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Review paper

Proteomics as an emerging tool in equine meat research: an overview

Antonella della Malva[a], Mohammed Gagaoua[b]*, Agostino Sevi[a] and Marzia Albenzio[a]*

[a] *University of Foggia, Department of Agriculture, Food, Natural Resources and Engineering (DAFNE), Via Napoli 25, 71121 Foggia, Italy*
[b] *PEGASE, INRAE, Institut Agro, 35590 Saint-Gilles, France*

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ABSTRACT

Proteomics tools in the field of equine meat research have been very recently applied to explore the changes in the post-mortem muscle proteome and to discover biomarkers to monitor the variations in its different meat quality traits. The current advances achieved by proteomics in equine meat research are reviewed. Different proteomics techniques (sodium dodecyl sulphate-polyacrylamide gel electrophoresis; two-dimensional polyacrylamide gel electrophoresis; fluorescent two-dimensional difference gel electrophoresis; targeted proteomics; tandem-mass tag labeled proteomics; data-independent analysis proteomics) have been applied in the study of the equine muscle/meat. The studies revealed the biochemical pathways involved in the development of several donkey and horse (foal) meat quality variation. The current knowledge would be useful to develop high-quality products.

1. Introduction

In the last years, the increase in consumer health consciousness led to increased demand and consumption of alternative meat sources worldwide, with consumers choosing products with high nutritional value. Equine meat (from donkeys and horses) is widely recognized as a health-beneficial food due to its greater content of vitamins, minerals, conjugated fatty acids, and low fat and cholesterol content (*Marino et al.*, 2022). Despite its excellent nutritional value, preferences and perceptions of equine meat differ significantly due to the social, historical, ethical, and psychological characteristics of consumers (*Lopez-Pedrouso et al.*, 2023). In fact, recent trends show an increase in production and consumer demand for equine meat (*FAOSTAT*, 2021). Improving consumer confidence in equine meat consumption including its nutritional features could offer an opportunity for farming systems to enlarge the livestock species, to differentiate the market, and to promote environmental sustainability. In terms of sensory properties, equine meat is considered a tough and dark red meat, which drives its consumer acceptability, and consequently, the purchasing decision at the point of sale. Proteomics-based techniques have been employed to decipher the proteome changes and the underlying molecular mechanisms related to different meat organoleptic traits in different species (*Di Luca et al.*, 2011; *della Malva et al.*, 2017; *Lopez-Pedrouso et al.*, 2020; *Gagaoua et al.*, 2020; *Gagaoua et al.*, 2021; *Lamri et al.*, 2023). In the particular case of equids, proteomics has been very recently applied, and to the best of our knowledge, in less than 10 studies (Table 1). Such studies have as their ultimate goal the development of high-qual-

***Corresponding authors:** Antonella della Malva, *antonella.dellamalva@unifg.it*; Mohammed Gagaoua, *mohammed.gagaoua@inrae.fr*

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ity equine products and, more specifically, the goal of better understanding the biochemical pathways behind the variability of equine meat quality. Therefore, this paper aims to briefly introduce the basis of proteomic approaches in the assessment of equine meat quality variation by highlighting the applications of this powerful tool in both donkey and horse meat research with a focus on i) post-mortem changes and underlying mechanisms and ii) meat quality traits.

2. Brief overview of proteomics in meat research

In the field of meat research, proteomics is an adapted tool for in-depth characterization and to explore the biochemical processes taking place during the conversion of muscle into meat or during the post-mortem time (*D'Alessandro & Zolla*, 2013; *Purslow et al.*, 2021; *Gagaoua et al.*, 2022). Several strategies and methodologies can be used. Gel-based approaches (sodium dodecyl sulphate-polyacrylamide gel electrophoresis, SDS-PAGE; two-dimensional polyacrylamide gel electrophoresis, 2D-PAGE; fluorescent two-dimensional difference gel electrophoresis, 2D-DIGE) coupled with mass spectrometry (MS) are widely applied and have, so far, been the main methods used to investigate muscle proteome changes (For review: *Gagaoua et al.*, 2022). However, in recent years, gel-free quantitative techniques (label-free or label-based approaches) have gradually taken over, leading to high accuracy and sensitivity in the quantification of proteins, including intact proteoforms and post-translational protein modifications (*Li et al.*, 2021; *Lamri et al.*, 2023). Furthermore, the tremendous progress in powerful bioinformatics tools and software have recently enabled the expansion and identification of new features related to several meat quality traits (*Kiyimba et al.*, 2022). Generally, the muscle undergoes several dynamic modifications during the animal's life due to intrinsic and extrinsic factors. Several studies on beef evidenced that those factors are responsible for huge variations in the muscle proteome, which consequently impact the final quality traits of muscle foods (*Sierra et al.,* 2021; *Di Luca et al.*, 2022; *Gagaoua et al.*, 2022). Proteomics technologies were successfully applied to decipher several of these changes and mechanisms, including in the field of biomarkers discovery to monitor meat tenderness, color, and water holding capacity (*Gagaoua et al.,* 2022; *Gagaoua and Picard*, 2022).

