SEARCH FOR PROTEOMIC BIOMARKERS RELATED TO BOVINE PRE-SLAUGHTER STRESS USING LIQUID ISOELECTRIC FOCUSING (OFFGEL) AND MASS SPECTROMETRY

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ABSTRACT

Proteome changes derived from animals that have suffered pre-slaughter stress are a fact. In this study, Proteomic analysis was carried out on 20 bovine loin samples from Asturiana de los Valles and crossbreds cattle previously classified as normal and DFD meat at 24 h post-mortem using pH measurements. Sarcoplasmic sub-proteome of *Longissimus thoracis* at 24 hours post-mortem was fractionated by the use of liquid isoelectric focusing (OFFGEL) in the pH range 3-10, followed by SDS-PAGE analysis of each retrieved fraction. The protein fractionation profile showed high reproducibility along the different sample groups. Five protein bands showed significant differences ($p<0.05$) between the two groups, allowing discrimination between them. Proteins present in these bands, which were identified by LC-MS, were actin, phosphoglucosylase-1, alpha-crystallin B, heat shock protein beta-6 and heat shock protein beta-1.

The **significance** of this study relies on the optimization of OFFGEL fractionation as a promising technology to search for reliable biomarkers of pre-slaughter stress. This method separates proteins along different liquid fractions according to their isoelectric point; the obtained fractions can be further characterized by SDS-PAGE or directly identified by LC-MS. This achievement stands out as an alternative to the use of 2-DE electrophoresis in protein separation and analysis.

Key words: DFD meat; pre-slaughter stress; OFFGEL; protein biomarkers; mass spectrometry.

1. INTRODUCTION

Proteomics has thoroughly sought a deep understanding of gene expression and protein translation. This discipline concerns protein interactions or modifications (phosphorylation, denaturation, methylation, etc) but also the biochemical pathways that take place under different conditions in living organisms [1]. It has been widely used to search for protein biomarkers related to meat tenderness, colour and water holding capacity (WHC) [2]. However, few research efforts have been put in the discovery of pre-slaughter stress biomarkers leading to discriminate among meat from either normal or stressed animals as stated by Franco et al. [3], reporting for the first time the usefulness of Proteomics to study pre-slaughter stress condition (PSS) in Rubia Gallega cattle breed.

PSS arises from both intrinsic and extrinsic factors of slaughtered animals. Among the intrinsic factors, physiology, age, sex and genetics are among the most relevant. Regarding extrinsic stressors such as temperature, handling activities, human presence, lairage time or feed/water deprivation in the abattoir [4] they are not only prevalent but also manageable. Before slaughter, animals exposed to PSS consume their glycogen reservoirs to produce ATP through glycolysis. This perturbs glycolysis after slaughter, lowering its rate due to the prior glycogen reduction and generating not enough lactic acid to enable a normal pH reduction, thus giving rise to higher pH values like 6.0 and above, which evolves in an abnormal conversion of muscle into meat [5]. This condition produces dark, firm and dry (DFD) meat, which causes economical losses in the meat industry because of its lower quality attributes such as alterations in tenderness during aging, higher WHC or spoilage by microorganism contamination [6]. To reduce the incidence of DFD meat, current practices performed by meat producers, food industry and governments consider the improvement of animal welfare regarding their transportation, lairage, handling at abattoirs and slaughter methods.

Although there are many biochemical indicators (glucose, lactate, pH, etc.) that can be quickly measured to assess animal stress [7], they do not provide further information about biological changes occurring due to PSS. In this respect, Proteomics has become a powerful tool to unveil reliable protein biomarkers to detect meat quality defects [8].

