Identification of Biomarkers of Stress in Meat of Pigs Managed under Different Mixing Treatments

Mamen Oliván[1*], Verónica Fernández-Suárez[1], Fernando Díaz-Martínez[1], Verónica Sierra[1,2], Ana Coto-Montes[2], Beatriz de Luxán-Delgado[2], Raquel Peña[3], Anna Bassols[3], Emma Fàbrega[4], Antoni Dalmau[4] and Antonio Velarde[4]

[1]Área de Sistemas de Producción Animal, SERIDA, Apdo.13, 33300 Villaviciosa, Asturias, Spain.
[2]Departamento de Morfología y Biología Celular, Facultad de Medicina, Universidad de Oviedo, C/ Julián Clavería s/n, 33006 Oviedo, Spain.
[3]Departament de Bioquímica i Biologia Molecular , Servei de Bioquímica Clínica Veterinària, Facultat de Veterinària, Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, Spain.
[4]Animal Welfare Subprogram, IRTA, Veïnat de Sies s/n, 17121 Monells, Girona, Spain.

Authors' contributions

This work was carried out in collaboration between all authors. Authors MO, AB and AV designed the study. Authors EF and AD were responsible of animal management. Authors VFS, FDM, VS, BDLD and RP was performed sample analysis and data acquisition. Authors MO and VS performed the statistical analysis. Author MO wrote the manuscript. Authors MO, ACM, AB and AV performed data interpretation. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Aims: The objective of this work was to apply the proteomic approach for identification of animal-based stress biomarkers during pig muscle maturation.
Study Design: Pigs were subjected to management treatments that may promote stress, like

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Corresponding author: Email: mcolivan@serida.org;

mixing unfamiliar animals on farm and/or during transport and lairage before slaughter. Three pre-slaughter treatments were applied: UF-UTL (Unmixed on Farm - Unmixed during Transport and at Lairage), MF-UTL (Mixed in Farm - Unmixed during Transport and at Lairage) and MF-MTL (Mixed in Farm - Mixed during Transport and at Lairage).

Methodology: Fifteen entire male pigs were used in this experiment. At slaughter, blood samples were taken for biochemical analysis. Meat quality traits (pH, electrical conductivity, drip loss, meat colour) were measured and muscle samples from the *Longissimus thoracis* were taken within the first 24 h post-mortem and used for analysis of total antioxidant activity (TAA) and sarcoplasmic protein profile study by SDS-PAGE.

Results: Mixing treatments (MF-UTL and MF-MTL) provoked faster muscle pH post-mortem decline (P= .009), lower total antioxidant activity (P< .001) and changes in some key proteins, like the heat shock 70kDa protein-1B (P= .040), glyceraldehyde-3-phosphate dehydrogenase "GAPDH" (P= .047), lactate dehydrogenase-A "LDH-A" (P< .001) and adenylate kinase isoenzyme-1 "AK-1" (P< .001). Furthermore, serum molecules related to fat metabolism (LDL-cholesterol and triglycerides) and changes of muscle proteins involved in the adenine nucleotide metabolism (AK-1) and glycolysis regulation (piruvate kinase "PK" and GAPDH) allowed accurate prediction of muscle pH and discriminated correctly 87% of meat samples into "no-mixing" or "mixing" treatments.

Conclusions: Early monitorization (within 24 h after slaughter) of selected biomarkers could be used in the meat industry as a tool for detection of situations of pre-slaughter stress related to animal mixing practices. However more research is needed in order to validate these results on a broader animal population.

1. INTRODUCTION

Over the last decades, animal production systems have been intensified in order to be more efficient and to increase production. In this context, different housing strategies and management practices on farm and previous to slaughter are being studied as they could have a great influence on performance, animal welfare and product quality.

The welfare status of farm animals is becoming an important aspect of overall food quality from the consumer point of view in terms of ethical quality. However, animal welfare assessment is not a simple issue and requires a multi-criteria approach [1]. Furthermore, individual differences in agonistic abilities, emotional reactivity and animal responsiveness to stress make even more difficult to establish the welfare status of every single individual, which could differ from the average status of the animal group. Then, there is increasing interest in the development of reliable animal-based methodologies to assess individual perceptions of stress and its possible effect on animal's health and welfare status and consequently on the ultimate meat quality.

Mixing unfamiliar animals is a common practice in pig production, but it may lead to injuries and social stress within the group. Previous reports have shown that mixing of unacquainted pigs is usually followed by fighting in order to create a new hierarchy [2,3]. Also, the removal of one or more animals in a pen may disrupt the already established rank orders and lead to fighting or stress in the remaining animals [4].

Exposing an animal to a pre-slaughter stressor will initiate a cascade of physiological reactions that may have an effect on meat quality, mostly negative. This phenomenon is well described in pigs, and a growing body of evidence suggest that pre-slaughter stress explains a large portion of the variation in the post-mortem muscle metabolism, and consequently of meat quality traits [5-7].

Most of the processes occurring in the muscle post-mortem are regulated by proteases and other proteins. Thus proteomics could be a useful tool for understanding biochemical pathways and cellular events involved in the process of meat quality acquisition and also for the identification of biomarkers of animal stress. To date, several studies have investigated proteins as potential biomarkers of meat quality, using proteomic approaches [8-11], some of them focused on the animal's susceptibility to stress [12,13]. However, the high inconsistency of results reflects the complexity of the processes involved.

The objective of this work was to apply the proteomic approach to increase our understanding of the effect of mixing unfamiliar pigs at different stages of the production process on the individual animal's responsiveness to stress and therefore on its physiological responses at slaughter and on the post-mortem muscle metabolism, in order to identify candidate animal-based biomarkers of pre-slaughter stress.

This study is a part of a broader investigation in which the effect of mixing and slaughter strategy on behaviour, welfare, performance and meat quality was evaluated. The results of animal behavior, welfare and productivity [4] and biomarkers of autophagy and oxidative stress [14] have already been published.

