# Role of the endoplasmic reticulum in the search for early biomarkers of meat quality

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ABSTRACT

Defects in meat quality such as dark, firm and dry (DFD) beef have been related to high levels of oxidative stress that produce cellular alterations that may affect to the process of meat quality acquisition. Despite the important role of endoplasmic reticulum (ER) in the cellular response to oxidative stress, its function in the muscle-to-meat conversion process has not yet been studied. In this study, differences in muscular antioxidant defense and the unfolded protein response (UPR) of the ER in CONTROL (normal $pH_{24}$) and dark, firm, and dry (DFD, $pH_{24} \geq 6.2$) beef at 24 h *post-mortem* were analyzed to understand the changes in the muscle-to-meat conversion process related to meat quality defects. DFD meat showed poor quality, lower antioxidant activity ($P < 0.05$) and higher UPR activation ($P < 0.05$), which indicates higher oxidative stress what could partly explain the occurrence of meat quality defects. Therefore, the biomarkers of these cellular processes (IRE1α, ATF6α, and p-eIF2α) are putative biomarkers of meat quality.

## 1. Introduction

In cattle, the appearance of meat quality defects such as dark, firm, and dry (DFD) beef causes consumer rejection, economic losses in the meat sector and food waste. DFD beef exhibits a defective *post-mortem* muscle maturation process resulting in meat with high ultimate pH (pHu), unappealing dark color, abnormal texture and higher spoilage (Loudon et al., 2018; Mahmood, Turchinsky, Paradis, Dixon, & Bruce, 2018; Ponnampalam et al., 2017).

These defects have been related to different types of pre-slaughter stressors including adverse weather conditions, husbandry practices, handling, transport and lairage conditions, duration of the fasting period, thirst, and fatigue (Muchenje, Dzama, Chimonyo, Strydom, & Raats, 2009). Moreover, it is known that the effect of slaughter conditions on the muscle metabolism and the ultimate meat quality depends on the physiological state of the animal, including its individual reaction to stress (Bourguet et al., 2010; Terlouw et al., 2021). It is important to note that the animal's stress state depends on its evaluation of the

situation, not on the situation itself. Therefore, the stress perception by each animal is an individual and subjective experience that is difficult to detect and prevent. For this reason, meat scientists are focused on the identification of molecular biomarkers related to the *post-mortem* muscle metabolism that have significant effects on the muscle-to-meat conversion process and may be detected in the carcass at early *post-mortem* times (Ponnampalam et al., 2017).

After animal slaughter and exsanguination, muscle cells face anoxia, which drastically reduces cellular energy production by inhibiting the mitochondrial electron transport chain. This situation alters the redox balance between oxidant and antioxidant molecules, increasing production of reactive oxygen species (ROS), which are highly reactive free radicals that are produced as byproducts during oxidative phosphorylation and produce oxidative stress (Li et al., 2011). Although different physiological, environmental, and breeding conditions give rise to ROS, it is widely accepted that ROS levels can increase dramatically during stressful situations (Xing, Gao, Tume, Zhou, & Xu, 2019). Any imbalance between these molecules and antioxidant mechanisms can culminate as

cellular oxidative stress, which can cause damage and molecular changes in DNA, proteins, and lipids, with detrimental effects on the ultimate meat quality (Díaz et al., 2020; Díaz-Luis et al., 2020; González-Blanco et al., 2021). In response to this oxidative challenge, muscle cells that at the early *post-mortem* still keep their capacity to trigger different mechanisms to restore homeostasis, may enroll in different pathways for recycling and replacement of damaged molecules, organelles, or cells to overcome the damage; therefore, the muscle tissue may engage different cell death subroutines, such as apoptosis or autophagy (Sierra & Olivan, 2013).

Previous research has demonstrated that different conditions that may cause pre-slaughter stress (PSS) such as different rearing systems (intensive *vs* extensive), pre-slaughter management systems (mixing or not with unfamiliar animals at farm and/or transport and lairage) and individual emotional state (cognitive bias positive or negative) increase oxidative stress and trigger cell death processes (autophagy and/or and apoptosis) in the muscle cells, which affects to the muscle-to-meat conversion process (Díaz et al., 2020; Díaz-Luis et al., 2020; Fuente-Garcia et al., 2019; García-Macia et al., 2014; Potes et al., 2017; Rubio-González et al., 2015). Other authors have also demonstrated that oxidative stress alters the function of different subcellular compartments, leading to mitochondrial dysfunction and abnormal calcium metabolism, which may play a role in meat quality defects such as dark cutting (Xing et al., 2019).

The search for novel biomarkers of meat quality has led to the study of other cellular organelles involved in the regulation of cellular stress. This is the case for the endoplasmic reticulum (ER), which has been shown to malfunction in human muscles under stressful conditions (Estébanez, De Paz, Cuevas, & González-Gallego, 2018). PSS significantly increases cellular oxidative stress, which can alter the normal ER function in animal muscles. However, despite the important role of ER in the cellular response to oxidative stress, its function in the muscle-to-meat conversion process has not been studied. The ER is the main organelle involved in protein synthesis, folding, and transport, as well as intracellular calcium storage in eukaryotic cells, and is to be considered crucial in regulating cellular responses to stress (Wang & Kaufman, 2016). An imbalance in the redox state may disrupt calcium homeostasis in the ER, leading to the accumulation of unfolded or misfolded proteins. Previous studies have revealed that misfolded proteins in the ER can affect their normal physiological functions and induce ER stress, which promotes the unfolded protein response (UPR) (Cao & Kaufman, 2012; Schröder & Kaufman, 2005). This multidimensional response is initiated in the ER by three transmembrane proteins that promote different pathways: inositol-requiring protein 1 (IRE1α), activating transcription factor 6 (ATF6α), and RNA-dependent protein kinase-like ER/eukaryotic initiation factor 2α (PERK/eIF2α) (González-Blanco et al., 2022; Walter & Ron, 2011). Each pathway activates multiple mechanisms to restore homeostasis and achieve cell survival. In addition, recent studies have indicated that the ER cooperates with other important cellular organelles, such as the mitochondria and nucleus, in apoptotic processes (Breckenridge, Germain, Mathai, Nguyen, & Shore, 2003), and a relevant role of the ER in autophagic pathways has also been considered (Lemasters, 2005).

Therefore, the aim of this study was to analyze differences in the muscle cellular oxidative status and ER stress response in CONTROL (normal pH$_{24}$) and DFD beef to elucidate the role of ER in the muscle-to-meat conversion process and identify novel early biomarkers of beef quality.

