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Short communication

Assessment of caspase activity in *post mortem* muscle as a way to explain characteristics of DFD beef

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ABSTRACT

Pre-slaughter stress can lead to 'dark cutting' defective meat characterized by a high ultimate pH (pHu) in cattle, causing high economical losses in the meat industry. Therefore, the study of biochemical mechanisms related to animal stress response is a fact that needs to be addressed. The aim of this work was to determine caspase 9 and 3/7 activities in high and normal pHu muscle samples at 24 h *post mortem* as a way to reveal stress situations in different Spanish local breeds (Asturiana de los Valles, Retinta and Rubia Gallega) and crossbred animals (n = 83). Either breed or pHu factors had an effect in both caspase activities, showing the high pHu group the highest caspase activity independently of the studied breed. In contrast, activity levels among breeds were slightly different for each caspase and between the high and normal pHu group in the case of caspase 3/7. Overall, this study demonstrated that caspase activity can be a good indicator to detect stress situations that might lead to defective high pHu meats.

1. Introduction

During the last decades, meat industry has moved towards the production of animals that are efficient feed converters, fast-growing and have high lean meat content with minimum production costs. This can negatively influence animal welfare and animals' stress status in relation to non-adequate animal handling practices, and consequently, increasing the appearance of meat quality defects such as *dry, firm and dark* (DFD) or 'dark-cutting' meat (Ferguson and Warner, 2008; Ponnampalam et al., 2017). DFD meats are associated with any factor that lead to the depletion of muscle glycogen reserves prior to slaughter in reaction to acute stress. This causes the reduction of the substrate availability and modifies *post mortem* glycolytic metabolism, resulting in less lactic acid and high ultimate pH (pHu) (McVeigh et al., 1982; Tarrant, 1989). In cattle, dark-cutting or high pHu meats cause consumers' rejection due to undesirable flavor, altered tenderness and short shelf-life (Adzitey and Nurul, 2011; Newton and Gill, 1981; Wulf et al., 2002). This has a cost of around 55 and 20 million dollars per annum for Australian (Jose et al., 2015) and British (Adzitey and Nurul, 2011) industry, respectively. There is no official information about incidence of this problem in Spain, but available data indicate that may affect around 12–14% of bovine carcasses (Mach et al., 2008). Taking in mind its implications in terms of food quality and safety, but also in economic benefits for meat industry, an appropriate assessment of reliable indicators of pre-slaughter stress is currently necessary.

Measurement of pHu is the most common indicator for establishing the incidence of DFD meats since it is directly related to animals' pre-slaughter stress (PSS) (Chambers et al., 2001; Loudon et al., 2018). However, usefulness of pHu assessment is compromised since high values do not necessarily certify the occurrence of true DFD meats (Ponnampalam et al., 2017). New insights addressing the search of new stress indicators are needed to understand biochemical mechanisms underlying PSS condition and to overcome uncertainties from classic pHu determinations in meat (Ponnampalam et al., 2017). In this line, Fuente-Garcia et al. (2021a) proposed, for the first time, the study of caspase activity over *post mortem* time as a way to understand influence

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Table 1
pHu values of normal and high pHu meat samples from each studied breed.

Breed*		Mean	SEM**
AV	Normal	5.46	0.125
	High	6.42	
CB	Normal	5.65	0.110
	High	6.56	
RE	Normal	5.61	0.061
	High	5.99	
RG	Normal	5.50	0.105
	High	6.18	

*AV: Asturiana de los Valles; CB: Crossbred (Asturiana de los Valles x Friesian); RE: Retinta; RG: Rubia Gallega.
**SEM, standard error of the mean.

of pre-slaughter handling practices (feeding system and transport/lairage conditions) on the stress status of several bovine breeds. Caspases are a family of cysteine dependent peptidases that are mainly synthesized as inactive zymogens (pro-caspases); however under internal (metabolic and/or hypoxic stress) and/or external stimuli they initiate a series of controlled reactions that ultimately lead to apoptosis or programmed cell death (Boatright and Salvesen, 2003; Grilo and Mantalaris, 2019; Taylor et al., 2008). They can be classified according to their point of entry into the cell death pathway as: a) initiator caspases (caspases 8, 9 10 and 12) and b) executioner caspases (caspases 3, 6 and 7). Previous studies have also associated the activation of these enzymes with stress situations reporting higher caspase 1 activity in stressed mice (Towers et al., 2019) and activation of caspase 3 in Zebrafish in response to heat stress (Alderman et al., 2018). In bovine cattle, Díaz-Luis et al. (2021) found a higher expression of the caspase 3 large subunit in DFD beef samples at 24 h *post mortem*.