Skeletal muscle houses a huge variety of proteins with different roles such as structural, defensive/stress and metabolic functions [9]. Some of them have been suggested as stress biomarkers, as the case of creatine kinase (CK) and lactate dehydrogenase (LDH) in animals that have suffered pre-slaughter stress [10]. Increased levels of these enzymes in plasma and blood would be indicative of muscle damage due to stressor factors [11]. Moreover, Heat Shock Proteins (HSPs) normally involved in defence mechanisms, have been also studied in relation to stress. It should be emphasized that HSPs play a major role as essential molecular chaperones under environmental and physiological stress conditions [12,13].

Regarding protein fractionation techniques, the most common approach was sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by two-dimensional polyacrylamide gel electrophoresis (2-DE) [14]. Despite the popularity of gel-based strategies, novel gel-free alternatives have recently been proposed to overcome drawbacks of traditional fractionation concerning costs in routine analysis (mainly 2-DE) and limitations for covering the entire proteome (small biomolecules) of samples. One of these emerging technologies is the liquid isoelectric focusing (OFFGEL) that separates proteins according to their isoelectric point (pI) [15]. Thus, a multi-compartment chamber with 12 or 24 wells is placed on top of an immobilized pH gradient gel (IPG) strips that provides the pH gradient. Solubilised proteins migrate across IPG strips until meeting the pH corresponding to their respective pI and stop. Time required for fractionation depends on sample complexity, reaching up to 24 h. Retrieved liquid fractions containing fractionated/enriched proteins can be submitted to further gel-based separation steps or directly digested and analysed by liquid chromatography coupled to mass spectrometry (LC-MS).

Advantages in fractionation/enrichment of protein samples using OFFGEL were recently demonstrated [16,17] and promising results were also achieved when it was implemented in studies assessing safety, quality and origin of meat and meat products [18,19,20]. However, its use in the fractionation/enrichment of muscle proteome is still limited.

This work aims to study changes in the sarcoplasmic proteome of meat to elucidate which proteins and biochemical mechanisms are involved in PSS. Then, normal and

DFD samples of bovine *Longissimus thoracis* (LT) muscle were analysed by OFFGEL fractionation/enrichment followed by SDS-PAGE and LC-MS analysis. The proposed methodology would facilitate the elucidation of different proteins as reliable PSS biomarkers that could be useful to assess quality of commercialized meat and meat products.

2. MATERIALS AND METHODS

2.1. Animal samples

In this research, a total of **twenty** (n=20) beef samples were analysed. Eight 'normal' meat samples with pH values lower than 5.5 at 24 h post-mortem were collected from Asturiana de los Valles (AV) breed. Another **twelve** samples were collected from crossbred animals (**AV x Friesian**) from which six samples were considered as 'DFD' meat (**PSS-CB: pre-slaughter stressed crossbred animals**), with pH values over 6.0, and **the other six were considered as 'normal' meat (N-CB: normal crossbred animals)**, with pH values below 6.0. These made a 'normal' meat group of **14** samples, and a 'DFD' meat group of 6 samples. The pH measurements were taken at the *Longissimus thoracis* (LT) muscle of the 6th rib at 24 h post-mortem.

Animals were slaughtered and dressed in commercial abattoirs at the age of 12 to 18 months (yearling bulls) following the safety and welfare conditions established by the European Union (Council Regulation (EC) No 853/2004 and No 1099/2009). At 2 h post-mortem, a LT muscle sample from the 13th rib was excised from the left half of each carcass. After muscle sampling, the epimysium was carefully dissected, and about 10 g of each muscle sample were taken. Samples were then transported to the laboratory in refrigerated conditions and, at 24 h post-mortem, they were frozen in liquid nitrogen and stored at -80 °C until analysed.