## 2. Materials and methods

### 2.1. Animals

A total of 1133 yearling bulls from the autochthonous beef breed 'Asturiana de los Valles' (AV) were monitored at several accredited abattoirs from the Asturias region, where animals were slaughtered according to current EU regulations (Council Regulation [EC] No. 1099/2009). After slaughter, carcasses were transferred to a cold room at 3 °C, and at 24 h *post-mortem*, the ultimate pH (pH$_{24}$) was measured at the 13$^{th}$, 10$^{th}$, and 6$^{th}$ rib levels of the *Longissimus thoracis et lumborum* (LTL) muscle of the left half of the carcass using a pH meter (InLab Solids Go-ISM, Mettler-Toledo S.A.E., Barcelona, Spain) fitted with an insertion glass electrode and an automatic temperature compensation probe. Before each measurement, the pH meter was calibrated using the standard buffer solutions of pH 4.0 and pH 7.0 Hamilton DuraCal™ (Hamilton, Bonaduz AG, Switzerland). The average of triplicate measures was used to classify the carcasses into two groups: CONTROL beef with normal pH$_{24}$ (5.4 $\leq$ pH$_{24}$ $\leq$ 5.6) and extreme DFD beef with high pH$_{24}$ (pH$_{24}$ $\geq$ 6.2). The pH$_{24}$ threshold for DFD carcasses was set to 6.2 to ensure unambiguous classification of defective meat (Adzitey & Nurul, 2011). When a DFD carcass was detected, other carcass of the same origin, age, and slaughter batch, but with normal ultimate pH$_{24,}$ was analyzed as control. A total of 32 beef samples (16 pairs of extreme DFD and CONTROL samples) were collected on different sampling days.

### 2.2. Muscle sample collection

Muscle samples (20 g) were collected from the LTL of 32 beef carcasses (16 DFD and 16 CONTROL) at the 13$^{th}$ rib level 24 h *post-mortem* for the analysis of oxidative and ER stress biomarkers and were immediately stored at $-80$ °C until analysis. For analysis, the LTL muscle was collected between the 6$^{th}$ and 13$^{th}$ ribs, transported to the laboratory, and divided into 2.5 cm steaks to determine beef quality traits. Instrumental color measurements were determined on the first steak and in the second, the water-holding capacity determination. The third steak was cut under sterile conditions and divided into three portions for subsequent microbiological analysis of total viable counts (TVC), *Enterobacteriaceae* (ENT), lactic acid bacteria (LAB), and yeast (YS) at 3, 7, and 14 d *post-mortem*. The next three steaks were used for meat toughness measurement using a Warner-Bratzler shear force test at 3, 7, and 14 d *post-mortem*. Finally, the last steak was divided into three portions for proteomic analysis after 3, 7, and 14 d. Steaks were vacuum-packed in polyamide 20 μm/polyethylene 70 μm bags and aged in dark under 4 °C. Following aging, steaks were frozen at $-20$ °C ($-80$ °C for proteomics) for subsequent analysis.

### 2.3. Meat quality measurements

Meat quality measurements were performed on individual LTL muscles extracted from every 32 carcasses. Meat color was recorded at three random points on the exposed cut surface of the LTL muscle using a Minolta CM-2300d spectrophotometer (Konica Minolta Inc., Osaka, Japan). An aperture size of 8 mm with a D65 illuminant and a 10° standard observer were used throughout the experiment. The CIE lightness ($L^*$), redness ($a^*$), yellowness ($b^*$), Chroma ($C^* = \sqrt{(a^{*2} + b^{*2})}$) and Hue ($h^* = \tan^{-1}(b^*/a^*)$) were recorded after 60 min of blooming and the average value of three determinations was used (American Meat Science Association, AMSA).

Water-holding capacity (WHC) of fresh meat was determined by duplicates of fresh samples (1.5 g) taken 24 h *post-mortem*, following the centrifugal method for drip loss described by Jauregui, Regenstein, and Baker (1981) with little modifications. Two pieces of Whatman filter paper (9 and 5 cm) were weighted and afterwards used to form a thimble. 1.5 $\pm$ 0.3 g sample of ground muscle was added to the thimble. The sample in the thimble was then centrifuged in a 50 ml polycarbonate centrifuge tube at 1950 x *g*, 20 min at 4 °C. The filter paper and sample were then removed from the tube with tweezers, the meat "cake" removed from the filter paper, and the paper reweighed. All samples were run in duplicate, and the expressible moisture reported as percent weight lost from original sample.

Meat toughness was calculated using the Warner-Bratzler shear force (WBSF) test after 3, 7, and 14 d on cooked meat, as described by Díaz

et al. (2020). Briefly beef was cooked at 75 °C for 30 min in a water bath and eight cores (1 cm$^2$ in cross-section) from each steak were subjected to a perpendicular cut using the TA.XT Plus instrument (Stable Micro Systems, London, UK). The results were expressed as the mean WBSF maximum load (N) value for each steak.

For microbiological analysis, the beef samples were processed based on ISO 7218 (International Organization for Standardization, 2007). First, each vacuum-packed beef sample was opened (after 3, 7, and 14 d of aging respectively), a portion of 10 g was excised and aseptically transferred using sterile tweezers into a masticator bag, and 90 mL of sterile (0.1 %) buffered peptone water solution (PW; Oxoid, Unipath Ltd., Basingstoke, UK) was added. The mixture was homogenized in a stomacher (IUL Instruments, Barcelona, Spain) during 2 min. For microbial counts, decimal dilutions were placed on Petri dishes. TVC was determined on Plate Count Agar (PCA; Oxoid, Unipath Ltd., Basingstoke, U.K.) and incubated at 30 °C for 72 h (ISO 4833-2:2013). ENT was determined on Violet Red Bile Glucose Agar (VRBG; Merck, Darmstadt, Germany), with samples incubated at 37 °C for 24 h (ISO 21528-2:2017). LAB were determined on Man, Rogosa, Sharpe medium Agar (MRS; Oxoid, Unipath Ltd., Basingstoke, UK), with samples incubated at 30 °C for 72 h in a ST 6120 culture incubator (ISO 15214–1998). YS was determined on Symphony Agar (Biokar), with samples incubated at 25 °C for 72 h in a culture incubator (BKR 23/11–12/18 alternative analysis method for agribusiness certified by AENOR).

After incubation, microbial counts were performed as described in ISO 7218:2007. All plates were counted, and data were transformed into logarithms of the number of colony-forming units per gram of sample (log CFU/g).

### 2.4. Extraction of sarcoplasmic proteins

Sarcoplasmic extracts were obtained from individual 0.5 g muscle samples ($n = 32$) that were homogenized in 4 mL TES buffer (10 mM Tris pH 7.6, 1 mM EDTA pH 8.0, 0.25 M sucrose and 0.6% protease inhibitor cocktail (P8340, Sigma-Aldrich, MO, USA), using a Polytron PT1200 E (Kinematica Inc., Luzern, Switzerland) two times for 15 s at maximum speed, and the homogenate was centrifuged (20 min at 20000 x *g*) at 4 °C (Bjarnadóttir, Hollung, Frgestad, & Veiseth-Kent, 2010). After extraction, the supernatants of the 16 individual DFD samples were randomly divided into two groups (of eight pooled samples each); the 16 CONTROL sample supernatants were also pooled in two groups of eight samples each. At the end of the extraction procedure, four different pools were obtained (two different pools of CONTROL samples and two of DFD samples). These pools were aliquoted and stored at −80 °C. Three replicates of each sample extraction were prepared and pooled as explained previously. The final protein content in each pool was estimated using the Bradford method.

### 2.5. Muscular antioxidant defense

*Post-mortem* muscle antioxidant defense was measured in each of the extraction replicates of the different pools (CONTROL and DFD). Total superoxide dismutase activity (SOD; EC 1.15.1.1), which was based on the inhibition of hematoxylin autoxidation by the colored compound hematein, was measured in the sarcoplasmic fraction using a method described by Martin, Dailey, and Sugarman (1987). Catalase activity (CAT; EC 1.11.1.6) was assayed according to the method from Lubinsky and Bewley (1979), using $H_2O_2$ as a substrate and measures its breakdown and conversion into $O_2$ and $H_2O$.