Our main goal was to study the activity of initiator caspase 9 and executioner caspases 3/7 on both high and normal pHu loin samples of different Spanish cattle breeds and crossbreeds animals at early *post mortem* times (24 h). This would contribute to understand the influence of pre-slaughter stress on the occurrence of high pHu meats linked to defective meats and to be an early predictor of this condition in bovine cattle.

2. Materials and methods

2.1. Chemicals and reagents

DTT (Dithiothreitol) was from Scharlab (Scharlab S.L., Barcelona, Spain). HEPES (N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid) sodium salt and CHAPS (3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate) hydrate were from Sigma (Sigma-Aldrich Co., St. Louis, MO, USA). Ac-LEHD-AMC was from Cayman (Cayman Chemical, Ann Arbor, MI, USA). Ac-DEVD-AMC was from AAT Bioquest (AAT Bioquest Inc., Sunnyvale, CA, USA). Water was of ultrapure grade from Millipore (EMD Millipore Co., Billerica, MA, USA).

2.2. Sample collection

In this work, a total of sixty-three (n = 63) beef samples were obtained from different Spanish local breeds: Asturiana de los Valles (AV, n = 18), Retinta (RE, n = 14) and Rubia Gallega (RG, n = 11). Additionally, another twenty samples (n = 20) were collected from crossbred (CB) animals (Asturiana de los Valles x Friesian). Male calves were handled in accordance with Directive 2010/63/EU (2010) and slaughtered at their 14–15 (AV, RE and CB) or 10 (RG) months of age in commercial abattoirs of each region following safety and welfare conditions according to European Union regulations (Council Regulation (EC) No 1099/2009).

At 24 h *post mortem*, approximately 10 g of *Longissimus thoracis et lumborum* (LTL) muscle sample were excised at 13th rib level from the left-half carcass of each animal, frozen in liquid nitrogen and stored in Falcon tubes at −80 °C until further analysis. Muscle samples were sorted into two different groups according to their pHu values: 'high' pHu samples (9 AV, 10 CB, 7 RE and 5 RG) having pHu values higher than 5.9. The rest of samples were classified as 'normal' samples (9 AV, 10 CB, 7 RE and 6 RG) showing pHu values below 5.9 (Table 1). The pHu measurements were done at the LTL muscle of the 6th rib level at 24 h *post mortem* using a penetration electrode (CRISON pH/mV-meter 506, CRISON Instruments SA, Spain).

2.3. Extraction of sarcoplasmic proteins

Protein extraction was carried out as described by Fuente-Garcia et al. (2021c). Briefly, half gram of each type of meat sample (high and normal pHu), was homogenized in 2 mL of 10 mM HEPES extraction buffer, pH 7.5, and centrifuged at 20,000*g* for 20 min at 4 °C. The supernatant was filtered through 0.45 μm PVDF syringe filter (Fisher Scientific, Madrid, Spain) and stored at −80 °C until analysed.

2.4. Fluorogenic determination of caspase 9 and 3/7 activities

The caspase enzyme reaction took place in a Nunc™ 96-microtiter-well plate (Fisher Scientific, Madrid, Spain) following the procedure proposed by Fuente-Garcia et al. (2021c). Briefly, each sarcoplasmic protein extract (50 μL) was pre-incubated for 30 min in the presence of DTT (20 μL) in each well of the microtiter plate. Enzyme reaction started with the addition of 50 μL of 0.1 mM of Ac-LEHD-AMC (caspase 9) or Ac-DEVD-AMC (caspases 3/7) substrate dissolved in HEPES-CHAPS buffer. Fluorescence intensity was measured for 30 min at 37 °C using a CLARIOstar microplate fluorometer (BMG LABTECH GmbH, Ortenberg, Germany) in the excitation and emission wavelengths of 360 ± 15 and 480 ± 20 nm, respectively. Analysis were performed in triplicate and results were expressed as mean values of Relative Fluorescence Units (RFU) from the three analytical replicates.