2.2. Extraction and quantification of sarcoplasmic proteins

Half a gram of each type of meat, normal and DFD, was homogenized in 4 mL of extraction buffer (10 mM Tris pH 7.6, 1 mM EDTA, 0.25 M Sucrose) containing 25

μL of inhibitors cocktail (P8340, Sigma-Aldrich Co., St. Louis, MO, USA) using an Ultra-Turrax Yellow Line Di 25 (IKA-Werke GmbH, Staufen, Germany). The homogenate was centrifuged at 20,000 *g* for 20 min at 4 °C using a Beckman Coulter centrifuge (Brea, CA, USA), being the obtained supernatant filtered through a 45 μm PVDF syringe filter and stored at -80°C until analysed. Protein quantification of samples was assessed using the Bio-Rad Protein Assay Kit (Hercules, CA, USA) following the Bradford method and using a calibration curve ranging from 0.05 to 0.8 mg/mL of BSA.

2.3. Liquid Isoelectric Focusing (OFFGEL)

Sarcoplasmic proteins were fractionated according to their *pI* into 12 liquid fractions using 13 cm IPG strips with a linear gradient in the pH range 3-10 (GE healthcare, Uppsala, Sweden). One mg of each sarcoplasmic extract was diluted to a final volume of 2 mL with 1.25X protein OFFGEL stock solution (50 % v/v glycerol, 7 M urea, 2 M thiourea, 65 mM DTT and IPG buffer pH 3-10, GE healthcare, Uppsala, Sweden) and used for protein fractionation/enrichment using an Agilent 3100 OFFGEL fractionator (Agilent Technologies, Waldbronn, Germany) following the manufacturer's instructions. IPG strips were fixed onto the tray and rehydrated with 1.25X protein OFFGEL stock solution. Fractionation was performed at 20 °C with a constant current of 50 μA to reach 20 kVh for about 20 h (depending on salt concentration of samples). After focusing, the 12 fractions were separately collected and stored at -80 °C.

2.4. SDS PAGE and Image analysis

SDS-PAGE analyses were carried out in duplicate (analytical replica) for each of the [twenty](#) fractionated muscle extracts. OFFGEL protein samples were diluted (50:50, v/v) with a sample buffer solution (0.5 M Tris-HCl pH 6.8, 50% v/v glycerol, 10% w/v SDS, 0.2 M DTT and 0.04 w/v bromophenol blue) and heated at 95 °C for 4 min to denaturalize proteins. Samples were centrifuged (2,000 *g*, 1 min) before loading onto 1.5 mm x 8 cm x 9 cm 12 % polyacrylamide gels. Each pair of gels was

simultaneously run in a Hoefer Mighty Small II SE260 electrophoresis unit (San Francisco, CA, USA) at a constant current of 50 mA for two hours. After electrophoresis, gels were fixed into 12 % trichloroacetic acid solution for 1 h, then washed two times with bidistilled water for 10 min. Finally, gels were stained with colloidal Blue Coomassie [21] overnight and finally destained with bidistilled water. For scanning gels, LAS-1000 Luminescent Image Analyzer with Intelligent Dark Box II (FUJIFILM, Barcelona, Spain) was used. The intensity of each band was determined using the freeware Gel Analyzer 2010 software (<http://www.gelanalyzer.com>). Individual band intensities were normalized according to the total band volume per gel. Estimated pI and molecular mass (Mr) of each band was determined from their position in the OFFGEL fractions and their migration in the polyacrylamide gel, respectively, comparing to commercial standards ranging from 14 to 200 kDa (Cat. No. 161-0317, Bio-Rad, Hercules, CA, USA).