### 2.6. Western blotting

Ninety μg of protein per sample was mixed with Laemmli sample buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and denatured for 5 min by boiling at 100 °C. Afterwards, samples were loaded in the gels and fractionated using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 200 V, and the proteins were transferred onto polyvinylidene fluoride membranes (PVDF; Immobilon TM-P; Millipore Corp., MA, USA) at 350 mA. The membranes were blocked with 10 % (*w/v*) of skim milk dissolved in Tris-buffered saline (TBS) (50 mM Tris-HCl and 150 mM NaCl, pH 7.5), and then incubated at 4 °C overnight with the corresponding primary antibodies: ATF6α (sc-22,799, Santa Cruz Biotechnology, CA, USA), IRE1α (3294, Cell Signalling, Danvers, MA), p-eIF2α (3398, Cell Signalling). All antibodies were pre-diluted in Tris-buffered saline (TBS) containing 5 % (w/v) skim milk or bovine serum albumin (BSA), as appropriate. Then, the membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich, Missouri, USA) and diluted in TBS buffer with skim milk or BSA 2 % (w/v) for 1 h at 25 °C. Immunoconjugates were detected using a chemiluminescent horseradish peroxidase substrate (WBKLS0500, Millipore Corp., Darmstadt, Germany) and Image Studio Lite 5.2.5 software (LI-COR Biosciences, NE, USA) was used to quantify the optical density of the bands. The densitometry values were expressed as semi-quantitative optical density (in arbitrary units) of the blot bands, normalized to total protein Ponceau as a loading control due to variations in the typical constitutive protein levels (GAPDH, β-actin, and α-tubulin) in the *post-mortem* muscle (Fortes et al., 2016) (*Fig. S1*). Three replicates were performed for each sample pool.

### 2.7. Statistical analysis

Statistical analyses were performed using the SPSS software (version 22.0; SPSS Inc., Chicago, IL, USA). The normality of variables was tested using the Kolmogorov-Smirnov test. Meat quality attributes were analyzed in the 32 beef samples collected using linear mixed models (LMM) with quality type (CONTROL *vs* DFD) as a fixed effect and animal and sampling day as random terms. For variables measured at different *post-mortem* times, such as WBSF and microbiological analysis, the lineal mixed models include quality type (CONTROL *vs* DFD), *post-mortem* time (3, 7 and 14 d) and their interaction (quality type x *post-mortem* time) as fixed effects and animal and sampling day as random terms. Pairwise comparison between means was enabled by least significant difference at the 0.05 level. The oxidative status and ER stress response were analyzed by LMM with quality type (CONTROL *vs* DFD) as fixed effect and pool and extraction replicates as random terms.

Relationships between variables were estimated by means of multiple linear regression models for the prediction of pH$_{24}$ (which was the variable used to classify CONTROL and DFD beef). Independent variables (drip loss, *L\**, *a\**, *b\**, *C\**, *h\** WBSF, TVC, ENT, LAB, YS, CAT, SOD, IRE1α, ATF6α and p-eIF2α) were included or removed iteratively from the model in each step (by the stepwise method), according to their significance ($P < 0.05$), thus rejecting variables with very low tolerance (linearly related to another included in the analysis). The purpose of the model was to determine which of these variables contributed to the defective pH *post-mortem* decline (pH$_{24}$) as described below:

$$Yi = \beta 0 + \beta j Xij + \varepsilon ij.$$

where *Yi* is the dependent variable (pH$_{24}$) measured for the $i$th sample ($i = 1, ..., n$), $\beta 0$ is the intercept, $\beta j$ is the regression parameter associated with the $j$th biomarker ($j = 1, ..., n$), *Xij* is the percentage contribution of the $i$th sample to the $j$th biomarker and $\varepsilon ij$ is the random error inherent to each sample, which is assumed to be independent and normally distributed. The predicted ability of the obtained models was evaluated in terms of the coefficient of determination (R$^2$) and the standard error of prediction (SEP). Moreover, Principal component analysis (PCA) was performed to study the relationships among meat quality, muscular antioxidant defense, and UPR response.

## 3. Results and discussion

### 3.1. Meat quality traits

Table 1 shows the results for drip loss and meat color attributes in the two groups analyzed, namely CONTROL ($5.4 \leq pH_{24} \leq 5.6$) and DFD ($pH_{24} \geq 6.2$). DFD meat had a darker, brownish, and saturated color ($L*$ and $b*$ ($P < 0.05$), $a*$ ($P < 0.001$), and $C*$ ($P < 0.01$)) and lower drip loss ($P < 0.001$).

Meat color depends on the concentration and chemical state of the pigment myoglobin and on the muscle structure and the scattered light. Hughes, Clarke, Purslow, and Warner (2017) found that in high pH *Longissimus thoracis* beef, muscle fibers were swollen, which limit their ability to scatter light what ends in darker meat. The lower drip loss (indicating higher water-holding capacity) of DFD beef found in this study, is also related to the higher $pH_{24}$. The water-holding capacity of normal beef (CONTROL) is lower because its $pH_{24}$ is closer to the iso-electric point of myofibrillar proteins (for example, myosin pI = 5.4); therefore, their net charge is equalized, which reduces their water retention capacity (Huff-Lonergan & Lonergan, 2005).

Table 2 shows the effect of $pH_{24}$ sample type (CONTROL *vs* DFD), the *post-mortem* time (3, 7, 14 d) and their interaction on meat toughness (WBSF) and microbiology results. The two factors and their interaction were significant for all variables. DFD beef showed significant lower toughness ($P < 0.01$) than CONTROL. In addition, significant differences were observed between the CONTROL and DFD groups in the microbial loads of TVC, ENT, LAB, and YS after 7 d ($P < 0.05$) and 14 d ($P < 0.001$) of aging, while no differences were found at 3 d *post-mortem*.

These findings agree with previous studies that have described DFD meat as having darker color, higher water-holding capacity, altered meat tenderization (gummy flesh of unpleasant texture), and faster microbial spoilage than normal-pH meat (Adzitey & Nurul, 2011; Holman, Kerr, Morris, & Hopkins, 2019; Ijaz et al., 2020). Furthermore, these defects have been related to modifications of the *post-mortem* muscle metabolism that affect to the muscle-to-meat conversion process (Díaz-Luis et al., 2020).

In this work DFD beef was significantly more tender ($P < 0.01$) than CONTROL (normal $pH_{24}$) beef throughout the whole maturation process. It is important to note that DFD beef did not show a normal *post-mortem* tenderization pattern. There was a significant decrease ($P < 0.01$) of WBSF with storage time in CONTROL beef, whereas WBSF showed low and steady values for DFD throughout the *post-mortem* period (Fig. 1). These results are consistent with previous studies that found a curvilinear relationship between meat ultimate pH and tenderness for beef. Then, Lomiwes, Farouk, Frost, Dobbie, and Young (2013) found that shear force values increased progressively until $pH_{24}$ reached values of 5.9, after which the WBSF decreases as $pH_{24}$ increases. Similarly, Ijaz et al. (2020) compared beef from the LTL of normal (pH $\leq$ 5.70), atypical DFD ($5.70 < pH \leq 6.09$), or typical DFD (pH > 6.09), and found higher ($P < 0.05$) and more variable shear force values in atypical DFD samples during aging, whereas typical DFD showed lower shear force values with no significant differences from normal beef. A likely explanation for this phenomenon could be that high $pH_{24}$ (closer to

physiological pH) enhance the action of calpains that have the ability to degrade myofibrillar and cytoskeletal proteins and contribute to a faster meat tenderization compared with normal ultimate pH found in *post-mortem* muscle (5.4 – 5.6) (Bhat, Morton, Mason, & Bekhit, 2018).