2.5. Statistical analysis

The General Linear Model of ANOVA was used to determine if there were significant differences ($P \leq 0.05$) in the caspase activity (9 and 3/7) at 24 h *post mortem*. Caspase activities were analysed using breed (Asturiana de los Valles, crossbreed, Retinta and Rubia Gallega) and pHu (high and normal pHu meat samples) as fixed factors. Normality and homoscedasticity of the variables were checked. Additionally, least square means (LSD) of dependent variables for the levels of breed and pHu fixed factors were compared using the LSD test and results were represented in box-plots. Significance level was declared at $P \leq 0.05$. Statistical analyses were performed using SPSS statistical software (version 25.0, New York, USA).

3. Results and discussion

Determination of meat pHu is critical for meat industry since authorities worldwide consider that values beyond 6.0 at 24 h *post mortem* are intimately associated to animals suffering PSS condition and occurrence of DFD meats (Chambers et al., 2001; Loudon et al., 2018; Polati et al., 2012). Thus, additional indicators such as caspase 9 and 3/7 activities were considered in order to relate them to changes in pHu values, and then, to evaluate their ability to explain stress situations. It is well-known that animals suffering higher stress levels would reach higher apoptosis levels compared to animals managed under less stressful conditions (Alderman et al., 2018; Towers et al., 2019). In our previous studies, we reported that caspase activity varied depending on *post mortem* time, being 24 h *post mortem* the most accurate period capable to discriminate among samples collected from several breeds managed under different feeding and transport/lairage conditions

Table 2
Statistical significances of studied factors and binary interactions.

Fixed effects and interactions	Caspase 9	Caspase 3/7
Breed	***	***
pHu	***	***
Breed x pHu	ns	***

***$P \leq 0.001$; ns: not significant.

(Fuente-Garcia et al., 2021a).

Results of statistical significances of studied factors and binary interactions are reported in Table 2. In general, the activity of both caspases was affected by breed ($P \leq 0.001$) and pHu ($P \leq 0.001$), and the behavior of both caspases was quite similar as observed in Figs. 1A and 2. However, there was an interaction between breed and pHu for caspase 3/7 activity ($P \leq 0.001$).

Regarding the effect of breed on caspase 9 activity at 24 h *post mortem* (Fig. 1B), AV showed a significantly higher caspase activity compared to CB and RG ($P \leq 0.05$), while intermediate activities were observed for RE breed ($P > 0.05$). Observed activity data variability was also higher in AV breed, with highest maximum and lowest minimum activities, compared to other breeds. These results are partially in accordance to those reported by Fuente-Garcia et al. (2021a), where AV and RE breed showed significantly higher caspase 9 activities at 24 h *post mortem* compared to RG breed. The higher caspase 9 activity in AV and RE at 24 h *post mortem* could be indicative of higher stress levels reached before slaughter. Some studies have reported the relationship between breed excitable temperament and stress susceptibility on these breeds (Ponnampalam et al., 2017). While RE breed has widely described as tempered breed giving rise to high stress levels (García and Cruz, 2005), AV and RG breed have been considered non-aggressive breeds (Eusebi et al., 2020; Ruiz Tena, 1999). It is noteworthy to mention that AV breed is known as double-muscle or hypertrophied breed (Oliván et al., 2004) and, in this respect, it has been considered more susceptible to stress due to their regularly limited mobility and reduced muscle capillary density as compared to normal cattle (Fiems, 2012). Concerning the effect of pHu, significantly higher caspase activity at 24 h *post mortem* was observed in high compared to normal pHu meat samples (Fig. 1C). Although there are dearth of studies that analyse caspase 9 activity in relation to meat pHu, results would be compared to those reported for caspase 3/7 activity. In this regard, some authors have revealed differences in caspase 3 regulation levels between normal and high pHu samples, reporting higher expression of caspase 3 large subunit in high compared to normal pHu meat samples at 24 h *post mortem* (Díaz-Luis et al., 2021). Meanwhile, other authors reported that some heat shock proteins (HSPs) such as alpha-crystallin B and heat shock protein beta-1 and beta-6, were over abundant in high pHu meat samples (Fuente-Garcia et al., 2019, 2021b). This finding seems to be in accordance with the fact that stress can induce the synthesis of HSPs that may delay the apoptosis process over *post mortem* time. Taking into account that caspase activation occurs immediately after animal exsanguinations and then it decreases over time, the idea of anti-apoptotic role exerted by heat shock proteins is becoming stronger.