2.5. In-gel digestion of selected protein bands

Those bands showing statistically significant intensity differences between normal and DFD samples were digested with trypsin to elucidate their protein profiles. Thus, bands were excised from the gel and placed into 0.5 mL Eppendorf tubes. Bands were washed twice in 50 mM ammonium bicarbonate for 10 min in continuous agitation and solvent discarded by pipetting. Bands were dried until turning shrink and opaque by washing twice with 100% ACN. Once the liquid was removed by pipetting, the remaining liquid was evaporated using a Savant SPD121P Speed Vac concentrator equipped with a RVT400 refrigerated vapour trap (Thermo Scientific, San Jose, CA, USA). Dried samples were digested with 15 µL of 12.5 µg/mL sequencing grade modified trypsin (Promega, Madison, WI, USA) solution and mixed with 50 µM ammonium bicarbonate (pH 8.5) overnight at 37 °C with continuous gently shaking. Peptides were recovered by washing samples twice with water/ACN (50:50, v/v) acidified with 0.1 % TFA, sonicated for 15 min, the supernatants poured into clean Eppendorf tubes and evaporated as previously mentioned. Finally, samples were redissolved in 40 µL of a 0.1 % aqueous TFA solution and poured into LC-MS vials.

219 **2.6. Mass spectrometry analysis**

220 Twenty-five μL of each sample prepared as described in section 2.5 were injected
221 in a Thermo Surveyor LC system equipped with a refrigerated autosampler and
222 coupled to a Thermo LCQ Advantage (Thermo Scientific, San Jose, CA, USA) ion
223 trap MS instrument loading an electrospray (ESI) probe. Separation of peptides was
224 carried out on a Jupiter Proteo reversed-phase C18 column (15.0 cm $\times$ 0.5 mm;
225 Phenomenex, Torrance, CA, USA). Mobile phases consisted of solvent A containing
226 0.1 % formic acid (v/v) in bidistilled water and solvent B containing 0.1 % formic acid
227 (v/v) in ACN. Chromatographic analysis was at 23 $^{\circ}\text{C}$ and separation conditions
228 consisted of a stepwise isocratic gradients including 15 min of 0 % B, 5 min of 20 %
229 B, 10 min of 40 % B and finally 10 min of 100 % B at a flow rate of 30 $\mu\text{L}/\text{min}$, then
230 returning to initial conditions having 0 % of solvent B.

231 Operating parameters of the ion trap detector were the following: electrospray
232 ionization in positive mode; capillary temperature, 250 $^{\circ}\text{C}$; spray voltage, 4.0 kV;
233 capillary voltage, 15.0 V. First scan event was full MS detection in the 400–2000
234 m/z range. The second scan event was a data dependent MS/MS analysis of the
235 most intense ions with charges from +2 to +4 with the following parameters: repeat
236 count for most intense ion, 3; repeat count duration, 0.5 min; exclusion duration, 10
237 min; normalized collision energy, 35 %; minimum MS signal for MS/MS analysis,
238 1×10^5 . Both scan events shared the same number of microscans and maximum
239 injection time that were 3 and 300 ms, respectively. Control of the LC-MS system
240 and data acquisition was done through a PC loading the Thermo Xcalibur v2.0
241 (Thermo Scientific, San Jose, CA, USA) software. Peptide identification was
242 achieved by the interpretation of generated MS/MS data against Uniprot KB
243 v2017_11 (www.uniprot.org) and NCBIprot v20170428 protein databases using
244 Mascot v2.3 as search engine with the following parameters: enzyme, trypsin; no
245 fixed or variable modifications but “Error tolerant” option enabled; mass accuracy
246 was set to 1.2 and 0.8 Da for MS and MS/MS analyses, respectively; The option
247 “Mammalia” was selected as taxonomy restriction parameter; decoy option was
248 used to estimate the false positive rates by means of False Discovery Rate
249 threshold from 1 %. For selection of protein family identifications obtained from

Mascot, only those having a protein score derived from individual ion scores indicating identity or extensive homology ($p < 0.05$) were considered as true protein identifications.

2.7. Statistical and protein functional analysis

Obtained results were analysed using univariate and multivariate statistics. The univariate Mann-Whitney non-parametric test was applied to find significant differences between normal and DFD sample groups ($p < 0.05$) using SPSS 23.0.0 software (SPSS Inc., Chicago, IL, USA). The intensity of each of the selected protein bands of the fourteen (normal) and six (DFD) individuals were compared. For multivariate analysis of data, principal component analysis (PCA) was utilised using the XLSTAT 2010.5.02 software (Addinsoft, Paris, France). The two-dimensional coordinate system defined by the first two principal components was used to study the distribution of each animal according to the intensity of selected protein bands.