Our results also showed faster spoilage of DFD beef (Fig. 2) for all the microorganisms studied. It is known that a muscle $pH_{24}$ higher than 6 shortens the shelf life of meat, as a high ultimate pH creates an ideal environment for rapid microbial growth of potent spoilage organisms that are inhibited at the usual ultimate pH of meat (Gill & Gill, 2005). Moreover, there will also be a significant reduction in shelf life because microorganisms, in the absence of glucose, use amino acids as an energy source when bacterial numbers are low; thus, spoilage becomes evident at lower cell densities in DFD (owing to its faster glucose exhaustion) than in CONTROL beef and produces inappropriate odors and faster microbial degradation (Gill & Gill, 2005; Newton & Gill, 1981; Shange, Makasi, Gouws, & Hoffman, 2018).

### 3.2. Muscular antioxidant defense

The loss of homeostasis in the *post-mortem* muscle due to the un-balance of the endogenous antioxidant defenses towards the generation of excessive ROS and other reactive compounds, promote oxidative stress and leads to the damage of cellular components such as proteins, lipids, and nucleic acids (Bekhit, Hopkins, Fahri, & Ponnampalam, 2013). These oxidative processes are major non-microbiological factors involved in quality deterioration of meat and may affect to quality traits such as color, flavour, juiciness and tenderness. In this work, the muscular antioxidant defense was characterized by the activity of two main antioxidant enzymes, CAT and SOD, that contribute to detoxify ROS and protect cells against oxidative stress in the skeletal muscle (Descalzo et al., 2007; Gatellier, Mercier, & Renerre, 2004; Pradhan, Rhee, & Hernández, 2000). It has been found that they effectively counteract the harmful effects of ROS on proteins from diverse meats such as pork (Chen, Zhou, Xu, Zhao, & Li, 2010), beef (Utrera, Parra, & Estévez, 2014) and poultry (Delles, Xiong, True, Ao, & Dawson, 2014).

Figure 3 shows the CAT and SOD activities in the sarcoplasmic fractions of CONTROL and DFD beef samples. These results revealed that DFD meat showed lower antioxidant capacity with lower activities of CAT ($P < 0.05$; Fig. 3A) and SOD ($P < 0.001$; Fig. 3B) than CONTROL meat at 24 h *post-mortem*.

To the best of our knowledge, there is still little information about the role of the antioxidant enzymes in the *post-mortem* conversion of the muscle into meat and its relationship with main meat quality defects. The results obtained in this study showed lower enzymatic activities of CAT and SOD in DFD muscles at 24 h *post-mortem* that leads to an increase in oxidative damage that may compromise lipids and proteins susceptibility to oxidative reactions during storage. In fact, previous studies showed lower CAT activity and a significant increase in lipid peroxidation in DFD beef (González-Blanco et al., 2021). Similarly, ROS can alter muscle proteins affecting their hydrophobicity, conformation and solubility and causing an altered susceptibility of protein substrates to proteolytic enzymes, affecting the final quality of meat (Lund, Heinonen, Baron, & Estévez, 2011). Therefore, the differences between DFD

**Table 1**

The effect of $pH_{24}$ sample type (CONTROL *vs* DFD) on drip loss and color parameters (mean $\pm$ SEM).

| Variable | Time *post-mortem* | CONTROL ($5.4 \leq pH_{24} \leq 5.6$) (n = 16) | DFD ($pH_{24} \geq 6.2$) (n = 16) | SEM | *P-value* |
|---|---|---|---|---|---|
| Drip loss (%) | 48 h | 30.32 | 21.74 | 0.46 | 0.000 |
| Meat Color | | | | | |
| $L*$ | 48 h | 37.60 | 30.41 | 1.79 | 0.030 |
| $a*$ | 48 h | 10.75 | 7.41 | 0.6 | 0.000 |
| $b*$ | 48 h | 12.29 | 7.08 | 1.48 | 0.019 |
| $C*$ | 48 h | 15.36 | 9.13 | 1.37 | 0.004 |
| $h*$ | 48 h | 44.96 | 39.50 | 2.93 | 0.090 |

DFD: dark, firm, and dry; $C*$: Chroma; $h*$: Hue angle.

**Table 2**
The effect of pH$_{24}$ sample type (CONTROL *vs* DFD), *post-mortem* time (3, 7 and 14 d *post-mortem*) and their interaction on meat quality traits (mean ± SEM).

| Variable | pH$_{24}$ sample type (*T*) | | | Post-mortem time (t) | | | | P-value | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | CONTROL (5.4 ≤ pH$_{24}$ ≤ 5.6) (n = 16) | DFD (pH$_{24}$ ≥ 6.2) (n = 16) | SEM | 3 d | 7 d | 14 d | SEM | *T* | *t* | *T x t* |
| Meat toughness (WBSF, N) | 67.24 | 45.71 | 1.81 | 62.22 | 55.75 | 51.46 | 2.213 | 0.000 | 0.004 | 0.001 |
| TVC (log CFU/g) | 3.73 | 4.72 | 0.11 | 2.81 | 3.55 | 6.32 | 0.133 | 0.000 | 0.000 | 0.002 |
| ENT (log CFU/g) | 1.93 | 2.95 | 0.20 | 1.45 | 1.92 | 3.95 | 0.243 | 0.001 | 0.000 | 0.002 |
| LAB (log CFU/g) | 2.49 | 3.59 | 0.16 | 1.53 | 2.62 | 4.97 | 0.191 | 0.000 | 0.000 | 0.000 |
| YS (log CFU/g) | 3.67 | 4.59 | 0.13 | 2.59 | 3.69 | 6.11 | 0.160 | 0.000 | 0.000 | 0.004 |

DFD: dark, firm, and dry; *T x t*: Interaction between pH$_{24}$ type and *Post-mortem* time. TVC: total viable counts; ENT: *Enterobacteriaceae*; LAB: lactic-acid bacteria; YS: yeast; WBSF: Warner-Bratzler shear force.
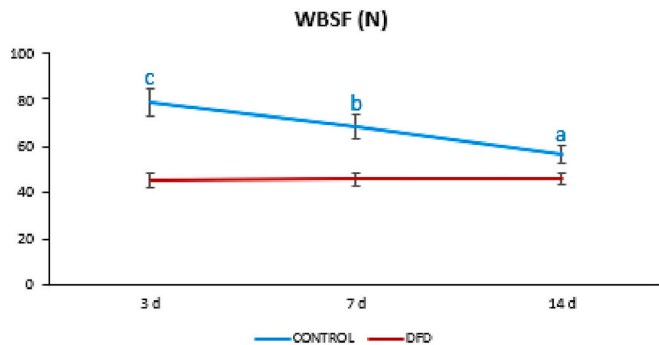


**Fig. 1.** *Post-mortem* evolution of Warner-Braztler shear force (WBSF) showing the meat tenderization pattern. Different letters indicate significant differences (*P* < 0.01) at 3, 7 and 14 d *post-mortem* for CONTROL (blue) and DFD (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and CONTROL beef regarding drip loss and texture could be partially explained by an imbalance between antioxidant enzymes and ROS in the muscle cells of DFD beef.