2. MATERIALS AND METHODS

2.1 Animals and Experimental Management

Fifteen entire male piglets ((Large White x Landrace) x Duroc) were used in this experiment and subjected to rearing treatments that may promote stress, like mixing unfamiliar animals on farm and/or during transport and lairage prior to slaughter. These pigs took part of a bigger experiment in which 96 animals were managed under two rearing treatments during the fattening period: half of them were in a wean-to-finish regime, and the others were mixed as in conventional farms practice (for complete explanation of management and housing conditions, see [4]).

When reaching 120 kg weight, pigs were slaughtered at an experimental abattoir located at a distance of 1 km (5-10 minutes lorry drive) and the handling and transport was gentle to avoid additional stress. During the phase of pre-slaughter management, two different treatments were applied: some animals were not mixed with animals from other pens during transport and lairage previous to slaughter (Unmixed group during transport and lairage, "UTL") and others were mixed with unfamiliar animals (Mixed group during transport and lairage, "MTL"). Lairage time ranged from 30 min to 2 h. Within the whole experiment of 96 animals, 15 pigs from the same slaughtering batch were randomly selected for the biochemical and proteomic study, in order to standardize slaughtering conditions, having 5 animals in each of three different management treatments:

-UF-UTL (Unmixed on Farm - Unmixed during Transport and at Lairage)
-MF-UTL (Mixed on Farm - Unmixed during Transport and at Lairage)
-MF-MTL (Mixed on Farm – Mixed during Transport and at Lairage)

2.2 Sample Collection and Meat Quality Measurements

Blood was collected at exsanguination in tubes without anticoagulant. Serum was obtained by centrifugation at 2000xg for 10 min and kept in aliquots at -80ºC.

The left side of each carcass was used to assess meat quality. Muscle pH was measured at 45 min (pH45) and at 24 h (pH24) post-mortem on the *Semimembranosus* (SM) muscle, using a Crison portable pH-meter equipped with a xerolyt electrode. Electrical conductivity (EC) was also measured at 24 h post-mortem on the SM using a Pork Quality Meter (PQM-I, INTEK Aichach, Germany). Meat samples (20 g) were taken from the *Longissimus thoracis* (LT) muscle immediately after slaughter (t= 0 h) and after 4 h, 8 h and 24 h of meat conditioning at 4ºC, for analysis of electrophoretic protein profile and total antioxidant activity (TAA). These muscle samples were frozen in liquid nitrogen and stored at -80ºC.

Meat colour was recorded on three 10 mm diameter spots on the exposed cut surface of the LT muscle at the last rib level at 24 h post-mortem. Indicators of lightness (L*), redness (a*) and yellowness (b*) were taken using a Minolta C2002 Spectrophotometer, illuminant C (Konica Minolta Inc., Madrid, Spain), and the average value of the three spots was used.

Meat drip loss (% exudates) was determined in duplicate on 25 mm diameter fresh samples taken from the LT at 24 h post-mortem and placed in a special container (Meat juice collector, Sarstedt, Germany), according to the method of Rasmussen and Andersson [15] with small modifications.

2.3 Biochemical and Physiological Parameters

The measurement of serum metabolic parameters was carried out with the Olympus AU400 Chemistry Analyzer (Beckman Coulter Inc., Barcelona, Spain). Glucose (hexokinase method), cholesterol (CHOP-PAP-method),

high density lipoprotein (HDL)-cholesterol (Immunoinhibition method), low density lipoprotein (LDL)-cholesterol (Selective protection method), triglycerides (GPO-PAP method), lactate (LDH method), total protein (Biuret method), haptoglobin (Phase Haptoglobin kit) and creatine kinase ("CK", IFCC method) were determined using the Olympus System Reagents (Olympus Diagnostica GmbH, Dublin, Ireland). Non-esterified fatty acids (NEFAs) were determined with NEFA-C reagent (Wako Chemicals GmbH, Neuss, Germany), 3-hydroxybutyrate with Ranbut and Glutathione Peroxidase (GPx) with Ransel (both from Randox Laboratories Ltd., Crumlin, UK). All parameters were analyzed in duplicate. Muscle Total Antioxidant Activity (TAA) was determined at 0 h post-mortem using the ABTS/H2O2/HRP method described by de Gonzalo-Calvo et al. [16]. Results are expressed in equivalents of mg Trolox/mg protein.

2.4 Sarcoplasmic Protein Extraction and Electrophoresis

Sarcoplasmic proteins were extracted from 2 g of muscle homogenized in 15 ml of extraction buffer (pH=7.4) containing 0.1M Phosphate buffer using an Ultra-Turrax T25 mixer (IKA, Staufen, Germany) three times for 15 s at maximum speed. Homogenates were centrifuged at 1000xg for 30 min at 4°C. Supernatants were filtered through cheesecloth and samples were frozen at -20°C. Protein concentrations were determined by the Bradford method [17], using bovine serum albumin as standard. 120µg of proteins were denatured by mixing with sample buffer (62.5 mM Tris/HCl pH 6.8, 2% SDS, 20% glycerol, 5% mercaptoethanol, 0.025% of bromophenol blue) and heated at 95ºC for 5 min, and loaded to 1mm dual vertical slab gels (Xi Protean II, Bio-Rad Laboratories Inc., CA, USA) for one-dimensional sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). The resolving gel contained 11% and the stacking gel 4% of 30% (w/v) acrylamide:bisacrylamide and a mixture of Tris/HCl (375 mM) pH 8.8, milli-Q water, SDS 10% (w/v), ammonium persulphate 10% (w/v), and 0.1% TEMED. Pre-stained molecular weight standards (Precision Plus ProteinTM All Blue Standards, Bio-Rad Laboratories Inc., Hercules, CA) were added on each gel. Gels were run at 80 volts for 2 h, 160 volts for 2 h, 250V 10 h and 500 volts for 20 min (Universal PowerPack 500, Bio-Rad), stained in a mixture of 30% (v/v) methanol, 10% (v/v) acetic acid and 0.01% (w/v) Coomassie Brilliant Blue R-250 and destained using a mixture of 40% (v/v) methanol and 10% (v/v) acetic acid. Three gels were produced per sample.

2.5 Image Analysis and Protein Identification by Peptide Mass Fingerprint

Stained gel images were captured using a GS-800 Imaging Densitometer (Bio-Rad) and analyzed using image analysis software Quantity One 5.5.1 (Bio-Rad). To account for slight variations in protein loading, the density protein bands was expressed as relative intensity.

