) was mixed in RIPA buffer containing 200 mmol/L Tris/HCl (pH 7.4), 130 mmol/L NaCl, 10 % (v/v) glycerol, 0.1 % (v/v) SDS, 1 % (v/v) Triton X-100 and 10 mmol/L $MgCl_2$ together with anti-proteases and anti-phosphatases (Sigma-Aldrich, St. Louis, MO, USA) by a TissueLyser II (Qiagen, Tokyo, Japan). The suspended solids were separated by centrifugation at 14,000g at 4 °C for 20 min. In the solution, protein concentration was measured using an RC-DC kit (Biorad Lab., Hercules, CA, USA) following the manufacturer's instructions. To eliminate the impurities from protein fractions, the protein aliquots of 100 μg were subjected to one-dimensional SDS-PAGE. The electrophoresis was performed until the protein is concentrated on the top of the resolving gel (10 %) in a single band. This band was then carefully excised using sterile scalpels and cut into small pieces, followed by a washing with Milli-Q water and 50 mM ammonium bicarbonate in 50 % methanol. Subsequently, the dehydration was carried out with acetonitrile and a vacuum centrifuge. Finally, the protein sample was reduced with 10 mM DTT in 50 mM ammonium bicarbonate at 60 °C for 30 min and alkylated with 55 mM iodoacetamide solubilized in ammonium bicarbonate at room temperature for 30 min in darkness. Afterwards, the protein solution was digested by trypsin (Promega, Madison, USA) at a concentration of 20 ng/μL in 20 mM ammonium bicarbonate at 37 °C for 16 h. The final solution of peptides was mixed with 0.1 % formic acid and stored at −20 °C until LC-MS/MS analysis.

2.3.2. Generation of the reference spectral library

A mixture of 4 μg of peptides from each sample was prepared and analysed by a shotgun data-dependent acquisition proteomic approach by micro-LC-MS/MS. The peptides were separated by the micro-LC system Ekspert nLC425 (Eksigen, Dublin, CA, USA) using an YCM-TriartC18 column (150 μm × 0.3 mm, 12 nm, s-3 μm) (YMC CO, Japan) at a flow rate of 5 μL/min. The solvent A (water, 0.1 % formic acid) and solvent B (acetonitrile, 0.1 % formic acid) were mixed in a linear gradient from 5 % to 95 % B for 30 min, at 90 % B for 5 min and 5 % B for 5 min in an equilibration step. The mass spectrometer coupled model Triple TOF 6600 (SCIEX, Framingham, MA, USA) operates with a data-dependent acquisition mode following the parameters of 250 ms survey scans from 100 to 1500 m/z (25 ms of acquisition time) for a total cycle time of 2.8 s. The MS raw files were used to search in Uniprot Swiss-Prot databases using ProteinPilot software v.5.0.1. (SCIEX, Framingham, MA, USA). The false discovery rate (FDR) was set to 1 for peptides and proteins with a confidence score above 99 %.

2.3.3. Protein quantification

For accurate quantification, SWATH-MS was carried out by a DIA

method. A total of 20 samples from the four groups (five biological replicates in each group) were analyzed. Therefore, 4 μg of peptides were subjected to liquid chromatography using the same conditions as previously described. Regarding detection, the mass spectrometer operated with a 50 ms survey scan and the MS/MS analysis in a cyclic manner using an acquisition time of 50 ms in a total time of 6.3 s. A cycle included 65 scans per SWATH window of variable width (1 *m/z* overlap) covering the 400 to 1250 *m/z*. The data extraction and alignment of spectral were performed by PeakView v.2.2. (SCIEX, Framingham, MA, USA). These data matched the reference spectral library from the section above using the following settings: 10 peptides/protein and 7 fragments/peptide and FDR below 1 %. The protein quantification was calculated according to the sum of peak areas from all peptides and fragment ions of the protein. Moreover, two technical replicates were used for each meat sample.

2.4. Statistical analysis

For the statistical analysis of the meat quality attributes, a two-way ANOVA was performed for all variables considered, using the General linear model procedure of the SPSS package (SPSS 23.0, Chicago, IL). A fixed effect of breed and finishing feed was included in the model. The model used was:

$$Yij = \mu + Bi + Fj + (BxF)ij + \varepsilon ij.$$

where Yij is the observation of dependent variables, μ is the overall mean, Bi is the effect of breed, Fj is the effect of finishing feed, (BxF)ij is the interaction between breed and finishing feed and εij is the residual random error associated with the observation. Interaction term was initially included in the model, but finally it was not included in the table of results because there was no statistical significance ($P > 0.05$) for any of the evaluated variables.

For proteomics data analyses, a student's *t*-test was applied to compare groups in pairs identifying the proteins with significant differences, using a p-value scoring of 0.05 and a fold change of 1.5 as a cut-off. The correlations among individual protein quantifications and meat quality traits were determined by Pearson's linear correlation coefficient at two levels ($P < 0.01$ and $P < 0.05$) using standardized data (z-scores). Subsequently, the differentially abundant proteins within breed (Burguete and Jaca Navarra) were used to generate heatmaps using clustering analysis on XLSTAT 2.01 (Addinsoft SARL, París, France). The Euclidean distances were used based on z-scores.

The correlations between protein abundances and meat quality traits (shear force, color and intramuscular fat) were determined using Pearson's linear correlation coefficient. Only the proteins which showed a p-value < 0.05 and a coefficient of correlation higher than 0.7 or lower than −0.7 were considered in the manuscript.

For bioinformatics analyses, STRING v10.5 open source software was used to analyze the protein–protein interactions. Metascape® was further used for in-depth analyses. Gene Ontology (GO) processes enrichment on the list of proteins that were correlated with the investigated meat quality traits.