### 3.3. ER stress: UPR

Because of the essential role of proteins in the skeletal muscle maintenance, three independent pathways involved in the UPR of the ER stress response were analyzed in the sarcoplasmic fraction of DFD and CONTROL beef samples (Fig. 4). These analyses were performed in two different pools of samples per condition, with the aim to study the overall trend of each category (CONTROL *vs* DFD). This design decreases the biological differences between individuals (replicates) but it clearly increases the power of detection of a biological trend at the global level of the group (Sentandreu et al., 2021). According to Rosenthal and Schisterman (2008), pooling the specimens reduces the effective variance of the biomarker. This can decrease the proportion of observations below the detection threshold and therefore increase the amount of information that can be extracted from the data. This is especially useful
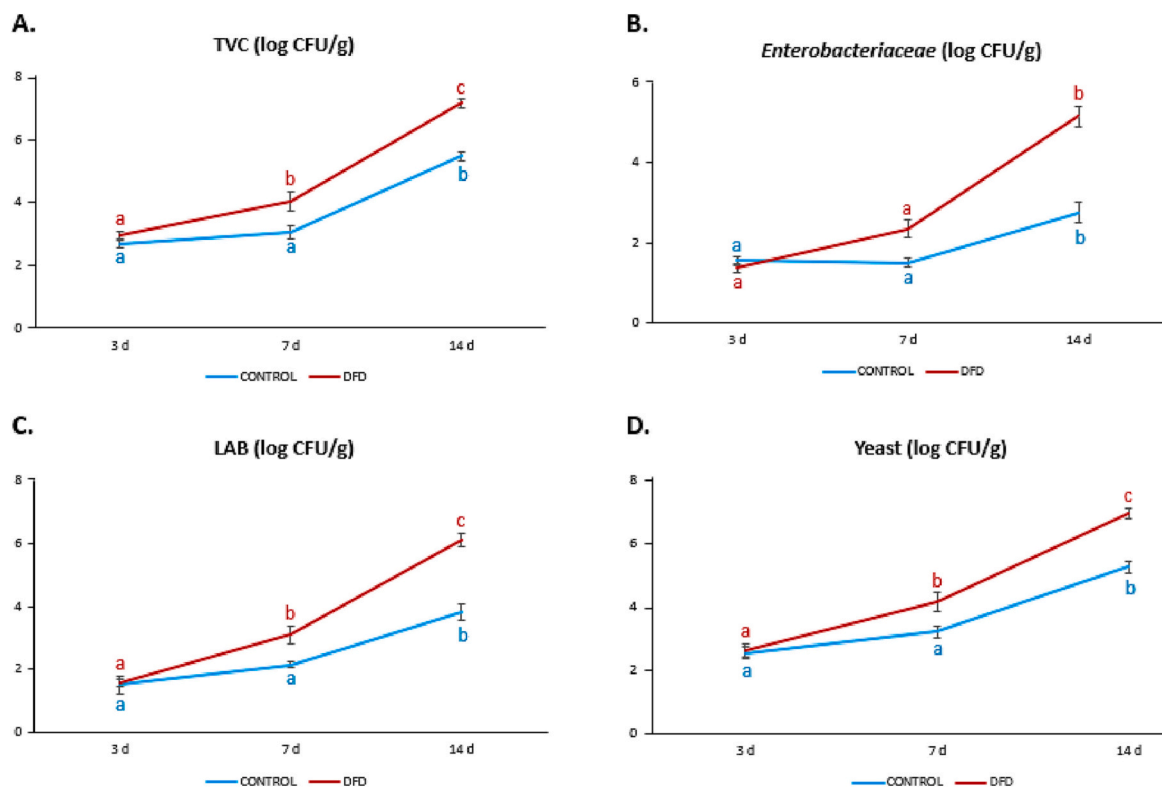


**Fig. 2.** *Post-mortem* evolution of microbiological analysis for A) total viable counts (TVC), B) *Enterobacteriaceae*, C) lactic acid bacteria (LAB), and D) yeast. Different letters indicate significant differences (*P* < 0.05) at 3, 7 and 14 d *post-mortem* between CONTROL (blue) and DFD (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
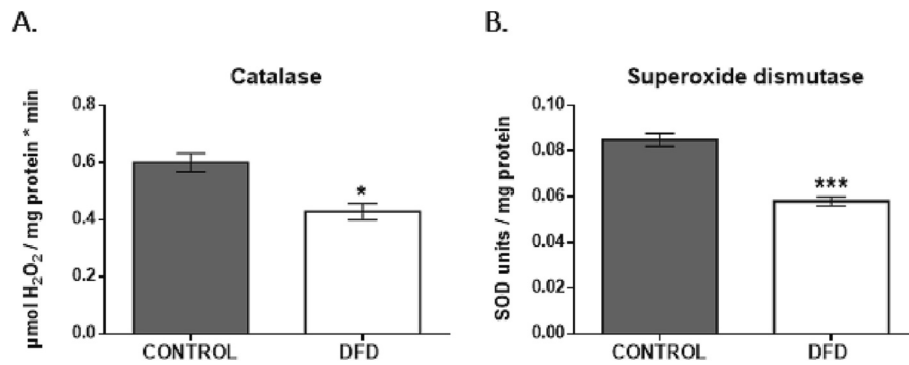
**Fig. 3.** Antioxidant enzymes activity. A) Catalase (expressed as μmol $H_2O_2$/min mg protein) and B) Superoxide dismutase (expressed as SOD units/mg of protein) activity of the sarcoplasmic fraction from CONTROL (grey bars) and DFD (white bars) beef samples. Data are represented as the mean ± SEM. *, $P < 0.05$; ***, $P < 0.001$.
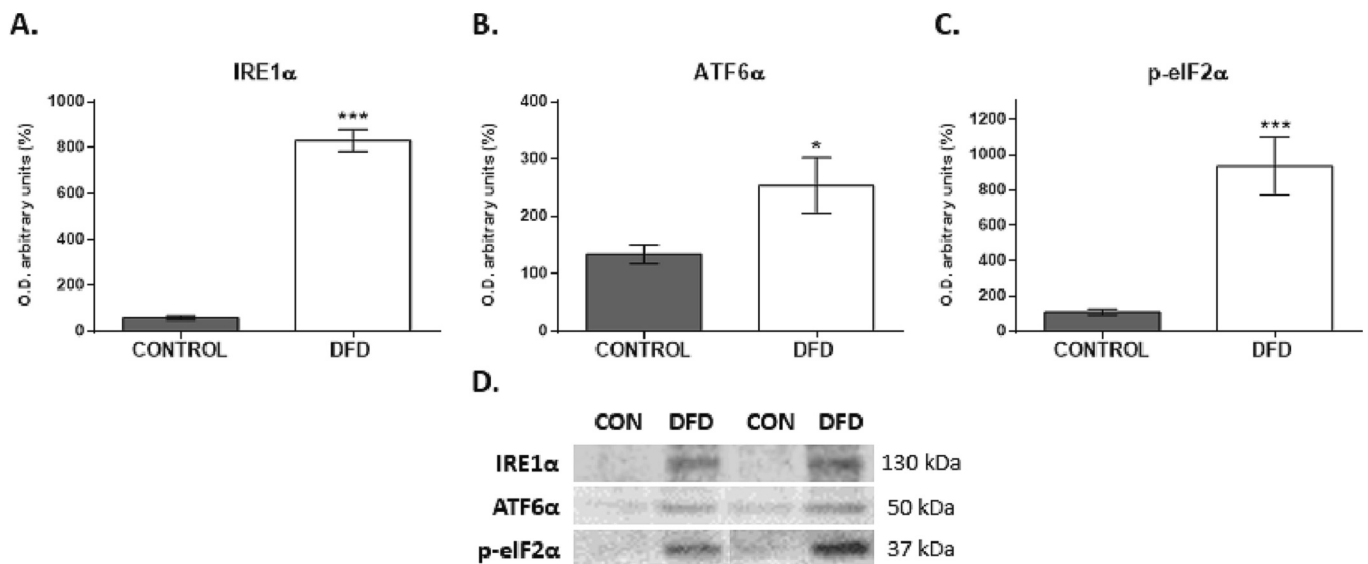