Bands of interest were manually excised and sent for identification to the proteomics laboratory of Inbiotec S.L. (León, Spain), where the proteins were digested following the method of Havlis et al. [18] and processed for further analysis as indicated by Jami et al. [19]. The samples were analyzed with a 4800 Proteomics Analyzer matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF/TOF) mass spectrometer (ABSciex, MA, USA). A 4700 proteomics analyzer calibration mixture (Cal Mix 5, ABSciex) was used as external calibration. All MS spectra were internally calibrated using peptides from the trypsin digestion. The analysis by MALDI-TOF/TOF mass spectrometry produced peptide mass fingerprints, and the peptides observed (up to 65 peptides per spot) were collected and represented as a list of monoisotopic molecular weights with a signal to noise (S/N) ratio greater than 20 using the 4000 Series Explorer v3.5.3 software (ABSciex). All known contaminant ions (trypsin- and keratin- derived peptides) were excluded for later MS/MS analysis. Hence, from each MS spectra, the 10 most intensive precursors with S/N greater than 20 were selected for MS/MS analyses with CID in 2-kV ion reflector mode and precursor mass windows of ±7 Da. The default calibration was optimized for the MS/MS spectra.

For protein identification, Mascot Generic Files combining MS and MS/MS spectra were automatically created and used to interrogate a non-redundant protein database using a local license of Mascot v 2.2 from Matrix Science through the Global Protein Server v 3.6 (ABSciex). The search parameters for peptide mass fingerprints and tandem MS spectra obtained were set as follows: i) NCBInr (2012.06.26) sequence databases were used; ii) taxonomy: All entries (18713758 sequences,

6412106995 residues); iii) fixed and variable modifications were considered (Cys as S carbamidomethyl derivative and Met as oxidized methionine); iv) one missed cleavage site was allowed; v) precursor tolerance was 100 parts per million and MS/MS fragment tolerance was 0.3 Da; vi) peptide charge: 1+; and vii) the algorithm was set to use trypsin as the enzyme. Protein candidates produced by this combined peptide mass fingerprinting/tandem MS search were considered valid when the global Mascot score was greater than 85 with a significance level of P < .05.

2.6 Statistical Analysis

The effect of mixing treatment (UF-UTL, MF-UTL or MF-MTL) on meat quality traits, biochemical and proteomic variables was analyzed by Analysis of Variance (ANOVA) using the General Linear Model (GLM) procedure of SPSS (v 15.0 2006, SPSS Inc, Chicago, USA). For variables measured at different post-mortem time (sarcoplasmic protein profile) the model included the effects of mixing treatment, post-mortem time and their interaction as main effects. Once the interaction between mixing treatment and time was discarded, the effect of mixing treatment or the effect of time (with animal as random factor), were tested. When significant, differences were analysed by means of the Tukey post-hoc test (Games-Howell test when variances were not homogeneous).

Multiple linear regression models were calculated for prediction of main meat quality traits (pH45, EC24, L*, drip loss) and independent variables (metabolites detected in the serum at slaughter, muscle TAA and the muscle sarcoplasmic profile obtained at 0 h, 4 h, 8 h and 24 h post-mortem) were selected by the stepwise method, which included or removed iteratively independent variables from the model according to their significance (P< .05). The purpose of the model was to determine which biomarkers (metabolites and peptide bands) contributed to the ultimate meat quality. The predictive ability of the obtained models was evaluated in terms of the coefficient of determination (R^2), root mean square error of prediction (RMSEP) and the residual predictive deviation (RPD) which is the ratio of prediction error (SEP) to range in reference values (SD) and is used to test the accuracy of prediction models.

In order to ascertain if bioindicators (biochemical variables and bands/peptides) included in the regression models could be used for discrimination of meat products according to the pre-slaughter animal management ("no-mixing" or "mixing"), discriminant analysis was performed using the dummy regression technique on the biochemical/electrophoretic profiles, by applying The Unscrambler® software (v 9.8, CAMO, Trondheim, Norway). The meat samples were identified with dummy variables (no-mixing =1; mixing = 2) and PLS (partial least squares) regression was used to generate a mathematical model that was cross-validated (leave one-out) to select the most relevant PLS components. According to this model, a meat sample was correctly classified as belonging to a specific management category (no-mixing/mixing) if the predicted value was within ±0.5 of the dummy value.

3. RESULTS AND DISCUSSION

3.1 Meat Quality Traits

There was a significant (*P*= .009) effect of the mixing treatment on the pH measured on the SM muscle at 45 minutes post-mortem (pH45, Table 1), with a faster pH decline in the treatments with animal mixing, either mixing on farm (MF-UTL) or mixing on farm and during transport and at lairage (MF-MTL), although ultimate pH was similar in all groups (pH24=5.5). This is in agreement with Støier et al. [20], who found that pre-slaughter handling (traditional vs low stress stunning) significantly resulted in changes of pH fall in pork meat within the first 6 h post-mortem, although the muscle pH became similar at 24 h post-mortem.

It has been shown that post-mortem pH and temperature kinetics of the muscle may influence the rate and extent of protein denaturation, oxidation and proteolysis, colour characteristics and water holding capacity of meat [21]. In our trial there was a tendency for more exudative meat (higher drip loss, *P*= .157) when animals were subjected to mixing treatments (MF-UTL and MF-MTL) compared to animals coming from the unmixed group (UF-US), although the difference was not significant (Table 1). This could be due to the small sample size but also could reflect high individual variability, especially in the MF-UTL group, where the standard deviation exceeded the mean. This high individual variation could reflect high variability in the animal's susceptibility to stress, which could be the reason for the inconsistencies found in the literature, with some works describing a negative effect of pre-slaughter stress on meat quality

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[21,22], while others did not find significant effects [23,24].