Each identified protein was classified according to its biological function by means of Gene Ontology (GO) term from AmiGO website (<http://amigo.geneontology.org/amigo/>). Protein-protein interactions from bands that showed significantly different abundances between normal and DFD samples were assessed using STRING v.10.5 freeware software (ELIXIR, Wellcome Genome Campus, Hinxton, Cambridgeshire, UK, <https://string-db.org>). *Bos taurus* species was used to perform the analysis.

3. RESULTS AND DISCUSSION

The twenty bovine meat samples analysed in this work were classified as normal and DFD according to pH values measured at 24 h post-mortem (data not shown). The main aim of this study was to compare normal and DFD animal's proteome using liquid isoelectric focusing in combination with SDS-PAGE and LC-MS.

After OFFGEL separation, 12 protein fractions were obtained for each sample along the assessed pH range (3 to 10). These were further analysed by SDS-PAGE in order to reveal the protein distribution along these fractions.

Figure 1 shows representative examples of SDS-PAGE gels of the fractionated sarcoplasmic sub-proteome from normal (1A) and DFD (1B) meats. Protein distribution profiles achieved by OFFGEL separation/enrichment were highly reproducible in all retrieved fractions, enabling the study of differences between normal and DFD groups. In both cases, acidic fractions (1 to 5) housed only few proteins in contrast to the most populated neutral and basic fractions (6 to 12).

The most noticeable differences in protein abundances between normal and DFD gels were observed in bands A to E (squared in **Figures 1A and 1B**). This finding was confirmed by densitometry analysis (**Figure 2**) that revealed significant differences ($p<0.01$) among both meat groups. So, abundance of band B was notoriously higher in normal samples, whereas band A was significantly less abundant in the normal group in comparison to DFD meat. Interestingly, it was observed that bands C, D and E were exclusive of the DFD group.

Further LC-MS analysis of bands with the same molecular mass clustered in neighboring fractions (bands B and E in **Figure 1**) was carried out to confirm same protein assignment for each particular band (**Table 1**). This outcome was previously reported [16] and seemed to be related to protein phosphorylation, which induces changes in the pI value of the protein. Both phosphoglucosyltransferase-1 (PGMT1; band B in **Figure 1**) and heat shock protein beta-1 (HSPB1; band E in **Figure 1**) have a phosphorylation site. Thus, their degree of phosphorylation could distribute them over different OFFGEL fractions according to their final pI value. Furthermore, PGMT1 participates in the transference of the phosphate group from glucose-1-P to glucose-6-P as well as HSPs can suffer a post-translational phosphorylation that may alter their pI value [22].

Figure 3 illustrates PCA representation of factor scores of each sample (animal) studied according to the intensity of selected protein bands (A to E). The first component (PC1) explained 76,23% of the total variability found among beef samples and clearly discriminated between normal and DFD groups in which AV and N-CB had negative factor scores, while PSS-CB had positive factor scores.

Among the normal sample group, all individuals (AV and N-CB) were mixed in the left side of the PC1 and it was observed there was no separation between AV and N-CB groups neither through PC1 nor PC2. Then, we can conclude that breed effect does not have remarkable influence on the normal sample group, being this factor not relevant in our study of searching for biomarkers related to PSS.

Functional analysis of identified proteins

As mentioned, LC-MS study characterized proteins from bands A to E, being classified (**Table 1**) according to their biological role as stress (alpha-crystallin B, heat shock protein beta-6 and heat shock protein beta-1), metabolic (phosphoglucosaminase-1) and structural (actin) proteins. Among those proteins and under normal conditions (pH below 6.0 after slaughter), actin is the only one that exhibits a poor aqueous solubility even if a small part of it can be extracted together with sarcoplasmic proteins [9,23,24]. In contrast, pH values of DFD meat (above 6.0) are far enough from the pI of actin (5.23), enhancing its solubility and turning more noticeable the presence of this protein in the soluble extracts of stressed animals (see **Figures 1** and **2**).