**Fig. 4.** Unfolded protein response signalling pathways. Western blot analysis for studying the ER stress pathways in CONTROL (grey bars) and DFD (white bars) beef samples. Bar chart showing the semiquantitative optical density (O.D.) (arbitrary units) of blot bands of A) inositol-requiring enzyme 1α (IRE1α), B) activating transcription factor 6α (ATF6α), and C) phosphorylated eukaryotic initiation factor 2α (p-eIF2α). D) Representative immunoblots of UPR markers. CON: CONTROL samples; DFD: Dark, firm and dry samples. Data are represented as the mean ± SEM. *, $P < 0.05$; ***, $P < 0.001$.

for studying biomarkers that may exist naturally in small quantities, such as oxidative stress biomarkers, as in this case.

The results obtained with this design showed higher expression levels of IRE1α ($P < 0.001$; Fig. 4A), ATF6α ($P < 0.05$; Fig. 4B), and p-eIF2α ($P < 0.001$; Fig. 4C) in DFD beef.

Certain conditions, such as glucose deprivation, oxidative stress, ATP depletion, and calcium imbalance, disrupt ER homeostasis and lead to the accumulation of unfolded or misfolded proteins within the ER lumen (Zhang & Kaufman, 2006). To survive this stress and avoid cell death in extreme cases, cells activate the UPR to restore ER function by up-regulating chaperone expression, attenuating the synthesis of new proteins, and removing those already produced (Ron & Walter, 2007). Although this is the first study to link UPR to muscle cell oxidative stress and the resulting defects in meat quality, many studies have demonstrated malfunctioning of the ER under stress conditions in human muscles (Estébanez et al., 2018). It is well known that prolonged exposure to hypoxia, similar to what occurs at slaughter due to the sudden cut-off of blood flow, causes ER stress (D'Hulst et al., 2013; Tagliavacca, Caretti, Bianciardi, & Samaja, 2012). Hypoxia activates PERK, which in turn phosphorylates eIF2α and inhibits translation initiation (Koumenis et al., 2002). The regulation of IRE1α and ATF6α by hypoxia has not been extensively studied and requires further

investigation. In our study, the three UPR pathways showed increased expression ($P < 0.05$) in the sarcoplasmic fraction of DFD beef 24 h *post-mortem*. PERK activation is typically the first indicator of UPR activation, and it phosphorylates eIF2α to induce the downregulation of protein synthesis (Brown et al., 2014). Similar results have been previously reported regarding PERK activation; for instance, an increase in PERK gene expression was observed in ground squirrel skeletal muscle during hibernation stress situations (Zhang et al., 2019) and an increase in phospho-eIF2α expression was observed in amyotrophic lateral sclerosis mouse skeletal muscle (Chen, Wang, & Chin, 2015). Thus, our data indicated that the redox imbalance observed in DFD meat could activate essential PERK-dependent mechanisms, reducing the number of unfolded proteins in the ER (Liu & Kaufman, 2003), in an attempt to restore cellular homeostasis.

The IRE1α pathway regulates the activation of degradation pathways, including ER-associated degradation (ERAD), in an attempt to rectify the accumulation of misfolded proteins (Yoshida, 2007). Upon ER stress, misfolded proteins bind to the luminal domain of IRE1α to activate UPR signalling (Karagöz et al., 2017). The association of misfolded proteins mediates conformational changes that result in the dimerization/oligomerization of IRE1α and subsequent autophosphorylation (Karagöz et al., 2017), promoting a key branch of the UPR

signalling pathway that contributes to the modulation of protein folding and ERAD. In this process, unfolded or misfolded proteins are trapped by the ERAD machinery and retro-translocated across the ER membrane into the cytosol (Hampton, 2002; Jarosch, Lenk, & Sommer, 2003), where they are ubiquitinated and degraded by the proteasome. Moreover, a third signalling pathway, ATF6α, is activated by ER stress causing a transcriptional upregulation of ER chaperone proteins, lipids, and multiple antioxidants (Walter & Ron, 2011; Wang et al., 2000). ER stress causes the inactive 90-kDa ATF6α precursor to relocalize to the Golgi, where it is cleaved by site-specific proteases into the active 50-kDa protein (Ye et al., 2000) which translocates to the nucleus and activates the promoters of ER chaperone genes (Okada, Yoshida, Akazawa, Negishi, & Mori, 2002). These newly synthesized chaperones refold misfolded proteins in the ER in an effort to relieve ER stress.

In accordance with our results, increased levels of IRE1α/ATF6α expression have been previously described in the skeletal muscles of heat-stressed rats (Sharma et al., 2021). Published reports have also shown that all three ER stress-responsive UPR pathways are activated in skeletal muscle under various stress conditions, such as acute exercise (Bohnert, McMillan, & Kumar, 2018). Prolonged UPR activity, which indicates that ER stress cannot be mitigated and homeostasis cannot be restored, is correlated with a more intense autophagic process (Yorimitsu & Klionsky, 2007). In line with this, previous studies have shown a more intense autophagic process in DFD than in CONTROL beef, and it has been hypothesized that this could delay the onset of apoptosis and cause an abnormal tenderization process and defects in meat quality (Díaz-Luis et al., 2020).

### 3.4. Relationship between meat quality traits and cellular biomarkers

Linear regression models were applied to detect the best combination of quality traits and stress biomarkers contributing significantly to the prediction of $pH_{24}$, which is the variable used to classify samples as CONTROL or DFD. Four significant models ($P < 0.05$) that allowed high explanation of the variance ($R^2 > 0.82$) were obtained as shown in Table 3. The best model in terms of coefficient of determination ($R^2 = 0.896$) and standard error of prediction (SEP = 0.157) was the Model 4, which included two quality attributes (drip loss, WBSF) and two proteins from the UPR response (IRE1α, p-eIF2α). The regression coefficients, standard errors and probabilities for this model are shown in Table 4. It is worth noting that the relationship of drip loss and WBSF with $pH_{24}$ was negative, while the expression of the two UPR proteins (IRE1α, p-eIF2α) was positively related to $pH_{24}$, which again demonstrate higher muscle ER stress as higher is the ultimate *post-mortem* pH.