3.2 Biochemical and Physiological Parameters

Biochemical and physiological parameters measured on serum and muscle samples at slaughter are presented in Table 2. There was a significant (*P*< .001) effect of mixing treatment on the total antioxidant activity (TAA) of the muscle at 0 h post-mortem, being higher in the tissue of animals from the unmixed treatment (UF-UTL=19.55 mg Trolox/mg protein) than in animals mixed at farm (MF-UTL= 11.62 mg/mg) or at farm and during transport and lairage (MF-MTL=12.40 mg/mg). This seems to indicate a healthier oxidative status of the muscle tissue of animals coming from the unmixed treatment, probably due to lower level of pre-slaughter stress.

Mixing treatments did not switch other physiological variables, although there was a marked increase in concentrations of glucose, haptoglobin and CK in blood serum, as the level

of animal mixing increased (UF-UTL<MF-UTL<MF-MTL) and also higher levels of triglycerides and hydroxybutyrate in the serum of animals coming from the double mixing treatment (at farm and previous to slaughter: MF-MTL). Then, it seems that social stress due to animal mixing could impose higher demand on the energy metabolism, which turned into higher levels of serum glucose (due to the gluconeogenic effects of glucocorticoids, as found by Becker et al. [25], associated with animal stress during transportation), CK (marker of skeletal muscle and good indicator of vigorous physical activity or tissue damage and fatigue [26,27]) and haptoglobin (bioindicator of animal stress during transport and a marker of inflammation [28,29]).

3.3 Electrophoretic Pattern of Muscle Extracts

SDS-PAGE gels allowed separation and quantification of a total of 27 bands of protein (260 to 23 kDa) (Fig. 1). Table 3 shows protein identification.

Table 1. Effect of mixing treatments on meat quality traits

	UF-UTL	MF-UTL	MF-MTL	SEM	*P*
pH45	6.63±0.10 a	6.41±0.16 b	6.33±0.12 b	0.058	.009
pH24	5.48±0.03	5.53±0.07	5.49±0.09	0.031	.490
EC24 (mS)	4.25±0.29	4.53±0.83	5.23±1.22	0.389	.226
Drip loss (%)	1.26±0.60	2.92±3.35	4.56±2.70	1.122	.157
L* (lightness)	48.38±1.24	49.78±1.98	49.74±2.08	0.807	.407

*Results are shown as means ±SD (standard deviation); Means in the same row followed by different letters are significantly different at P< .05; SEM: Standard error of means; EC: Electrical conductivity

Table 2. Effect of mixing treatments on serum and muscle biochemical parameters

	UF-UTL	MF-UTL	MF-MTL	SEM	*P*
Glucose (mg/dL)	121.84±23.77	129.76±31.73	139.70±40.21	14.579	.694
Cholesterol (mg/dL)	100.68±8.69	91.78±8.43	92.90±17.08	5.406	.470
HDL-cholesterol (mM)	1.106±0.11	0.998±0.16	1.006±0.18	0.068	.483
LDL-cholesterol (mM)	1.568±0.12	1.468±0.11	1.450±0.29	0.086	.596
Triglycerides (mg/dL)	63.40±8.29	55.40±9.29	70.0±29.05	8.161	.471
Lactate (mmol/L)	8.694±3.17	8.394±4.05	8.806±5.03	1.858	.987
Total protein (g/dL)	7.164±0.31	7.210±0.34	7.170±0.23	0.131	.964
Haptoglobin (mg/mL)	0.096±0.03	0.126±0.06	0.210±0.24	0.066	.470
Creatine Kinase (U/L)	1140.46±277.07	1444.28±682.39	1564.98±767.80	274.706	.547
NEFAs (mmol/L)	0.558±0.10	0.496±0.14	0.592±0.21	0.070	.625
Hydroxybutyrate (mmol/L)	0.085±0.02	0.085±0.02	0.097±0.03	0.011	.683
Glutathione peroxidase (U/L)	6115.0±543.21	5649.6±138.39	5670.4±730.74	237.795	.329
TAA 0h (mg Trolox/mg prot)	19.550±3.19 a	11.619±2.36 b	12.395±2.73 b	1.243	.001

*Results are shown as means ± SD (standard deviation); Means in the same row followed by different letters are significantly different at P< .05; SEM: Standard error of means; HDL: High density lipoprotein, LDL: Low density lipoprotein, NEFAs: Non-esterified fatty acids; TAA: Total antioxidant activity

Table 3. Protein identification of individual bands of sarcoplasmic extracts separated by SDS-PAGE

Band (MWe[a])	Identification	Accession no.[b]	MOWSE[c] scores	Seq. Cov.[d] (%)	Matched Queries	MWt[e]
B1 (316.5)	Filamin-C isoform 2 [Sus scrofa]	gi|311275459	876	26	50	289.6
B2 (244.0)	Myosin-2 [Sus scrofa]	gi|55741490	899	20	33	223.9
B3 (184.5)	Glycogen debranching enzyme [Bos Taurus]	gi|300794727	600	23	33	176.2
B4 (162.2)	Myosin-binding protein C, fast-type [Sus scrofa]	gi|335290041	628	35	32	128.4
B5 (113.1)	SERCA 1: Sarco/endoplasmic reticulum Ca^{2+}-ATPase 1 [Sus scrofa]	gi|324120946	831	33	30	110.4
B6 (95.1)	Glycogen phosphorylase, muscle form isoform 1 [Sus scrofa]	gi|335281566	1140	57	44	97.7
B7 (82.5)	Muscle 6-phosphofructokinase [Sus scrofa]	gi|95117652	666	48	26	82.4
B8 (76.5)	Transferrin [Sus scrofa]	gi|833800	464	16	18	78.9
B9 (72.3)	Heat shock 70kDa protein 8 "HSP70-8" [Sus scrofa]	gi|345441750	683	45	22	71.0
B10 (68.7)	Heat shock 70kDa protein 1B "HSP70-1B" [Sus scrofa]	gi|47523308	344	36	15	70.3
B11 (64.4)	Albumin [Sus scrofa]	gi|833798	1020	53	27	71.4
B12 (60.2)	Phosphoglucomutase-1 [Sus scrofa]	gi|350538593	751	41	19	62.0
B13 (56.4)	Pyruvate kinase isozymes M1/M2 "PK" [Sus scrofa]	gi|335292434	826	50	28	68.5
B14 (51.6)	ATP synthase subunit alpha, mitochondrial [Sus scrofa]	gi|297591975	467	37	15	55.3
B15 (48.8)	ATP synthase H+ transporting, mitochondrial F1 complex beta subunit "ATP5B"[Sus scrofa]	gi|89574051	297	54	14	47.1
B16 (44.8)	β-enolase [Sus scrofa]	gi|113205498	757	70	23	47.4
B17 (40.3)	Creatine kinase M-Type [Sus scrofa]	gi|194018722	851	65	23	43.3
B18 (37.2)	Fructose-bisphosphate aldolase A [Bos taurus]	gi|156120479	556	53	15	39.9
B19 (34.4)	Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) "GAPDH" (EC1.2.1.12)-pig	gi|65987	736	53	13	35.9
B20 (32.8)	L-lactate dehydrogenase A chain "LDH-A" [Sus scrofa]	gi|288860136	637	70	17	36.8
B21 (31.2)	Malate dehydrogenase precursor (EC 1.1.1.37), partial [Sus scrofa]	gi|164541	207	12	12	32.2
B22 (29.7)	four and a half LIM domain 1 protein, isoform C [Sus scrofa]	gi|47523806	566	67	19	35.6
B23 (27.4)	Carbonic anhydrase 3 [Sus scrofa]	gi|56711366	277	60	14	29.7
B24 (26.4)	Carbonic anhydrase 3 [Sus scrofa]	gi|56711366	655	57	20	29.7
B25 (25.4)	Triosephosphate isomerase 1 [Sus scrofa]	gi|262263205	872	77	17	26.9
B26 (24.0)	Myosin light chain MLC1f [Sus scrofa]	gi|117660874	556	57	18	21.0
B27(22.5)	Adenylate kinase isoenzyme 1 "AK-1" [Sus scrofa]	gi|350579688	623	69	19	23.6