3. Proteomics applications in horse meat research

Proteomics tools in horsemeat research have been applied with the objectives of monitoring and exploring the tenderization rate and the quality traits of different muscles during aging and also for achieving a better understanding of the muscle proteome differences among different breeds and diets (Table 1). A 2D-PAGE approach combined with liquid chromatography-tandem mass spectrometry (LC/MS-MS) was used by *della Malva et al*. (2019) to investigate the post-mortem changes in the myofibrillar muscle proteome and the tenderization rate during the aging time of different muscles (*Longissimus lumborum*, *Semimembranosus* and *Semitendinosus*) from Italian Heavy Draft Horses. The authors identified 22 myofibrillar and sarcoplasmic protein biomarkers to follow up on the influence of aging time on proteolysis. The 22 proteins belong, based on Gene Ontology analysis, to six biological cluster pathways, these being muscle contraction (GO: 0006936), NADH regeneration (GO: 0006735), regulation of ATP-dependent activity (GO: 0043462), muscle structure development (GO: 0061061), response to estradiol (GO: 0032355), and organophosphate biosynthetic process (GO: 0090407). In addition, *della Malva et al*. (2019) revealed differences in the rates of tenderization among muscles during aging, showing a greater accumulation of MYL1 and MYL2 fragments in *Semitendinosus* muscle with an extending aging time (14 days). When investigating the changes in the sarcoplasmic proteome in comparison with the meat organoleptic characteristics, another study by *della Malva et al.* (2022) found 24 muscle-specific protein patterns during aging, from which TPM1 and TPM2 cytoskeletal proteins were potential biomarkers of intense proteolysis for *Longissimus* muscle. The authors also indicated mitochondrial and glycolytic proteins (SOD, PGM1, and MB) as putative biomarkers to monitor the meat quality characteristics of horse *Semitendinosus* muscle.

Beldarrain et al. (2022) applied the OFFGEL proteomics-based approach on the Hispano-Breton *Longissimus thoracis et lumborum* (LTL) horse muscle to unveil changes in the myofibrillar proteome during three weeks of aging time. The authors indicated that aging-induced significant abundance changes of muscle structure proteins (MYL1, MYBPC1, TNNT3, and TNNI2) as key players of horse meat tenderization. To better understand the changes

found in the myofibrillar proteome of horse meat during aging, a 2D-DIGE approach was further applied by the same authors (*Beldarrain et al.*, 2023) to gain insights into the biochemistry of horse muscles and identify candidate protein biomarkers to monitor meat tenderness. Five putative protein biomarkers (TNNT3, MYBPC1, MYBPC2, ACTA1, and GAPDH) evidenced/expressed a great potential to monitor the changes in horse meat tenderization.

Targeted proteomics (SWATH-MS: Sequential Window Acquisition of all Theoretical Mass Spectra) has been recently applied by *Lopez-Pedrouso et al.* (2023) on two horse breeds (Burguete *vs* Jaca Navarra) finished with conventional concentrate and straw *or* silage and organic feed diets to identify biomarkers of multiple horsemeat quality traits (tenderness, color, and intramuscular fat). The authors built a database of 294 proteins, from which 23 proteins were candidate biomarkers of intramuscular fat content, while eight proteins, from the energy metabolism (ALDOA, CKM, TPI1, and PGMA2) and the muscle structure (ACTA1, MYBPH, MYL1, and TNNC1) pathways were identified as biomarkers to monitor the tenderization process. Regarding horse meat color determination, seven potential protein biomarkers related to energy metabolism (ALDOA, PKM, PFKM, and CKM), and oxidative stress (HSPA1A, SOD2, and PRDX2) were identified.