3. Results and discussion

3.1. Characterization of foal meat

Considering the chemical composition of the meat samples, the most outstanding characteristics are the low-fat (3.63–5.05 %) and high protein contents (20.03–20.64 %) as shown in Table 1. The values of intramuscular fat (5.10 ± 3.40 %) and protein (20.3 ± 0.26 %) were similar to those obtained from older horses (4 to 7 years old) (Stanisławczyk, Rudy, & Gil, 2020). Another important feature of foal meat is the low amount of cholesterol in comparison with lean meat of beef or pork (62 and 71 mg/100 g meat, respectively) (Ahmad, Imran, & Hussain, 2018) as confirmed in this study, with values varying from 38.90 to 41.70 mg/100 g of meat (Table 1). It has been reported that regular consumption of horse meat may contribute to reduce total and LDL cholesterol in healthy individuals (Del Bo' et al., 2013).

Concerning the textural parameters, values of cooking losses of 23.84–27.62 % and shear force of 26.5–37.19 N/cm^2 (Table 1) were obtained, that can be considered acceptable for consumption in terms of juiciness and tenderness. Additionally, the finishing diet based on concentrate increased the intramuscular fat and decreased the shear force in both breeds, which could improve the consumer acceptability. This is largely due to the horse breeds which are intended for meat

Table 1
Effect of breed (B) and animal feeding (F) on chemical composition, color parameters, pH, cooking losses and shear force of foal meat (values are expressed as mean ± standard deviation).

Parameter	Foal breed			Animal feed		
	Burguete	Jaca Navara	*p*-Value	Concentrate	Silage	*p*-Value
Chemical composition						
Moisture (%)	70.97 ± 1.36	71.92 ± 1.05	0.040	70.70 ± 1.32	72.19 ± 0.69	0.003
Intramuscular fat (%)	4.79 ± 1.30	3.88 ± 1.01	0.047	5.05 ± 1.19	3.63 ± 0.79	0.004
Protein (%)	20.64 ± 0.87	20.03 ± 0.63	0.111	20.36 ± 0.94	20.30 ± 0.69	0.863
Ash (%)	1.11 ± 0.03	1.21 ± 0.07	0.002	1.16 ± 0.07	1.16 ± 0.08	0.941
Cholesterol (mg/100 g wet meat)	38.90 ± 3.07	41.70 ± 3.77	0.079	39.07 ± 3.56	41.53 ± 3.45	0.118
Color parameters						
Luminosity (L*)	39.29 ± 1.73	37.11 ± 2.75	0.052	38.84 ± 2.94	37.56 ± 1.91	0.234
Redness (a*)	14.24 ± 0.76	13.84 ± 1.19	0.410	14.13 ± 1.23	13.95 ± 0.75	0.698
Yellowness (b*)	13.54 ± 0.70	12.12 ± 1.04	0.002	13.13 ± 1.14	12.54 ± 1.09	0.156
Chroma (C*)	19.66 ± 0.84	18.43 ± 1.15	0.016	19.32 ± 1.27	18.77 ± 1.04	0.250
Hue (h°)	43.56 ± 1.79	41.23 ± 3.41	0.085	42.89 ± 3.31	41.90 ± 2.52	0.443
% Myoglobin	17.05 ± 3.05	21.47 ± 3.74	0.008	18.94 ± 2.94	19.58 ± 5.03	0.664
% Metmyoglobin	27.42 ± 1.75	26.78 ± 2.31	0.519	27.17 ± 1.60	27.04 ± 2.46	0.890
% Oxymyoglobin	55.13 ± 1.37	52.18 ± 2.75	0.009	53.81 ± 2.41	53.50 ± 2.90	0.754
Fe (mg/100 g wet meat)	1.93 ± 0.30	2.39 ± 0.43	0.013	2.26 ± 0.50	2.06 ± 0.35	0.260
Quality parameters						
Ultimate pH	5.55 ± 0.07	5.59 ± 0.05	0.140	5.61 ± 0.05	5.53 ± 0.06	0.004
Cooking losses (%)	24.08 ± 3.36	27.39 ± 2.42	0.005	23.84 ± 2.78	27.62 ± 2.75	0.002
Shear force (N)	28.39 ± 7.51	35.31 ± 6.74	0.004	26.51 ± 6.41	37.19 ± 4.90	0.000

The interactions breed × animal feed were not significant (P > 0.05), therefore they are not shown.

production along with the finishing feed and their early slaughter age. It is worthy to mention that this last factor has an important effect on tenderness, as reported in earlier studies using horses at the age of 4 to 7 years, which presented tougher meats with a shear force of 96.7 ± 4.01 N/cm2 (Stanisławczyk et al., 2020). Moreover, both considered effects had in this study a significant ($P \leq 0.004$) influence on meat shear force. Nevertheless, most of the quality parameters evaluated were more defined by breed than finishing diet, as expected. These results are in line with those previously discussed in a former study (Cittadini et al., 2022).

In this study, Burguete meat had ~~a~~ higher ($P < 0.05$) intramuscular fat percentage than those of Jaca Navarra~~,~~ which could be related to the distinct carcass characteristics and morphology of these two breeds (Cittadini et al., 2021). Therefore, these animals had also lower ($P < 0.01$) myoglobin values, which could be due to the different temperament, physical activity and, consequently, metabolic muscle properties of these two equine breeds. Data, additionally, showed a tendency among groups for cholesterol values ($P < 0.1$), where Burguete meat had the lowest contents in comparison to Jaca Navarra.

Concerning diet, samples from the C group reported the greatest ($P < 0.01$) fat percentages, probably due to the composition of this diet, which contained higher fat amounts than the S group (Cittadini et al., 2022). In the same line, the C group improved the texture properties, that showed significantly lower ($P < 0.001$) shear force values.

The above results were further confirmed by the PCA analysis (Fig. 1). In the PCA, the first two principal components (PC) explained 52.15 % of the total variation, with principal component 1 (PC1) accounting for 35.31 % and PC2 for 16.85 %, respectively. In detail and, considering the four groups, it seemed that Burguete foals fed with conventional concentrates (C group) accumulated the greatest amounts of intramuscular fat and the lowest values of cholesterol. Furthermore, they were related to lightness (L*) and yellowness (b*) and stood out for their enhanced meat quality attributes, such as the reduced toughness, which could increase consumers' acceptability. On the contrary, Jaca Navarra foals supplemented with silage reported the opposite trend. These results are in accordance with (Cittadini et al., 2022) who demonstrated that foal meat from Burguete finished with a traditional concentrate showed the highest quality standards in terms of tenderness and juiciness.