As for caspase 9 activity, the activity of caspase 3/7 was significantly higher in meat samples having high pHu compared to meat samples with normal pHu in all studied breeds ($P \leq 0.05$) (Fig. 2). Moreover, this

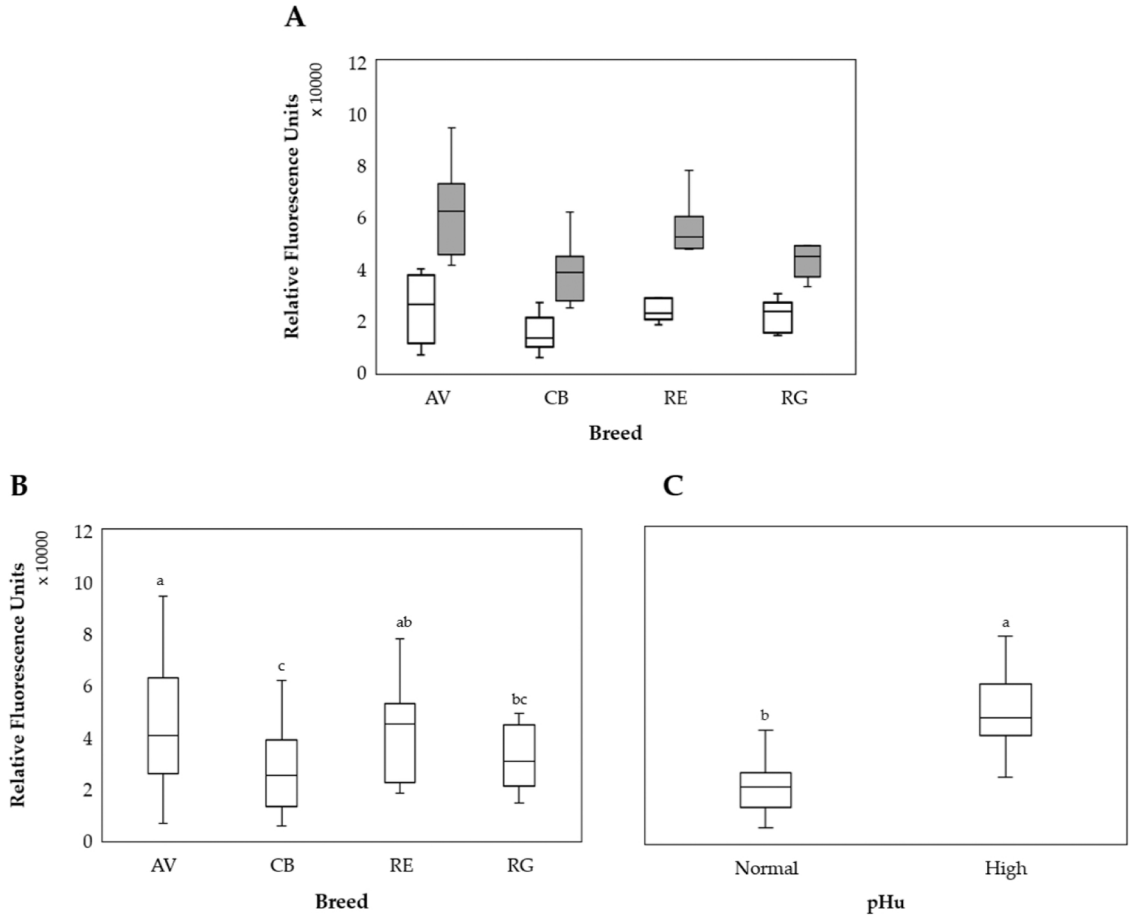

Fig. 1. Box-plot with upper and lower whiskers representing the effect of (A) breed and meat pHu interaction (□ normal pHu meat samples; ■ high pHu meat samples) (B) breed and (C) meat pHu on caspase 9 activity at 24 h *post mortem*. Different letters indicate significant differences among breeds and meat pHu ($P \leq 0.05$).