Protein-protein interaction network of the five characterized bovine proteins is depicted in **Figure 4**. Phosphoglucosaminase-1 (PGM1) and heat shock protein beta-6 (HSPB6) remain out of the protein network, with no interaction between these proteins and the others. PGM1 plays an important role in the regulation of glycogen metabolism through the inter-conversion of glucose-1-phosphate to glucose-6-phosphate. This latter enters the glycolytic pathway to produce ATP in order to maintain cell homeostasis [25]. The catalytic site of phosphoglucosaminase-1 molecule contains a phosphorylated serine residue at position 117 of its polypeptide chain. The phosphoryl group is transferred to the C-6 hydroxyl group of glucose 1-phosphate to form glucose 1,6-biphosphate. The C-1 phosphoryl group of this intermediate is then shifted to the same serine residue, resulting in the formation of glucose 6-phosphate and the regeneration of the phosphoenzyme.

Heat shock protein beta-1 (HSPB1) is in the centre of the network node and is related to alpha-crystallin B (CRYAB) and actin (ACTA1). Although HSPB6 is not

linked to HSPB1 and CRYAB, this protein is well known in terms of its function, so we could conclude that the database lacks of information to relate this protein to the other two. All of them belong to the heat shock protein family and have been widely described as molecular chaperones involved in several mechanisms (protein assembly and disassembly, protein folding and unfolding, translocation, ...) and can interact with damaged proteins under stressed conditions to preserve their function [26]. Some authors have associated HSPB6 to meat tenderness [27] and apoptosis pathway [28], although the mechanism involved remains unclear.

Interaction between actin and heat shock protein beta-1 comes from the protective action exerted by HSPs under stress conditions as reported by Jia et al. [29], detailing their patronizing effect on actin filaments against aggregation promoted by oxidative stress. However, it has been stated that increased phosphorylation of mammalian HSPs can appropriately modify the oligomeric conformation of proteins, turning them a more stable resultant forms and protecting against stress [30].

Relationship between protein abundance and meat quality derived from PSS

Tenderness is one of the most relevant traits of meat quality together with juiciness and flavour, and many efforts have been carried out to understand the mechanisms involved [27]. This attribute is influenced by a wide variety of both pre-slaughter (animal species, breed, age, muscle type and handling activities) and post-mortem (pH, WHC, sarcomere shrinkage, temperature and proteolysis of myofibrillar proteins) factors [31]. Some studies reported that DFD meat from animals that suffered PSS was significantly more tender than normal meat at 24 h post-mortem [3,32]. Abnormally higher pHs (>6.0) reached in DFD meat may contribute to an increased tenderness in different ways. Firstly, raising the electrostatic repulsion between myofibrillar proteins confers less lateral shrinkage of muscle fibres [33]. Secondly, overall protein denaturation is lower and stimulates the water-binding capacity of myosin. We can conclude that both mechanisms characterize DFD meat with higher WHC.

There was reported the importance of phosphoglucomutase-1 on meat and fish quality regarding tenderness [8,34]. This enzyme seems to be downregulated in tender meats as demonstrated by Ciaramella et al. [35] in stressed catfish and by Picard et al. [36] in tender compared to tough beef steaks. In this line, other studies stated that DFD meat is more tender than normal meat at 24 h post-mortem [3,32]. All those results are in agreement with findings illustrated by **Figures 1** and **2** regarding higher abundance of PGM1 in normal compared to DFD samples. This can be understood considering the potential nine phosphorylation sites of PGM1, one of them involved in the glycogen pathway that seems to play an important role in meat tenderization [34]. In this study, this enzyme spread along three different OFFGEL fractions (lanes 6, 7 and 8 in **Figure 1** A and B) in both sample groups due to phosphorylation. More concretely, one peptide of PGM1 was observed to be phosphorylated at Ser-117 of the protein chain (**Table 2**) in these fractions for both normal and DFD meat. This suggested the activation of the catalytic site of PGM1 that enabled its participation into the aforementioned glucose interconversion via glycolysis to produce ATP. The lower abundance of this protein in DFD samples (**Figure 2**) could be related to glycogen depletion before slaughter due to PSS, notably reducing the metabolism of the enzyme.