[a] *MWe is the experimental molecular weight (kDa);* [b] *Accession number correspond to NCBInr database;* [c] *The MOWSE score is a numeric descriptor of the likelihood that the identification is correct. Protein scores greater than 69 are significant (P<0.05);* [d] *Percentage of coverage of the entire amino acid sequence;* [e] *MWt is the theoretical molecular weight (kDa)*
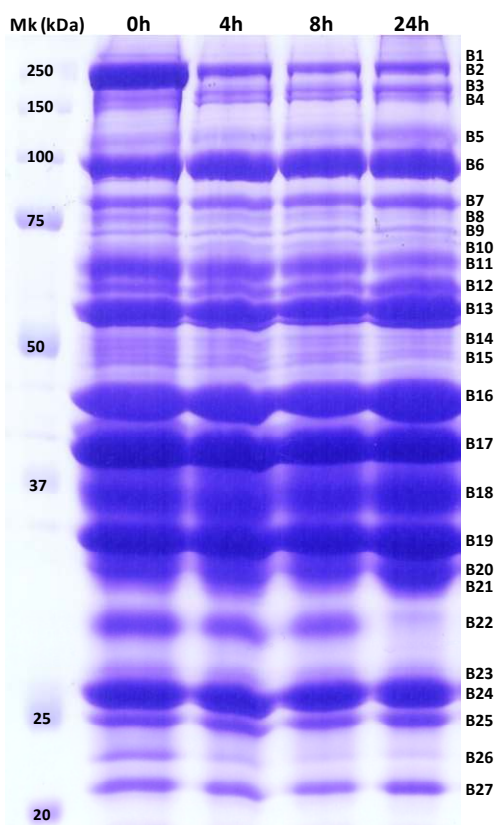
Fig. 1. SDS-PAGE gel image of a sarcoplasmic extracts of the muscle tissue at different post-mortem times (0 h, 4 h, 8 h, 24 h)

Mk, pre-stained molecular weight marker (Bio-Rad). Band names are denoted by B (band) followed by a number

It is worth noting that several protein bands (B1, B2, B8, B11, B17, B22, B23, B24, B26) showed decreasing pattern during meat aging in all treatments. Most of them have structural and maintenance functions in the muscle tissue, so its disappearance during the first 24 h post-mortem seems to be related to the cellular dismantlement and proteolysis. These were B1 (filamin-C isoform 2, P= .028), the myosin fragments B2 (Myosin-2, $P<$ .001) and B26 (Myosin light chain "MLC1f", P= .032), B8 (transferrin, P= .027), B11 (albumin, $P<$ .001) and B22 (four and a half LIM domains protein 1 isoform C "FHL1C", $P<$ .001). Four and a half LIM domains protein 1 isoform C "FHL1C", also known as cypher protein, is confined to the Z-line of skeletal muscle and its proteolysis has been correlated with the release of intact alpha-actinin from bovine myofibrils and would contribute to the weakening of the Z-line during meat aging [12].

Within the group of proteins with decreasing post-mortem pattern we also found a metabolic protein, B17 (Creatine kinase "CK", $P<$ .001), related to the glycolysing metabolism. This is in accordance with previous reports describing a faster reduction of CK in fast glycolysing pork meat [30,31].

In contrast, some protein bands showed increasing pattern with aging, like B5 (sarco/endoplasmic reticulum Ca2+-ATPase 1 "SERCA 1", P= .020), linked to the translocation of calcium from the cytosol to the sarcoplasmic reticulum lumen, and others related to the glycolysis pathway and the muscle-to-meat transition metabolism, like B6 (glycogen phosphorylase, $P<$ .001), B7 (muscle 6-phosphofructokinase, P= .043), B12 (phosphoglucomutase-1 "PGM-1", P= .010), B13 (Pyruvate Kinase "PK", $P<$ .001), B16 (β-enolase, $P<$ .001), B18 (fructose-bisphosphate aldolase A, $P<$ .001) and B20 (L-lactate dehydrogenase chain A "LDH-A", $P<$ .001), some of which have been identified as relevant in comparative proteomic studies between extreme groups of tenderness in beef [32]. Furthermore, most of them have been related to different situations of muscle degradation or meat conditioning, like β-enolase [8,33] or PK [11,34].