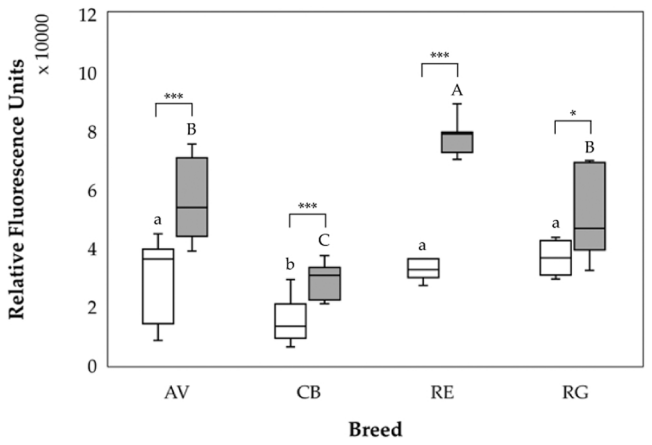

Fig. 2. Box-plot with upper and lower whiskers representing the effect of breed and meat pHu interaction on caspase 3/7 activity. Different lower case letters indicate significant differences among breeds in meat samples with normal pHu, and different capital letters indicate significant differences among breeds in meat samples with high pHu. Asterisks represent differences between the normal (□) and high (■) pHu meat samples for each studied breed. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

pattern was quite different in RE breed, showing strong differences in caspase 3/7 activity between normal and high meat samples, reinforcing again the idea of the major role played by bovine breed in caspase activity. It has been previously indicated the aggressive behavior and stress susceptibility of RE breed (García and Cruz, 2005) compared to AV and RG breeds, these latter having a calmer behavior (Eusebi et al., 2020; Ruiz Tena, 1999). On the other hand, crossbred animals showed the lowest caspase activity levels in both normal and high pHu meat samples, which could be related to the increased resilience of these animals to stress susceptibility. In this sense, some authors pointed out that crossbred animals yielded a better response to environmental changes and stressors compared to pure breeds (Mäki-Tanila, 2007; Peric et al., 2013). In terms of variability of the activity, in general this was similar for both types of assayed meats (normal and high pHu) except for RG breed, whose activity variability was greater in high pHu meat samples compared to normal samples and similar to AV breed (Fig. 2). Therefore, further research is necessary to better understand the influence of breed on caspase 3/7 activity in *post mortem* muscle.

4. Conclusions

Results reported in this work revealed that both caspase 9 and 3/7 activities were significantly higher in meat samples with high pHu compared to normal pHu independently of the studied breed. This demonstrates that caspase activity measured at 24 h *post mortem* can be a good indicator to characterize high pHu meat samples. However, differences among breeds varied for each studied caspase. In the case of caspase 3/7 these differences were dependent on pHu. Crossbred animals and Rubia Gallega breed showed lower and intermediate activity values, respectively, for both caspase activities and studied pHu groups as compared to the other two breeds. However, Asturiana de los Valles and Retinta breeds showed slightly different activity levels for each caspase and between high and normal pHu group in the case of caspase 3/7. In this regard, further research is necessary to better understand how the particular characteristics of each breed can affect the activity of studied caspases.

CRediT authorship contribution statement

Claudia Fuente-García: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. **Noelia Aldai:** Data curation, Writing – review & editing, Supervision. **Enrique Sentandreu:** Writing – review & editing. **Mamen Oliván:** Methodology. Methodology. **Daniel Franco:** Methodology. **Susana García-Torres:** **Miguel Ángel Sentandreu:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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