4. Proteomics applications in donkey meat research

Proteomics investigations in donkey meat has been applied to decipher the biological mechanisms governing the meat tenderness and intramuscular fat variation and, consequently, to reveal the underlying pathways including those for the identification of protein biomarkers of the desirable meat quality (Table 1). In the frame of understanding the tenderization rate and the proteins changes occurring during the aging time of Martina Franca donkey meat, a gel-based 2DE proteomics approach coupled with LC/MS-MS was applied by *della Malva et al.* (2022). The authors proposed the first repertoire of 15 meat tenderness biomarkers for the donkey meat species. Using bioinformatics, the candidate biomarkers were allocated to three interconnected pathways: nine proteins were from muscle contraction, structure pathway (MYH1, MYH2, ACTA1, MYLPF, MYL6B, MYL1, TNNC2, TPM1, and TPM2); five from energy metabolism (ATP-5PD, UQCRC1, COX5A, GAPDH, and CKM), and one protein from the response to stress pathway (HSPB1), thus evidencing a key insight into the pathways and processes involved in the tenderness development of donkey meat.

Intramuscular fat content plays a pivotal role in the quality of muscle foods, thus affecting the flavor, juiciness, and tenderness of the end product. A tandem-mass tag (TMT) labeled proteomics study conducted by *Tan et al.* (2022) identified 30 differentially abundant proteins strictly related to the intramuscular fat deposition of the donkey *Longissimus thoracis* muscle. The functional enrichment analysis confirmed that the main biological pathways are involved in lipid metabolism and adipogenesis, thus confirming their role in the biological mechanisms that regulate meat quality variation.

Chai et al. (2022) used a data-independent analysis (DIA) proteomics approach to investigate the proteome differences of donkey muscles (*Semitendinosus* (ST), *Longissimus thoracis* (LT), and *Gluteus maximus* (GM)) related to meat quality parameters. The pairwise comparisons of the ST/LT and GM/LT allowed identification of, respectively, 111 and 127 differentially abundant proteins involved mainly in the MARK signaling pathway, fat digestion and absorption, and regulation of actin cytoskeleton. The focus given by these studies on the strong role of different biological pathways in the post-mortem processes linked with the donkey meat quality variation emphasizes the need for future research to explain the molecular basis of variations in donkey meat quality for developing high-quality products from this sustainable species.

5. Future perspectives/Conclusion

The potential of proteomic tools to decipher and understand biological mechanisms and pathways underlying the equine meat quality variations has barely/recently been explored. The current few proteomics studies, developed on equids, allowed us to gain more insights about the biological mechanisms responsible for the variations in meat quality, in terms of tenderness, color, and intramuscular fat content. Several biological pathways have been discovered including proteins from the energy metabolism, muscle structure, oxidative stress, lipid metabolism, and adipogenesis, although further post-mortem muscle proteome studies and multi-omics approaches will be necessary to validate the biochemical pathways and the proposed candidate biomarkers, with the aim of monitoring equine meat quality

Table 1. Proteomics approaches in the selection of biomarkers related to meat quality in equids.