However, the effect of mixing treatment was significant on few protein bands, namely B10 (Heat Shock 70kDa protein 1B, "HSP70-1B", P= .040), B19 (GAPDH, P= .047), B20 (LDH-A, $P<$ .001) and B27 (Adenylate kinase isoenzyme 1 "AK-1", $P<$ .001). It is known that HSP70-1A/1B functions in the anti-apoptotic pathway and the inhibition of ROS formation [35] and it brings chaperone activity with HSP40 to ensure a good functioning of the muscle under oxidative stress conditions [36]. In this trial, HSP70 showed higher values in the muscle of animals mixed at farm (MF-UTL), with significant differences at 4 h post-mortem (P= .010). In contrast, LDH-A showed lower values in meat from animals mixed at farm (MF-UTL), being significantly different at 0 h (P= .007) and 24 h (P= .036) post-mortem. In a similar way, GAPDH, enzyme implicated in glycolysis, showed lower values in meat from animals managed under mixing treatments, both MF-UTL and MF-MTL.

With respect to AK-1 (B27), which catalyzes the reversible transfer of the terminal phosphate group between ATP and AMP, significantly higher values were found in meat from the group of unmixed animals (UF-UTL) at 4 h ($P<$ .001) post-mortem. In skeletal muscle, AK1 plays an

important role in the maintenance of the cellular energy homeostasis, therefore its higher level in meat from unmixed animals seems to indicate that animal mixing treatments (MF-UTL and MF-MTL) produced early post-mortem muscle cell dismantlement and hence earlier AK-1 exhaustion.

3.4 Relationship between Meat Quality Traits and Biochemical/ Electrophoretical Variables

Table 4 shows the best prediction model and sampling time obtained for every meat quality trait (dependent variable), and the significant biomarkers included. In the global data, pH45 was accurately explained (R^2=0.961, RPD=2.25) from the serum levels of LDL-cholesterol and triglycerides (both related to the metabolism of fats), and the muscle abundance at 4 h post-mortem of some peptides playing important roles in the adenine nucleotide metabolism (AK-1) and glycolysis regulation (PK and GAPDH). This agrees with previous reports that describe high influence of physiological stress on the lipid metabolism and/or the glycolytic potential of the muscle [37,38]. In our study, all significant variables included in the prediction model for pH45 contributed positively, except PK that showed negative relationship, which indicates higher enzyme levels in meat with lower pH at 45

minutes post-mortem, (i.e, meat showing higher or faster post-mortem glycolysing metabolism) which agrees with findings of Sierra et al. [11] in beef.

Meat exudation (EC24), was accurately predicted (R^2=0.994, RPD=3.26) at shorter post-mortem time (0 h) from the presence in the muscle extracts of some peptides involved in muscular contraction and/or the ATP synthesis/hydrolysis (myosin-2, SERCA 1, ATP-synthase-α mitochondrial) combined with results of fat metabolites in serum (NEFAs, hydroxybutyrate and triglycerides), thus showing a significant relationship between meat exudation (higher EC) and changes influenced by the dismantlement of the muscle structure and the metabolic homeostasis, as well as fat metabolism.

Other main meat quality traits, such as meat lightness (L*) and drip loss were also significantly (*P*< .001) predicted by different peptides detected in the muscle at 4 h (drip) or 24 h (L*) post-mortem (Table 4), but showed lower variance explanation (R^2<0.85) and the level of accuracy did not reach the value needed for analytical purposes, as RPD value was lower than 2. Thus, these regression models were rejected from further use for testing meat classification.

Table 4. Multiple linear regression models for the prediction of main meat quality traits (pH45, CE24, L*, drip-loss) based on biomarkers (biochemical and electrophoretical variables) detected in the muscle sarcoplasmic extracts

Dependent variable	Predictors included in the model (positive "+" or negative "-" contribution)	Post-mortem time point	$R^{2 \text{ (sign.)}}$	RPD	RMSEP
pH45	AK-1(+) LDL-cholesterol (+) PK (-) GAPDH (+) Triglycerides (+)	4h	0.961[***]	2.25	0.077
EC24	NEFAs (+) SERCA 1 (+) Myosin-2 (-) Hydroxibutyrate (+) ATP synthase subunit alpha, mitochondrial (+) Triglycerides (+) ATP5B (+)	0h	0.994[***]	3.26	0.272
L*	HSP70-8 (-) Haptoglobin (+) Carbonic anhydrase 3 (-)	24h	0.811[***]	0.65	1.216
Drip loss	Glycogen debranching enzyme (-) AK-1 (-) PK (+)	4h	0.828[***]	1.83	1.439