3.2. *Comparative analysis of proteome from meat samples from two breeds (Burguete and Jaca Navarra) under two finishing diets (concentrate and silage)*

The proteomic approach used in this study quantified 294 proteins. The heat map based on unsupervised clustering analysis is given in Fig. 2. A significant clustering around breeds was observed, meanwhile, a lower impact of finishing diet was observed on muscle proteome. This is confirmed by the grouping of the animals within each breed. The foals highlighted in blue grouped mainly the meat from Burguete breed, whereas the brown cluster consisted of Jaca Navarra breed meat samples. This demonstrates a proteomic pattern associated with the horse breed. Genotype has a prominent effect on farm animal features, which is reported to be linked to muscle proteomic. Indeed, proteomics was traditionally used to differentiate the breeds as a tool for product authentication (Rodríguez-Vázquez et al., 2020). Feeding and management strategies are also used to enhance meat quality, but with a lower impact on the final product than genotype (Alemneh & Getabalew, 2019). In general, animal feeding studies often focus on intensive and extensive production systems, using proteomic tools to differentiate them (de Melo et al., 2020; Fonseca et al., 2019).

Even though stronger proteomic differences were attributed to the breed effect, within each studied genotype, there were also observed variations in muscle protein abundances associated with the diet (Fig. 3). Regarding diet effect, our results were accurate and reproducible in both breeds. Firstly, the impact of finishing diet for conventional concentrates and straw (C) in comparison with silage (S) for 3–4 months was detected. In this case, the dietary treatments induced changes at the muscle proteome level, affecting the final meat quality traits. A volcano plot assessed differential protein expression displaying the protein changes due to this factor of variation. Red points represent the proteins with the largest statistically significant changes (fold-change ≥ 30; p-value ≤ 0.05) as a response to the ~~by~~ finishing diets. In detail, the points with red color located on the left represent the proteins significantly over-abundant in feeding with silage, meanwhile on the right represent the proteins over-abundant in feeding with concentrates and straw (C). In the Burguete breed, four proteins were significantly over-abundant due to the silage diet: ATP synthase subunit (ATP5PO), PDZ domain-containing protein (AHNAK), albumin (ALB) and annexin (ANXA5). Eight proteins were over-abundant in the traditional concentrate finishing diet: enoyl-CoA hydratase 1 (ECH1), AIR carboxylase (PAICS), alpha-2-HS-glycoprotein (AHSG), phosphoglycerate mutase (PGAM2), triosephosphate isomerase (TPI1), mono-ADP ribosylhydrolase 1 (MACROD1), nebulin (NEB) and malate dehydrogenase (MDH1).

For the Jaca Navarra breed, eight proteins were over-abundant as a consequence of silage feeding: hydroxiacylglutathione hydrolase (HAGH), heat shock protein beta 6 (HSPB6), voltage-dependent anion-selective channel protein 3 (VDAC3), selenium binding protein 1 (SELENBP1), peptidyl-prolyl cis–trans isomerase (PPIA), guandinoacetate *N*-methyltransferase (GAMT), acid phosphatase (ACP1) and fatty

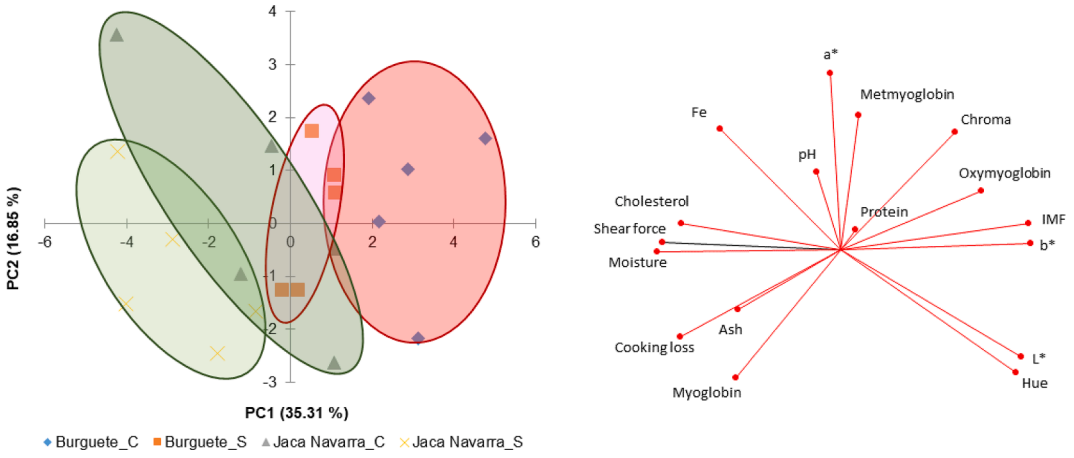

Fig. 1. Principal Component Analysis in foal meat from two breeds (Burguete and Jaca Navarra) finished with two diets (C = conventional concentrate and straw and S = silage and organic feed), using chemical composition and quality traits (moisture, intramuscular fat, protein, ash, cholesterol, luminosity = L*, redness = a*, yellowness = b*, chroma, hue, myoglobin, metmyoglobin, oxymyoglobin, Fe, pH, cooking losses and shear force).