Although the action of HSPs has been extensively studied in living muscle, their role in post-mortem muscle is still unclear. In this study, the three stress-related proteins indentified (bands C, D and E in **Table 1**) were only found in DFD meat samples (**Figure 1**). This finding seems to agree with evidence that stress can induce the synthesis of protective proteins such as HSPs to preserve cellular proteins against denaturation and possible loss of function [37] in living animals. Furthermore, there is an existing controversy regarding the pro- or anti-apoptotic role of HSPs, suggesting that their function in apoptosis may depend on its phosphorylation state. Ouali et al. [27] proposed apoptosis as a novel way to explain meat tenderisation and suggested that HSPs might delay meat aging, negatively affecting meat tenderness. Several mechanisms were also reported in which stress-generated HSPs could play diverse roles as anti-apoptotic factor by complexation with active caspases, protection of target proteins and restoration of the active structure of proteins [38]. This is in accordance to results obtained by Rosa et al. [39] for HSPB6 since they reported a negative correlation between the expression of this

protein and tenderness. In contrast, these same authors found that HSPB1 was positively correlated with beef tenderness in LT muscle, in accordance to results obtained by Morzel et al. [40] and Carvalho et al. [41]. As observed, despite many studies addressing HSPs, stress and meat tenderness, relevance of these proteins in post-mortem muscle and meat quality still remains unclear and deserves further investigations.

CONCLUSION

The comparison of sarcoplasmic sub-proteomes of normal and DFD meat samples was successfully achieved using liquid isoelectric focusing (OFFGEL) hyphenated to SDS-PAGE and LC-MS analyses. Five bands associated to proteins actin, phosphoglucomutase-1, alpha-crystallin B, heat shock protein beta-6 and heat shock protein beta-1, with different abundances and biological functions, were found for normal and DFD meat groups and firstly proposed as biomarkers of the PSS.

The innovative proteome fractionation approach presented in this work can constitute an interesting alternative to traditional 2-DE electrophoresis. Highly reproducible protein profiles achieved by implementing OFFGEL fractionation enabled the accurate comparison of the proteome from different meat quality groups.

This research may facilitate future studies addressing the biological role and post-translational modification of these biomarkers under specific stress conditions, as well as the direct implementation of the OFFGEL strategy in gel-free approaches supported by LC-MS analysis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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549

Figure captions

Figure 1. 12 % SDS-PAGE of fractions obtained after OFFGEL isoelectric focusing in the pH range 3-10 from two representative samples: 1A) normal and 1B) DFD meat. *Std*: Molecular standard.

Figure 2. Mean values of abundances (and standard deviations) of the five selected protein bands obtained by densitometry analysis (described in Table 1) in normal (dark, n=14) and DFD (light, n=6) meat sample groups. Error bars indicate the standard error of the mean for each group.

Figure 3. Animal sample distribution on the two-dimensional coordinate system defined by principal component (PC) 1 and 2. Each point represents an individual sample (♦ AV: Asturiana de los Valles animals; ■ N-CB: normal crossbred animals; ▲ PSS-CB: pre-slaughter stressed crossbred animals).