Proteomic approach	Muscle/cut (breed)	Considered variable(s)/ effects	Identified proteins/candidate biomarkers[1]	References
		Horse		
SDS-PAGE, 2DE, HPLC/ Q-TOF mass spectrometry, and Western Blotting	*Longissimus lumborum* (LL), *Semitendinosus* (ST) and *Semimembranosus* (SM) (Italian Heavy Draft Horse)	Aging – Muscle	MYL1, TNNT3, MYLPF, MYL3, TPM2, CKM, ENO2, AK1, PGK1, TPI1, GPX1	*della Malva et al.*, 2019
SDS-PAGE, 2DE, and HPLC/Q-TOF mass spectrometry	*Longissimus lumborum* (LL), *Semitendinosus* (ST) and *Semimembranosus* (SM) (Italian Heavy Draft Horse)	Aging – Muscle	PGM1, CKM, TPM1, TPM2, ENO3, ALDOB, GPD1, GAPDH, TPI1, AK1, MB, SOD1	*della Malva et al.*, 2022
OFFGEL, SDS-PAGE, LC-MS/MS	*Longissimus thoracis et lumborum* (LTL) (Hispano-Breton horse)	Aging	MYL1, TUBB4A, TNNT3, CRYAB, CKM, ENO3, ALDOA, GAPDH, LDHA, TNNI2, MYBC1, TPM2, ALDOA, CAPZA2, LDHA, MDH2, VDAC3, ATP5F1C, CA3, PGAM2, MYL3, ATP5PO, MYL1, MYLPF	*Beldarrain et al.*, 2022
2-D DIGE, LC-MS/MS and Immunoblotting	*Longissimus thoracis et lumborum* (LTL) (Hispano-Breton horse)	Aging	ACTA1, MYBPC2, MYBPC1, PYGM, HSPA1A, DLAT, ALB, MYBPC2, SDHA, DES, TNNT3, ALDOA, CKM, LDHA, MYOZ1, ENO3, PHB, NDUFS3, HSPB1, ATP5PD, GAPDH,	*Beldarrain et al.*, 2023
Shotgun data-dependent acquisition proteomic approach by micro-LC-MS/MS, Data-independent acquisition (DIA), SWATH-MS	*Longissimus thoracis and lumborum* (LTL) (Jaca Navarra and Burguete horses)	Breed – Feeding – Meat quality	**WBSF** *Burguete:* OBSCN, MYBPC1, MYL1, AHNAK, FLNC, NEB, SMTNL1, PDLIM5, CKM, MYOM1, LDB3, ACTN3, MSN, PAICS, ALDOA, ACTA1, HBA2, PGAM2, ARHGDIA, NME2, MYBPH, WARS1, TTN, GSTO1, MYBPC2, MYOM2, PFN1, TPI1, MACROD1 *Jaca Navarra:* TNNC2, CKM, EEF1G, NDUFV2, ADSS1, FABP4); **Lightness (L*)** *Burguete:* IGL, PGK1, ALDOA, CKM, MACROD1, ORM1, ACYP2, GSTP1, PCMT1, SDHB, PKM, ALB, A1BG, ATP5F1B, UAB1, GSTO1, PDLIM5, CES1 *Jaca Navarra:* HIBADH, BIN1, CKM, HSPA1A, PCMT1, SOD2, EEF1A2, GAPDH, GOT2, MYBPH, GOT1); **Redness (a*)** *Burguete:* ECH1, VDAC2, PFKM, EEF1G, DES, SOD2, TRIM72, PGP, *Jaca Navarra:* HBA2, PPIA, GOT1, GLNA1, PRDX2, MYL1, CS, PHGDH), **Yellowness (b*)** *Burguete:* A1BG, TRIM72, VCL, ALDOA, ANXA7, ST13, NPEPPS, PSMA4, ORM1, CSRP3, MACROD1, DES *Jaca Navarra:* PCMT1, MYBPC1, ARHGDIA, AHCY); **IMF** *Burguete:* ACTA1, MSN, HSPD1, MYH1, PDLIM3, NME2, ART3, ALDH2 *Jaca Navarra:* HSPA5, FBP1, TF, LDHB, ANXA1, STIP1, EEF1G, A1BG, GPD1, TNNC2	*Lopez-Pedrouso et al.*, 2023

Proteomic approach	Muscle/cut (breed)	Considered variable(s)/ effects	Identified proteins/candidate biomarkers[1]	References
		Donkey		
SDS-PAGE, 2DE, LC-MS/MS, and Western Blotting	*Longissimus thoracis et lumborum* (LTL) (Martina Franca donkey)	Aging	MYLPF, TPM2, TPM1, MYL1, ACTA1, GAPDH, TNNC2, MYH1, MYH2, UQCRC1, HSPB1, MYL6B, COX5A, ATP5PD, CKM	*della Malva et al.*, 2022
Tandem-mass-tag, LC-MS/MS	*Longissimus dorsi* (LD) (Liaoxi donkey)	Meat quality	MAP4K4, PDLIM3, ADGRV1, ACTB, H2AJ, FLOT1, TPM1, UBR4, TRMT61A, PRMT3, C9, RPL32, MPC2, MBP, FHL3, LHPP, TAPT1, F11R, RPL27A, GLDN, PYCARD, COG3, CFAP251, SPAG8, GALNS, ROCK2, PRKCQ, K9KFH7, LUC7L2, ATP5MJ	*Tan et al.*, 2022
Data-independent acquisition (DIA) and nano-LC–MS/MS	*Longissimus thoracis* (LT), *Gluteus maximus* (GM), and *Semitendinosus* (ST) (Dezhou donkey)	Muscle	111 proteins in the ST/LT and 127 proteins in the GM/LT muscles comparison were differentially expressed	*Chai et al.*, 2022

[1]The full names of the proteins (gene names) are retrieved from Uniprot database (https://www.uniprot.org).

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