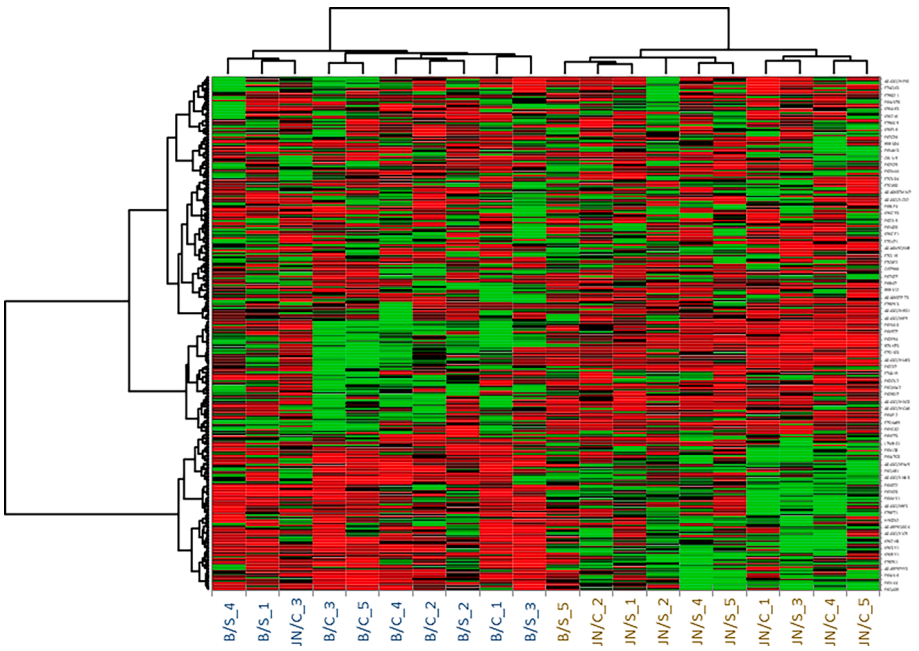

Fig. 2. Heat map analysis based on the 294 quantified proteins The values of protein quantifications were transformed in the base 2 logarithm scale for five biological replicates. The *longissimus thoracis* and *lumborum* muscle from two breeds (Burguete and Jaca Navarra) and two finishing diets (C = conventional concentrate and straw and S = silage and organic feed) were used for the analysis. Bright green and red represents the higher and lower abundance of proteins, respectively. Two main clusters (blue and brown) were merged from protein abundance showing similar proteomic profiles for each breed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

acid-binding protein 3 (FABP3). In the case of concentrate feeding, two proteins were over-abundant ATP synthase subunit beta (ATP5F18) and Smoothelin like 2 (SMTNL2).

At first glance, it may appear that the effect of the two finishing diets on the proteome of Burguete and Jaca Navarra foals cannot be compared. However, an in-depth assessment showed that variations in the diet affected energy metabolism, mainly glucose and lipid metabolism within the two breeds. In Burguete meat samples, several proteins such as MDH1, PGAM2 and TPI1 from carbohydrate metabolism mainly consisting of glycolysis, tricarboxylic acid cycle and oxidative phosphorylation were affected. According to our results, both ATP synthase members (ATP5F18 and ATP5PO) were influenced by dietary changes in both breeds. In the case of the TCA cycle, the pyruvate is converted into acetyl coenzyme A producing NADH. Thus, NADH is used in the respiratory chain complexes to produce large amounts of ATP by means of ATP synthase (Bonora et al., 2012). On the other hand, fatty acids play critical roles in the energy metabolism of mammals. Our results showed significant differences in lipid transporter fatty acid-binding protein 3 (FABP3) from Jaca Navarra foals and albumin (ALB) from Burguete ones, which are related to lipid metabolism. FABP3 plays an important role in skeletal muscle participating in fatty acids uptake and cytosolic transport showing a high binding affinity for palmitic, oleic and stearic acids. The ALB can transport long-chain fatty acids and consequently plays a role in the fatty acid mobilization along the antioxidant activity in plasma. Differences in the regulation of the lipid metabolism which determine the final fatty acid composition of the muscle have important consequences on quality traits (Kim, Markkandan, Lee, Kim, & Yoo, 2020; Puig-Oliveras et al., 2014). In general terms, the shift in metabolism provoked by the type of diet underlies a proteomic-specific pattern associated with quality parameters of foal meat as in the case of other species.

3.3. Proteomic analysis related to meat quality traits (tenderness, colour and intramuscular fat) from Burguete and Jaca Navarra breeds

The relationship between the most important meat quality traits and protein abundances is displayed in Table 2. This table depicted the proteins with absolute values of correlation coefficients above 0.7. We identified 35, 67 and 21 proteins to be significantly correlated with shear force, color and intramuscular fat, respectively.

The prediction and control of meat tenderness using protein biomarkers is currently of great importance. Our results showed a significant association between tenderness (shear force) with 29 and 6 proteins in the case of Burguete, and Jaca Navarra, respectively. Among them, some proteins were from muscle filament sliding such as ACTA1 ($r = -0.745$), TNNC2 ($r = 0.858$), MYBPC1 ($r = -0.881$), MYBPC2 ($r = -0.715$), MYL1 ($r = -0.860$), TTN ($r = -0.721$), NEB ($r = 0.848$) and ACTN3 ($r = -0.782$). Furthermore, many of the proteins associated with tenderness resulted to be structural proteins including MYOM1 ($r = -0.796$), MYOM2 ($r = -0.715$), MYBPH ($r = -0.727$) and OBSCN ($r = -0.922$). Regarding the sign of correlation, most of them presented negative correlations meaning that tender meat have a higher abundance of mostly structural proteins according to other species (López-Pedrouso et al., 2019). It is known that tenderness is related to the breakdown of myofibrillar structure caused by *postmortem* proteolysis. According to Gagaoua, Terlouw, et al. (2021), Gagaoua, Warner et al. (2021), ACTA1, MYBPH, MYL1 and TNNC1 could be considered as very robust candidate biomarkers of beef tenderness.

Several other proteins correlated negatively with tenderness are involved in energy metabolism such as ALDOA ($r = -0.752$), CKM ($r = -0.797$), TPI1 ($r = -0.707$) and PGAM2 ($r = -0.739$). During the *pre-rigor* metabolism, energy metabolism mainly glycolysis contributes to tenderness development. Muscle metabolism is influenced by animal nutrition, pre-slaughter stress, muscle type and other factors leading to modifications in glycogen level and mitochondria status. In meat tenderness, the variation in proteolysis is a more critical factor than genetic considerations (Warner, Greenwood, Pethick, & Ferguson, 2010). Biochemical processes related to proteolysis of structural proteins particularly ACTA1, MYBPH, MYL1 and TNNC1 as well as ALDOA, CKM, TPI1 and PGMA2 involved in energy metabolism have been proved to be the most trusted biomarkers in the field of tenderness (Gagaoua, Terlouw, et al., 2021; Gagaoua, Warner et al., 2021).