To obtain an overall view of the relationships between the meat quality traits, muscular antioxidant defense, and ER stress, a PCA was carried out on the variables with higher correlation loadings (over 70 % of explained variance). Fig. 5 shows the biplot obtained by PCA between the variables (loadings) and meat samples (scores). The first principal components (PC1 and PC2) explained 76 % of the total variance. The PC1 explained 63 % of the variance and separated on the positive axis the DFD meat samples, with higher $pH_{24}$ values, higher microbiological load of TVC, ENT, LAB, and YS (at 7 and 14 d *post-mortem*), and an

**Table 3**
Multiple linear regression models for variables that significantly contribute to $pH_{24}$ prediction.

| Model | Predictors | $R^2$ | SEP | Significance |
|---|---|---|---|---|
| 1 | Drip Loss | 0.821 | 0.205 | *** |
| 2 | Drip loss + IRE1α | 0.855 | 0.185 | ** |
| 3 | Drip loss + IRE1α + WBSF14 | 0.883 | 0.167 | ** |
| 4 | Drip loss + IRE1α + WBSF14 + p-eIF2α | 0.896 | 0.157 | * |

$R^2$: coefficient of determination; SEP: standard error of prediction; WBSF14: Warner-Bratzler shear force measured at 14 d *post-mortem*. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

**Table 4**
Meat quality traits and cell stress biomarkers that significantly contributed to $pH_{24}$ prediction in Model 4.

| Model 4-$pH_{24}$ | Coefficients | Std. error | P-value |
|---|---|---|---|
| Intercept | 6.888 | 0.438 | 0.000 |
| Drip Loss | −0.300 | 0.015 | 0.046 |
| IRE1α | 0.423 | 0.000 | 0.003 |
| WBSF14 | −0.186 | 0.003 | 0.014 |
| p-eIF2α | 0.179 | 0.000 | 0.044 |

Coefficients: regression coefficients, Std. error: Standard error.

overexpression of IRE1α and p-eIF2α at 24 h *post-mortem*. The positive association indicated by Model 4 between high $pH_{24}$ (DFD samples) and IRE1α and p-eIF2α is clearly ratified by the PCA. Previous studies suggests that all three transmembrane proteins (IRE1α, ATF6α, and PERK) are co-activated in response to ER stress (Walter & Ron, 2011). However, in addition to transcriptional responses that largely serve to increase protein folding capacity in the ER, PERK (through phosphorylation of eIF2α) and IRE1α function as positive feedback loops, decreasing the load of proteins entering the ER by reducing translation. Precisely these two proteins are the ones that have shown a strong positive relationship with high $pH_{24}$ and thus DFD meat.

The CONTROL samples were grouped on the negative axis of PC1, with normal values of WBSF (at 3, 7, and 14 d *post-mortem*) and drip loss and higher antioxidant enzyme activities of SOD and CAT, that is, variables that indicate an adequate muscle-to-meat conversion process. Agreeing with this, drip loss and WBSF (14 d) was found to be negatively related to $pH_{24}$ in the linear regression models. In contrast, antioxidant enzymes (SOD, CAT) show a positive relationship with drip loss, which agree with previous studies that have associated a reduced antioxidant enzymes activity with an abnormal water-holding capacity (Mir, Rafiq, Kumar, Singh, & Shukla, 2017; Sohaib et al., 2017). In this work, DFD beef has shown abnormal meat tenderization process, with very low and steady values of WBSF along *post-mortem* maturation. In beef, higher oxidative stress and protein oxidation has been related to higher WBSF values (Estévez, 2011). However, studies on *in vitro* muscle cells pointed out that mild oxidation may enhance myofibrillar protein degradation via calpain and caspase-3 (Smuder, Kavazis, Hudson, Nelson, & Powers, 2010). It is important to consider the degree of oxidation when discussing the relationship between tenderness and proteolytic activity, since extensive oxidation may lead to more compact protein structures, while moderate oxidation may unfold proteins structures and make them more accessible for the activity of enzymes (Bao & Ertbjerg, 2019). Moreover, high $pH_{24}$ may enhance the action of calpains that contribute to faster meat tenderization (Bhat et al., 2018). Consequently, it seems that high $pH_{24}$ combined with high oxidative stress may contribute to the abnormally low texture of DFD meat by promoting early protein fragmentation and preventing the normal tenderization process.

It is widely known that antioxidants try to reduce ROS and prevent ER stress-induced oxidative damage and activation of the UPR (Malhotra et al., 2008). According to this, in the PCA the antioxidant enzymes are located in the negative axis, together with CONTROL samples, while the expression of UPR proteins is located in the positive axis, thus, showing their negative relationship.

Multivariate analysis revealed a clear separation between CONTROL and DFD beef samples based on the different variables analyzed. Overall, these results indicate that high-pH meat showed poor ultimate meat quality, lower muscle cell antioxidant activities and an increased ER stress response (UPR), with higher levels of IRE1α, ATF6α, and p-eIF2α, in an attempt of the cell to restore the muscle homeostasis. These results contribute to the identification of proteins with a relevant role in *post-mortem* muscle metabolism that are involved in the muscle-to-meat conversion process, which can be used as new early putative biomarkers of meat quality (Fig. 6).