Figure 4. Protein-protein interaction network of five selected protein bands in normal and DFD meats using String 10.5. The network nodes (circles) represent proteins, the edges represent protein-protein functional associations, line colour indicates the type of interaction and line thickness denotes the association strength (threshold: 0.4, medium confidence level). ACTA1: Actin; HSPB1: Heat shock protein beta-1; CRYAB: Alpha-crystallin B; HSPB6: Heat shock protein beta-6; PGM1: Phosphoglucomutase-1.

1 **Table 1. Proteins from bovine *Longissimus thoracis* muscle showing significant differences between normal and DFD meat that were identified by**
2 **LC-ESI-MS/MS using the MASCOT search engine and NCBI nr and Uniprot KB protein databases. All identified proteins belonged to *Bos taurus***
3 **species.**

Band	Protein Identification/ Gene ^a	Theoretical ^a / apparent Mr (kDa) ^b	Theoretical ^a / apparent pI (pH value in the OFFGEL fraction)		Accession number ^c (UNIPROT)	Mascot Score ^a	Protein coverage ^a (%)	p-value ^d	Biological function
A	Actin ACTA1	42.02/50.80	5.23	5.05-5.53	P68138	388	25	<0.01	Structural maintenance
B	Phosphoglucomutase-1 PGM1	61.55/71.20	6.36	6.50-7.47	Q08DP0	366	19	<0.01	Regulation of glycogen metabolism
C	Alpha-crystallin B CRYAB	20.02/20.73	6.76	7.47-7.95	P02510	388	43	<0.01	Stress resistance
D	Heat shock protein beta-6 HSPB6	17.46/19.15	5.95	6.02-6.50	Q148F8	418	61	<0.01	Stress resistance
E	Heat shock protein beta-1 HSPB1	22.38/25.92	5.98	5.53-6.50	Q3T149	613	43	<0.01	Stress resistance and actin organization

4 ^a Protein and gel identification, theoretical Mr and pI, Mascot Score, Coverage and N°. of peptides identified were removed from Swiss-Prot/UniProt KB
5 database.

6 ^b Apparent Mr was calculated through band position of the gel.

7 ^c Accession number was derived from UNIPROT database.

8 ^d p-value was estimated using Mann-Whitney test.

Table 2 Phosphorylated peptides identified from PGM1 in both normal and DFD meat from the analysis of MS/MS data using MASCOT search engine and NCBIprot and Uniprot KB protein databases. Underlined amino acids correspond to those that have suffered phosphorylation.

Fraction	Protein identified	Specific sequence showing a phosphorylated position ^a	Peptide Score ^b	N° of phospho-peptides ^c	Precursor ion m/z	Charge	Probability (%) ^d
6	PGM1	¹⁰⁸ AIGGIILTA <u>S</u> HNPGGPNGDFGIK ¹³⁰ + phosphorylation (S117)	57	2	1144.15	2+	81.78
7	PGM1	¹⁰⁸ AIGGIILTA <u>S</u> HNPGGPNGDFGIK ¹³⁰ + phosphorylation (S117)	56	4	1143.27	2+	59.50
8	PGM1	¹⁰⁸ AIGGIILTA <u>S</u> HNPGGPNGDFGIK ¹³⁰ + phosphorylation (S117)	44	2	1143.71	2+	100

^a Superscripts indicate the position of the peptide into the PGM1 protein sequence.

^b Peptide score was derived from the highest score value among all phosphorylated peptides identified.

^c N° of identified peptides that were phosphorylated at serine 117.

^d It is referring to the probability that the modification occurs in that specific amino acid of the sequence that it has been studied.

A

B

Highlights

- Soluble bovine muscle sub-proteome from normal and pre-slaughter stressed animals was analysed to study protein changes.
- Five proteins showed significant statistical differences in abundance between normal and stressed animals.
- Alpha-crystallin B, HSPB6 and HSPB1 were the most reliable biomarkers related to stress condition.
- OFFGEL fractionation gets up as a novel approach to study biochemical changes related to pre-slaughter stress in cattle.