Overall, these data agree with the information depicted in the string network within the clusters displayed in Fig. 4. There are two main protein clusters, one related to energy metabolism (ALDOA, CKM, PGAM2, TPI1 and NME2) and the other one involving structural proteins (ACTA1, TNNC2, MYBPC1, MYBPC2, MYL1, TTN, NEB and ACTN3).

Regarding color parameters, the concentration of myoglobin and its oxidation state play a key role in chromatic parameters (Gagaoua,

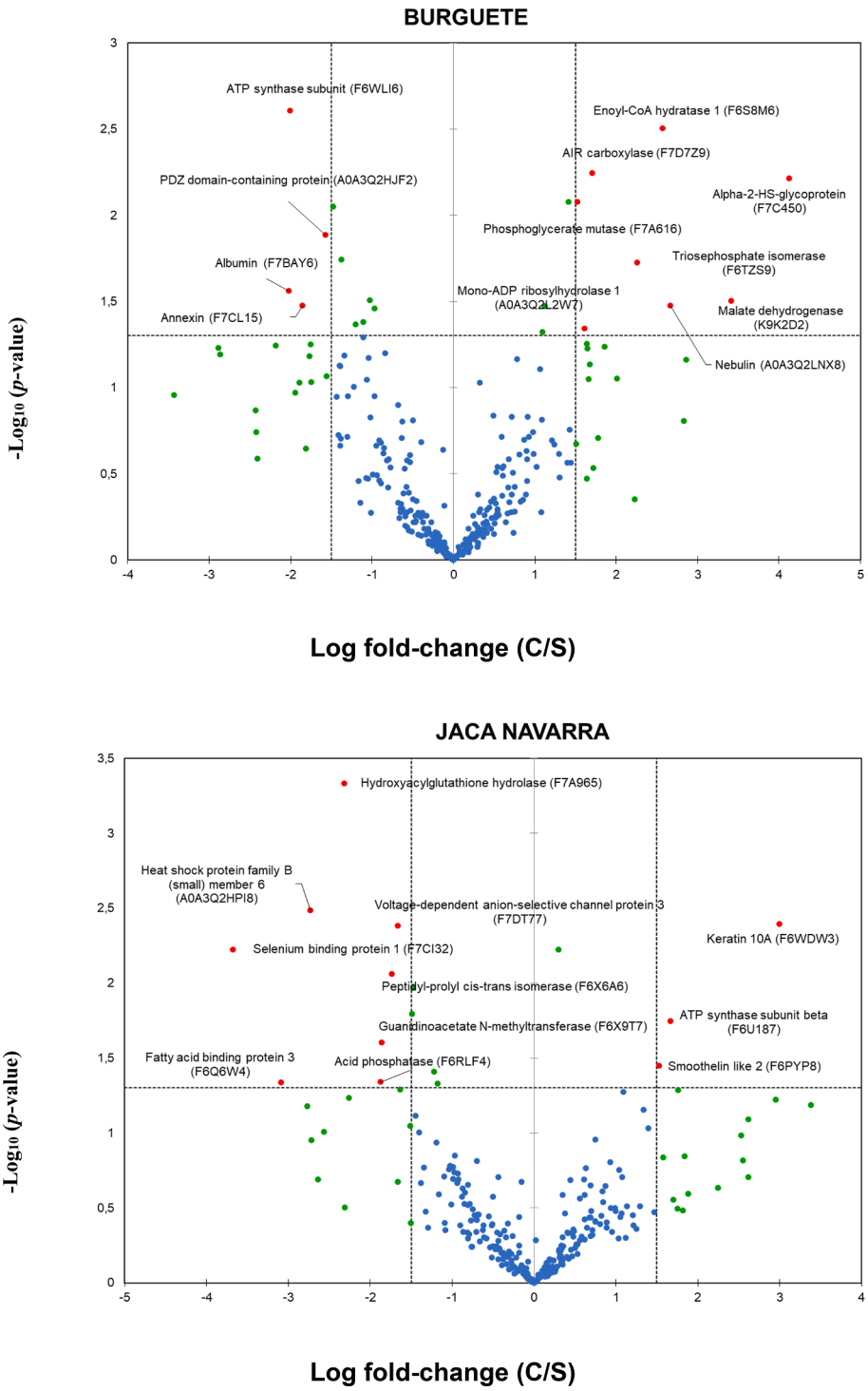

Fig. 3. Volcano plots in the comparison of two finishing diets (C = conventional concentrate and straw and S = silage and organic feed) for two breeds (Burguete and Jaca Navarra). The points with red colour located on the left represent the proteins significantly overexpressed in feeding with S, meanwhile on the right represent the proteins overexpressed in feeding with C. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Warner et al., 2021; Purslow, Warner, Clarke, & Hughes, 2020). The level of myoglobin is also impacted by other oxidative enzymes. In particular, horse meat has a dark red color with a bluish sheen connected with a its large amount of myoglobin. Other proteins such as haemoglobin and cytochrome *C* also contribute to meat color (Seong et al., 2019). The light scattering is mainly due to variations in the myofilament lattice spacing, sarcomere length and sarcoplasmic protein distribution (Purslow et al., 2020). It can be highlighted that lightness would be determined by modifications in the surface charge of myofibrillar proteins in connection with myofilament lattice spacing governed by factors such as pH, protein solubility, and integrity of cytoskeletal proteins and myofilaments proteins (Purslow et al., 2020). All these facts demonstrate the complex relationships between proteins and meat color. As can be seen in Table 2, a large amount of the proteins (70 proteins) was significantly correlated with color instrumental parameters. Among these, several proteins from energy metabolism, calcium homeostasis and oxidative stress were correlated with L* such as HSPA1A (r = 0.819 in Jaca Navarra), SOD2 (r = −0.802 in Jaca Navarra), ALDOA (r = 0.852 in Burguete), PKM (r = 0.745 in Burguete), CKM (r = 0.836, 0.834 in Burguete and Jaca Navarra); correlated with a* SOD2 (r = 0.707 in Burguete), VDAC2 (r = −0.786 in Burguete), PRDX2 (r = 0.734 in Jaca Navarra), PFKM (r = −0.717 in Burguete); and

Table 2
Correlation between protein abundance and quality traits (shear force, color parameters and intramuscular fat) of foal meat from Burguete and Jaca Navarra breeds.