3.5 Testing the Use of Biomarkers for Identification of Meat Obtained under Animal Mixing Management

Fig. 2 shows the predicted value obtained for the dummy variable (no-mixing =1; mixing = 2) calculated for every meat sample (n=15) by discriminant analysis based on the selected biomarkers of meat quality (pH45 and EC24), and its acceptability range (±0.5). The model predicting pH45 (Fig. 2a) could successfully discriminate 86.7% of the meat samples according to the pre-slaughter animal treatment: "no-mixing" (predicted dummy variable within the acceptation range 1±0.5 for 80% of meat samples) and "mixing" treatments "MF-UTL" and "MF-MTL" (predicted dummy variable within the range 2±0.5 for 93.3% of samples), while biomarkers included in the prediction model for EC24 (Fig. 2b) failed to correctly classify meat samples, as all of them were grouped into the "mixing" treatment (predicted dummy variable included within the acceptance range 2±0.5 for 100% of meat samples).
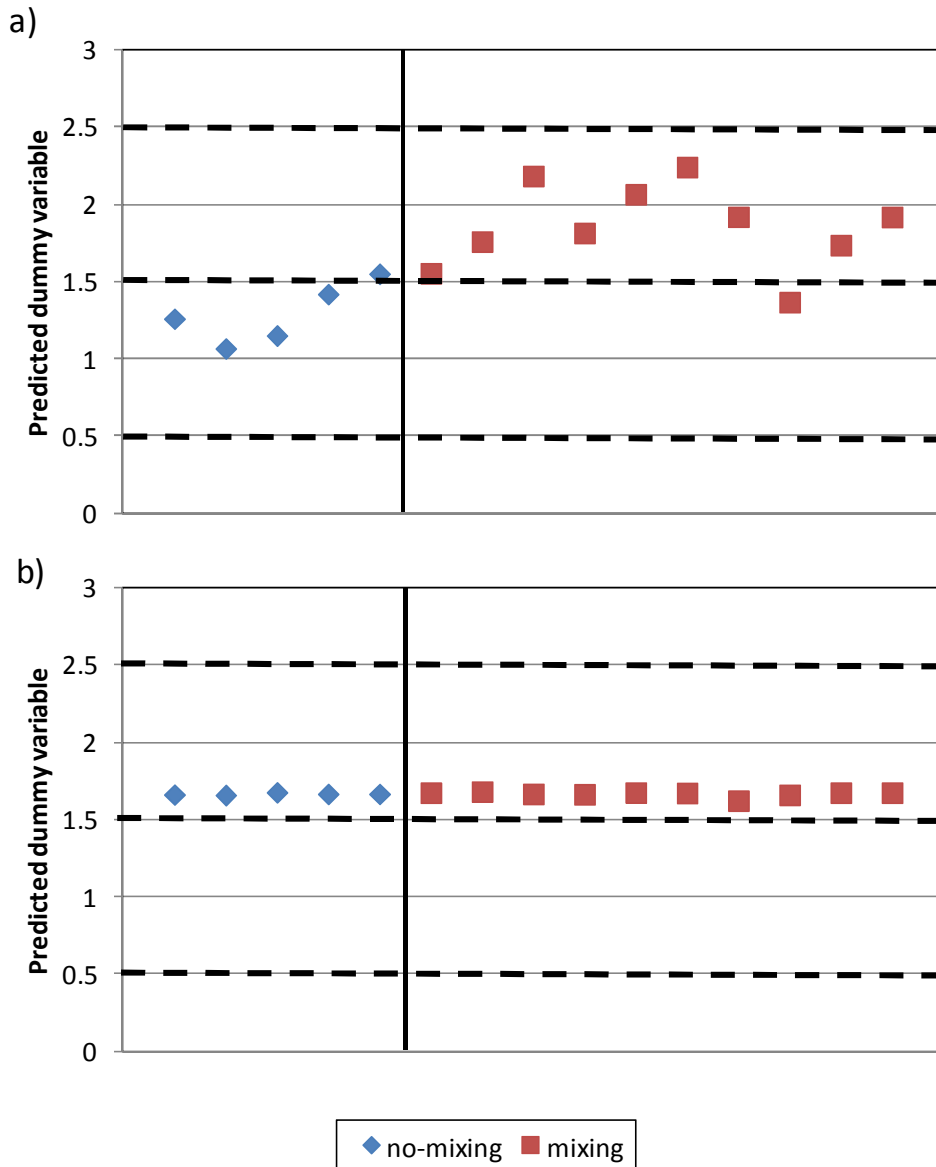

Fig. 2. Discriminant analysis of meat samples based on biomarkers included in the best predictive regression for: a) pH45, b) EC24

4. CONCLUSION

From these results we can suggest that some of the biochemical and proteomic variables measured at slaughter or within the first 24 h post-mortem could be used for the detection of situations of pre-slaughter stress related to animal mixing practices. In particular, the detection of higher levels of serum molecules related to the fat metabolism (LDL-cholesterol and triglycerides) together with changes in peptides playing a crucial role in the adenine nucleotide metabolism (AK-1) and the glycolysis regulation (PK and GAPDH) of the muscle, allowed a good identification of animals coming from pre-slaughter mixing treatments, at farm and/or during transport and lairage. However, it is worthwhile to mention that the obtained prediction model has been calculated in a small size population and more research is needed in order to validate these results on a broader animal population.

ETHICAL APPROVAL

This experiment complied with the appropriate laws and institutional guidelines for animal pre-slaughter management, as common routines allowed in animal production were applied.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Velarde A, Dalmau A. Animal welfare assessment at slaughter in Europe: Moving from inputs to outputs. Meat Sci. 2012;92:244-51.
2. Arey DS, Franklin MF. Effects of straw and unfamiliarity on fighting between newly mixed growing pigs. Appl Anim Behav Sci. 1995;45:23-30.
3. Andersen IL, Andenaes H, Boe KE, Jensen P, Bakken M. The effects of weight asymmetry and resource distribution on aggression in groups of unacquainted pigs. Appl Anim Behav Sci. 2000;68:107-20.
4. Fàbrega E, Puigvert X, Soler J, Tibau J, Dalmau A. Effect of on farm mixing and slaughter strategy on behaviour, welfare and productivity in Duroc finished entire male pigs. Appl Anim Behav Sci. 2013; 143:31-9.
5. Hambrecht E, Eissen JJ, Newman DJ, Smits CHM, Verstegen MWA, den Hartog LA. Preslaughter handling effects on pork quality and glycolytic potential in two muscles differing in fiber type composition. J Anim Sci. 2005;83:900-7.
6. Bertram HC, Oksbjerg N, Young JF. NMR-based metabonomics reveals relationship between pre-slaughter exercise stress, the plasma metabolite profile at time of slaughter, and water-holding capacity in pigs. Meat Sci. 2010;84:108-13.
7. Gajana CS, Nkukwana TT, Marume U, Muchenje V. Effects of transportation time, distance, stocking density, temperature and lairage time on incidences of pale soft exudative (PSE) and the physico-chemical characteristics of pork. Meat Sci. 2013;95: 520-5.
8. Lametsch R, Karlsson A, Rosenvold K, Andersen HJ, Roepstorff P, Bendixen E. Postmortem proteome changes of porcine muscle related to tenderness. J Agric Food Chem. 2003;51:6992-6997.
9. Jia X, Veiseth-Kent E, Grove H, Kuziora P, Aass L, Hildrum KI, et al. Peroxiredoxin-6-A potential protein marker for meat tenderness in bovine longissimus thoracis muscle. J Anim Sci. 2009;87:2391-9.
10. Di Luca A, Mullen AM, Elia G, Davey G, Hamill RM. Centrifugal drip is an accessible source for protein indicators of pork ageing and water-holding capacity. Meat Sci. 2001;88:261-70.
11. Sierra V, Fernández-Suárez V, Castro P, Osoro K, Vega-Naredo I, García-Macia M, et al. Identification of biomarkers of meat tenderisation and its use for early classification of Asturian beef into fast and late tenderizing meat. J Sci Food Agric. 2012;92:2727-40.
12. Morzel M, Chambon C, Hamelin M, Santé-Lhoutellier V, Sayd T, Monin G. Proteome changes during pork meat ageing following use of two different pre-slaughter handling procedures. Meat Sci. 2004;67:689-96.
13. Kim NK, Park HR, Lee HC, Yoon D, Son ES, Kim YS, et al. Comparative studies of skeletal muscle proteome and

transcriptome profilings between pig breeds. Mamm Genome. 2010;21:307–19.