Under this situation of increased oxidative stress and higher levels of
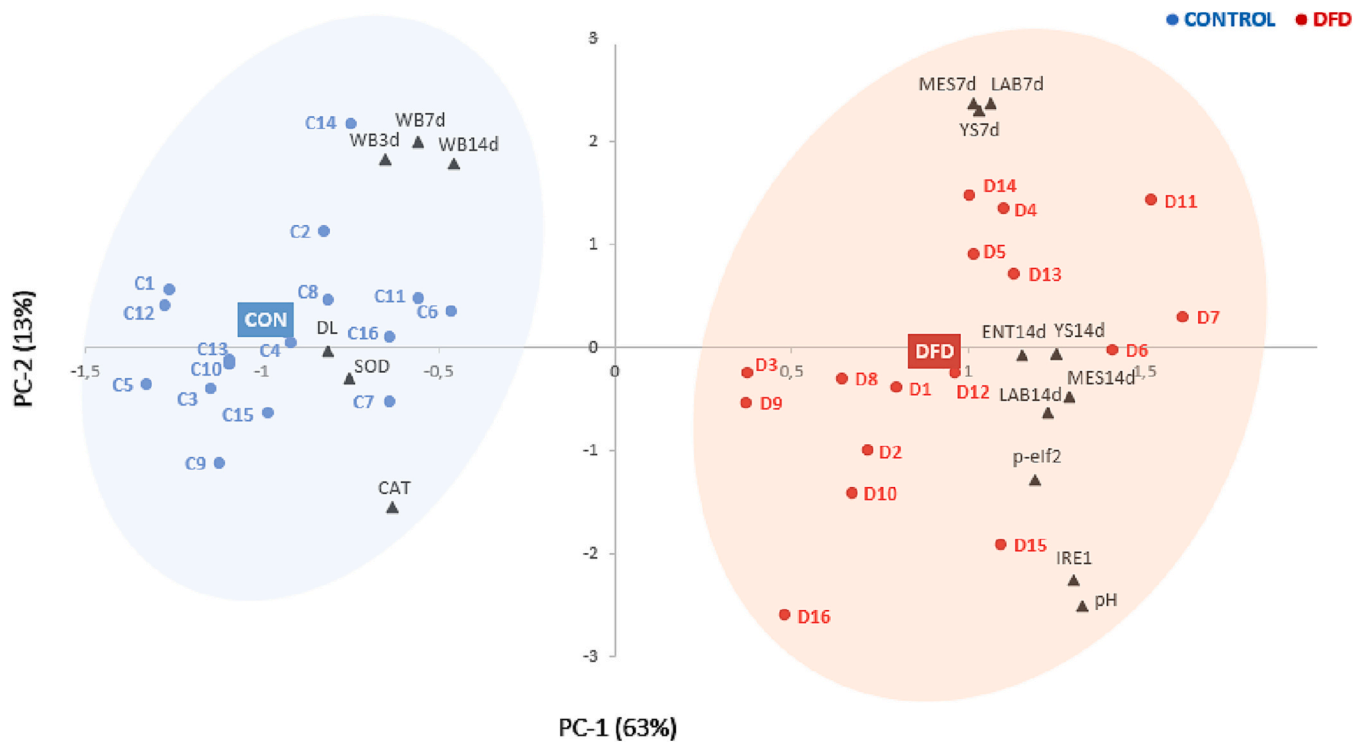
**Fig. 5.** Biplot of variables and individuals (beef samples). The centroids of the animal type are shown in squares denoted with codes: CON (CONTROL) and DFD (dark, firm and dry). Individual samples are shown in blue bullets (C-number for CON) and red bullets (D-number for DFD). WB3d: Warner-Bratzler shear force at 3 d *post-mortem*, WB7d: Warner-Bratzler shear force at 7 d *post-mortem*, WB14d: Warner-Bratzler shear force at 14 d *post-mortem*; DL: Drip loss; CAT: Catalase; SOD: Superoxide dismutase; TVC7d: Total viable counts at 7 d *post-mortem*, TVC14d: Total viable counts at 14 d *post-mortem*; ENT7d: *Enterobacteriaceae* load at 7 d *post-mortem*, ENT14d: *Enterobacteriaceae* load at 14 d *post-mortem*; LAB7d: Lactic-acid bacteria load at 7 d *post-mortem*, LAB14d: Lactic acid bacteria load at 14 d *post-mortem*; YS7d: Yeast load at 7 d *post-mortem*, YS14d: Yeast load at 14 d *post-mortem*; IRE1α: inositol-requiring enzyme 1α; p-eIF2α: phosphorylated eukaryotic initiation factor 2α. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
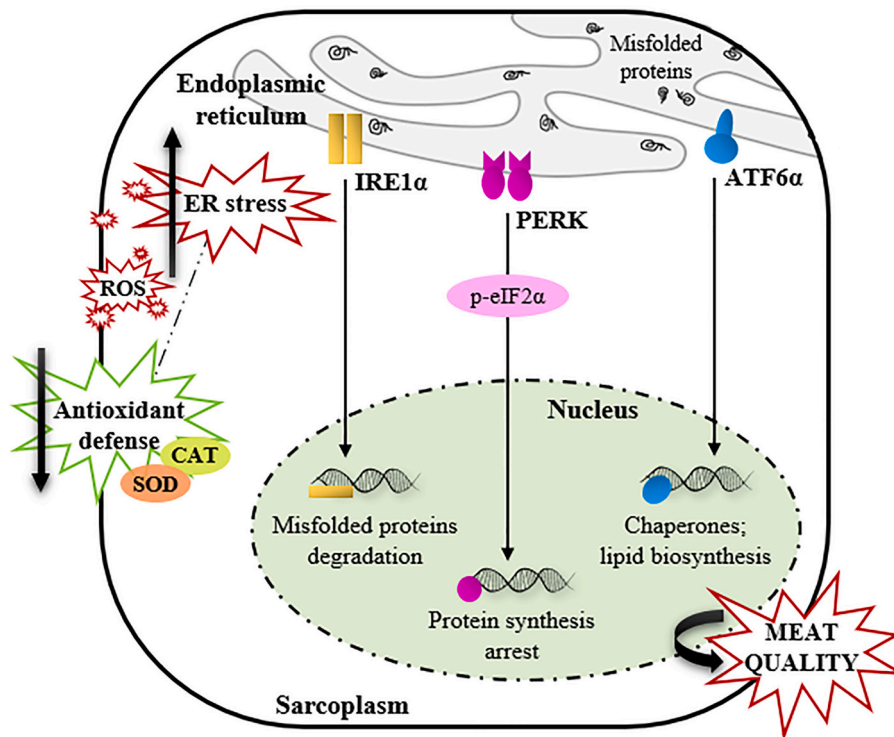


**Fig. 6.** Scheme of the endoplasmic reticulum response to oxidative stress in *post-mortem* muscle cells. ER: Endoplasmic Reticulum; ROS: Reactive oxygen species; CAT: catalase; SOD: superoxide dismutase; IRE1α: inositol-requiring enzyme 1α; ATF6α: activating transcription factor 6α; p-eIF2α: phosphorylated eukaryotic initiation factor 2α.

UPR, muscle cells from DFD may promote an intense autophagic response and release antiapoptotic factors as a survival mechanism to counteract stress insults. In fact, an increased autophagic response has been previously described in relation to pre-slaughter stress (Díaz et al., 2020). Under normal circumstances, the stressful situation of slaughter and exsanguination enrolls muscle cells into an apoptotic process, however we hypothesize that in DFD beef this process is altered due to the earlier activation of autophagy, affecting the normal process of muscle-to-meat conversion what can partly explain meat quality defects.

## 4. Conclusions

This study revealed that high $pH_{24}$ beef (DFD) showed lower antioxidant activity (SOD and CAT) and an increased ER stress response, showing Unfolded Protein Response (UPR), with higher levels of IRE1α, ATF6α, and p-eIF2α than normal $pH_{24}$ beef (CONTROL), which together show higher level of cellular stress in the DFD beef, which alters the muscle-to meat conversion process. These results reveal the relevant role of UPR activation in the defense of the muscle tissue against an increased oxidative stress and the putative use of the main biomarkers of the cellular processes involved (IRE1α, ATF6α, and p-eIF2α) as early biomarkers of meat quality defects. However, future research is needed to validate these biomarkers in a wider range of studies, including exposure to PSS, different animal types and management systems.

## Statement of authorship

We hereby declare that we are the sole authors of this original article, and that we have not used any sources other than those identified as references. We declare that we have not submitted this original article to any other journals and that it is not under consideration for publication elsewhere.

## CRediT authorship contribution statement

**Laura González-Blanco:** Methodology, Investigation, Data curation, Writing – original draft, Visualization. **Verónica Sierra:** Conceptualization, Methodology, Formal analysis, Data curation, Writing – original draft, Visualization, Funding acquisition. **Yolanda Diñeiro:** Investigation. **Ana Coto-Montes:** Conceptualization, Writing – review & editing, Supervision. **Mamen Oliván:** Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition.

## Declaration of Competing Interest

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

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meatsci.2023.109224.

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