Pearson correlation of differential abundant proteins with shear force					
	Protein name	Gene name	Uniprot ID	Coefficient, r	p-value
BURGUETE	Obscurin. cytoskeletal calmodulin and titin-interacting RhoGEF	OBSCN	F6WQX2	−0.922	< 0.001
	Myosin binding protein C1	MYBPC1	F6Y6F3	−0.881	0.001
	Myosin light chain 1	MYL1	F7AX54	−0.860	0.001
	PDZ domain-containing protein	AHNAK	A0A3Q2HJF2	0.859	0.001
	Filamin C	FLNC	F6ZWZ3	−0.850	0.002
	Nebulin	NEB	A0A3Q2LNX8	0.848	0.002
	Smoothelin like 1	SMTNL1	F6R563	0.828	0.003
	PDZ and LIM domain 5	PDLIM5	A0A5F5PRT5	0.804	0.005
	Creatine kinase	CKM	F7BR99	−0.797	0.006
	Myomesin 1	MYOM1	F7CNU9	−0.796	0.006
	LIM domain binding 3	LDB3	F6QY18	0.794	0.006
	Actinin alpha 3	ACTN3	E7D103	−0.782	0.008
	Moesin	MSN	F6PUX2	0.771	0.009
	AIR carboxylase	PAICS	F7D7Z9	−0.766	0.010
	Fructose-bisphosphate aldolase	ALDOA	A0A3Q2HN78	−0.752	0.012
	Actin alpha 1	ACTA1	F7CZ92	−0.745	0.013
	GLOBIN domain-containing protein	HBA2	Q28383	0.745	0.013
	Phosphoglycerate mutase	PGAM2	F7A616	−0.739	0.015
	Rho GDP dissociation inhibitor alpha	ARHGDIA	F6W039	0.734	0.016
	Nucleoside diphosphate kinase	NME2	F6YY66	−0.730	0.017
	Myosin binding protein	MYBPH	F6VIP2	−0.727	0.017
	Tryptophan–tRNA ligase	WARS1	A0A3Q2KTL8	0.725	0.018
	Titin	TTN	A0A5F5PKC5	−0.721	0.019
	Glutathione transferase	GSTO1	F6SA20	−0.718	0.019
	Myosin binding protein *C*2	MYBPC2	F6VIG2	−0.715	0.020
	Myomesin 2	MYOM2	A0A3Q2I9B4	−0.715	0.020
	Profilin	PFN1	F6UJ33	−0.708	0.022
	Triosephosphate isomerase	TPI1	F6TZS9	−0.707	0.022
	Mono-ADP ribosylhydrolase	MACROD1	A0A3Q2L2W7	−0.705	0.023
JACA	Troponin C	TNNC2	F7CGE8	0.858	0.002
	Creatine kinase (Fragment)	CKM	A0A0R5SAL6	−0.793	0.006
	Elongation factor 1-gamma	EEF1G	A0A452GER3	−0.784	0.007
	NADH dehydrogenase [ubiquinone] flavoprotein 2.	NDUFV2	F7B7S1	0.740	0.014
	Adenylosuccinate synthetase isozyme 1	ADSS1	A0A3Q2GSI4	−0.735	0.015
	Adipocyte-type fatty acid-binding protein	FABP4	F6YN05	−0.711	0.021

Pearson correlation of differential abundant proteins with L*					
	Protein name	**Gene name**	**Uniprot ID**	**Coefficient, r**	**p-value**
BURGUETE	Immmunoglobulin lambda light chain variable region	IGL	A0A0A1E9B4	−0.709	< 0.001
	Phosphoglycerate kinase	PGK1	F7D1R1	0.867	0.001
	Fructose-bisphosphate aldolase	ALDOA	A0A3Q2HN78	0.852	0.002
	Creatine kinase	CKM	F7BR99	0.836	0.003
	Mono-ADP ribosylhydrolase	MACROD1	A0A3Q2L2W7	0.833	0.003
	Alpha-1-acid glycoprotein 2	ORM1	F7CQ86	−0.827	0.003
	Acylphosphatase	ACYP2	A0A3Q2HXC0	0.794	0.006
	GST class-pi	GSTP1	F6VSN2	0.776	0.008
	Protein-L-isoaspartate O-methyltransferase	PCMT1	K9K455	−0.769	0.009
	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit	SDHB	Q0QEY2	−0.746	0.013
	Pyruvate kinase	PKM	B3IVM0	0.745	0.013
	Albumin	ALB	F7BAY6	0.735	0.016
	Alpha-1B-glycoprotein	A1BG	F6VJR6	0.724	0.018
	ATP synthase subunit beta	ATP5F1B	F6U187	−0.724	0.018
	Ubiquitin-activating enzyme E1	UBA1	F7C6F2	−0.718	0.019
	Glutathione transferase	GSTO1	F6SA20	0.710	0.021
	PDZ and LIM domain 5	PDLIM5	A0A5F5PRT5	−0.705	0.022
	Carboxylic ester hydrolase	CES1	A0A3Q2LAR7	0.702	0.024
JACA	3-hydroxyisobutyrate dehydrogenase	HIBADH	F6XXT9	−0.862	0.001
	Bridging integrator 1	BIN1	A0A3Q2LCX2	−0.843	0.002
	Creatine kinase	CKM	F7BR99	0.834	0.003
	Heat shock 70 kDa protein 1A	HSPA1A	F7DW69	0.819	0.004
	Protein-L-isoaspartate O-methyltransferase	PCMT1	K9K455	−0.802	0.005
	Superoxide dismutase	SOD2	F6U991	−0.802	0.005
	Elongation factor 1-alpha	EEF1A2	F6Q4R1	0.795	0.006
	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	A0A0K8TPT3	−0.794	0.006
	Aspartate aminotransferase	GOT2	F6YXZ8	−0.769	0.009
	Myosin binding protein H	MYBPH	F6VIP2	−0.748	0.013
	Aspartate aminotransferase	GOT1	A0A5F5Q2J1	−0.747	0.013