14. Rubio-González A, Potes Y, Illán-Rodríguez D, Vega-Naredo I, Sierra V, Caballero B, et al. Effect of animal mixing as a stressor on biomarkers of autophagy and oxidative stress during pig muscle maturation. Animal. 2015;9:1188-94.

15. Rasmussen AJ, Andersson M. New method for determination of drip loss in pork muscles. In Proceedings of the 42nd International Congress of Meat Science and Technology; Lillehammer, Norway. 1996;286–287.

16. De Gonzalo-Calvo D, Neitzert K, Fernández M, Vega-Naredo I, Caballero B, García-Macia M, et al. Differential inflammatory responses in aging and disease: TNF-alpha and IL-6 as possible biomarkers. Free Radical Biol Med. 2010; 49:733-7.

17. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72:248-54.

18. Havlis J, Thomas H, Sebela M, Shevchenko A. Fast-response proteomics by accelerated in-gel digestion of proteins. Anal Chem. 2003;75:1300-6.

19. Jami MS, Barreiro C, García-Estrada C, Martín JF. Proteome analysis of the penicillin producer *Penicillium chrysogenum*: Characterization of protein changes during the industrial strain improvement. Mol Cell Proteomics. 2010; 9:1182-98.

20. Støier S, Aaslyng MD, Olsen EV, Henckel P. The effect of stress during lairage and stunning on muscle metabolism and drip loss in Danish pork. Meat Sci. 2001;59: 127-31.

21. Rosenvold K, Andersen HJ. The significance of pre-slaughter stress and diet on colour and colour stability of pork. Meat Sci. 2003;63:199-209.

22. Terlouw EMC, Porcher J, Fernandez X. Repeated handling of pigs during rearing. II. Effect of reactivity to humans on aggression during mixing and on meat quality. J Anim Sci. 2005;83:1664–72.

23. Warriss PD, Brown SN. The physiological responses to fighting in pigs and the consequences for meat quality. J Sci Food Agric. 1985;36:87–92.

24. Nanni Costa L, Lo Fiego DP, Dall'Olio S, Davoli R, Russo V. Combined effects of pre-slaughter treatments and lairage time on carcass and meat quality in pigs of different halothane genotype. Meat Sci. 2002;61:41-7.

25. Becker BA, Mayes HF, Hahn GL, Nienaber JA, Jesse GW, Anderson ME, et al. Effect of fasting and transportation on various physiological parameters and meat quality of slaughter hogs. J Anim Sci. 1989;67: 334-41.

26. Miranda-de la Lama GC, Rivero L, Chacón G, Garcia-Belenguer S, Villarroel M, Maria GA. Effect of the pre-slaughter logistic chain on some indicators of welfare in lambs. Livest Sci. 2010;128:52-9.

27. Knowles TG, Warriss PD. Stress physiology of animals during transport. In: Livestock Handling and Transport, 2nd ed. Grandin T, editor. Wallingford: CAB International; 2000.

28. Saco Y, Docampo MJ, Fàbrega E, Manteca X, Diestre A, Lampreave F, et al. Effect of stress transport on serum haptoglobin and Pig-MAP in pigs. Anim Welfare. 2003;12:403-9.

29. Salamano G, Mellia E, Candiani D, Ingravalle F, Bruno R, Ru G, et al. Changes in haptoglobin, C-reactive protein and pig-MAP during a housing period following long distance transport in swine. Vet J. 2008;177:110-5.

30. Joo ST, Kauffman RG, Kim BC, Park GB. The relationship of sarcoplasmic and myofibrillar protein solubility to colour and water-holding capacity in porcine longissimus muscle. Meat Sci. 1999;52: 291-7.

31. Ryu YC, Choi YM, Kim BC. Variations in metabolite contents and protein denaturation of the longissimus dorsi muscle in various porcine quality classifications and metabolic rates. Meat Sci. 2005;71:522-9.

32. Picard B, Gagaoua M, Micol D, Cassar-Malek I, Hocquette JF, Terlouw CEM. Inverse relationships between biomarkers and beef tenderness according to contractile and metabolic properties of the muscle. J Agric Food Chem. 2014;62: 9808–18.

33. Chosa E, Sekimoto T, Sonoda N, Yamamoto K, Matsuda H, Takahama K, et al. Evaluation of human beta-enolase as a serum marker for exercise-induced muscle damage. Clin J Sport Med. 2003;13:209-12.

34. Lametsch R, Roepstorff P, Bendixen E. Identification of protein degradation during post-mortem storage of pig meat. J Agric Food Chem. 2002;50:5508-12.

35. Laufen T, Mayer MP, Beisel C, Klostermeier D, Mogk A, Reinstein J, et al. Mechanism of regulation of Hsp70 chaperones by Dna J cochaperones. Proc Natl Acad Sci USA. 1999;96:5452-7.

36. Guillemin N, Juire C, Cassar-Malek I, Hocquette JF, Renand G, Picard B. Variations in the abundance of 24 protein biomarkers of beef tenderness according to muscle and animal type. Animal. 2011;5:885-94.

37. Hambrecht E, Eissen JJ, Newman DJ, Smits CHM, Verstegen MWA, den Hartog LA. Preslaughter handling effects on pork quality and glycolytic potential in two muscles differing in fiber type composition. J Anim Sci. 2005;83:900-7.

38. White HM, Richert BT, Latour MA. Impacts of nutrition and environmental stressors on lipid metabolism. In: Lipid Metabolism. Valenzuela R, editor. InTech (Open access); 2013.

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