(*continued on next page*)

Table 2 (*continued*)

Pearson correlation of differential abundant proteins with shear force				
Protein name	Gene name	Uniprot ID	Coefficient, r	p-value
Creatine kinase	CKMT2	F6X8T2	−0.739	0.015
Myosin binding protein C1	MYBPC1	F6Y6F3	0.723	0.018
Medium-chain specific acyl-CoA dehydrogenase	ACADM	K9K3Y1	−0.719	0.019
Myosin binding protein *C*2	MYBPC2	F6VIG2	0.717	0.020
10 kDa heat shock protein	HSPE1	F6T0A6	−0.715	0.020
Macrophage migration inhibitory factor	MIF	F6S243	−0.705	0.023

Pearson correlation of differential abundant proteins with a*

	Protein name	Gene name	Uniprot ID	Coefficient, r	p-value
BURGUETE	Enoyl-CoA hydratase 1	ECH1	F6S8M6	0.901	< 0.001
	Voltage-dependent anion-selective channel protein 2	VDAC2	F6TLU0	−0.786	0.007
	ATP-dependent 6-phosphofructokinase	PFKM	A0A3Q2GST7	−0.717	0.020
	Elongation factor 1-gamma	EEF1G	A0A452GER3	0.712	0.021
	Desmin	DES	F7AQ43	−0.710	0.021
	Superoxide dismutase	SOD2	F6U991	0.707	0.022
	Mitsugumin-53	TRIM72	A0A5F5PUG7	−0.706	0.023
	Phosphoglycolate phosphatase	PGP	F6ZBD7	0.701	0.024
JACA	GLOBIN domain-containing protein	HBA2	Q28383	0.907	<0.001
	Peptidyl-prolyl cis–trans isomerase	PPIA	F6X6A6	−0.852	0.002
	Aspartate aminotransferase	GOT1	A0A5F5Q2J1	0.851	0.002
	Globin A1	GLNA1	A0A1K0FUE2	0.734	0.016
	Peroxiredoxin	PRDX2	F7BFT1	0.734	0.016
	Myosin light chain 1	MYL1	F7AX54	−0.719	0.019
	Citrate synthase	CS	F6XMS1	0.711	0.021
	Malate dehydrogenase	PHGDH	K9K2D2	0.700	0.024

Pearson correlation of differential abundant proteins with b*

	Protein name	Gene name	Uniprot ID	Coefficient, r	p-value
BURGUETE	Alpha-1B-glycoprotein	A1BG	F6VJR6	0.829	0.003
	Mitsugumin-53	TRIM72	A0A5F5PUG7	−0.778	0.008
	Metavinculin	VCL	F6ZSZ5	−0.769	0.009
	Fructose-bisphosphate aldolase	ALDOA	A0A3Q2HN78	0.763	0.010
	Annexin	ANXA7	F7ALC9	0.758	0.011
	ST13 Hsp70 interacting protein	ST13	A0A5F5PN24	−0.754	0.012
	Aminopeptidase	NPEPPS	F7BXA6	−0.748	0.013
	Proteasome subunit alpha type	PSMA4	F6Z688	−0.739	0.015
	Alpha-1-acid glycoprotein 2	ORM1	F7CQ86	−0.729	0.017
	Cysteine and glycine-rich protein 3	CSRP3	A0A3Q2HGW3	−0.717	0.020
	Mono-ADP ribosylhydrolase 1	MACROD1	A0A3Q2L2W7	0.713	0.021
	Desmin	DES	F7AQ43	−0.702	0.024
JACA	Protein-L-isoaspartate O-methyltransferase	PCMT1	K9K455	−0.887	0.001
	Myosin binding protein C1	MYBPC1	F6Y6F3	0.780	0.008
	Rho GDP dissociation inhibitor alpha	ARHGDIA	F6W039	−0.777	0.008
	Adenosylhomocysteinase	AHCY	A0A3Q2HUY8	0.751	0.012

Pearson correlation of differential abundant proteins with IMF

	Protein name	Gene name	Uniprot ID	Coefficient, r	p-value
BURGUETE	Actin alpha 1	ACTA1	F7CZ92	0.871	0.001
	Moesin	MSN	F6PUX2	−0.769	0.009
	60 kDa chaperonin	HSPD1	K9K3I2	0.742	0.014
	Myosin-1	MYH1	A0A3Q2HB31	0.734	0.016
	PDZ and LIM domain 3	PDLIM3	F6RZN9	−0.717	0.019
	Nucleoside diphosphate kinase	NME2	F6YY66	0.707	0.022
	NAD(P)(+)–arginine ADP-ribosyltransferase	ART3	F6W836	0.706	0.023
	Aldehyde dehydrogenase	ALDH2	F6SL95	0.703	0.023
JACA	78 kDa glucose-regulated protein	HSPA5	F6YG82	0.871	0.001
	Fructose-bisphosphatase	FBP1	F6YTD3	−0.867	0.001
	Beta-1 metal-binding globulin	TF	A0A5F5PRC5	0.842	0.002
	L-lactate dehydrogenase	LDHB	C6L1J5	0.815	0.004
	Annexin	ANXA1	K9K271	0.767	0.010
	Hsc70/Hsp90-organizing protein	STIP1	F7E1X2	0.755	0.012
	Elongation factor 1-gamma	EEF1G	A0A452GER3	0.735	0.015
	Alpha-1B-glycoprotein	A1BG	F6VJR6	0.725	0.018
	Glycerol-3-phosphate dehydrogenase	GPD1	A0A3Q2HV72	−0.721	0.019
	Troponin *C*2	TNNC2	F7CGE8	−0.710	0.021

(*continued on next page*)

Table 2 (*continued*)

Pearson correlation of differential abundant proteins with shear force				
Protein name	Gene name	Uniprot ID	Coefficient, r	p-value
Heat shock 70 kDa protein 1A	HSPA1A	F7DW69	0.709	0.022
Moesin	MSN	F6PUX2	0.706	0.023
Nucleoside diphosphate kinase	NME2	F6YY66	0.704	0.023

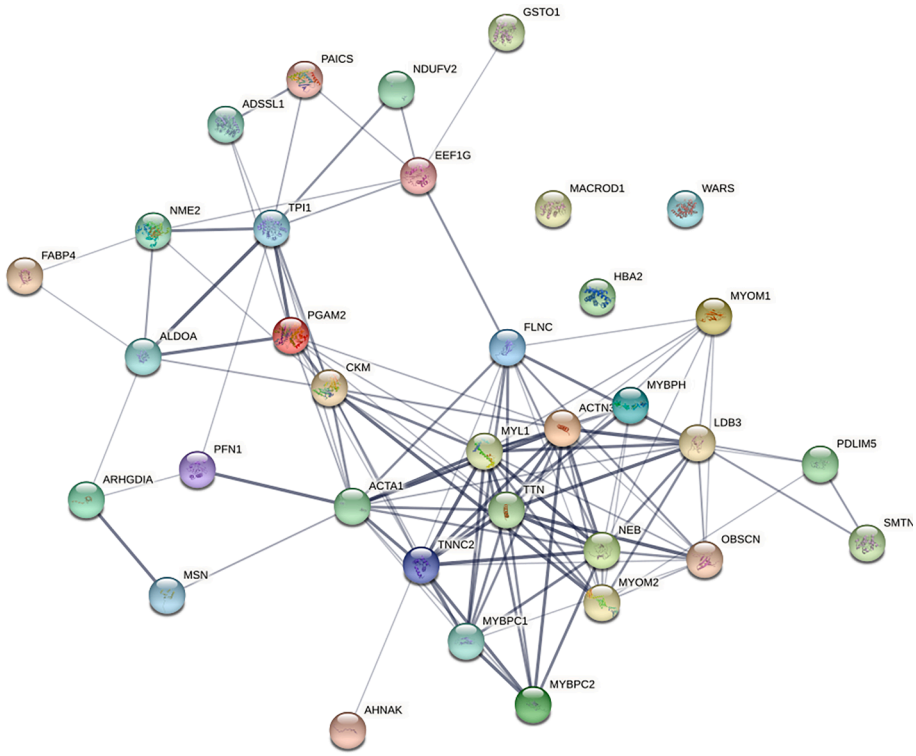

Fig. 4. Protein-Protein Interaction network of the 33 proteins correlated with shear force using Search Tool for the Retrieval of Interacting Genes/proteins (STRING). These proteins of foal meat also were differentially abundant between Burguete and Jaca Navarra.

correlated with b* ALDOA (r = 0.763 in Burguete). Among them, glycolytic enzymes were identified (ALDOA, PKM and PFKM) as well as CKM that buffers intracellular ATP concentrations through the storage and usage of high-energy phosphate species in the form of phosphocreatine. These results evidenced that these glycolytic proteins have a strong effect on color parameters resulting in ALDOA as the most important biomarker since it simultaneously influences both L* and b*. This agrees with the integromics study of Gagaoua et al. (2020) on beef color that shortlisted this putative biomarker as of great interest for further validation. Other proteins involved in oxidative stress (SOD2 and PRDX2) may affect the variation in different ways including the oxidative status of myoglobin, hence changing the chromatic traits. It also demonstrated that the expression of heat shock proteins is related to protection against oxidative stress (Kalmar & Greensmith, 2009). Additionally, these biomarker candidates were confirmed in beef using an integromics meta-analysis (Gagaoua et al., 2020).

In the case of intramuscular fat, 21 proteins were correlated as shown in Table 2. In general, there is a connection among tenderness, intramuscular fat and water holding capacity. As shown in Table 2, five proteins resulted of importance: ACTA1 (r = 0.871 in Burguete), TF (r = 0.842 in Jaca Navarra), FBP1 (r = −0.867 in Jaca Navarra), LDHB (r = 0.815 in Jaca Navarra) and HSPA5 (r = 0.871 in Jaca Navarra) for intramuscular fat. But they are not correlated with shear force. Thus, it is worthy to reveal in this study that proteomic differences jointly affect these important quality parameters. This fact may be related to the low-fat amount present in foal meat, avoiding the potential connection with tenderness.

4. Conclusions

The present study reports that foal meat from Burguete seemed to have superior quality than Jaca Navarra in terms of tenderness, and color lightness. Additionally, the diet treatment with concentrate, increased the intramuscular fat along with a decrease in shear force, which could favour an increase in the consumer acceptability of horse meat.

The proteomic approach applied in this study revealed significant changes and differences at the muscle level because of the breed effect. These were more pronounced than those caused by the finishing diet type. The metabolic shifts related to the finishing diet evidenced several ~~a~~ candidate protein biomarkers such as ATP5P0/ATP5F18 (ATP synthases), ALB, PGAM2, TPI1, MDH1 and FABP3.

In terms of meat quality traits, several protein biomarkers were further identified. For tenderness, the main mechanisms were related to muscle structure with key proteins (ACTA1, MYBPH, MYL1 and TNNC1) including few others involved in energy metabolism (ALDOA, CKM, TPI1 and PGMA2). Regarding meat color, proteins from energy metabolism (ALDOA, PKM, PFKM and CKM), and proteins from oxidative stress and protein folding (HSPA1A, SOD2 and PRDX2) can be considered putative candidates for foal meat color determination. Additionally, 23 proteins were found as possible biomarkers for intramuscular fat content that need further investigation and validation.

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CRediT authorship contribution statement

María López-Pedrouso: Conceptualization, Writing – original draft, Writing – review & editing. **José M. Lorenzo:** Conceptualization. **Aurora Cittadini:** Formal analysis, Writing – review & editing. **María V. Sarries:** Formal analysis, Writing – review & editing. **Mohammed Gagaoua:** Conceptualization, Writing – review & editing. **Daniel Franco:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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